Properties of a Cyclodextrin-specific, Unusual Porin from Klebsiella oxytoca*

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The function of CymA, 1 of the 10 gene products involved in cyclodextrin uptake and metabolism by Klebsiella oxytoca, was characterized. CymA is essential for growth on cyclodextrins, but it can also complement the deficiency of a lamB (maltoporin) mutant of Escherichia coli for growth on linear maltodextrins, indicating that both cyclic and linear oligosaccharides are accepted as substrates. CymA was overproduced in both cyclic and linear oligosaccharides are accepted as substrates. CymA was overproduced in both cyclic and linear oligosaccharides. CymA was overproduced in both cyclic and linear oligosaccharides that are capable of binding to CymA. CymA assembled a channel for entry into the periplasm. In this report, we show that CymA is an atypical porin with novel properties specialized to transfer cyclodextrins across the outer membrane.

Cyclodextrins (CDs) are cylindrically shaped oligosaccharides made up of six (α-CD), seven (β-CD), or eight (γ-CD) glucose units. Since the hydroxyl groups border the outside of the ring, CDs are hydrophilic and readily soluble whereas the cavity of the cylinder is hydrophobic.

CDs are formed from starch by several microorganisms via the action of cyclodextrin-glucanotransferases, which are secreted into the medium (2). The extracellular CDs can be used for growth on cyclodextrins by the respective organisms as carbon and energy source.

In the case of Klebsiella oxytoca, the utilization pathway has been studied in detail and it could be shown that the products of at least 10 genes, designated cymA to cymJ, are involved (3). Four of them are constituents of a periplasmic binding protein-dependent uptake system (3), from which CymE is the binding protein (4). CymF and CymG are integral membrane components, and CymD is the ATPase. There is convincing evidence that α- and β-CDs are taken up as intact entities (3, 4) and that they are linearized in the cytoplasm by the product of the cymH gene that is a cyclodextrinase (5). CymE, CymF, CymG, and CymD are homologs of the components of the paradigmatic maltose uptake system, MalE, MalF, MalG, and MalK, respectively (3, 6). The metabolism of γ-CDs is dependent on the activity of the cyclodextrin-glucanotransferase (3), so γ-CDs must be converted into α- or β-CDs or linearized (7, 8) to be taken up.

How the bulky molecules of α-CD and β-CD traverse the outer membrane was still unknown. Because of their outer diameter of 1.37 (α-CD) and 1.53 (β-CD) nm and their physical properties, it was a priori improbable that they use the LamB channel for entry into the periplasm. In this report, we show that α-CDs cross the outer membrane of K. oxytoca and also of recombinant Escherichia coli cells via a specific porin that has been identified as the product of the cymA gene. We demonstrate that this porin also accepts linear maltodextrins as passenger molecules. The purification of CymA and its intriguing properties are reported.

**Experimental Procedures**

Strains and Plasmids, Media, and Growth Conditions—Bacterial strains and plasmids are listed in Table I. Media and growth conditions were as described previously (3, 4).

Standard Genetic Procedures—Standard genetic procedures were adopted from Miller (10) and Sambrook et al. (11). Enzymes for recombinant DNA techniques were used according to the recommendations of the manufacturers (Roche Molecular Biochemicals GmbH, Mannheim, Germany; Amersham Pharmacia Biotech, Freiburg, Germany; New England Biolabs, Schwalbach, Germany). The oligonucleotide primers used were synthesized by MWG (Ebersberg, Germany).

Construction of Plasmid pCYMA2—Plasmid pCYMA2 is a pSU2719 derivative (12) which carries a K. oxytoca DNA fragment containing the full length of the cymA gene of K. oxytoca M5a-I. The cymA gene was cloned via PCR using phosphorylated oligonucleotides derived from the nucleotide sequence of the homologous gene from Klebsiella pneumoniae (13). Oligonucleotide LB331 is a 24-mer (5′-ATGATTAGTACTCTGCACACACTTT-3′) and starts with the ATG of the

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‡ The abbreviations used are: CD, cyclodextrin; OPOE, octyl-polyoxyethylene; DMPC, dimyristoylphosphatidylcholine; PCR, polymerase chain reaction.

