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Pore-forming Activity of the Tsx Protein from the Outer Membrane of *Escherichia coli*

DEMONSTRATION OF A NUCLEOSIDE-SPECIFIC BINDING SITE*

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The Tsx protein from the outer membrane of *Escherichia coli* is known to be involved in the permeation of nucleosides across the outer membrane under limiting substrate conditions. We purified Tsx from an *E. coli* strain that overproduces Tsx. The purified protein was still functional since it could neutralize the Tsx-specific bacteriophage T6 *in vitro*. When the purified Tsx was reconstituted into a lipid bilayer, there was a large increase of the membrane conductance, indicating pore-forming activity of Tsx *in vitro*. This increase could be strongly blocked with adenosine and to a much lesser extent with cytidine. Titration of the pore conductance with adenosine or cytidine suggested the presence of a binding site for nucleosides in the Tsx pore, with a K_s of 6×10^{-4} and 2×10^{-2} M for adenosine and cytidine, respectively. We propose that the Tsx protein functions *in vivo* as a pore that specifically facilitates the permeation of nucleosides across the outer membrane due to its binding site for nucleosides.

The outer membrane of *Escherichia coli* acts as a molecular filter for hydrophilic substrates (for reviews see Refs. 1–4). Its diffusion properties are due to the presence of a major class of proteins called porins (5). Normally, porins such as OmpF and OmpC show little specificity for solutes (6, 7) and sort the molecules primarily according to their molecular weight. However, under certain growth conditions, the outer membrane also contains porins with marked specificity (for reviews, see Refs. 8 and 9). One of these, the maltose-inducible LamB protein, functions as a general transmembrane diffusion channel and, in addition, preferentially mediates the permeation of maltose and maltodextrins across the outer membrane at low substrate concentration (<0.1 mM) (10, 11). This specificity results from the previously reported maltose- and maltodextrin-binding site of LamB (12–15).

The Tsx protein is a component of the outer membrane of *E. coli* (8, 9) and serves as the receptor for bacteriophage T6 and colicin K (16–18). Expression of its structural gene, *tsx*, is under double negative control of the *deoR*- and *cytR*-encoded repressors and is regulated positively by the cAMP·CAP complex (19–21).[1] The DeoR and CytR proteins also control expression of the nucleoside-uptake systems NupC and NupG located in the cytoplasmic membrane (22–25) and several nucleoside-catabolizing enzymes (26). The coregulation of Tsx synthesis with the systems for nucleoside uptake and metabolism (for reviews, see Refs. 27 and 28) is of functional importance, since the Tsx protein is involved in the permeation of nucleosides across the outer membrane. This was demonstrated first by Hantke (29), who found that in *tsx* mutants the uptake of several nucleosides is impaired. Further analysis (19, 25, 30) revealed a remarkable specificity in the Tsx-mediated permeation of nucleosides across the outer membrane. The rate of uptake for adenosine and thymidine is strongly reduced in *tsx* mutants, while the rate of uptake of cytidine in such a strain is almost identical to that in a *tsx*$^+$ strain. The importance of the Tsx protein for nucleoside uptake becomes apparent only at low (<1 μM) substrate concentrations (19, 25). At higher concentrations of substrate, the Tsx protein becomes dispensable, and the nucleosides permeate the outer membrane by diffusion through the nonspecific porins (31). Furthermore, in *ompB* mutants that lack the major pore-forming proteins OmpC and OmpF, the Tsx protein seems to facilitate the diffusion of serine, glycine, and phenylalanine across the outer membrane (32). Tsx protein has been purified and its amino acid composition analyzed (21, 33), but its primary structure is unknown.

It has been hypothesized, but not directly shown, that the Tsx protein introduces a permeability pathway into the outer membrane by forming a pore (19, 25, 29, 30). To test this hypothesis and to investigate further the nucleoside specificity of the Tsx pore, we purified the Tsx protein and reconstituted it *in vitro* into lipid bilayers. We show here that the incorporation of Tsx into this membrane results in a strong increase in conductance. The increase can be blocked by the addition of adenosine and, to a much smaller extent, by cytidine. Our results are consistent with the view that the Tsx protein forms a pore in the outer membrane, and they provide evidence for the presence of a nucleoside-specific binding site within the pore that is essential for the facilitated permeation process.

EXPERIMENTAL PROCEDURES

Materials—DEAE-Sephacel was from Pharmacia LKB Biotechnology, Inc. A low molecular weight protein calibration kit (Sigma Chemie, München, Federal Republic of Germany) was used as molec-

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[1] E. Bremer, P. Gerlach, and A. Middendorf (1988) *J. Bacteriol.* **170**, in press.

ular weight standard. Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad, München, Federal Republic of Germany). Triton X-100 was purchased from Fluka AG (Buchs, Switzerland) and DNase I from Boehringer (Mannheim, Federal Republic of Germany). All salts were of analytical grade and were obtained from Merck (Darmstadt, Federal Republic of Germany). Adenosine and cytidine were purchased from Sigma.

