Crystal structure of the bacterial nucleoside transporter Tsx

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Tsx is a nucleoside-specific outer membrane (OM) transporter of Gram-negative bacteria. We present crystal structures of *Escherichia coli* Tsx in the absence and presence of nucleosides. These structures provide a mechanism for nucleoside transport across the bacterial OM. Tsx forms a monomeric, 12-stranded β -barrel with a long and narrow channel spanning the outer membrane. The channel, which is shaped like a keyhole, contains several distinct nucleoside-binding sites, two of which are well defined. The base moiety of the nucleoside is located in the narrow part of the keyhole, while the sugar occupies the wider opening. Pairs of aromatic residues and flanking ionizable residues are involved in nucleoside binding. Nucleoside transport presumably occurs by diffusion from one binding site to the next.

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Introduction

The outer membrane (OM) of Gram-negative bacteria forms a protective permeability barrier around the cells, and serves as a molecular filter for hydrophilic substances. For this reason, channels are present in the OM to mediate the transport of nutrients and ions across the membrane into the periplasm. Depending on the mode of transport, these channels can be divided into three classes (Nikaido, 1994, 2003): general porins, substrate-specific transporters, and active transporters. The general porins such as OmpC, OmpF, and PhoE are passive pores that do not bind their substrates. They form trimeric, water-filled pores in the OM, through which relatively small (<600 Da) solutes diffuse, driven by their concentration gradient (Nikaido, 1994, 2003). For nutrients that are present at low (μ M) concentrations in the extracellular environment, passive diffusion is no longer efficient, and transport occurs via substrate-specific

and active transporters. The latter class of transporters, to which the iron-siderophore receptors FepA and FhuA belong (Clarke *et al.*, 2001; Braun and Braun, 2002), bind their substrates with high (nM) affinity and transport them against a concentration gradient. This process requires energy, which is provided by the inner membrane (IM) protein TonB (Postle and Kadner, 2003; Ferguson and Deisenhofer, 2004). The substrate-specific transporters contain low-affinity (μ M to mM) substrate-binding sites that are saturable and allow efficient diffusion of substrates at very shallow concentration gradients (Hantke, 1976; McKeown *et al.*, 1976; Nikaido, 1994). The nucleoside transporters belong to this class, exemplified by the *Escherichia coli* Tsx protein, which is the product of the *tsx* gene (receptor for phage T-six). Other examples of substrate-specific transporters are LamB (maltose and maltodextrins) (Luckey and Nikaido, 1980), ScrY (sucrose) (Hardesty *et al.*, 1991), OprB (glucose) (Trias *et al.*, 1988), and OprD (basic amino acids) (Trias and Nikaido, 1990). Among these, only the structures of the sugar transporters LamB and ScrY have been solved (Schirmer *et al.*, 1995; Forst *et al.*, 1998).

Both eukaryotes and prokaryotes can take up nucleosides to serve as carbon and nitrogen sources, and as precursors for nucleic acid synthesis (Hantke, 1976; McKeown *et al.*, 1976; Acimovic and Coe, 2002). In Gram-negative bacteria, the first step in this process is transport across the OM into the periplasm, mediated by the Tsx family of proteins. After traversing the periplasmic space, the nucleosides are transported across the IM into the cytoplasm by the transporters NupC and NupG (Westh Hansen *et al.*, 1987; Munch-Petersen and Jensen, 1990; Craig *et al.*, 1994; Norholm and Dandanell, 2001). These proteins use the proton gradient across the IM for transport, similar to lactose permease (LacY) (Abramson *et al.*, 2003). Since no periplasmic nucleoside-binding proteins have been found, transport across the IM is presumably an efficient process that maintains a low concentration of nucleosides inside the periplasmic space, preserving the driving force for transport (Munch-Petersen *et al.*, 1979; Westh Hansen *et al.*, 1987; Craig *et al.*, 1994). This contrasts with the sugar transporter systems, where, after transport across the OM, periplasmic proteins such as the maltose-binding protein function as sinks and deliver the substrate to ATP-binding cassette (ABC) transporters in the IM.

