Expression of porin from *Rhodopseudomonas blastica* in *Escherichia coli* inclusion bodies and folding into exact native structure

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Abstract The homotrimeric membrane channel porin from *Rhodopseudomonas blastica* was expressed without signal sequence in *Escherichia coli*. The protein assembled in inclusion bodies in the cytosol, from which it could be recovered using urea and detergents. After purification by anion-exchange chromatography, the protein crystallized under wild-type conditions. The X-ray structure was determined at 2.2 Å resolution, and a comparison with the known wild-type structure showed that the recombinant porin is identical at the atomic level. The method yields porin and designed mutants thereof in 100 mg amounts, allowing for detailed functional and mechanistic studies.

Key words: Porin; Membrane channel; Inclusion bodies; Naturation; X-Ray analysis; *Rhodopseudomonas blastica*

1. Introduction

Porins are water-filled channels spanning the outer membrane of Gram-negative bacteria. They are permeable for small polar solutes with exclusion limits around 600 Da, but exclude nonpolar molecules of comparable sizes. Usually, porins are found as homotrimeric proteins with molecular masses ranging from 30 to 50 kDa per subunit. They are particularly stable toward heat, detergents, and proteases [1–4]. A structure analysis of the major porin from *Rhodobacter capsulatus* showed that the pore is formed by a 16-stranded β-barrel [5]. As shown in Fig. 1, the same applies for the major porin from *Rhodopseudomonas blastica* reported here [6,7] as well as for porins OmpF and PhoE from *E. coli* [8].

For translocation through the inner membrane, porins require an N-terminal signal sequence which is about 15–25 amino acids in length [9]. Since the expression of porin genes with signal sequences caused lysis of the host cells, several groups omitted them and obtained reasonable expression levels [10–12]. After a number of failures using signal sequences, we did the same and deleted this sequence in a heterologous expression of porin from *R. blastica* in *E. coli*. The expressed protein was naturated, purified and crystallized. A structure determination and comparison with the known wild-type structure followed. The resulting efficient production system for native-like recombinant porin allows for mutational studies intended to clarify the mechanism of diffusion through the channel.

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Abbreviations: C_8E_4, *n*-octyltetraoxyethylene; LDAO, *N,N*-dimethyldodecyl-amine-*N*-oxide; PAGE, polyacrylamide gel electrophoresis.

2. Materials and methods

2.1. Subcloning into an expression vector

The gene for the *R. blastica* porin was taken by PCR from plasmid pBluescript-II-KS-por [6,13]. The selected primers were 5'GGAATTGCATATGATCAGTCTGAACGGCTATGG with an *Nde*I site (including start codon) and 5'GGGGATCCTTCGTTCCGGTCTGTCTG with a *Bam*HI site. The reaction was carried out with Recombinant *Pfu* DNA Polymerase (Stratagene) in an OmniGene HB-TR3-CM thermocycler (Hybaid).

The PCR product was purified and concentrated using a PCR purification kit (Qiagen). It was digested with restriction endonucleases *Nde*I and *Bam*HI (New England Biolabs). A corresponding digestion was applied to plasmid pET-3b (Novagen). Both fragments were run on an 1% agarose gel and isolated using a gel extraction kit (Qiagen). The fragments were then ligated by a standard T4 ligase procedure (Boehringer-Mannheim) to obtain plasmid pET-3b-por. After transformation in *E. coli* XL1-Blue (Stratagene), a plasmid preparation (Qiagen) with subsequent *Nde*I, *Bam*HI digest showed the correct fragment lengths.

2.2. Protein expression and isolation of inclusion bodies

About 100 ng pET-3b-por DNA were transformed in *E. coli* BL21(DE3)pLysS (Novagen), and a single colony was inoculated into 10 ml SB medium (20 g/l casein hydrolysate and 10 g/l yeast extract both from Gibco BRL, 5 g/l NaCl, 2.5 g/l K_2HPO_4, 1 g/l $MgSO_4 \cdot 7H_2O$, pH 7.0) containing ampicillin (100 mg/l) and chloramphenicol (50 mg/l). After incubation for 8 h at 37°C, the culture was transferred to four 1 litre flasks, each containing 250 ml of the same medium. Incubation was continued in a shaking incubator at 37°C until an optical density of 0.8 at 578 nm was reached. At this point, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After shaking for 6 more hours, cells were harvested by centrifugation (Sorvall GS-3, 10,800 × *g*, 15 min), resuspended in 3 ml of TEN buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl) per g of cells (wet weight), and then frozen overnight.