Bacterial Strains and Strain Construction—The *E. coli* K-12 strain P400 (F⁻ *thr leu argE proA thi mtl xyl ara galK lacY rpsL supE non*) and its *tsx*-200 derivative, strain P407, have been described previously (34, 35). Strain CH8 was constructed from its parent P400 by stepwise selection for mutants resistant against phages K3h30 (OmpA-specific, 36), hy2 (OmpC-specific, 37), K20 (OmpF-specific, 35), and λ*vir* (LamB-specific, 38). This strain remained sensitive to the Tsx-specific phage T6. Phage-resistant mutants were isolated by mixing 0.1 ml of cells from a freshly grown culture with 0.2 ml of a high titer (10^{11}–10^{12} pfu/ml)[2] phage lysate. After 30 min of incubation at 37 °C to allow phage adsorption, the mixture was spread onto a DYT-agar plate (39) and incubated overnight at 37 °C. Nonmucoid colonies were purified twice on the same medium, and the resistance of the strains was retested by cross-streaking against the phage used to isolate the mutants. To confirm the loss of the phage receptor protein, cell envelopes were prepared (40) from 40-ml overnight cultures grown in DYT medium (39). Cell envelope proteins were solubilized in sample buffer containing 2% SDS by boiling for 10 min, and aliquots were electrophoresed on a 12% SDS-polyacrylamide gel. Strains P400 and P407 were grown with aeration in LB medium (39); all other strains were grown in the richer DYT medium. High titer phage lysates were prepared as decribed previously (39).

SDS-Polyacrylamide Gel Electrophoresis—The SDS-polyacrylamide gel system (12% acrylamide) of Lugtenberg *et al.* (41) was used to examine outer membrane proteins. The gels were stained either with Coomassie Brilliant Blue or silver nitrate (42, 43).

Purification of Tsx Protein—One liter of prewarmed DYT medium was inoculated with 125 ml of an overnight culture of strain CH8 and incubated at 37 °C with aeration in a 5-liter Erlenmeyer flask until the culture reached a density of 0.8 (OD_{600}). Cells from 4-liter cultures were collected by centrifugation (8,000 × *g* for 10 min) and resuspended in 20 ml of 10 mM Tris-HCl (pH 8.0) containing 20 mg of DNase I. The bacteria were disrupted by passing them three times through a French pressure cell at 20,000 p.s.i., and unbroken cells were removed by centrifugation (1,200 × *g* for 10 min). The supernatant was layered on a two-step sucrose gradient (70% sucrose (w/v), 54% sucrose (w/v)) in 10 mM Tris-HCl (pH 8.0) and centrifuged at 8 °C for 16 h at 80,000 × *g*. The outer membrane fraction was collected, diluted 1:5 in double-distilled water and centrifuged for 1 h at 140,000 × *g*. The pellet was resuspended in 5 ml of 2% Triton X-100, 10 mM Tris-HCl (pH 8.0), and outer membrane vesicles were sonified for 30 s on ice with a Branson Sonifier B-12. The protein concentration was approximately 8 mg/ml, as determined by the Bio-Rad protein assay using bovine albumin as a standard. The membrane vesicles were pelleted by centrifugation (140,000 × *g* for 1 h), resuspended in 5 ml of the same solution, sonified, and recentrifuged. Outer membrane proteins were solubilized at room temperature in 5 ml of 2% Triton X-100, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and the solution was centrifuged (140,000 × *g*) for 1 h. The supernatant, containing the outer membrane proteins (2 mg/ml), was loaded onto a DEAE-Sephacel column (0.9 × 30 cm) that had been equilibrated with column buffer (0.1% Triton X-100, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA). The column was washed successively with 40 ml of column buffer and 40 ml of column buffer containing 0.1 M NaCl. Tsx protein was eluted with 100 ml of a linear salt gradient from 0.1 to 0.2 M NaCl in column buffer at a flow rate of 10 ml/h. Fractions of 0.7 ml were collected, and the Tsx-protein-containing fractions eluted between 0.15 and 0.18 M NaCl. Approximately 1 mg of purified Tsx protein was obtained.

Neutralization of Phage T6 in Vitro—The ability of phage T6 to adsorb to the purified Tsx protein was determined as follows: Tsx-containing fractions eluted from the DEAE-Sephacel column were pooled (10 ml). The pooled fractions were then dialyzed for 48 h against 5 liters of dialysis buffer (0.1% Triton X-100, 10 mM Tris-HCl (pH 8.0)) to remove the EDTA and NaCl present in the eluate, because we found that the presence of EDTA (10 mM) in the column buffer prevented the formation of plaques of phage T6. The dialysis buffer was changed twice. 100 μl of a phage lysate (approximate 10^3 phages) was mixed with increasing concentrations (2.8–21 μg) of the dialyzed Tsx protein solution (70 μg/ml) in a final volume of 500 μl and incubated for 2 h at room temperature. To determine the number of unadsorbed phages, we added 50 μl (10^9 cells/ml) of a freshly grown culture of strain P400 to the reaction mixture and incubated it for 30 min at room temperature. 3 ml of LB top agar was then added, and the mixture was plated onto a LB plate. After incubation for 16 h at 32 °C, the number of phage plaques was determined.