Relatively little is known about the Tsx proteins, which are found in a number of Gram-negative bacteria and which are essential for the uptake of nucleosides and deoxynucleosides at low (sub μ M) substrate concentrations (Fsihi *et al.*, 1993). Tsx does not seem to play a role in the transport of free nucleobases or monophosphate nucleosides (McKeown *et al.*, 1976; van Alphen *et al.*, 1978; Benz *et al.*, 1988). Reconstituted purified Tsx forms a very low-conductance channel (10 pS at 1 M KCl), indicative of a narrow pore (Benz *et al.*, 1988; Maier *et al.*, 1988). In addition to being a channel for (deoxy)nucleosides, *E. coli* Tsx functions as a receptor for a number of bacteriophages and colicin K (Hantke, 1976; Manning and

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Reeves, 1976; Nieweg and Bremer, 1997; Nikaido, 2003). Txs also transports albicidin, a relatively high-molecular weight (~850 Da) antibiotic produced by *Xanthomonas albilineans* (Birch and Patil, 1985; Birch *et al.*, 1990; Nieweg and Bremer, 1997).

In order to clarify the mechanism of nucleoside transport across the bacterial OM, we have determined crystal structures of Txs alone and with different bound nucleosides. Txs forms a monomeric β -barrel consisting of 12 β -strands, with a narrow and long central pore. The structures of Txs in complex with nucleosides show several distinct binding sites, and suggest a mechanism for transport of nucleosides across the bacterial OM.

Results

Overall structure of Txs

E. coli Txs was expressed as a native protein, and purified by a combination of metal-affinity chromatography, gel filtration, and ion-exchange chromatography (see Materials and methods). The protein elutes as a monomer in gel filtration, and, in contrast to many other OM proteins, it is relatively easy denatured by SDS (Maier *et al.*, 1988); Txs does not require heat denaturation to migrate at its expected molecular weight of ~30 kDa on SDS gels. The crystal structure was solved by single-wavelength anomalous dispersion (SAD) using seleno-methionine (SeMet)-substituted protein (Table I). The two molecules in the asymmetric unit are arranged in opposite orientations (Supplementary Figure 1A); moreover, the crystal packing within the lattice does not show any molecular interface that could be physiologically relevant. These observations suggest that Txs functions as a monomer. The Txs molecules in the crystals have contacts mediated by both the hydrophobic, membrane-embedded parts of the protein and the polar, solvent-exposed loops. The final model includes ~90% of the residues in the mature protein, with the exception of residues 1–8 at the N-terminus and residues 59–74 in loop L2, which are disordered. An overview of the Txs structure is shown in Figure 1, and a schematic topology model in Supplementary Figure 1B. Txs has 12 antiparallel β -strands that form a flattened cylin-

der with a cross-section of $\sim 30 \times 15$ –20 Å. The front of the cylinder consists of transmembrane strands S1–S6, and the back of the cylinder consists of strands S7–S12 (Figure 1). On the narrowest side of the barrel, side chains of several residues in the front and back directly interact with each other. These interactions presumably stabilize the narrow barrel. The overall structure of Txs resembles that of other OM proteins of which the structures have been solved, with long extracellular loops and short periplasmic turns. A notable exception is the stretch consisting of residues S85–F99, which forms a relatively long periplasmic loop (P3) that includes a short α -helix. This region was predicted to form two short transmembrane β -strands and a short extracellular loop (Nieweg and Bremer, 1997), and is the reason that 14 β -strands were predicted instead of the observed 12 β -strands.

The channel pore

A molecular surface, viewed in a direction perpendicular to the plane of the membrane, shows that Txs has a continuous channel that is located slightly off-center in the molecule (Figure 2A). With 12 β -strands, Txs is the smallest known OM protein that functions as a channel. The cross-section of the channel pore is shaped like a keyhole, with dimensions of 10–12 Å in the long direction and 3–5 Å in the short direction. The narrow pore is caused by the flattened shape of the cylinder (Figure 1), and by the fact that the β -strands at the front (S1–S6) are bent inwards in the center of the membrane to constrict the channel even further (Figure 2B).

