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Crystallization and Preliminary X-ray Diffraction Analysis of ScrY, a Specific Bacterial Outer Membrane Porin

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The sucrose-specific outer membrane porin ScrY of *Salmonella typhimurium* was isolated from *Escherichia coli* K-12 strain KS 26 containing the plasmid pPSO112. The protein was purified to homogeneity by differential extraction of the cell envelope in the presence of the detergents sodium dodecyl sulfate and lauryl (dimethyl)-amine oxide (LDAO). The porin had apparent molecular weights of 58 kDa and 120 kDa for the monomer and for the trimer, respectively, on SDS/PAGE. The purified trimers were crystallized using poly(ethylene glycol) 2000 and the detergents octylglucoside (OG) and hexyl-(dimethyl)-amine oxide (C6DAO). X-ray diffraction of the crystals showed reflections to 2·3 Å. The space group of the crystals was $R3$ and the lattice constants of the hexagonal axes were $a = b = 112{\cdot}85$ Å and $c = 149{\cdot}9$ Å. The crystal volume per unit of protein molecular weight was 3·47 Å^3/Da.

Keywords: porin; sucrose transport; membrane protein; crystallization; *E. coli*

The outer membrane of Gram-negative bacteria acts as a molecular sieve for hydrophilic solutes (Benz, 1988; & Bauer, 1988). Responsible for these molecular sieving properties are major classes of outer membrane proteins called porins (Benz, 1988; Benz & Bauer, 1988). Porins form SDS-resistant trimers, which contain three transmembrane channels (Benz & Bauer, 1988). Porins can be classified as general or as specific according to their action.

General porins show only slight differences in permeability for cations and anions and otherwise sort the solutes mainly according to molecular mass due to their specific exclusion limits. All specific porins studied to date contain binding sites for one class of solutes such as nucleosides, carbohydrates or anions and are inducible upon growth limitations (Benz, 1988). A prominent example of a specific porin is LamB of *Escherichia coli* and of other enterobactericeae (Benz, 1988). LamB is part of the maltose- and maltodextrin uptake system. LamB on the other hand is a relatively ineffective channel for sucrose.

The investigation of the metabolic pathway of sucrose in enteric bacteria led to the discovery of a single copy plasmid pUR400 in *Salmonella typhimurium* (Schmid *et al.*, 1982), which confers to its host the ability to utilize sucrose as the sole carbon source (Schmid *et al.*, 1982, 1988). The plasmid encodes components of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system for uptake and phosphorylation of sugars (Dills *et al.*, 1980; Postma & Lengeler, 1985). Five different genes have been localized on this plasmid. One of them is scrY (Schmid *et al.*, 1982, 1988) with a total length of 1515 base-pairs (Hardesty *et al.*, 1991; Schmid *et al.*, 1991). Its gene product of molecular mass 53 kDa is localized in outer membrane (Schmid *et al.*, 1988). *In vivo* and *in vitro* experiments have confirmed the idea that ScrY is a porin with a similar function to LamB, i.e. that it contains a binding site for carbohydrates. Although the affinity for the malto-oligosaccharides is somewhat smaller (Benz *et al.*, 1987; Schülein *et al.*, 1991).

The gene scrY from pUR400 and from *Klebsiella pneumoniae* has been sequenced (Hardesty *et al.*, 1991; Schmid *et al.*, 1991). Comparison of the amino acid sequence with that of LamB of *E. coli* showed 21% conserved amino acids throughout the primary sequence of both proteins with the exception of a 71 amino acid extension at the N terminus

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of ScrY. It is not likely that this extension has anything to do with the sugar binding to ScrY as it is highly variable between the known ScrY sequences of *S. typhimurium* and *K. pneumoniae* (Hardesty *et al.*, 1991; Schmid *et al.*, 1991). So far it is also unknown if the extension has anything to do with the unusually high single channel conductance of ScrY as compared with LamB and other specific porins (Benz, 1988; Benz *et al.*, 1987; Schülein *et al.*, 1991).