Membrane Experiments—The methods used for black lipid bilayer experiments have been described previously (44). The instrumentation consisted of a Teflon chamber with two aqueous compartments. Circular holes in the wall separating the two compartments had an area of either 1 mm^2 (for macroscopic conductance measurements) or about 0.1 mm^2 (for single-channel experiments). Membranes were formed across the holes by painting on a 1% solution of diphytanoyl phosphatidylcholine (Avanti Biochemicals, Birmingham, AL) in *n*-decane. The temperature was kept at 25 °C throughout the experiment. The aqueous solutions of adenosine and cytidine used were unbuffered (pH about 6). The Tsx protein was inactivated rapidly in aqueous salt solutions. To prevent this inactivation, we added the protein to the aqueous phase either immediately prior to membrane formation or after the membranes had turned completely black. The membrane current was measured at different voltages by using a pair of matched calomel electrodes with salt bridges, which were inserted into the aqueous solutions on both sides of the membrane. The macroscopic conductance measurements were performed with a Keithley model 602 electrometer. The current through the membrane in the single-channel experiments was boosted with a current amplifier (Keithley 427), monitored with a storage oscilloscope (Tektronix 5115), and recorded on a strip chart recorder.

RESULTS

Isolation of a Tsx-overproducing Strain—Under standard laboratory growth conditions, Tsx is only a minor protein component of the outer membrane of *E. coli* (8, 9). For unknown reasons, the *E. coli* K-12 strain P400 overproduces Tsx to the extent that it comprises about 8% of the membrane

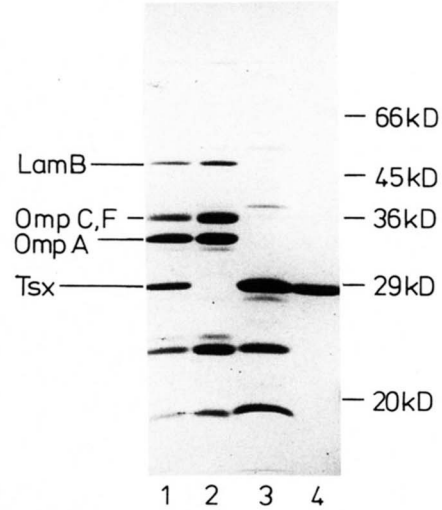

FIG. 1. **SDS-polyacrylamide electrophoresis of outer membrane proteins.** Outer membranes of strain P400 (*lane 1*), P407 (*lane 2*), and CH8 (*lane 3*) were isolated by sucrose density gradient centrifugation, proteins were solubilized by boiling for 10 min in sample buffer containing 2% SDS, and approximately 50 μg of protein from each strain was applied to a 12% SDS-polyacrylamide gel. *Lane 4* shows a sample of purified Tsx protein (approximately 4 μg) after DEAE-Sephacel chromatography. The positions of the outer membrane proteins LamB, OmpC, OmpF, OmpA, and Tsx are indicated. The gel system used does not resolve the OmpC and OmpF proteins. The following molecular weight marker proteins were used: bovine albumin (M_r 66,000), ovalbumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000), trypsinogen (M_r 24,000), trypsin inhibitor (M_r 20,100), and lactalbumin (M_r 14,000). The gel was stained with Coomassie Brilliant Blue.

[2] The abbreviations used are: pfu, plaque-forming units; SDS, sodium dodecyl sulfate; S, siemens.

proteins (18). To facilitate the purification of Tsx, we isolated a derivative of strain P400 that lacks or is severely deficient in the major outer membrane proteins OmpA, OmpC, OmpF, and LamB. This strain was constructed by stepwise selection for mutants resistant to bacteriophages K3h30, hy2, K20, and λ*vir*, which use these outer membrane proteins as part of their receptor (35–38). Consequently, the outer membrane of strain CH8 contained the Tsx protein in greatly enriched amounts. Fig. 1 shows the outer membrane protein profile of CH8 (*lane 3*) together with those of its parent P400 (*lane 1*) and P407, a *tsx* derivative of P400 (*lane 2*). The membrane proteins were solubilized in SDS at 100 °C and electrophoresed on a 12% SDS-polyacrylamide gel. From such gels we estimated the apparent molecular weight of the Tsx protein to be 28,000 (Fig. 1). The same molecular weight was found when the membrane proteins were solubilized for 30 min at room temperature prior to electrophoresis (data not shown).

Purification of the Tsx Protein—Outer membranes of strain CH8 were isolated by sucrose density gradient centrifugation. The outer membrane proteins were solubilized in the nonionic detergent Triton X-100 containing EDTA, and the proteins were separated chromatographically on a DEAE-Sephacel column. After washing the column with buffer and a solution of 0.1 M NaCl, we applied a linear salt gradient between 0.1 and 0.2 M NaCl ("Experimental Procedures"). Fig. 2 shows the elution profile of the separated proteins. The protein content of individual fractions was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). Tsx protein was found in the second major peak and eluted from the DEAE column between approximately 0.15 and 0.18 M NaCl (Fig. 2). Highly purified Tsx protein was recovered as shown in Fig. 1, *lane 4* (gel stained with Coomassie Brilliant Blue) and in Fig. 3, *lanes 6–8* (gel stained with silver nitrate). In the main fractions containing pure Tsx protein, the protein concentration was estimated to be 70 μg/ml; thus we obtained approximately 1 mg of pure Tsx protein from a 4-liter culture of strain CH8.