The Txs pore is lined by a number of aromatic residues. Five of these are located at the front, where they form a 'greasy slide' (Schirmer *et al.*, 1995). Going from the extracellular side to the periplasmic side, these residues are Y152, F77, Y51, Y53, and F42 (Figure 2B). The remaining aromatic residues that line the channel are located in the back. Going from the extracellular side to the periplasmic side, these are F27, F186, and F170. Interestingly, these three residues are paired with the aromatic residues F77, Y51, and F42 in the front sheet, respectively. The three pairs of aromatic residues, many of which are conserved among Txs homologs (Figure 3), form the narrow part of the keyhole-shaped pore.

Table I Data collection and refinement statistics

Data set	SeMet (8BM)	Thymidine (X25) soak	Uridine (X-25) (co-crystallization)
Resolution (Å)	30.0–3.0	30.0–3.1	30.0–3.1
Unique reflections	58328	27650	27972
Redundance	9.1 (9.2) ^a	6.1 (6.2)	7.4 (7.5)
Completeness	96.1% (80.4%)	97.1% (91.9%)	97.2% (92.0%)
$\langle I/\sigma \rangle$	12.1 (4.7)	12.5 (5.8)	10.4 (5.9)
R_{merge}^b	0.113 (0.442)	0.088 (0.433)	0.114 (0.506)
Reflections for $R_{\text{work}}^c/R_{\text{free}}^d$	54021/4307	25578/2072	25887/2085
$R_{\text{work}}/R_{\text{free}}$	26.2%/28.8%	26.9%/29.7%	27.6%/29.9%
R.m.s.d. bond length (Å)	0.0077	0.0081	0.0084
R.m.s.d. bond angle (deg)	1.459	1.498	1.503
Mean <i>B</i> -factor	68.3	62.8	60.0
Ramachandran plot (most favored/ additionally allowed)	84.3%/14.8%	83.1%/15.9%	80.1%/18.5%

^aValues in parentheses refer to data in the highest resolution shell (3.10–3.00 Å and 3.21–3.10 Å in the data sets with and without nucleoside, respectively).

^b $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the average intensity.

^c $R_{\text{work}} = \sum_{hkl} |F_{\text{obs}} - k|F_{\text{calc}}| / \sum_{hkl} F_{\text{obs}}$.

^d R_{free} is the same as R_{cryst} for a selected subset (10%) of the reflections that was not included in prior refinement calculations.

Figure 1 Overview of the Tsx structure. Stereo ribbon diagrams showing the Tsx backbone from the side (top panels) and 90° rotated, viewed from the extracellular side (bottom panels). The approximate positions of the membrane (M) boundary are indicated as horizontal lines, with the extracellular side (E) on top and the periplasmic side (P) on the bottom. The locations of extracellular loops and periplasmic turns are indicated. The transmembrane strands S1–S6 are labeled in the side view. All figures were made using RIBBONS (Carson, 1991).

Figure 2 Nucleoside-binding sites in the Tsx channel. (A) Surface representation viewed from the extracellular side colored as in Figure 1, showing the keyhole shape of the Tsx pore (left panel). The right panel shows a close-up of the channel with the nucleoside bound at Nuc1. (B) Cut-away side view in stereo with a difference ($f_o - f_c$) map contoured at 3σ , showing the nucleoside-binding sites in thymidine-soaked crystals. The nucleosides that could be built in the density at Nuc1 and Nuc2 are shown in green. The aromatic residues that line the channel and that are involved in nucleoside binding are indicated in cyan. The front (F) and the back (B) of the barrel are indicated.

Nucleoside-binding sites in Tsx

Channel conductance experiments on Tsx reconstituted in liposomes suggested that Tsx can bind different nucleosides (Benz *et al*, 1988; Maier *et al*, 1988). In order to identify the nucleoside-binding site(s), we performed co-crystallization and soaking experiments of Tsx with a range of nucleosides and deoxynucleosides. Diffracting crystals were obtained

from soaks with thymidine (3.1 Å), deoxyadenosine (3.8 Å), and deoxyuridine (3.6 Å), and from co-crystallization experiments with uridine (3.1 Å) and inosine (3.5 Å). Electron density difference maps obtained from Tsx in the presence of different nucleosides revealed two well-defined patches of electron density at similar positions inside the channel, corresponding to nucleoside-binding sites. We designate