After thawing, benzonase (50 U; Merck) was added and the suspension was stirred at room temperature for 30 min. The cells were broken by 20 min sonication (model 7100, Measuring and Scientific Equipment) and the inclusion bodies were separated by centrifugation (SS-34; 4300 × *g*, 1 h). The pellet was resuspended in 60 ml of Triton buffer (2% (v/v) Triton X-100, 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 mM NaCl) and incubated at 37°C overnight under shaking. Triton was removed by centrifugation (SS-34; 4300 × *g*, 20 min), resuspension in 60 ml of TEN buffer (2 h shaking at 37°C), and a further centrifugation (SS-34; 4300 × *g*, 20 min), yielding a white pellet.

2.3. Naturation and purification

The white pellet was resuspended in 60 ml of freshly made Urea buffer (8 M urea in TEN buffer), incubated for 2 h at 37°C under mild shaking, and centrifugated (SS-34, 48,000 × *g*, 20 min). The supernatant was expanded to 125 ml using Urea buffer, further diluted with 125 ml of LDAO-10 buffer (10% (w/v) *N,N*-dimethyldodecyl-amine-*N*-oxide (LDAO) in TEN buffer), and sonicated for 30 min (Sonorex, Bandelin). The resulting solution was loaded with a flow rate of 60 ml/h onto a Q-Sepharose Fast Flow column (2.0 × 11.5 cm; Pharmacia), which had been equilibrated with LDAO-0.2 buffer (0.2% (w/v) LDAO, 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 3 mM NaN_3). After washing with LDAO-0.2 buffer, the column was equilibrated with

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Fig. 1. Stereoview of homotrimeric porin from *R. blastica* with β-strands and a short α-helix depicted as ribbons. The view is from the external medium at about 30° away from the molecular 3-fold axis, which is perpendicular to the membrane plane. Each chain consists of 289 amino acid residues forming a 16-stranded antiparallel (all next-neighbor) β-pleated sheet [6,7]. The N- and C-termini are labelled, not all of them are directly visible.

C_8E_4 buffer (0.6% (w/v) *n*-octyltetraoxyethylene (C_8E_4), 20 mM Tris-HCl pH 7.2, 100 mM LiCl, 3 mM NaN_3).

The protein was subsequently eluted with a 100–600 mM LiCl gradient in C_8E_4 buffer. Fractions of 2 ml were collected, analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and pooled. The pooled fractions were concentrated (Centriprep-30, Amicon) and dialyzed against C_8E_4 buffer containing 300 mM LiCl. The enzyme concentration was determined photometrically using $\varepsilon_{280} = 47{,}080$ $M^{-1} \cdot cm^{-1}$ as derived from the sequence [14].

2.4. Crystallization and structure analysis

The crystallization procedure followed Kreusch et al. [7]. Crystals appeared after 2 days and grew within 1–2 weeks to sizes of about (500 μm^3). They belong to space group R3 with $a_{hex} = b_{hex} = 104.0$ Å, $c_{hex} = 124.6$ Å like the wild-type and diffract to high resolution. Data were collected on a rotating anode X-ray generator (model RU200B, Rigaku) using a multi-wire area detector (model X1000, Siemens). The data were processed with program XDS [15]. The structure was refined using program X-PLOR [16].

3. Results and discussion

3.1. Selection of expression system

We made several attempts to express the *R. blastica* porin in *E. coli* and to isolate it from the outer membrane as described [6]. In all studied plasmid/gene combinations we used the signal sequence from *R. blastica*. In addition, we tested a construct carrying the pelB signal sequence [17] (pET-22b, Novagen). However, all experiments resulted in premature lysis of the host cells yielding insufficient amounts of recombinant porin. Therefore, we dispensed with the signal sequence and expressed into inclusion bodies [10–12] using plasmid pET-3b [18] with the strong T7 promoter. One of the PCR primers was designed such that the N-terminal glutamate was replaced by a methionine for starting gene translation. Adding a methionine before Glu1 was ruled out because the amino

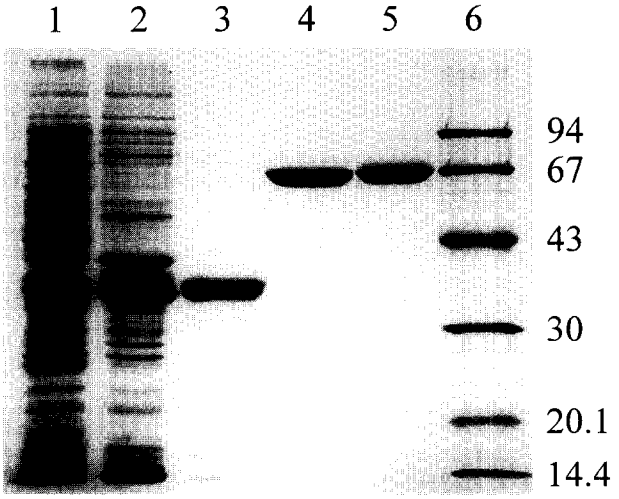

Fig. 2. SDS-PAGE of porin from *R. blastica* (lane 1), cell lysate (lane 2), inclusion bodies resuspended in Urea buffer (lane 3), porin fractions pooled, concentrated and dialyzed after anion-exchange chromatography (heated) (lane 4), same as lane 3, but not heated (lane 5), wild-type porin from *R. blastica* (not heated) (lane 6), protein size markers in kDa.