Purified Tsx Protein Shows Receptor Activity for Phage T6—It is known that Tsx solubilized in Triton X-100 is able

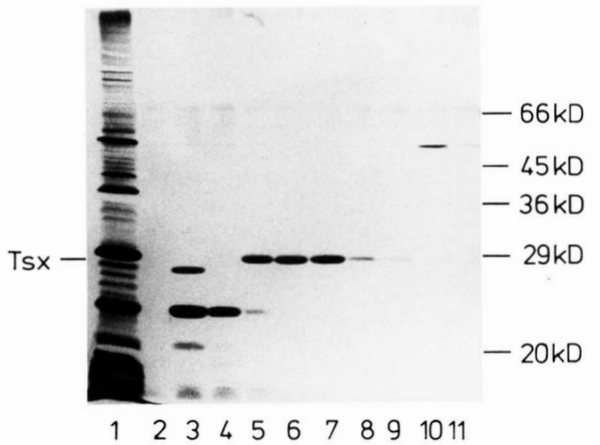

FIG. 3. **Protein profile of individual fractions after DEAE-Sephacel chromatography.** From each fraction, 60-μl samples were mixed with 10 μl of sample buffer containing 4% SDS, boiled for 10 min, and electrophoresed on a 12% SDS-polyacrylamide gel. The gel was stained with silver nitrate. *Lane 1* shows a sample of the outer membrane proteins from strain CH8 applied to the DEAE-Sephacel column. Samples from the following column fractions were analyzed: fraction 104 (*lane 2*), fraction 121 (*lane 3*), fraction 133 (*lane 4*), fraction 146 (*lane 5*), fraction 152 (*lane 6*), fraction 162 (*lane 7*), fraction 173 (*lane 8*), fraction 180 (*lane 9*), fraction 190 (*lane 10*), and fraction 196 (*lane 11*). These fractions were used to determine the specific membrane conductance shown in Fig. 2.

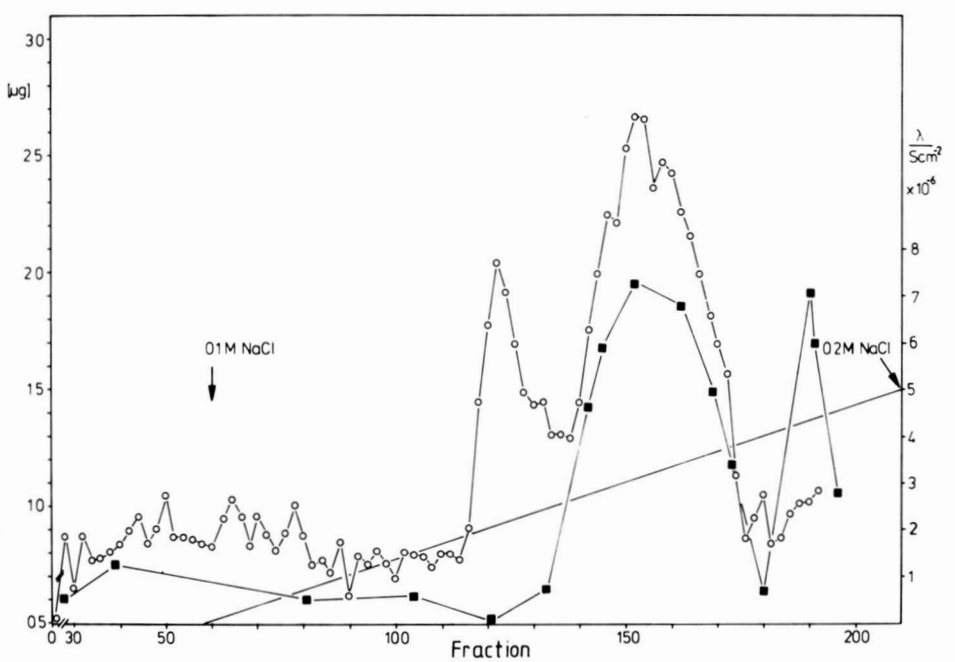

FIG. 2. **DEAE-Sephacel chromatography of outer membrane proteins of strain CH8 and measurement of specific membrane conductance.** A 10-ml solution of outer membrane proteins was applied to a DEAE-Sephacel column. The column was washed with column buffer and 0.1 M NaCl, and the proteins retained on the column were eluted with a linear salt gradient. Fractions of 0.7 ml were collected and their protein contents (○) were determined with the Bio-Rad protein assay. The reaction was monitored with a Gilford spectrophotometer at a wavelength of 595 nm. The specific membrane conductance (■) was measured by lipid bilayer experiments. 50-μl portions from indicated fractions were diluted 1:1 in 1% Triton X-100, and 20 μl of this mixture were added to an aqueous solution of 10 ml of 1 M KCl bathing a lipid bilayer. The bilayer was formed from diphytanoyl phosphatidylcholine dissolved in *n*-decane. The specific membrane conductance was recorded after 20 min, and each point shown (■) represents the average of two to three independent measurements.

to neutralize phage T6 *in vitro* and that the presence of SDS destroys this phage receptor activity (21). Thus, the ability to neutralize phage T6 is a sensitive test for a functional Tsx protein. We tested our purified protein in the following manner. Increasing amounts (2.8–21 μg) of purified Tsx protein were incubated for 2 h with approximately 10^3 pfu of phage T6, and the number of unadsorbed bacteriophages was determined by plating the mixture onto a lawn of strain P400. As shown in Fig. 4, the purified protein has receptor activity for phage T6. This neutralizing activity is phage-specific since λ*vir*, which uses the LamB protein as receptor, was not neutralized by the Tsx protein (Fig. 4). The ability of phage T6 to bind to the purified protein suggested that we had recovered Tsx in a functional form. Neutralization of phage T6 *in vitro* requires an active receptor protein and lipopolysaccharide (33), indicating that our Tsx preparation contained still lipopolysaccharide. However, lipopolysaccharide plays no role in the primary adsorption of phage T6 *in vivo* but is apparently required in a later step of the infection process (19, 48). Such differences in the *in vivo* and *in vitro* requirements for the neutralization of phage T6 could account for the low T6 receptor activity (approximately 10 μg of Tsx was required to inactivate approximately 500 phages) of our Tsx preparation (Fig. 4). Alternatively, some of the Tsx protein could be inactive as a phage receptor. The T6 receptor activity of our preparation cannot be due to undetected contaminating polypeptides since it has clearly been established that Tsx is the receptor protein for phage T6 (18, 21).