Figure 3 CLUSTALW 1.8 alignment of Tsx homologs; EC, *Escherichia coli*; EA, *Enterobacter aerogenes*; SE, *Salmonella enterica*; BB, *Bdellovibrio bacteriovorus*; PA, *Pseudomonas aeruginosa*; VC, *Vibrio cholerae*; AG, *Anopheles gambiae*; SO, *Shewanella oneidensis*. The observed secondary structure is shown at the bottom, with strands indicated as dark blue arrows and the α -helix in loop P3 in red. Residues that have greater than 50% identity are shown in yellow, residues that have greater than 50% similarity are shown in gray. Aromatic (o) and ionizable (#) residues that are involved in nucleoside binding are indicated with red symbols.

these two sites Nuc1 and Nuc2, with Nuc1 being closest to the extracellular environment (Figure 2B). The Tsx side chains are virtually superimposable between the various

structures, even for residues that are involved in nucleoside binding. Taking into account the resolution of the various data sets, we only refined those of the thymidine soak and the

uridine co-crystallization (Table I). For the uridine data set, the density at the Nuc1 site has a much higher intensity than that at the Nuc2 site, while, in the case of thymidine, the densities at Nuc1 and Nuc2 are of about equal intensity (Supplementary Figure 2A).

The electron density at the Nuc1- and Nuc2-binding sites in the thymidine-soaked crystals is well defined and allows initial positioning and subsequent refinement of two nucleoside molecules (see Materials and methods). Viewed from the top, it can be seen that both nucleosides are lying along the center of the channel, with the base located in the narrow part of the keyhole and the sugar in the wider part (Figure 2A, right panel). In both binding sites, the orientation of the base relative to the sugar moiety is *anti*, with the pyrimidine O2 pointing away from the sugar moiety (Saenger, 1983). The sugar moiety adopts somewhat different orientations in both sites due to a small rotation around the glycosyl (C1'–N) bond, made possible by its location in the wider part of the pore.

Both aromatic and ionizable residues contribute to nucleoside binding at Nuc1 and Nuc2 (Figure 4). The aromatic residues stack against the base moieties and make van der Waals interactions with the sugar moieties. Interestingly, the stacking interactions with the base moieties are made exclusively by aromatics located in the back of the barrel (F27, F186, and F170). The ionizable residues form hydrogen bonds with hydroxyl groups of the base and sugar moieties. Stacking interactions of aromatic residues combined with hydrogen bonding by ionizable residues have also been observed for the binding of sugars by LamB and ScrY (Schirmer *et al*, 1995; Forst *et al*, 1998).

For the thymidine molecule located at Nuc1, the pyrimidine base stacks against the side chain of F27, and the sugar

moiety makes van der Waals interactions with the side chain of F77, which is absolutely conserved between Tsx homologs (Figure 3). In addition, there are three hydrogen bonds involved in binding the nucleoside at this site; these include a hydrogen bond between the side chain of D55 and the O5' sugar hydroxyl, and between the side chain of R32 and the O3' sugar hydroxyl (Figure 4). Only residue D55 is absolutely conserved in Tsx proteins. In addition to the hydrogen bonds to the sugar moiety, there is a likely hydrogen bond between the side chain of Y152 and the O2 hydroxyl of the base. In the case of the second binding site Nuc2, residues Y51 and F186 stack against the base, whereas Y53 makes van der Waals interactions with the sugar (Figure 4). These three aromatic residues are highly conserved between Tsx homologs. A hydrogen bond is present between the side chain of R234 and the O4' oxygen of the sugar. For the base moiety, there is a hydrogen bond between K168 and the O4 hydroxyl, and possibly between the E79 carboxyl and the O2 hydroxyl (Figure 4). Among these residues, E38 and E79 are highly conserved (Figure 3). For both Nuc1 and Nuc2, the electron density maps suggest that additional interactions between ionizable residues and the nucleosides are present, mediated by ordered water molecules. An example is the putative water-mediated hydrogen bond between the side chain of E40 and the O5' sugar hydroxyl in Nuc2 (Figure 4). At the current resolution of 3.1 Å, however, it is difficult to assign water molecules with confidence.