The general diffusion pore from *Rhodobacter capsulatus* is the first porin and the second integral membrane protein for which the three-dimensional structure has been solved at high resolution by X-ray crystallography (Deisenhofer & Michel, 1989; Weiss *et al.*, 1991*a*,*b*). Similar structures are not known for specific porins, although LamB has also been crystallized by Garavito *et al.* (1984). An orthorhombic form has been found by Stauffer *et al.* (1990) to diffract to 3 Å (1 Å = 0·1 nm), so that data collection is possible.

For this study *E. coli* KS26 was constructed by bacteriophage $P1_{vir}$ transduction (Miller, 1972). The donor strain was RAM191 [MCR106 Δ(*ompC*) 178 zei-198 :: Tn*10*] (Misra & Benson, 1988), and the recipient strain was PLB2360 [MC4100 Δ(*lamb*) 106 (*ompF'–lacZ*$^+$) Hyb 16-13] (Benson & Decloux, 1985). Transductants were selected on LB-medium containing tetracycline (12·5 μg/ml). The resulting strain KS26 lacked the outer membrane porins OmpF, OmpC and LamB. It was transformed with the plasmid PSO112 (*scr*R$^+$, *lac*I^Q, *tac*P, *scr*Y$_P$, *scr*Y$^+$, Ap^R, Spo^R) (Schmid *et al.*, 1991). The plasmid is a derivative of pBR322 and contains the gene *scr*Y after its promoter *scr*Y$_P$ in tandem with the tac promoter. The plasmid also contains both the *scr*R repressor gene and the *lac*I^Q allele. Using this expression vector *scr*Y can be expressed at high level by simultaneous induction both with D-fructose (the real inducer of *scr*R) and IPTG†. Since high expression of ScrY is lethal to the cells, they have to be harvested 90 min after induction.

The bacteria from an overnight preculture of KS26 pPSO112 in LB supplemented with 12·5 μg tetracycline/ml and 100 μg spectinomycin/ml were diluted 1 : 100 into 250 ml of the same medium. After 4 h of growth the expression of ScrY was induced by the addition of 10^{-3} M-IPTG and of 0·2% D-fructose. After further growth for 1·5 h the cells were harvested by centrifugation (10 min, 1000 *g*) and washed with 50 mM-Tris·HCl (pH 7·2). The cells were resuspended in 5 ml of 50 mM-Tris·HCl (pH 7·7) and passed three times through a French pressure cell at 900 lb in^2. Unbroken cells were removed by centrifugation at 1000 *g* for 10 min. The supernatant was centrifuged at 100,000 *g* for 1 h. The pellet was resuspended in 2 ml of a buffer containing 0·2% SDS, 10 mM-Tris·HCl (pH 7·7), 2 mM-$MgSO_4$ and centrifuged (100,000 *g*, 1 h). This procedure was repeated once. The pellet was resuspended in 2 ml of a buffer containing 2% of the neutral detergent Genapol-X80 (Fluka AG, Neu-Ulm, FRG), 10 mM-Tris·HCl (pH 7·7), 2 mM-$MgSO_4$ and centrifuged at 100,000 *g* for 1 h. The pellet was resolved in 2 ml of a buffer containing 0·2% of the zwitterionic detergent LDAO (Fluka AG, Neu-Ulm, Germany), 10 mM-Tris·HCl, 2 mM-$MgSO_4$ (pH 7·7) and centrifuged at 100,000 *g* for 1 h. The final pellet was solubilized with 2 ml of a buffer containing 0·6% LDAO, 10 mM-Tris·HCl, 2 mM-$MgSO_4$ (pH 7·7). After centrifugation (100,000 *g*, 1 h) the supernatant contained about 1 mg ScrY/ml (checked with 12% SDS/PAGE, stained with Coomassie blue; see Fig. 1, lane f). The pellet was dissolved in the same buffer, which contained 0·6% LDAO, 10 mM-$MgSO_4$. Another centrifugation led to a supernatant, which contained also ScrY (see Fig. 1, lane g).

Protein concentrations were determined with a Coomassie Brilliant Blue staining technique (Bio-Rad Laboratories GmbH, Munich, Germany).