Tsx-induced Increase in Macroscopic Membrane Conductance of Lipid Bilayer Membranes—To test the pore-forming properties of Tsx, we reconstituted the purified protein into lipid bilayers (44). When the Tsx protein was added in small

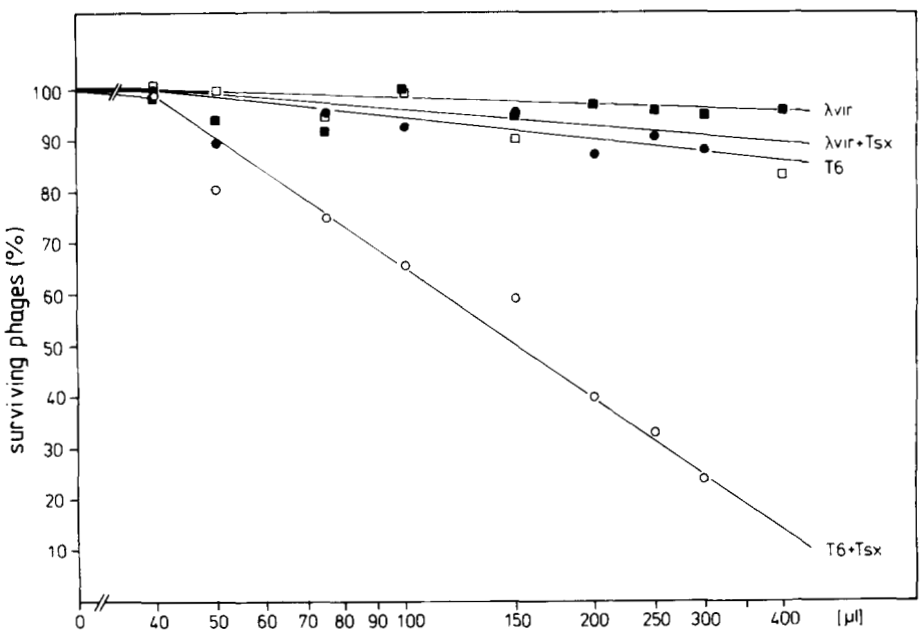

FIG. 4. **Neutralization of phage T6 by purified Tsx protein.** Increasing amounts of a solution of the purified Tsx protein (about 70 μg/ml in 0.1% Triton X-100, 10 mM Tris-HCl (pH 8.0)) were incubated for 2 h with approximately 10^3 pfu of a high titer lysate of phage T6 (○) and λ*vir* (□). In each assay, the number of unadsorbed phages was determined by plating these mixtures onto lawns of strain P400. As a control, both phage T6 (●) and phage λ*vir* (■) were incubated without added Tsx protein in the same buffer.

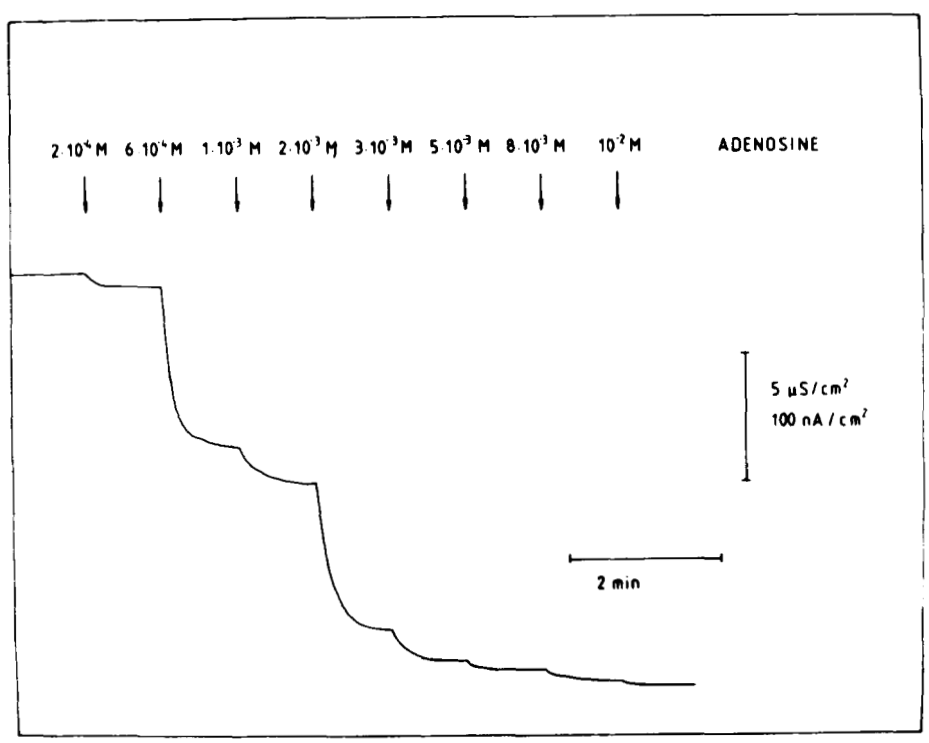

FIG. 5. **Titration of Tsx-induced membrane conductance with adenosine.** The membrane was formed from diphytanoyl phosphatidylcholine dissolved in *n*-decane. The aqueous phase contained 200 ng of Tsx/ml, 1 M KCl, and adenosine at the concentrations shown at the *top* of the figure. The applied voltage was 50 mV, and the temperature was 25 °C.