In addition to the well-defined electron density at Nuc1 and Nuc2, there is a weaker but significant ($\sim 3\sigma$) patch of positive difference density on the extracellular side of the membrane (Figure 2B, Supplementary Figure 2A). This density is too prominent to belong to a water molecule or any of the other components of the mother liquor, and therefore

Figure 4 Detailed stereoviews of the nucleoside-binding sites at Nuc1 (top panels) and Nuc2 (bottom panels), viewed in approximately the same orientations as in Figure 2A and with $2f_o - f_c$ density contoured at 1.6σ in pink. Hydrogen bonds between nucleosides (green) and residues that line the channel (cyan) are indicated as dashed lines. Nitrogen atoms are shown in blue and oxygen atoms in red. Putative water molecules are shown as red spheres.

likely corresponds to another bound nucleoside molecule. Due to a lack of features, the density did not allow building of a nucleoside with confidence. We propose that this site may be an initial, lower-affinity nucleoside-binding site (Nuc0). Aromatic residues that could potentially interact with a nucleoside bound at this site are Y152 and W242, whereas ionizable residues that could contribute to binding of a nucleoside in Nuc0 include N151 and R204.

Variation in nucleoside binding at Nuc1

Comparison of the binding of thymidine and uridine at Nuc1 shows that there are small differences in the orientation of the two nucleosides (Supplementary Figure 2B). Overall, the two different nucleosides are rotated relative to each other around the O4 hydroxyl of the base moiety, which occupies an almost identical position in both structures. As a result, the ribose moiety in uridine has moved somewhat (2–3 Å) towards the periplasmic side of the channel (Supplementary Figure 2B). More specifically, the O3' hydroxyl group of the sugar moiety of uridine makes a strong hydrogen bond with the carboxyl group of D55. In thymidine, this hydrogen bond is made instead with the O5' hydroxyl of the sugar. In addition, there is a hydrogen bond of the O3' hydroxyl in thymidine with the R32 side chain. In the case of uridine, this hydrogen bond is absent, but instead a hydrogen bond is present between the O5' hydroxyl and the side chain of R234, located on the opposite side of the channel (Supplementary Figure 2B). The observed variations in nucleoside binding may be relevant to the function of Tsx (see Discussion).

Agreement of the structure with biochemical data

By employing a selection scheme for mutants that developed resistance to the antibiotic albicidin, several point mutants that were defective in nucleoside uptake were isolated (Fsihi *et al*, 1993). The biochemical properties of all mutants are explained by the structure (Figure 5). The single-substitution mutants G28R and S217R were found to have the most profound effect, completely blocking nucleoside uptake at low substrate concentrations. G28 is located in loop L1, and lines the channel wall on the extracellular side of the membrane, in the vicinity of Nuc1 (Figure 5). The side chain of an arginine residue introduced at this position would point into the channel and block access of the nucleoside. The side chain of S217, located in strand S10, points inwards and forms a hydrogen bond with the side chain of R234, which is one of the ligands for the nucleoside in Nuc2. In addition, the side chain of S217 is close to F186, which is also involved in nucleoside binding. Replacing S217 with an arginine will very likely result in clashes with both F186 and R234, which are highly conserved, and will severely affect nucleoside binding.

The mutation F27L results in a significantly decreased level of nucleoside uptake. Even though F27 is involved in van der Waals interactions with the thymidine base moiety (see the preceding section), the aromatic character of this residue is apparently not essential for nucleoside binding at Nuc1. This notion is supported by the fact that F27 is not absolutely conserved in Tsx homologs (Figure 3). Other single amino-acid mutations that resulted in lower levels of nucleoside transport are G239D and G240D, which are located in loop L5. Inspection of the structure shows that the G239D mutation would likely affect the conformation of residues in loop

Figure 5 Mutagenesis of Tsx (Fsihi *et al*, 1993; Nieweg and Bremer, 1997). Cut-away side view, showing residues F27, G28, S217, G239, and G240 in green, with nitrogen atoms in blue and oxygen atoms in red. Residues F186 and R234 that are involved in nucleoside binding are shown in gray. The region corresponding to residues 199–206 that is involved in bacteriophage binding is shown in red.

L1, some of which (F27) contribute to nucleoside binding. G240 is located close to G28 at the channel entrance (Figure 5), and therefore its replacement with Asp could result in lower levels of nucleoside transport.