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The extract was clarified by centrifugation at 6,000 g. The supernatant (fraction SOM) was dialyzed against buffer C (10 mM potassium phosphate, pH 7.5, plus 3 mM NaN₃) and applied to a Superdex 200 HiLoad gel filtration column. The column was washed with 80 ml of buffer C containing 1 M NaCl. The fractions containing highly enriched CymA were concentrated by ultrafiltration, and the gel filtration chromatography with the Superdex 200 HiLoad gel filtration column was repeated. The fractions containing the purified CymA were concentrated by ultrafiltration to a concentration of 1 mg/ml, supplied with 3 mM NaN₃, and stored at 4 °C.

**Protein Sequencing of CymA**—The N-terminal sequence of purified CymA was determined in an Applied Biosystems pulsed liquid-phase sequencer 477A equipped with an on-line phenylthiohydantoin-amino acid analyzer 120 A (Applied Biosystems).

**Fourier Transform Infrared Spectroscopy**—The secondary structure composition of CymA was estimated with spectra obtained by Fourier transform infrared spectroscopy using the attenuated total reflection technique. Spectra were measured in a Nicolet PTIR 740 spectrometer at 2 cm⁻¹ resolution. Samples containing 30–70 μg of protein were dried onto Germanium crystals. Spectra were collected by adding 1024 scans for each determination and transformed without apodization for further analysis. Hydrogen-deuterium (H-D) exchange was performed on the Ge crystals in a particularly constructed chamber by flushing the sample with N₂/D₂O at 25 °C. The exchange was monitored online by taking spectra (8 scans added) every 15 s to 5 min. After 30 min the exchange was apparently completed, and a final spectrum (1024 scans) was measured. The secondary composition was judged by comparing the CymA spectrum with spectra of porins and other reference proteins whose structures are known and by quantitative analysis of the amide I band as described in Ref. 18.

**Two-dimensional Crystallization of CymA**—Two-dimensional crystallization was performed using the dialysis method and apparatus described by Paul et al. (19). CymA was dissolved in 10 mM potassium phosphate buffer solution, pH 7.5, plus 250 mM NaCl, 0.6% OPOE, and 3 mM Na₂S. The solution was supplemented with dimyristoylphosphatidylcholine (DMPC) solubilized in the same detergent such as to adjust protein-to-lipid ratios from 0.23 to 8.9 (w/w). The final volume was 200 μl, the final protein concentration 0.67–0.95 mg/ml, and the final OPOE content between 0.48% for the highest and 1.92% for the lowest protein-to-lipid ratio. A sample without protein served as a control for membrane formation, a sample without DMPC as a test for the capability of CymA to form crystals without additional lipid. The dialysis buffer consisted of 20 mM HEPES, pH 7.5, plus 3 mM Na₂S; the dialysis membrane had an exclusion cutoff of 15–20 kDa. Dialysis was performed at 33 ± 1 °C for 72 h at a buffer flow rate of 0.12 ml/min per dialysis chamber (200 μl). Membrane formation was monitored online by light diffraction (19), and the experiment was ended when the diffraction curve had approached its plateau for at least 24 h. The samples were inspected in the light microscope and investigated by electron microscopy afterward. All samples were stored at 4 °C.

**Electron Microscopy and Image Processing**—Aliquots of 5 μl were applied to carbon-coated copper grids made hydrophilic by means of glow discharge. The grids were washed with pure water to remove buffer salts prior to negative staining with 2% unbuffered uranyl acetate solution. Electron micrographs were taken in a Philips EM420 at nominal magnification ×36,000. Micrographs were digitized with an Eikonix 1412 camera applying a pixel size of 0.43 nm at the specimen level. Image processing was performed with a Silicon Graphics workstation using the SEMPER 6.4 system (20, 21) for analyzing and averaging two-dimensional crystalline membrane structures using the correlation averaging approach (22). Reference images for averaging were prepared by window filtering the Fourier transforms. Only the periodic
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TABLE II

| Strain | Genotype | G1–3 | G4 | G5 | G6 | G7 |
|--------|----------|------|----|----|----|----|
| MC4100/pSU2719 | lamB<sup>+</sup> | + + + | + + + + + + | + + + | + + + | + + + |
| GM7/pSU2719 | lamB<sup>+</sup> | + + + - - | - - - | - - - | - - - | - - - |
| GM7/pCYMA2 | lamB<sup>+</sup> , cymA<sup>+</sup> | + + + + + + + | + + + + + + + | + + + + + + + | + + + + + + + |