quantities (100 ng/ml) to the aqueous solution bathing a lipid bilayer membrane, the specific conductance of the membrane increased by many orders of magnitude. The time course of this change was similar to that described previously for other bacterial porins, including the sugar-specific LamB channel (1, 15, 45, 46); *i.e.* the increase in conductance was rapid for the first 15–20 min and then continued at a much slower rate. The conductance increase occurred regardless of whether Tsx was added to only one side or to both sides of the membrane. The addition of the detergent Triton X-100 alone at the same concentration used in the experiments with the protein did not lead to a significant increase in the membrane conductance. Since a steady state conductance level could not be reached in the experiments with the Tsx protein, the dependence of the specific membrane conductance on the protein concentration in the aqueous phase was somewhat difficult to determine. However, meaningful comparison was possible when we used the conductance value at a fixed time (20 min) after the addition of Tsx, when most of the conductance increase had occurred (data not shown). We found a linear relationship between the protein concentration in the aqueous phase and the membrane conductance. These results therefore suggest, that Tsx can form a pore *in vitro*.

Binding of Nucleosides to Tsx—To test the specificity of the Tsx channel for nucleosides, we performed multichannel experiments with membranes of large surface in the following way. Tsx protein was added to a black lipid bilayer membrane formed from diphytanoyl phosphatidylcholine at a concentration of about 200 ng/ml. 20 min after the addition of Tsx, when the rate of conductance increase had slowed considerably, small amounts of different adenosine solutions at final concentrations of between 0.2 and 10 mM were added to the aqueous solutions with stirring to allow equilibration. As shown in Fig. 5, the membrane conductance decreased as a function of the adenosine concentration. By assuming that during the binding of a nucleoside molecule inside the Tsx channel no ions can pass through, we were able to calculate the binding constant from a Lineweaver-Burk plot (Fig. 6). The straight line in Fig. 6 corresponds to a half-saturation constant of 0.6 mM (*i.e.* a stability constant of 1,500 M^{-1}). Similar experiments were also performed with the nucleoside cytidine (Fig. 7), and the half-saturation constant was found to be 20 mM (a stability constant of 50 M^{-1}). The difference in the half-saturation constants corresponds to results of *in vivo* studies, which showed that Tsx facilitated the permeation of adenosine, but not cytidine, across the outer membrane at low substrate concentration (19, 25, 29). It should be noted that the binding of adenosine and cytidine to the channel (*i.e.* the blocking of the channel) was fully reversible. Removal of the adenosine in experiments similar to those described above led to a restoration of the initial membrane conductance (before the addition of the nucleosides).

Single-channel Experiments—We tried to perform single-channel experiments with the Tsx protein similar to those performed earlier with the general diffusion pores OmpF and OmpC and the sugar-specific LamB channel (6, 15, 45). However, we found that the single-channel conductance of the Tsx pore was considerably smaller than that of these other pores (6) and was therefore difficult to determine precisely. We estimated the single-channel conductance of Tsx to be about 10 pS in 1 M KCl. The permeation of ions through LamB is blocked completely by the presence of its specific substrates maltose and maltodextrins (15). To determine whether the same phenomenon occurs in Tsx, we measured the influence of adenosine on single-channel conductance. No single channel could be detected when 10 mM adenosine was added, indicating that the binding of nucleosides to the Tsx protein blocks the permeation of ions through Tsx.

Pore-forming Activity Apparently Unrelated to Tsx—We tested individual fractions eluted from the DEAE-Sephacel column for pore-forming activity in macroscopic membrane conductance experiments (Fig. 2). Fractions from the first major protein peak showed no pore-forming activity (Fig. 2; Fig. 3, *lanes 3* and *4*). Samples from the second major protein peak, containing primarily Tsx protein, led to a strong increase in the specific membrane conductance in proportion to the amount of Tsx protein used (Fig. 2; Fig. 3, *lanes 5–9*); this membrane conductance could be blocked by the addition of adenosine (data not shown). We also observed a second increase in the membrane conductance (Fig. 2) that could not be reduced by the addition of adenosine, indicating that the pore-forming activity was not due to Tsx. These fractions contained some Tsx and two minor proteins with an apparent molecular weight of 47,000 and 52,000 (Fig. 3, *lanes 10* and

