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Crystallization and preliminary *X*-ray analysis of porin from *Rhodobacter capsulatus*

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Porin monomers of the phototrophic bacterium *Rhodobacter capsulatus* were purified. Crystals were obtained from a solution of porin solubilized with the detergent octyltetraoxyethylene within 5 days using the vapor phase equilibration technique. The crystals were rhombohedral with an edge length of 0.4 mm. The space group was trigonal R3 with unit cell constants of $a=b=95$ Å, $c=147$ Å. Reflexions were observed to 3.2 Å.

Porin; Membrane protein crystallization; X-ray diffraction; (*Rhodobacter capsulatus*)

1. INTRODUCTION

The outer membrane of Gram-negative bacteria contain one or more pore producing proteins (porins), which allow the uptake of small hydrophilic molecules and the excretion of waste products. The porins usually have molecular masses between 30 and 50 kDa and possess specific exclusion limits. Although they are described as 'general diffusion pores', some of them are weakly selective for either cations such as OmpF and OmpC, or anions such as PhoE. In the case of Enterobacteriaceae, the porins form homo-oligomeric trimers of identical subunits (reviews [1,2]). Porins are extraordinarily stable with respect to denaturation by detergents, organic solvents, heat and proteases when compared to other membrane proteins [2]. Most of the porins so far investigated are associated with the underlying peptidoglycan layer.

With respect to secondary structure porins possess an extraordinary high content of β-sheet structure [3–5], while other membrane proteins usually possess several transmembrane α-helices, which are loosely connected by hydrophilic loops. In β-sheet structure a network of hydrogen bonds between the amide bonds of all amino acids of the apposed peptide chains is formed. In contrast, in α-helices the amino acid side groups can form hydrogen bonds only with side groups of apposed α-helices. Intuitively this results in a much weaker stabilization of helical packings as compared to β-sheet structures.

The extraordinary stability of porins due to β-sheet structure may be the reason that matrix porin (OmpF) from *Escherichia coli* was the first membrane protein of which crystals were grown which diffracted to high resolution [5,6]. The crystals were obtained from a solution of detergent-solubilized trimers of porin [7]. Garavito et al. [8] later reported the crystallization of maltoporin (LamB) and OmpA from *E. coli* and showed that the LamB crystals diffracted to 6 Å. Worobec et al. [4] report the purification of crystallization-grade protein P from *Pseudomonas aeruginosa*.

Proteins with pore-forming properties were recently identified in the outer membranes of the closely related phototrophic species *Rhodobacter*

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capsulatus and *R. sphaeroides* [9–11]. Both porins were found to have the characteristic properties of other porins. The porin of *R. capsulatus* has a diameter of 1.6 nm [11]. The protein is heat-modifiable with an apparent M_r of ~36 kDa for the monomer. Porin from *R. sphaeroides* in contrast to matrix porin can be purified in monomeric form [10]. We purified porin monomers from *R. capsulatus*. It appeared interesting to see if different crystal forms or crystals of better quality could be obtained from these monomeric porin solutions as compared to the trimeric porin solutions of matrix porin. As we will report here, we obtained a crystal form similar to the hexagonal one reported by Garavito et al. [6].

2. MATERIALS AND METHODS

2.1. *Miscellaneous methods*

Protein was assayed using the Lowry method [12]. SDS polyacrylamide gel electrophoresis was performed according to Laemmli [13].

2.2. *Source and cultivation of bacteria*

R. capsulatus 37b4 was obtained from the Institut für Biologie II, Mikrobiologie der Universität Freiburg i.Br., FRG. Mass cultures were grown chemotrophically at 32°C in R8ÄH medium [14] containing 0.3% yeast extract. Cultures were also grown phototrophically in the same medium at a light intensity of ~2000 lux.