**Identification of CymA as the Putative Cyclodextrin Porin**—The sequence analysis of the products of the cym genes had indicated that three of them (CymA, CymC, and CymE) contain signal peptides (3). CymE has been identified as the cyclodextrin-binding protein in the periplasm (4), and CymC was shown to be dispensable for the utilization of cyclodextrins as sole carbon source (3). The only candidate left for the porin function, therefore, was CymA. Previous evidence for such a role was that an *E. coli* strain transformed with the 10 cym genes from *K. oxytoca* was able to grow on the expense of CDs but not when the cymA gene was deleted. In addition, the lamB gene from *E. coli* could not complement this deficiency (3).

We also introduced an in-frame deletion into the chromosomal cymA gene from *K. oxytoca* (yielding strain CYMA2). The mutant was not able to grow on CDs as sole carbon and energy source, but it was still able to grow on maltolhexaose, indicating that lamB was functionally expressed. This was confirmed by cloning the lamB gene from *K. oxytoca* via PCR and by demonstrating that it could complement the lamB deficiency of an *E. coli* mutant to grow on maltolhexaose (data not shown).

It was further analyzed whether the presence of CymA is required solely for growth on CDs or whether it can also support utilization of maltodextrins. A lamB mutant from *E. coli* (strain GM7) was, therefore, transformed with plasmid pCYMA2 that expresses cymA. Table II shows that the presence of the cymA gene confers the capability to the lamB mutant to grow on maltodextrins with four or more glucose units. Growth on glucose, maltose, or maltotriose also occurred in the absence of the cymA gene since these substrates are taken up via alternative porins (32, 33).

**Overproduction and Purification of CymA—**CymA was over-produced in *E. coli* strains transformed with plasmid pCYMA, and the protein was purified as detailed under “Experimental Procedures.” Fig. 1 displays the course of purification as analyzed by SDS-polyacrylamide electrophoresis of the pooled fractions after each purification step. A symmetrical peak of highly enriched CymA was eluted from the last column; the yield was 5.5 mg of protein out of 15 liters of culture.

**Secondary Structure Properties of CymA—**The N-terminal amino acid sequence of the purified protein was determined to be ASDQR, which confirms that the signal sequence has been cleaved off (Fig. 2A). An alignment of the sequence with those of porins with established structure and function did not reveal any conspicuous similarities. Inspection of the primary sequence using a previously proposed formalism (36) suggested that it contained possibilities for amphipathic β-strands, which is typical for outer membrane porins (37) (data not shown). A clear β-sheet structure is also visible at the C terminus (Fig. 2B), which contains the ultimate phenylalanine residue, which has been shown to be important for insertion of porins into the outer membrane (38). The consensus of the sequence -X-h-X-h-X-h-X-Y-X-F matches that of CymA.

To determine the secondary structure composition of CymA by experimental means, infrared spectra of the native and deuterated protein (H-D exchange by exposure to D<sub>2</sub>O vapor) were measured. The shape of the amide I band (1700–1600 cm<sup>−1</sup>) of the CymA spectrum (Fig. 3) is very typical for outer membrane proteins, and porins in particular, which consist of 55–60% antiparallel β-sheet forming a β-barrel (36). The prominent peak at 1628 cm<sup>−1</sup> is indicative for a high content of β-strands (60 ± 5% of the total amino acids, analysis not shown) and the shoulder at 1693 cm<sup>−1</sup> for β-turns, i.e. both together for antiparallel β-sheet. The loss of the underlying absorption band around 1655 cm<sup>−1</sup> upon deuteration and its shift to wave numbers below 1650 cm<sup>−1</sup> are indicative for loops and folds without preferred secondary structure characteristics (18). The α-helix content is very small and is assessed to be less than 10%. The spectra of both solubilized protein and CymA reconstituted into DMPC membranes did not differ.