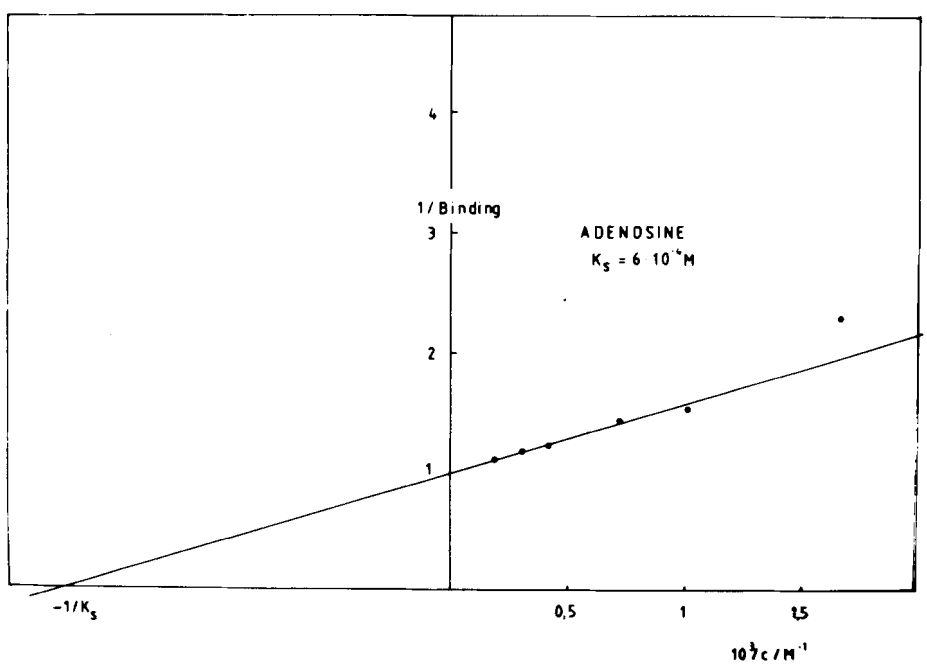

FIG. 6. **Lineweaver-Burk plot of the inhibition of the Tsx-induced membrane conductance by adenosine.** It is assumed that the binding of adenosine to the binding site of the pore completely inhibited the flux of ions through the Tsx channel.

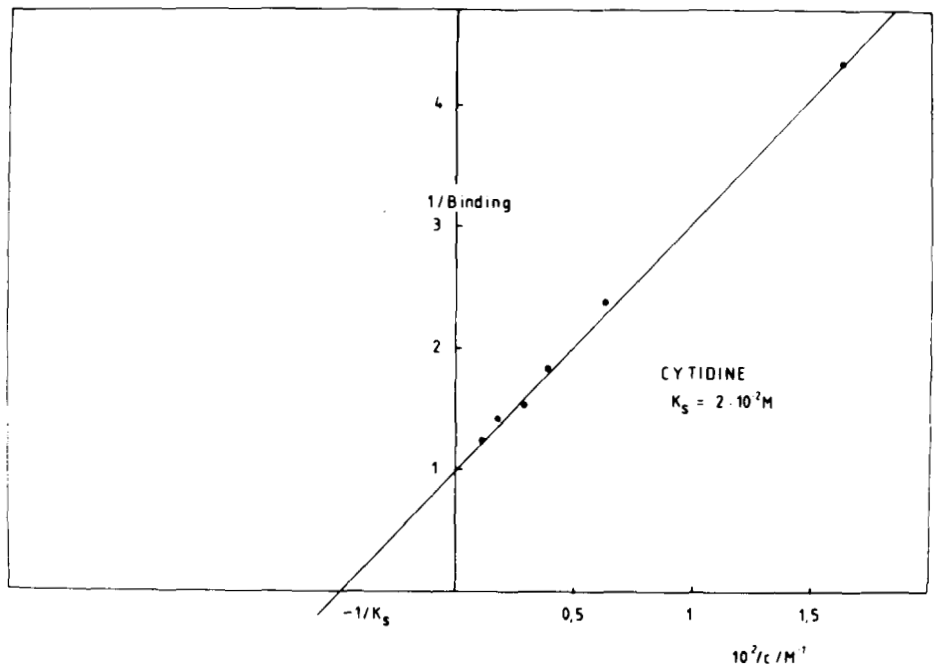

FIG. 7. **Lineweaver-Burk plot of the inhibition of Tsx-induced membrane conductance by cytidine.** It is assumed that the binding of cytidine to the binding site of the pore completely inhibited the ion flux through the Tsx channel.

11). Since our preliminary data,[3] suggest that the 47,000-dalton protein is not LamB (47,932 daltons; 47) one of these minor proteins might be a thus far unreported pore-forming protein in the outer membrane of *E. coli*.

DISCUSSION

Other investigators have used multistep procedures for the purification of Tsx; but, their procedures have either been reported incompletely (33) or yielded only partially purified Tsx protein (21). In this study, we report a simple purification scheme to isolate functional Tsx protein from the outer membrane of *E. coli*. The purified protein has enabled us for the first time to analyze the proposed pore-forming activity of Tsx by *in vitro* reconstitution into lipid bilayer membranes. Our results suggest that the Tsx protein can form a pore *in vitro* and that it contains a binding site for nucleosides.

Under standard laboratory growth conditions, the Tsx protein is only a minor polypeptide component of the outer membrane (8, 9). We therefore isolated a mutant that strongly overproduces Tsx to facilitate its purification. We obtained pure Tsx protein as judged by examination of SDS-polyacrylamide gels stained with Coomassie Brilliant Blue and with silver nitrate. Various values (25,000 and 26,000 daltons) for the apparent molecular weight for the Tsx protein have been described (21, 29, 32). From its mobility on a 12% SDS-polyacrylamide gel we have estimated an apparent molecular weight of 28,000 for Tsx after solubilization at 100 °C for 10 min in 2% SDS. This estimate is in close agreement to the results of Yamato and Hinz (33) who reported a molecular mass of 29,000 ± 2,000 daltons after sedimentation equilibrium ultracentrifugation in the presence of a detergent. Our molecular weight estimate was the same whether solubilization took place at room temperature or 100 °C. This behavior differs from that of other pore-forming proteins of *E. coli* which show temperature-dependent mobility changes on SDS gels as a consequence of their SDS-resistant trimeric structure (1-5). So far it is uncertain whether the active Tsx channel is also an oligomer. In any case, it is known that the phage receptor function of purified Tsx protein is destroyed by SDS (21). Therefore, our inability to detect Tsx multimers might simply reflect a strong sensitivity of Tsx oligomers to SDS.