Prior to crystallization the ScrY-containing

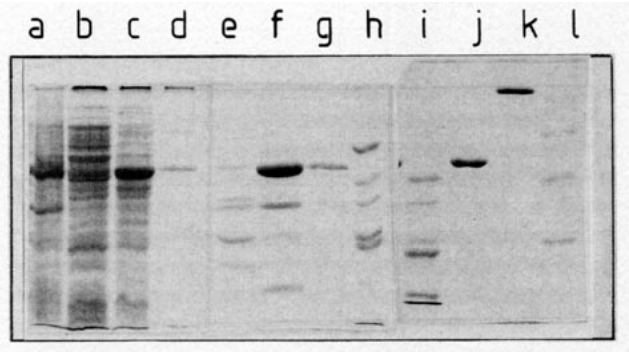

Figure 1. SDS/PAGE (12%, stained with Coomassie Blue) of the different purification steps of ScrY. Lane a, whole membranes, lane b, supernatant of the first wash of the cell envelope fraction with the buffer containing 0·2% SDS. Lane c, supernatant of the second wash with the same buffer. Lane d, supernatant of the first solubilization step with the buffer containing 2% Genapol-X 80. Lane e, supernatant of 0·2% LDAO, 2 mM-$MgSO_4$. Lane f, supernatant of 0·6% LDAO, 2 mM-$MgSO_4$. A contaminating band of OmpA, migrating at 36 kDa apparent mass is seen. Lane g, supernatant of 0·6% LDAO, 10 mM-$MgSO_4$. Lanes h, i, molecular mass markers (66 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20 kDa). Lane j, eluate from the anion-exchange column. The samples of lanes a to j and lane l were boiled for 10 min. Lane k, as lane j but without boiling. The protein solution of crystallization thus contains trimers of ScrY. Lane l, molecular mass markers (205 kda, 116 kda, 97 kda, 66 kDa, 45 kDa, 29 kDa).

† Abbreviations used: PEG, poly(ethylene glycol); OG, β-D-octylglucopyranoside, C7G, heptyl-β-D-glucopyranoside; LDAO, lauryl(dimethyl)-amine oxide; C6DAO, hexyl(dimethyl)-amine oxide; HT, 1,2,3-heptanetriol; CHAPSO, 3-[(3-Cholamidopropyl)-dimethylammonio]-2-hydroxy-propansulfonate; EDTA, ethylenediamine-N,N,N',N'-tetra-acetic acid; IPTG, isopropyl-α-D-thiogalactoside; SDS/PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; V_M, crystal volume per unit of protein molecular weight.

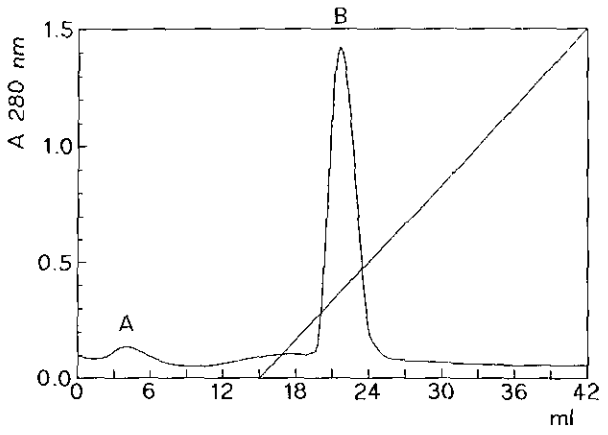

Figure 2. Anion exchange chromatography of ScrY. Fractogel EMD-DEAE was packed into a column with a gel bed volume of 2 ml, equilibrated, loaded with the ScrY obtained by LDAO extraction and washed as described in the text. A linear LiCl gradient was then applied to the column which eluted the porin as a narrow band at 250 mM (indicated with letter B). Another small peak (indicated as A) contained no protein.