The H-D exchange was extremely rapid as judged by spectra taken at 10 s, 60 s, 120 s, and higher up to 30 min after turning on the N<sub>2</sub>/D<sub>2</sub>O stream (data not shown). In fact, the final spectrum of
CymA Does Not Form Trimers in Solution or in Lipid Membranes—A typical property of bacterial porins is the trimeric structure of high thermal stability (39). Classical porins such as OmpF from E. coli or Omp32 from Comamonas acidovorans (40) at least partially retain their trimeric structure after incubation in SDS at 30 °C and are only denatured upon heating in SDS buffer at temperatures above 80 °C (41). As illustrated by Fig. 4, CymA is migrating as a monomer in SDS gels after incubation at 30 °C for 10 min as well as upon heating at boiling temperature. Although the gel was overloaded to render gelatinization of the cytoplasmic membrane components; lane S200 (2), eluate of the second Superdex gel filtration column; lane S200 (1), eluate of the first Superdex gel filtration column; lane Q-Sepharose, eluate of the Q-Sepharose column; lane P100, eluate of the Mono-Q ion-exchange column; lane S6, molecular mass standard; proteins were stained with Coomassie Brilliant Blue.

Fig. 2. A, signal sequence of CymA. The arrow indicates the cleavage site. B, alignment of C termini of porins and CymA. h indicates an hydrophobic residues.

CymA embedded in lipid membranes (Fig. 3) is almost indistinguishable from the very first one in the time series, meaning that the loops were easily accessible to D2O and were not shielded by membrane lipids or buried inside the protein.

CymA was solubilized in sample buffer containing SDS at 30 °C or 100 °C for 10 min. As a control, the trimeric porin Omp32 from C. acidovorans was treated under the same conditions (lanes 4 and 5). M, molecular mass standard; proteins were stained with Coomassie Brilliant Blue.

Fig. 3. Infrared spectra of CymA in the amide I and II band region before and after 30 min of H-D exchange. CymA was reconstituted in DMPC membranes. The prominent peak at 1628 cm−1 (at 1624 cm−1 after H-D exchange) and the shoulder at 1693 cm−1 are characteristic for a high content of antiparallel β-sheet. The peak at 1740 cm−1 originates from C=O stretching vibrations of the fatty acid ester bonds in the phospholipid used for membrane formation.

To clarify whether CymA forms trimeric complexes in lipid membranes as bacterial porins used to do, we reconstituted CymA in DMPC membranes by means of the dialysis approach (19). CymA formed tubules in presence of DMPC at molar lipid-to-protein ratios between 10:1 and 285:1. If considerably less lipid was added, CymA precipitated upon dialysis. The flattened tubules had dimensions of about 3 μm in length and 30–80 nm in width (Fig. 5). They showed faint striations perpendicular to the longitudinal axis, indicating a regular arrangement of molecules. While long tubules occurred at high lipid-to-protein ratios without exception, the tubular membranes tended to apparently “unwind” in form of ribbons if the lipid concentration was low (Fig. 5). These membrane ribbons showed strong reflections in reciprocal space and were selected for image processing while the tubules showed superimposed sets of reflections that were more difficult to analyze. The diffraction spots refer to a lattice in real space with the parameters a = 16.1 nm, b = 3.8 nm, and γ = 93° (Fig. 5). The angle γ was close to 90° in most of the membrane sheets analyzed. This observation and the fact that the odd-numbered reflections with the indices h0 and 0k in the power spectrum were usually very weak or completely missing, are compatible with the properties of the two-sided plane group c12 and a unit cell containing four CymA molecules or asymmetric structural units, respectively (23). The averaged unit cells show stained channels or cavities that are not arranged in trimers, being typical for other bacterial porins (42, 43), but occur in pairs representing molecules in upside-down orientation with respect to each other (central pair in the unit cell shown in Fig. 5). The two-dimensional crystal type and the structure of the...
projected molecule are consistent with the characteristics of a monomeric protein not forming symmetric complexes in the membrane.

The diameter of the CymA molecule is 3.8–4.0 nm according to the lattice parameters, the heavily stain-filled pore cross-section about 1.2–1.4 nm as assessed from the averages. The latter value is close to the resolution limit of the average, being 1.4 nm, and should therefore be taken as an estimate. If CymA has a cylindrical shape, the pore diameter is constant along the channel, and the height of the molecule is 5.0–5.5 nm, corresponding to the dimensions of the E. coli porins OmpF, PhoE, and maltoporin (determined with the atomic structures available in the Brookhaven Protein Data Bank), the calculated mass of one structural unit in Fig. 5 is about 42–46 kDa (23). This estimate is in reasonable accordance with the molecular mass of one CymA polypeptide, i.e., 38 kDa, and it confirms that the repetitive units in the averaged image indeed represent CymA monomers.