In another study, the eight-residue peptide segment corresponding to residues 199–206 was removed from the *E. coli* Tsx protein (Nieweg and Bremer, 1997). The resulting mutant conferred resistance to bacteriophages, but not to colicin K. In addition, the mutant protein still transported nucleosides efficiently. The structure shows that the deleted segment forms the tip of loop L5, one of the longest extracellular loops in the protein (Figures 1 and 5). It seems reasonable that removal of this loop segment would not interfere with nucleoside transport, but would prevent binding of bacteriophages to the extracellular side of the mutant Tsx protein.

Discussion

Mechanism of nucleoside transport by Tsx

The structures of Tsx with bound nucleosides demonstrate that there are at least three distinct binding sites in the channel (Nuc0, Nuc1, and Nuc2; Figure 2B, Supplementary Figure 2A). Substrate binding occurs both through hydrophobic contacts with pairs of aromatic residues located on opposite sides of the channel, and through hydrogen-bonding interactions with ionizable residues. Binding and release of nucleosides by the weak binding sites would result in the movement of the substrate through the channel, with the net direction of transport determined by the nucleoside concentration gradient across the OM. Since both the base and the sugar moiety of the nucleoside contribute to substrate binding, nucleobases and sugars may not provide enough binding interactions on their own, explaining the inability of Tsx to

transport these substrates (McKeown *et al*, 1976; van Alphen *et al*, 1978; Benz *et al*, 1988).

The nucleoside-binding site that is located farthest towards the periplasm (Nuc2) is located in the channel in the middle of the membrane (Figure 2B). Therefore, there may be a need for another binding site, located further towards the periplasm. The structure suggests that part of this binding site could be provided by the aromatic residue pair F42–F170, located in the front and back of the barrel, respectively (Figure 2B). The channel is somewhat wider at this point, making the distance between the two aromatic residues slightly larger ($\sim 8 \text{ \AA}$) than between the aromatic residue pairs at Nuc1 and Nuc2 ($\sim 6.5\text{--}7 \text{ \AA}$). The only conserved residue that could form hydrogen bonds with a nucleoside bound at this site is K44 (Figure 3). The absence of difference density suggests that the affinity of this potential binding site (Nuc3) for substrate may be substantially lower than that of Nuc1 and Nuc2, which might facilitate release of the nucleoside into the periplasm.

Comparison of the structures of Tsx with bound thymidine and uridine shows that there are differences in binding of different nucleosides (Supplementary Figure 2B). There may be several reasons for the observed differences. First of all, Tsx has to accommodate both purine and pyrimidine nucleosides, which have different structures and sizes. In addition, the favored conformations of nucleoside ribose and deoxyribose moieties in solution are different (Saenger, 1983). Secondly, substrate binding may be intrinsically flexible, with a relatively large number of similar, shallow free-energy minima present in the channel. This would result in a relatively smooth free-energy profile between binding sites, facilitating substrate sliding through the channel (Forst *et al*, 1998; Dutzler *et al*, 2002; Schwarz *et al*, 2003).

Differences between Tsx and other transporters

The size and shape of the Tsx channel set it apart from the sugar transporters and the general porins. These other transporters, which have barrels composed of either 16 or 18 β -strands, have wide periplasmic and extracellular funnels, resulting in an hourglass shape of the channels. The funnels lead to a relatively short constriction (5–10 $\text{\AA}$ in length perpendicular to the plane of the membrane) located in the center of the membrane, with a circular cross-section. The constriction is formed by the peculiar conformation of loop L3, which folds inwards into the channel lumen. In Tsx, it seems that the pore is not constricted by any extracellular loops that fold inwards. This makes sense, since the 12-stranded barrel of Tsx is much narrower than the 18-stranded barrels of the sugar transporters and the 16-stranded barrels of the general porins.