2.3. *Isolation of porin from cell envelopes*

Freshly harvested cells were disrupted mechanically by means of a cooled (4°C) vibrogen shaker (Type Vi 2, E. Bühler, Tübingen, FRG) for 15 min at full speed with glass beads at a weight per weight ratio of 1:2. The cell homogenate was passed through a glass filter (Type 6-1, Schott, Mainz, FRG) to remove the glass beads. Whole cells were separated by centrifugation at low speed (2000 × *g*, 10 min). Cell envelopes were obtained from the supernatant at 63000 × *g* for 30 min. They were washed three times with Tris-Cl buffer (20 mM Tris-Cl, pH 8.0). Porin was obtained from cell envelopes by differential extraction in the presence of SDS. The cell envelopes were first extracted in a buffer (pH 8.0) containing 2% SDS, 10% glycerol, 20 mM Tris-Cl at 50°C for 30 min. The pellet of the following centrifugation (113000 × *g*, 20°C, 1 h) was resuspended in the same buffer (plus 0.5 M NaCl) at 37°C for 30 min. After centrifugation (113000 × *g*, 20°C, 1 h) the supernatant was enriched with the porin, which was further purified by gel filtration.

Porin solutions in 2% (w/v) SDS were dialysed against bidistilled water with 3 mM sodium azide at room temperature. The dialysis buffer was changed twice each day. After each dialysis period the dialysis buffer was tested for its SDS content using the assay of Hayashi [15].

Porin solutions were concentrated to 2% of their original volume with an Amicon ultrafiltration cell, equipped with a YM10 membrane. The protein solution was then dialysed against 20 mM Tris-Cl, pH 7.2, 300 mM LiCl, 1 mM sodium azide, 5 mM EDTA, 0.6% (w/v) octyltetraoxyethylene (C8E4) (Bachem, Bubendorf, Switzerland) twice for 20 h at 4°C. The protein was again concentrated to 50% of its volume and centrifuged with a Beckmann Airfuge for 1 h. Finally the protein was purified by gel filtration chromatography with Sephacryl S200 (Pharmacia Fine Chemicals, Freiburg i.Br., FRG). The column had a diameter of 10 mm and a gel bed volume of 32 ml. The porin fractions were collected and concentrated to a concentration of 10 mg/ml by ultrafiltration as described above.

2.4. *X-ray diffraction*

Crystals were mounted in 1 mm quartz capillaries (Müller, FRG) which were sealed. Photographs were taken on a precession camera (reciprocal lattice explorer, STOE, Darmstadt, FRG), set at 125 mm crystal-to-film distance. The source of Cu K_α X-rays was a rotating anode generator (Siemens AG, FRG). The crystals were maintained at 11°C using a flow of cold air.

3. RESULTS AND DISCUSSION

The polypeptide pattern of the purified protein shows one band at an apparent molecular mass of 36 kDa (fig.1, lane 2) typical for porin monomers [9,10].

Crystals were obtained by the vapor diffusion technique. A porin solution of 6.5 mg protein/ml containing 12% (w/v) polyethyleneglycol (PEG) 600 (Merck, Darmstadt, FRG) was made by mixing with appropriate amounts of a 70% (w/v) PEG stock solution. The latter solution was made by dissolving PEG in the above-mentioned dialysis buffer. The presaturated protein solution was equilibrated with a 21% (w/v) PEG 600 reservoir solution made with the aforementioned buffer. Rhombohedral crystals with an edge length of 0.4 mm grew within 5 days. The crystals were birefringent.

Crystals were collected and washed with the reservoir solution three times by centrifugation in a bench-top centrifuge. The pelleted crystals were dissolved in water and dialysed against water for 20 h. The dialysed protein as well as the supernatant after the last centrifugation of the crystals were subjected to SDS polyacrylamide gel electrophoresis. In fig.1 the supernatant was applied to the gel (lane 4). The absence of stainable material shows that the crystals were washed free of dissolved proteins. As seen in lane 3, the dissolved crystals showed one band at 36 kDa, there was no band at 68 kDa. The porin crystals, thus, dissolved to porin monomers [9,10].