**CymA Is an Outer Membrane Channel Permeable for Ions—**

When CymA was added in small concentration (10–100 ng/ml) to the aqueous phase on one or both sides of a black lipid bilayer membrane, we observed an increase of the specific membrane conductance by several orders of magnitude. Control experiments in the presence of the same concentration of the detergent Genapol but without the protein demonstrated that the detergent alone did not lead to any appreciable increase of the membrane conductance. Single-channel experiments in the presence of small concentrations of CymA (5 ng/ml) demonstrated that the conductance increase was caused by the formation of small ion-permeable channels. Fig. 6 shows a single-channel recording taken from a lipid bilayer membrane in the presence of 5 ng/ml CymA. Interestingly, the CymA channels did not show the steplike appearance of general diffusion porins, which exhibit normally very little current noise in reconstitution experiments using lipid bilayer membranes (26). Instead, we observed a strong current noise, which was dependent on the number of reconstituted channels in a membrane. This means that part of the CymA channels, probably one or several loops between two successive β-strands, do not possess defined positions within the channel-forming unit and undergo rapid transitions between different states thus modulating the ion flux through the channel. Single-channel experiments were also performed at different pH in the aqueous phase (pH 5–9). However, also in these cases, we observed the rapid current fluctuations of the open channels, which made it rather difficult to evaluate the single-channel conductance of the CymA channels.

Fig. 7 shows the histogram of conductance fluctuations of the CymA channel in 1 M KCl. The channel distribution showed a maximum between 200 and 600 picosiemens. However, we observed also many other conductance steps probably caused by the rapid fluctuations of the CymA channels and the occurrence of multiple steps, which resulted in a broad histogram of single-channel conductance distributions. The dependence of the average single-channel conductance on the concentration of electrolyte in the aqueous phase was also difficult to obtain because of the considerable current noise of the open channels. Experiments at different KCl concentrations suggested that the single-channel conductance was a linear function of the electrolyte concentration. This means the CymA channel does probably not contain a binding site for potassium or chloride ions inside the channel.

**Titration of CymA-induced Conductance with α-CD and Evaluation of the Stability Constant of Carbohydrate Binding—**

CymA confers to K. oxytoca the capacity to grow on cyclodextrins (3, 4). To study the possibility that this porin has a binding site for the CDs similar to LamB for maltooligosaccharides (27, 28), we performed titration experiments with CymA reconstituted into lipid bilayer membranes. The measurements were performed in the following manner. CymA was added to black lipid bilayer membranes in a concentration of about 100 ng/ml in an aqueous phase containing 1 M KCl. The applied voltage was 10 mV; T = 20 °C.
ng/ml, and the membrane conductance started to increase after a lag time of a few minutes caused by slow aqueous diffusion of the protein. Simultaneously, the current noise increased considerably similar to the situation in single-channel experiments. At 30 min after the addition of the protein, the rate of conductance increase caused by reconstitution of CymA into the membrane had slowed down considerably. Then the experiment demonstrated in Fig. 8 started. Small amounts of concentrated α-CD solutions were added to the aqueous phase to both sides of the membrane, with stirring to allow equilibration. The results demonstrate that the membrane conductance decreased as a function of the α-CD concentration. Furthermore, the current noise of the recording on the strip chart recorder also decreased considerably. The data of Fig. 8 and of similar experiments were analyzed using the following equation derived here and previously from Equation 3. It describes the block of ion current through a one-site, two-barrier channel caused by substrate binding (27).

\[
G_{\text{max}} = \frac{G(c) G_{\text{max}}}{G_{\text{max}} - K_c c} \quad \text{(Eq. 4)}
\]

\(G_{\text{max}}\) is the maximum membrane conductance before the first addition of the α-CD. \(G(c)\) is the membrane conductance at a given α-CD concentration \(c\). Equation 4 means that the titration curve given in Fig. 8 can be analyzed using a Lineweaver-Burke plot as shown in Fig. 9. The straight line corresponds to a stability constant, \(K_c\), of 35,300 \(\text{M}^{-1}\) (half-saturation constant \(K_S = 28 \mu\text{M}\)). The mean value of the stability constant for α-CD-binding to the CymA channel was 29,000 ± 9,000 \(\text{M}^{-1}\) (\(K_S = 34 \mu\text{M}\)).