It has long been suspected (19, 25, 29, 30, 32) that Tsx is a pore-forming protein *in vivo*. Our successful reconstitution of purified Tsx protein into lipid bilayer membranes demonstrates that this outer membrane protein forms an ion-permeable channel *in vitro* and thus provides strong support for its proposed *in vivo* role. There is clear evidence that Tsx has an important function for the permeation of nucleosides at low substrate concentration (<1 μM) across the outer membrane. In *tsx* mutants the uptake of nucleosides, with the exception of cytidine and deoxycytidine, is impaired (19, 25, 29, 30). Furthermore, strains lacking OmpF porin, a major outer membrane protein, show reduced transport rates of adenosine of 15-20%, while *tsx* mutations cause a rate reduction of 90% (49). This specificity of Tsx for nucleosides is poorly understood. We show here that the Tsx protein contains a binding site for nucleosides, and we suggest that this nucleoside-binding site is responsible for the specificity of the Tsx channel. The increased membrane conductance caused by the incorporation of Tsx pores into lipid bilayer membrane could be reduced by the addition of adenosine. Furthermore, no single channel could be detected in the presence of adenosine. By titration of the pore conductance, we determined a K_s of 6×10^{-4} and 2×10^{-2} M for adenosine and cytidine, respectively. At present it is unclear whether these K_s values reflect different affinities of the two nucleosides for the same binding site or whether the Tsx pore carries two different binding sites. In further investigating the characteristics of the Tsx pore, we have found recently that Tsx forms slightly cation-selective channels and that other nucleosides can also block to different degrees the increase of the membrane conductance.[4]

Our *in vitro* results are in excellent agreement with the *in vivo* situation, where it has been demonstrated that the uptake of adenosine is strongly dependent on Tsx while the uptake of cytidine is not reduced in *tsx* mutants (19, 25, 29). The physiological relevance of this different dependence on Tsx for their permeation across the outer membrane is a matter

[3] C. Maier, E. Bremer, A. Schmid, and R. Benz, unpublished results.

[4] R. Benz, A. Schmid, C. Maier, and E. Bremer, manuscript in preparation.

of speculation. We note however that cytidine is the effector molecule of the CytR repressor, which strongly influences *tsx* expression (19, 28)[1] and also controls the synthesis of two nucleoside-specific transport systems and several nucleoside-catabolizing enzymes (27, 28). The apparent Tsx-independent permeation of cytidine across the outer membrane at very low concentration may allow cytidine to alert the cell to the presence of other exogenous nucleosides and to induce the expression of genes involved in their uptake and metabolism.

The single-channel conductance previously determined in lipid bilayer experiments for the general diffusion pores OmpC and OmpF were 1.5 and 1.9 nS, respectively, and that for the maltose- and maltodextrin-specific LamB channel was 160 pS (6, 15) in a 1 M KCl solution. Under the same experimental conditions, we found a considerably smaller single-channel conductance of 10 pS for the Tsx protein. As discussed by Hancock (50) and by Benz *et al.* (15), the diameters of a substrate-specific channel cannot be estimated from such small single-channel measurement.

In *E. coli*, there is another outer membrane protein (LamB) that acts as a general porin but shows a marked substrate specificity for maltose and maltodextrins (10–15). As with the *tsx* gene, expression of the structural gene for the LamB protein is coregulated with the system for the uptake and utilization of its specific substrates. The specificity of the LamB pore has also been attributed to the presence of a substrate-binding site. The reported K_s of maltotriose (4×10^{-4} M) for the binding site in LamB (13–15) is very similar to the K_s of adenosine (6×10^{-4} M) that we report here for the binding site in Tsx. The presence of a binding site is of physiological importance. A calculation of the flux of maltotriose through both the substrate-specific LamB channel and a general diffusion porin showed that at a maltotriose concentration of 10^{-4} M the LamB channel is much more efficient than the porin (15), although at a higher substrate concentration of substrate (10^{-1} M) the flux through the porin exceeds that through LamB.

Nucleosides occur in nature mainly as degradation products of nucleic acids. Their uptake is important for cell growth because they can serve as carbon and nitrogen sources and as precursors in nucleic acid synthesis (27). They are relatively small molecules (average molecular weight is around 250), and, at high concentrations, they can permeate the outer membrane by diffusion through the porins (31, 49). For reasons discussed in detail by Nikaido and Vaara (2), at low substrate concentration, the permeation of nucleosides across the outer membrane is rate-limiting for their overall uptake, because *E. coli* has two high affinity nucleoside transport systems, NupC and NupG, with apparent K_m values in the range of 0.3–0.6 μM (22–25, 27). Consequently, the presence of a nucleoside-specific Tsx pore should offer a significant growth advantage in very dilute environments.

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