

Purification, Characterization, Crystallization, and Preliminary X-Ray Results From *Paracoccus denitrificans* Porin

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ABSTRACT The porin from *Paracoccus denitrificans* ATCC 13543 was purified and crystallized. Two crystal forms were obtained from porin solutions with β -*D*-octylglucopyranoside as detergent. Crystals of form I belong to the monoclinic spacegroup C2 with unit cell dimensions $a = 112.2 \text{ \AA}$, $b = 193.8 \text{ \AA}$, $c = 100.5 \text{ \AA}$ and $\beta = 129.2^\circ$. There is 1 trimer per asymmetric unit. Crystals of form II are triclinic with $a = 89.7 \text{ \AA}$, $b = 98.8 \text{ \AA}$, $c = 112.5 \text{ \AA}$, $\alpha = 112.5^\circ$, $\beta = 101.8^\circ$, $\gamma = 106.7^\circ$ (2 trimers per asymmetric unit). Both crystal forms diffract to 3 \AA .

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INTRODUCTION

Porins are channel-forming proteins that span the outer membrane of Gram-negative organisms allowing the diffusion of small polar solutes across the membrane.¹ In general, bacterial porins are organized as trimers. Structure analysis of porin from *Rhodobacter capsulatus* showed that the polypeptide chain of each subunit traverses the membrane 16 times as antiparallel β -strands forming a large barrel.² Each subunit forms one channel; the trimer therefore contains three channels. The same architecture was observed for porins OmpF and PhoE from *E. coli*³ and the porin from *Rhodospseudomonas blastica*.⁴ Maltoporin differs from the nonspecific porins by containing an 18-stranded barrel.⁵

A transmembrane channel may be considered as the common structural motif of ionic channels from bacterial and eukaryotic membranes.⁶ While the outer membrane porins are made up of amphiphilic β -strands, the transmembrane domains of plasma membrane transporters generally consist of hydrophobic α -helices, often representing a 6 + 6 helix architecture.⁷ However, in eukaryotic cells the β -barrel design has been identified in at least one channel family, the mitochondrial porin, which forms large, open channels in the mitochondrial outer membrane.⁸ This is significant, as these organelles are considered descendants of bacterial symbionts.

In view of the endosymbiotic hypothesis of the evolutionary origin of mitochondria *Paracoccus denitrificans* occupies a unique position. It displays the most mitochondrial features compared with other aerobic bacteria⁹ and has been described in literature as “free living mitochondrion” or “protomitochondrion.”¹⁰ In contrast to the general trimeric organization of bacterial porins, the porin of *Paracoccus denitrificans* was originally described as a dimer.¹¹ Here we report the crystallization of the *Paracoccus* porin.

MATERIALS AND METHODS

Purification

Porin was purified from *Paracoccus denitrificans* ATCC 13543 according to a modified procedure.¹¹ Cells were broken by passage through a French pressure cell. The resulting homogenate was centrifuged at 160,000g for 45 min. In order to remove the inner membrane and some outer membrane proteins, the membrane pellets were treated with a buffer solution containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2% SDS, 35°C for 30 min. The pellet of the centrifugation step contained primarily porin bound to peptidoglycan. The porin was solubilized with Tris-HCl buffer (pH 8.0), containing 1 mM EDTA, 500 mM NaCl, and 2% of the detergent lauryl-(dimethyl)-amine oxide (LDAO), at 30°C for 60 min. The insoluble material was removed by another centrifugation step.

Further purification was achieved by anion exchange chromatography using Q-Sepharose (Pharmacia). After removing of NaCl by dialysis, the porin solubilisate was applied to a column, equilibrated with 20 mM Tris-HCl (pH 8.0), 1% (w/v) LDAO. Upon applying a linear NaCl gradient in the same buffer, porin eluted at 700 mM NaCl.

Analytical Ultracentrifugation

The ultracentrifugation experiments were performed as previously described.¹²

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Crystallization

Crystals were grown using the sitting drop method in microtiter plates. Sitting drops were prepared by mixing 5 μ l of the porin solution with 5 μ l of the corresponding reservoir solution. The protein concentration in the crystallization droplets was about 6 mg/ml.

X-Ray Crystallographic Studies

For X-ray exposure, the crystals were mounted in thin-walled glass capillaries (Mueller, Berlin, Germany). Still and precession photographs were taken with a rotating anode source (Siemens AG, Germany) operated at 35 kV, 20 mA and equipped with a precession camera (reciprocal lattice explorer, Stoe, Darmstadt, Germany). Native data sets were collected on a Stoe image plate detector system. The X-ray source was a rotating anode (Stoe) operating at 40 kV, 100 mA. For data reduction and determination of spacegroups, the XDS program¹⁸ was used.

RESULTS AND DISCUSSION

Analysis of the isolated porin by SDS-polyacrylamide gel electrophoresis showed major protein bands at apparent molecular masses of 81 and 33 kDa after solubilization with sample buffer at room temperature and 100°C, respectively. In general, the oligomeric species of porins are resistant to SDS denaturation. Incubation in sample buffer at 100°C results in the dissociation into monomeric subunits. The porin solution was contaminated by lipopolysaccharide (LPS) as detected by specific silver staining of SDS-gels.¹³ LPS was reported to interfere seriously with crystallization of bacterial porins.¹⁴ After further purification by anion exchange chromatography less than 1/50 mol LPS per mol of porin were present, according to the detection limit of the silver staining method for R-type LPS.¹⁵

Monodispersity of the protein-detergent complexes was demonstrated by sedimentation velocity experiments in an analytical ultracentrifuge. Sedimentation equilibrium experiments were performed in order to determine the mass of the native porin. A molecular weight of about 106,000 Da was obtained. Additional estimations with a gel filtration column (Sephacryl S-300, Pharmacia), calibrated with membrane proteins of known masses, and by native polyacrylamide electrophoresis in Triton X-100 were in accordance with a value of about 100,000 Da. With a monomer of 33,000 Da (determined by SDS-polyacrylamide gel electrophoresis) this suggests that the porin forms trimers as typical for bacterial porins. Therefore we cannot verify a dimeric structure of the *Paracoccus* porin, originally proposed¹¹ based on Ferguson plots and chemical cross-linking studies.