**DISCUSSION**

The CymA protein of *K. oxytoca* is required for growth of the organism on α-CD or β-CD as carbon source. Mutants with lesions in *cymA* lose this capacity, but they are still able to grow on linear maltodextrins that enter the periplasm via the LamB maltoporin. A defect in *lamB* abolishes this capacity (33); if such a *lamB* mutant is provided with a functional *cymA* gene, growth on maltodextrins is restored. Therefore, CymA has a role in the uptake of CDs and linear maltodextrins whereas LamB only accepts linear maltodextrins. *K. oxytoca* possesses both systems.

There is convincing evidence that CymA is a component of the outer membrane and that it functions as a porin specific for CDs. First, CymA is not solubilized from the total membrane fraction by sarcosyl, which is a property of outer membrane proteins (34). Second, CymA also requires a signal sequence for correct cellular location, indicating that it traverses the cytoplasmic membrane via the sec-dependent pathway (44). Third, the secondary structure composition is typical for outer membrane proteins and porins in particular (36). The antiparallel β-sheet very likely forms a β-barrel with a hydrophobic outer surface and a more hydrophilic inner side. Loop regions of CymA are easily accessible from the surrounding solution, even if the protein is reconstituted in lipid membranes, as judged from the kinetics of H-β-exchange experiments. Fourth, CymA possesses a central pore as rendered visible by electron micros-
duced membrane conductance by the facilitated diffusion of the CymA channel probably provides an advantage for the increasing concentrations of inhibition of the ionic current through the channel with inside the CymA channel. This was done by measuring the constant $K_{membrane} = 350 \text{ nS}$, for CymA binding to CymA of 35,300 $\mu\text{mol}$ ($K_e = 28 \mu\text{M}$). The membrane was formed from diphytanoyl phosphatidycholine/dodecan.

The single-channel conductance is similar to that of specific porins (45). Interestingly, the current traces following the stepwise increases showed a considerable current noise (see also Fig. 6), which may indicate that part of the protein, possibly surface exposed loops, have no defined location within the channel and modulate the current. This is presumably also the reason for the unusually broad channel distribution in the histograms.

We investigated the binding of $\alpha$-CD to the binding site inside the CymA channel. This was done by measuring the inhibition of the ionic current through the channel with increasing concentrations of $\alpha$-CD and assuming that a one-site two-barrier model is valid for the movement of the sugars through the CymA-channel (27). Implicit in the model is also that the affinity of the CD is vastly higher than the near-zero affinity through the CymA-channel (27). Implicit in the model is also that the two-barrier model is valid for the movement of the sugars. CDs are relatively stiff molecules, but they were found to adapt their structure upon binding to proteins (47). We assume that similar effects may facilitate the passage through the apparently narrow porin channel.

Electron microscopical investigations also revealed that CymA behaves distinctly different from classical porins, concerning the formation of oligomeric complexes in lipid membranes. CymA neither occurs in trimers in reconstituted membranes nor does it trimerize in solution. Solubilized porins from other sources usually crystallize in two dimensions with p3 symmetry or on p2-related rectangular lattices but always with a trimer as the “asymmetric” unit (42), whereas CymA formed two-dimensional crystals being related to p1-type plane groups and built from monomers not forming symmetric complexes. This is an unusual and unexpected finding for CymA, functioning as a specific porin. On the other hand, it is not unusual for outer membrane proteins in general. There are at least three classes of bacterial outer membrane proteins not occurring as trimers, i.e. the large $\beta$ family, consisting of eight-stranded $\beta$-sheet proteins including OmpA (48), the group of sid- dophore-specific pore proteins like PhuA (49), and the monomeric autotransporters (50). CymA is certainly not structurally related to the first two groups because of its size. However, at present we cannot clearly decide the relationship of CymA to other outer membrane proteins forming channels. It would be interesting to exactly determine the number of $\beta$-strands in CymA since general diffusion porins, specific porins, and the outer membrane proteins mentioned above can be distinguished by this property.

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