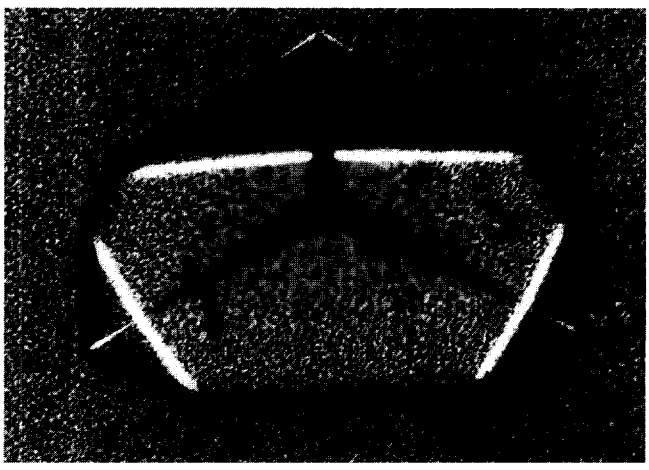

Fig. 3. Crystal of recombinant porin from *Rhodopseudomonas blastica*. The space group is R3, the size is about 600×500×400 μm^3.

Table 1
Refinement statistics of the crystal structures of recombinant and wild-type porin from *R. blastica*

	Recombinant	Wild-type
Resolution (Å)	1000–2.2	10–1.96
Crystallographic *R*-factor (%)	21.9	17.6
Free *R*-factor (%)	27.7	20.4
Number of reflections	21,269	35,620
Completeness (%)	81.5	97.8
Number of non-hydrogen atoms		
Protein atoms	2163	2164
Water molecules	122	247
C_8E_4-detergent atoms	0	63
RMS deviations		
Bond lengths (Å)	0.009	0.011
Bond angles (°)	1.5	1.8
Average temperature factors		
All atoms (Å^2)	23	28
Main chain atoms (Å^2)	21	23

group of Glu1 forms a structurally important salt bridge with C-terminal Phe-289′ of a neighboring subunit [7].

3.2. Subcloning of PCR product

The restriction sites *Nde*I and *Bam*HI were introduced as 5′-tags into the PCR primers. After digestion of both the PCR product and the vector as well as subsequent ligation, the resulting construct pET-3b-por was transformed into *E. coli* BL21(DE3)pLysS [18] harboring plasmid pLysS that reduces the T7 RNA polymerase background activity. Transformants were selected for ampicillin and chloramphenicol resistance. All analyzed clones expressed appreciable amounts of porin as detected by SDS PAGE (Fig. 2), and there occurred no cell lysis. After running a cell lysate on an SDS-PAGE and blotting it onto a polyvinylidene-difluoride (PVDF) membrane (Applied Biosystems), the recombinant porin band (identified by a wild-type standard) was cut out and sequenced. The sequence of the first 10 residues was NH_2-Met-Ile-Ser-Leu-Asn-Gly-Tyr-Gly-Arg-Phe-, which verified the protein identity as well as the Glu1 → Met substitution.

3.3. Naturation, purification and crystallization

Fermentation of *E. coli* BL21(DE3)pLysS harboring plasmid pET-3b-por produced inclusion bodies that contained porin with some contaminating host cell proteins. The inclusion bodies were solubilized with 8 M urea. Subsequent dilu-