Viewed from the side, it is clear the Tsx channel is not shaped like an hourglass with a short constriction. Instead, the channel has a much longer constriction, spanning almost the entire membrane (Figure 2B). The cross-section of the Tsx channel in the plane of the membrane is not circular, but shows a keyhole-like shape. The part of the channel that binds the base moiety of the nucleoside is only 3–5 $\text{\AA}$ wide over a length of circa 15 $\text{\AA}$. By contrast, the more circular cross-sections of the channel constrictions in the sugar transporters LamB and ScrY have diameters of ~ 7 and 8.5 $\text{\AA}$, respectively (Schirmer *et al*, 1995; Forst *et al*, 1998). The narrow Tsx channel restricts the conformational freedom

of the base moiety of the nucleoside, and is probably the reason that Tsx does not transport more bulky monophosphate nucleosides (McKeown *et al*, 1976; van Alphen *et al*, 1978; Benz *et al*, 1988). The sugar moiety of the nucleoside is located in the wider part of the keyhole-shaped channel, but even here the channel does not become wider than 7–8 $\text{\AA}$. The narrow, long channel explains in a qualitative way the observed very low single-channel conductance of Tsx (10 pS at 1 M KCl) (Maier *et al*, 1988).

Another difference between Tsx and the other crystallized passive transporters of the bacterial OM is that Tsx purifies and crystallizes as a monomer. This agrees well with previous studies, which failed to detect an oligomeric form of Tsx (Nikaido, 2003). The crystal structure strongly suggests that a monomer is the functional form of Tsx in the OM. For the trimeric OM proteins, the crystal structures show that the part of the barrel that is involved in trimer contacts is not long enough to span the apolar part of the membrane if the protein were present as a monomer. This is different in Tsx, where the entire hydrophobic part of the barrel is long enough to span the apolar portion of the membrane (Figure 1). This observation argues against a possible dissociation of an oligomeric Tsx protein during purification and crystallization. The reason for the difference in oligomeric state between Tsx and, for example, the sugar transporters is not clear. One possibility is that the relatively high affinity of the Tsx channel for its substrates (adenosine $K_d \sim 0.6 \text{ mM}$; Maier *et al*, 1988) would compensate for a less effective trapping of substrate compared to the larger, trimeric sugar transporters. In accordance with this, the sugar transporters have comparatively low affinities for their substrates (disaccharide $K_d \sim 10 \text{ mM}$; Benz *et al*, 1987). However, other factors such as the abundance of substrates in the extracellular environment and the levels of each type of protein within the OM may also contribute to the difference in oligomeric state between Tsx and the sugar transporters.

Possible implications for nucleoside transport in eukaryotes

Recently, two regions with sequence similarity between the prokaryotic Tsx proteins and the eukaryotic equilibrative nucleoside transporters (eENTs) were reported (Acimovic and Coe, 2002), leading to the proposal that they are distant homologs. The eENTs are a unique family of proteins widespread in eukaryotes. Like their prokaryotic counterparts, they facilitate substrate transport across membranes according to the concentration gradient, without a requirement for energy. The eENTs are essential for nucleotide synthesis in cells that are not able to make these compounds *de novo*, and they play an important role in adenosine-mediated regulation of many physiological processes. In addition, they mediate membrane transport of cytotoxic nucleoside analogs used in cancer and antiviral chemotherapy. Very little is known about their structures and mechanisms of nucleoside transport. Even though the eENTs do not form β -barrels, the distant relationship of the Tsx proteins to the eENTs raises the possibility that certain features of the nucleoside transport mechanism may be similar in both classes of transporters. It would therefore be interesting to test whether cytotoxic nucleoside analogs used in cancer and viral chemotherapy can enter bacterial cells via the Tsx channel. If this is the case,

it may be possible to use the Tsx protein for the design of drugs that are more effectively transported by eENTs.