To test for native structure, the porin was reconstituted into different asymmetric planar lipid bi-

layer systems with and without LPS and into symmetric phospholipid bilayers.¹⁶ The porin formed general diffusion pores without ion selectivity. The calculated pore diameter of 1.5 nm is in agreement with earlier estimations by liposome swelling assays.¹¹ Furthermore, single channel closing events in three steps are in accordance with a trimeric structure.¹⁶

A coarse crystallization screening with porin solubilized in various detergents yielded microcrystals in 3 cases, with 0.08% LDAO, 1.0% β -*D*-octylglucopyranoside (OG), and 0.2% decylmaltoside (DM) and with polyethylene glycol (PEG) 600 as precipitant. The presence of divalent cations in concentrations from 5 to 200 mM was necessary for crystal growth. For improvement of the crystal size, crystallization parameters such as pH, buffer type, ionic strength, ionic type were optimized. Crystals with dimensions of 200 \times 200 \times 100 μ m grew in a crystallization buffer containing 20 mM Tris-HCl (pH 7.5), 200 mM KCl, 10 mM CaCl₂, 1 mM NaN₃, and 1% OG within a few days. The initial protein solution contained 14 to 18% (w/v) PEG 600, the reservoir 28 to 32%. Still photographs showed reflections to 4 Å resolution. Crystals obtained in the detergents LDAO or DM showed only poor X-ray diffraction to about 8 Å. Further attempts to improve the resolution limit by changing the crystallization parameters or by adding of small amphiphiles¹⁷ such as β -*D*-hexylglucopyranoside or hexyl-(dimethyl)-amine oxide were not successful.

Given the importance of divalent cations during crystal growth we made porin preparations without adding EDTA in the two solubilization steps. Initially EDTA had been added with the intention to minimize the interactions between the porin and LPS molecules. The absence of EDTA did not alter the further purification, but obviously affected the quality of the crystals. Reflections were now observed to 3 Å.

The crystals belong to the triclinic space group, with unit cell dimensions $a = 89.7$ Å, $b = 98.8$ Å, $c = 112.5$ Å, $\alpha = 112.5^\circ$, $\beta = 101.8^\circ$, $\gamma = 106.7^\circ$ (crystal form II). Assuming a molecular weight of 106,000 Da (determined by analytical ultracentrifugation) the crystallographic asymmetric unit contains 2 trimers, with a resulting Matthews parameter V_M of 3.9 Å³/Da. When crystallization was conducted in the presence of 10–15% of PEG 400 or PEG 200 in the protein solution under otherwise identical conditions as above we obtained monoclinic crystals of the space group C2 (crystal form I, Fig. 1) with the same diffraction limit. The unit cell parameters are $a = 112.2$ Å, $b = 193.8$ Å, $c = 100.5$ Å, and $\beta = 129.2^\circ$. The asymmetric unit contains one trimer (calculated $V_M = 3.9$ Å³/Da).

In view of the close relationship between *Rhodobacter capsulatus* and *Paracoccus denitrificans* revealed by 16 S rRNA homology,¹⁹ we are try-

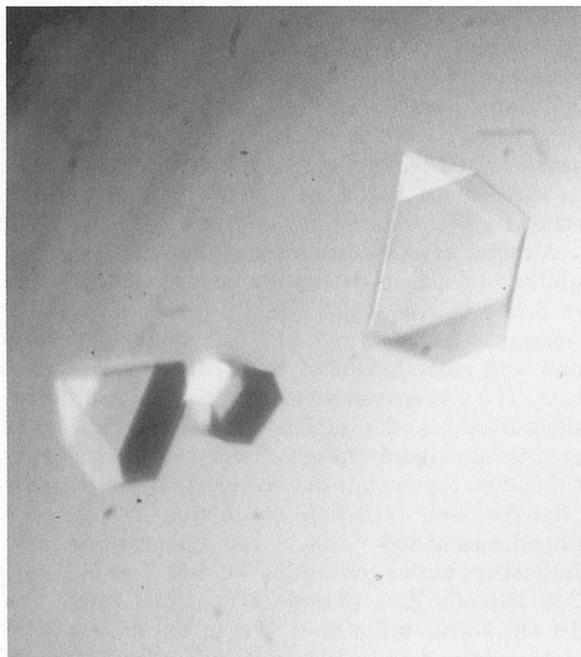


Fig. 1. Monoclinic crystals of *Paracoccus denitrificans* porin (form I). The crystals were grown using the sitting drop method. The reservoir conditions were 28% PEG 600, 10% PEG 200, 20 mM Tris-HCl 7.5, 200 mM KCl, 10 mM CaCl₂, 1 mM sodium azide, 1% OG. The maximal size observed was 250 × 250 × 100 μm.

ing to solve the structure by molecular replacement methods based on a model derived from the high-resolution structure of porin from *Rhodobacter capsulatus*.²

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