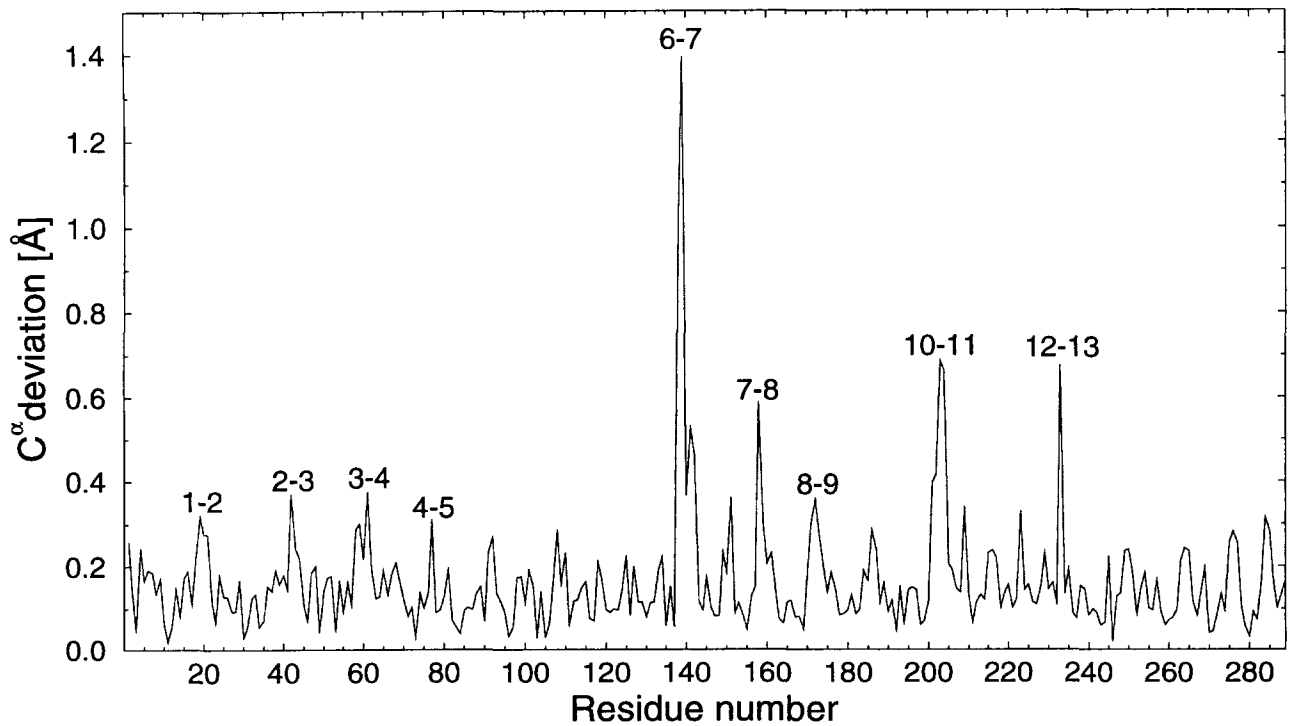

Fig. 4. Distribution of C^α deviations after best superposition of all C^α positions of recombinant and wild-type porin from *R. blastica*. The labels refer to loops between β-strands, for instance 1–2 is the loop between strands β1 and β2. Since the highest deviations occur at the weakest points of the wild-type structure [7], recombinant and wild-type porin are virtually identical (except for the exchange Glu1 → Met).

tion with LDAO-10 buffer probably initiated chain folding by insertion into detergent micelles. Interestingly enough, the yield of naturated porin depended on the inclusion body concentration in the urea/LDAO-10 mixture in agreement with Qi et al. [11].

For purification and also for removal of urea we used a Q-Sepharose column. A large fraction of the porin passed this column without binding, presumably because it was not correctly folded. For the porin bound at the column the detergent was then exchanged from LDAO to C_8E_4. The column was eluted with a 100–600 mM LiCl gradient where porin ran at 300 mM. Fractions containing pure porin were pooled, concentrated, and dialyzed. Typical yields were 20–25 mg protein/liter cell culture. The obtained protein was pure enough for crystallization. Crystals grew within 1–2 weeks to sizes suitable for X-ray structure analysis (Fig. 3). Their sizes corresponded to those of wild-type porin.

3.4. Structure analysis and comparison

The recombinant porin crystals diffracted to the same resolution as the wild-type. X-ray data were collected from one crystal and processed (Table 1). Because of isomorphism with wild-type crystals, we first calculated an $(F_{\mathrm{recomb,obs}}-F_{\mathrm{wt,calc}})\exp(i\alpha_{\mathrm{wt,calc}})$-difference Fourier map, which contained only few regions with density, among them density reporting the Glu1 → Met exchange. After modeling this exchange, the structure was refined using X-PLOR, which resulted in a model closely similar to the wild-type structure [7]. The root mean squares deviation of the C^{α} atoms after best superposition was 0.20 Å, which is within the limits of error. The distribution of these deviations is shown in Fig. 4. It has maxima at the loop positions where the wild-type model is of lower accuracy as indicated by low density correlation and high crystallographic temperature factors [7]. Accordingly, the quality of the recombinant porin structure corresponds to that of the wild-type.

3.5. Conclusion

Cytosolic expression of *R. blastica* porin in *E. coli* and successful folding into native trimers from inclusion bodies could be demonstrated. An X-ray structure analysis provided clear prove for the identity of recombinant and wild-type porin at the atomic level (except for exchange Glu1 → Met). Given the large production rates, recombinant porin and mutants thereof can now be used for studying the pore diffusion mechanism.

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