supernatants were subjected to anion exchange chromatography (Fractogel EMD-DEAE obtained from Merck, Darmstadt, FRG). The column was equilibrated with 20 mM-Tris·HCl (pH 8), 2 mM-$MgSO_4$, 3 mM-NaN_3 and either 0·08% LDAO or 1·2% (w/v) OG. After loading the protein to the column and washing it with 7·5 column volumes of equilibration buffer, the porin was eluted with 13·5 column volumes of a linear 0 M to 1 M-LiCl gradient. Fractions containing the eluting protein were concentrated using an ultrafiltration cell (Amicon GmbH, Witten, FRG equipped with YM30 membranes) to a concentration of 8 to 9 mg/ml and dialyzed against 20 mM-Tris·HCl (pH 8), 20 mM-$MgSO_4$, 3 mM-NaN_3, the desired concentration of LiCl and either 0·08% (w/v) LDAO or 1·2% (w/v) OG (denoted in the following as crystallization buffer).

ScrY elutes from the anion exchange column at 0·25 M-LiCl as a narrow band (see Fig. 2). Without boiling of ScrY in sample buffer prior to SDS/PAGE, ScrY showed a band at an apparent molecular weight of 120,000 (see Fig. 1, lane k). This converts to a band at 58,000 (see Fig. 1, lane j), when the sample was boiled. Similar results were reported by Schülein *et al.* (1991).

For crystallization we used the sitting-drop method using the depressions of microtiter plates (PS Mikrotestplatte, oberflächenbehandelt, Greiner und Söhne, GmbH, Nürtingen, Germany). 10 µl of the presaturated protein solution were added to one well, the remaining wells were used for the reservoir solution. The cover was sealed with Parafilm. During crystallization the plates were stored at 17 °C.

As precipitants for crystallization we used PEG of various molecular masses as well as ammonium sulfate. For optimization of the crystals a number of different parameters were varied, including pH, detergent for solubilizing ScrY, ionic strength and the type of ions added. We also tried various amphiphilic additives like HT (Fluka AG, Neu-Ulm, Germany), C6DAO (Oxyl GmbH, Dr. Schlude, Bobingen, Germany) or H7G (Calbiochem GmbH, Frankfurt, Germany).

For X-ray diffraction, the crystals were mounted in glass 1 mm capillaries (Müller, Berlin, Germany). Still- and precession photographs were made with a rotating anode X-ray source (Siemens AG, Germany) operated at 35 kV, 20 mA and equipped with a precession camera (reciprocal lattice explorer, STOE and Cie., Darmstadt, Germany).

With PEG600 and LDAO as detergent, we obtained no crystals. When LDAO was replaced by OG small needle-like crystals were observed.

Using PEG1000 and OG, 100 mM-LiCl and 2 mM-$MgSO_4$ were necessary to obtain thin crystalline plates. Precipitation of ScrY occurred at 18% (w/v) PEG1000. The optimal presaturation was in the range 12 to 16% (w/v), the optimal reservoir concentration range at 20 to 26% (w/v). CHAPSO at 1% (w/v), C6DAO at 3% (w/v) and C7G at 3% (w/v) all resulted in bigger crystals, which nevertheless were too small for X-ray diffraction experiments. In the presence of C6DAO the crystalline plates grew thicker as under other conditions.

The crystals obtained with PEG2000, OG, 100 mM-LiCl and 20 mM-$MgSO_4$, grew to larger size than those observed with PEG1000 and PEG600. Precipitation of ScrY occurred at 14% (w/v) PEG2000. The optimal presaturation was in the range 8 to 10·5% (w/v), the optimal reservoir concentration range at 12% to 15%. Without additives as well as in presence of C7G they still had the habit of platelets (400 µm × 200 µm × 20 µm) and diffracted to 7 Å. In the presence of C6DAO they showed an equantic habit and grew within 14 to 21 days to a size of 250 µm × 200 µm × 150 µm. A still photograph of these crystals (see Fig. 3) shows reflections to 2·3 Å.

Precession photographs allowed us to establish the space group as $R3$, which can be described by hexagonal axes. The lattice constants are $a = b = 112{\cdot}85$ Å, $c = 149{\cdot}9$ Å.