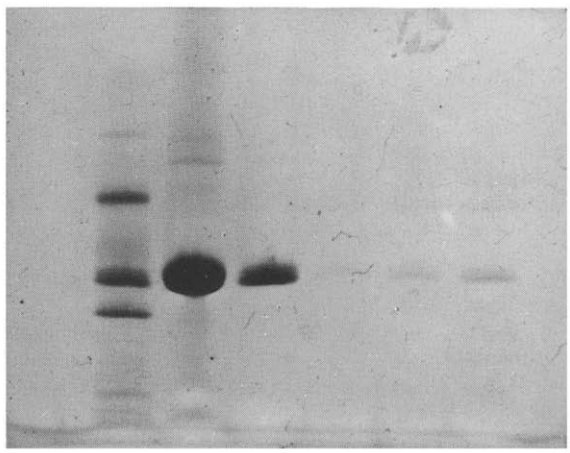

Fig.1. SDS-polyacrylamide gel of porin crystals. The crystals were washed three times as described in section 2. (a) Marker proteins with molecular masses of 29, 45 and 68 kDa. (b) The monomeric porin solution which was used for crystallization. (c) Porin crystals. One band is seen at an apparent molecular mass of approximately 36 kDa. (f,e,d) The three successive supernatants obtained in the process of washing the crystals.

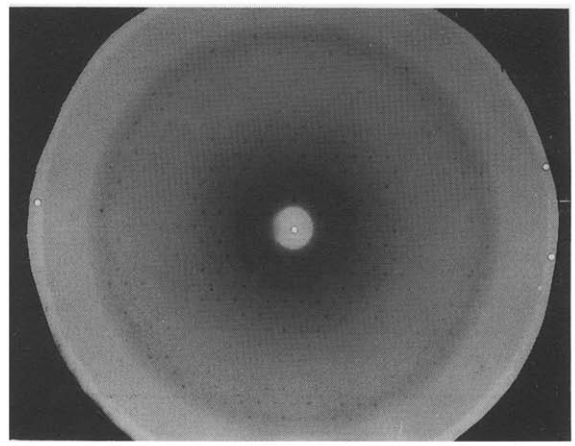

Fig.2. Precession photograph of the *hk*O plane of the crystals.

Precession photographs allowed us to establish the space group as R3 which can be described by hexagonal or by rhombohedral axes. The lattice constants are $a = b = 95$ Å, $c = 147$ Å and $a = 73$ Å ($\alpha = 82°$), respectively.

A precession photograph of the *hk*O plane ($\mu = 11°$) is shown in fig.2. A 6-fold symmetry, but no mm symmetry is seen, which excludes space group R32. Threefold symmetry is observed on higher levels. The *hOl* plane shows systematic absences which are consistent with the chosen rhombohedral unit cell.

In still photographs, reflexions were observed to 3.2 Å. Beyond 3.5 Å, however, only few reflexions are observed. The crystals were stable in the X-ray beam up to ~50 h.

Assuming one porin molecule of 33 kDa per asymmetric unit, a density of the crystal of V_m of 3.9 Å^3/dalton can be calculated. This value is similar to the values reported for matrix porin crystals of *E. coli*, especially to the hexagonal crystal form, which has a density of 3.76 Å^3/dalton [6]. The three-fold symmetry axis of the space group indicates, that porin of *R. capsulatus* 37b4 forms trimers in the crystal. The close similarity of the lattice constants to those found in 2-dimensional crystalline sheets of matrix porin [16] ($a = b = 92$ Å), also indicates that the R3 crystal form consists of hexagonal sheets, which are stacked on top of each other to form a cubic closest packing of trimers. This indicates that the porin from *R. capsulatus* in the outer membrane forms similar trimers as matrix porin from *E. coli*. These data are of particular interest, as for porins from both *R. capsulatus* and *R. sphaeroides* a dimeric structure was discussed and proven for the chemotrophic *Paracoccus denitrificans* by cross-linking studies [17]. *Paracoccus denitrificans* is closely related to *R. capsulatus* and *R. sphaeroides* as judged from 16S-RNA sequence analyses [18].

Similar work is in progress with porin from *R. sphaeroides, Thiobacillus versutus* and from *Paracoccus denitrificans*.

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