Materials and methods

Protein expression, purification and crystallization

Full-length *E. coli* Tsx, including the signal sequence and a C-terminal hexahistidine tag, was cloned into the pB22 expression vector, which is under control of the arabinose promoter (Guzman *et al.*, 1995) and was expressed in *E. coli* C43 cells (Aventis). The subsequent purification steps were all carried out at 4°C. Tsx was extracted from the OM by solubilization of the total membranes in a mixture of 1% LDAO/1% β -OG (Anatrace). The protein was purified by affinity chromatography using Co^{2+} resin (Talon) in the presence of 0.2% LDAO, followed by gel filtration on Superdex-200 (Amersham Pharmacia Biotech) in the presence of 0.05% LDAO. A Mono-Q column (Amersham Pharmacia Biotech) was used as a final purification and detergent exchange step, during which the LDAO was exchanged to 0.45% C8E4 (Anatrace). Finally, the protein was concentrated to ~10 mg/ml and dialyzed overnight against Mono-Q buffer (10 mM Tris/50 mM NaCl/10% glycerol/0.45% C8E4 (pH 8.0)) using dialysis membranes with a molecular weight cutoff of 50 kDa (Spectrum). SeMet-substituted protein was produced in wild-type C43 cells by inhibition of the methionine biosynthesis pathway (Van Duyne *et al.*, 1993), and purified as the native protein. No reducing agents or EDTA was added to the buffers, since these inhibited the formation of crystals. Native and SeMet-substituted Tsx was crystallized by hanging drop vapor diffusion by mixing 1–1.5 μ l protein solution and 1 μ l of reservoir solution containing 27–32% PEG 550 monomethylether (MME) as a precipitant and 50 mM Na-acetate (pH 4.3). Hexagonal bars appeared overnight and grew to maximum dimensions of $75 \times 75 \times 500 \mu\text{m}^3$ in a week. They belong to spacegroup P3₂21 and have two molecules in the asymmetric unit, corresponding to a very high solvent content of ~82% (Matthews coefficient $V_m = 6.2 \text{ \AA}^3/\text{Da}$) (Kantardjieff and Rupp, 2003). Crystals were cryoprotected by stepwise adding reservoir solution containing 20% glycerol to the drop, to a final glycerol concentration of ~17%. Crystals were flash-frozen in liquid nitrogen and data were collected at 100 K.

For nucleoside-binding experiments, stock solutions of adenosine, guanosine, cytidine, thymidine, uridine, inosine and their deoxy analogs were made either at high concentrations (25–100 mM) in 50–100% DMSO or in mother liquor. Both co-crystallization and soaking experiments were attempted, typically at ~10 mM nucleoside, for ~4 h in the case of soaking experiments. A number of data sets were recorded, but significant ($>3\sigma$) density for nucleosides in difference maps was only observed in the case of deoxyadenosine, deoxyuridine, inosine, thymidine and uridine (data not shown). Of these, only the data sets recorded for thymidine (soak) and uridine (co-crystallization) diffracted to a sufficient resolution (3.1 Å) to allow building of the nucleoside(s) in the density.

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Data collection and structure determination

The native and SeMet crystals diffracted to a resolution of 3.0 Å. Diffraction data were collected on beamline 8-BM (NE-CAT) at the Advanced Photon Source in Argonne and on beamline X25 at the National Synchrotron Light Source (NSLS) at the Brookhaven National Lab. Data were processed with HKL2000 (Otwinowski and Minor, 1997). The structure was solved by a SeMet SAD experiment recorded at the Se K-edge (Table I). The 10 selenium sites for the two Tsx molecules in the asymmetric unit were located, refined, and used for phasing with SOLVE (Terwilliger and Berendzen, 1999). After solvent flattening with RESOLVE (Terwilliger, 2003), an interpretable electron density map was obtained. Several cycles of model building in O (Jones *et al.*, 1991) and refinement using CNS (Brunger *et al.*, 1998) resulted in a final model of the native protein with an *R*-factor of 26.2% and an *R*_{free} of 28.7%. No electron density could be observed for residues 1–8, 59–74, and the last three residues of the hexa-histidine tag. The locations of the nucleoside-binding sites were obtained from difference maps using the structure factors of the native protein. The nucleosides were manually placed into the density, followed by rigid body and torsion angle refinement of the whole asymmetric unit in CNS. For thymidine, we used the anti-C2'-endo-deoxyribose puckering conformation, which is most favored in solution (Saenger, 1983), as a starting point for refinement. In the case of uridine, we used the anti-C3'-endo-ribose puckering conformation as a starting point. In both cases, small rotations around the glycosyl (C1'–N) bond and the ribose exocyclic C4'–C5' bond were allowed during refinement, since there is no phosphate attached to the C5' carbon atom. The final *R*_{free} values for the thymidine-Tsx and the uridine-Tsx structures are 29.7 and 29.9%, respectively (Table I).

Accession numbers

The coordinates of complexed and uncomplexed Tsx have been deposited in the Protein Data Bank with accession codes 1TLY (apo-Tsx), 1TLW (Tsx-thymidine), and 1TLZ (Tsx-uridine).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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