Assuming the mass of one ScrY molecule (53,000, deduced from the amino acid sequence of the mature protein) per asymmetric unit, a V_M value of 3·47 Å^3 dalton can be calculated. This value is close to the V_M reported for the $R3$ crystals of porin from *R. capsulatus* of 3·9 Å^3/dalton (Nestel *et al.*, 1989) and for the hexagonal crystal form of OmpF to 3·76 Å^3/dalton (Garavito *et al.*, 1983). ScrY in the crystal thus should be arranged similarly to porin from *R. capsulatus* (Nestel *et al.*, 1989). Trimers of ScrY should form hexagonal sheets, which are stacked on top of each other to form a cubic close-packed array. The 3-fold symmetry axis of the space group indicates that ScrY forms trimers in the crystal, as was also concluded from gels and conductivity measurements (Schülein *et al.*, 1991). It is obvious that the band migrating in SDS/PAGE

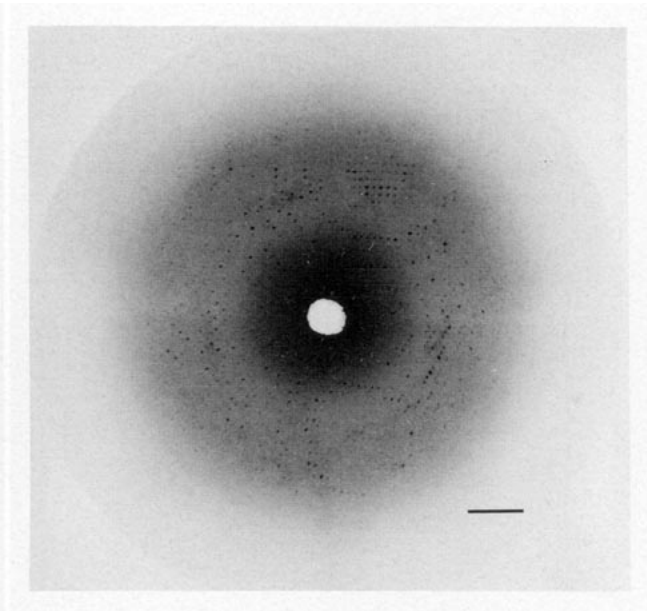

Figure 3. Still-photograph (exposure time 4 h) of a ScrY crystal grown in the presence of 1·2% (w/v) OG, 100 mM-LiCl, 5 mM-$MgSO_4$, 3 mM-NaN_3, 3% (w/v) C6DAO. The PEG2000 concentration in the presaturated and in the reservoir solution were 9% (w/v) and 13% (w/v) respectively. The crystals grew within 3 weeks at 17°C. The crystal-to-film distance was 75 mm. The bar represents 10 mm on the film. The reflexions extend to 2·3 Å.

after solubilization without boiling at an apparent molecular mass of 120,000 is the ScrY trimer.

Crystals with an equantic habit useful for X-ray diffraction experiments grew in the presence of 3% (w/v) C6DAO. This substance seems to act as a "small amphiphile" (Michel, 1983, 1991; Timmins *et al.*, 1991). Interesting, short chain alkanoylamine oxides have also been useful for the crystallization of purple bacterial B800-850 light-harvesting complexes (Welte *et al.*, 1985; Welte & Wacker, 1991).

For crystallization conditions slightly different from those given above, we observed crystals which grow in space group $P1$ with cell parameters close to those of the $R3$ crystals ($\alpha = 90°$, $\beta = 90°$, $\gamma = 120°$). Precession photographs of these crystals show a similar pattern to that of the $R3$ crystals but with additional reflections with indices that are absent in space group $R3$. Three-dimensional data sets of these crystals collected on an image-plate detector revealed the absence of a threefold axis along c, so that the space group cannot be described as $P3$. As we consider this space group with nine molecules/asymmetric unit as unfavorable, we did not try to optimize crystallization conditions. We are currently collecting native and heavy-atom data sets of the ScrY $R3$ crystals and hope that they and those of LamB (Stauffer *et al.*, 1990) will allow us to determine the structure of substrate-specific porin and of the sugar-binding site inside the channel.

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