Strategy for Membrane Protein Crystallization Exemplified With OmpA and OmpX

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ABSTRACT **The bacterial outer membrane proteins OmpA and OmpX were modified in such a manner that they yielded bulky crystals diffracting X-rays isotropically beyond 2 Å resolution and permitting detailed structural analyses. The procedure involved semi-directed mutagenesis, mass production into inclusion bodies, and (re)naturation therefrom; it should be applicable for a broader range of membrane proteins. Proteins 1999;34:167–172.**

Key words: crystallization; inclusion bodies; integral membrane proteins; OmpA; OmpX; (re)naturation

INTRODUCTION

In contrast to their water-soluble counterparts, membrane proteins seem to dislike crystallization, which gave rise to a broad discussion with even political implications.[1–4] Here we report the crystallization of two of these membrane proteins, the results of which suggest a novel approach for all of them. The strategy involves restrained mutagenesis at the polar surfaces (Fig. 1), which are often known from preceding biophysicochemical and molecular biological experiments, combined with mass production into inclusion bodies and (re)naturation therefrom.

The outer membrane protein A (OmpA)from *Escherichia coli*[5] is important for bacterial conjugation,[6] for maintaining structural stability,[7] and it functions as a receptor for bacteriophages[8] and colicines.[9] Moreover, it plays a role in neonatal meningitis caused by certain bacteria.[10] OmpA consists of 325 residues.[11] The N-terminal domain (OmpA171, residues 1 to 171) is located within the membrane, whereas the C-terminal domain is in the periplasm.[12] A model has been proposed suggesting that the membrane domain OmpA171 consists of an 8-stranded antiparallel β-barrel.[13] The model is supported by biochemical,[8] biophysical,[13,14] and molecular biological[15,16] data, but contradicted by a recent porin-like model proposing a 16-stranded β-barrel for the whole protein.[17] Small OmpA crystals have been reported some time ago.[18]

The outer membrane protein X (OmpX) from *E. coli* is homologous to membrane proteins of pathogenic bacteria like PagC of *Salmonella typhimurium* and Ail of *Yersinia enterocolitica*.[19,20] These proteins mediate the adherence of bacteria to mammalian cells and protect against the immune system. Models for OmpX suggest an 8-stranded antiparallel β-barrel.[21,22] OmpX consists of 148 residues and is presently the smallest reported outer membrane protein.[23]

MATERIALS AND METHODS

Conventional Protein Production

An *ompa*⁻ mutant of *E. coli* UH300[24] harboring plasmid pRD87/171 with the gene of OmpA171[15] was cultivated in 9 L LB-medium with 0.5% (w/v) glucose, 100 µg/mL ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 10 h at 37°C pH 7.0 to an OD_{578} of 1.4. The harvested cells (about 60 g) were suspended in 150 mL buffer-P (20 mM Tris-HCl pH 8.5, used in all following steps), lyzed by sonication, centrifuged (70 min, 48,000*g), pre-extracted at 38°C with 2% (w/v) Triton X-100 (Fluka, Buchs, Switzerland) and with 2% (w/v) Genapol X-80 (Fluka), and subsequently at 4°C with 0.2% (w/v) LDAO (Fluka). The protein OmpA171 was then extracted at 4°C from the remainder using 0.5% (w/v) LDAO. Supernatants were pooled, concentrated, dialyzed against 0.08% (w/v) LDAO, and loaded onto a 6 mL Resource-Q ion exchange column (Pharmacia, Uppsala, Sweden). The detergent was exchanged by washing with 60 mL 0.5% (w/v) n-octyltetraoxyethylene (C_8E_4, Bachem, Heidelberg, Germany). The protein was then eluted with a 0–200 mM NaCl gradient and further purified on a Superdex-200 gel permeation column (Pharmacia).

Some of the ion exchange fractions (data not shown) contained an additional band at ${}^{app}M_r$ 16,300. These fractions were separated and identified as OmpX by N-terminal sequencing and electrospray mass spectrometry.

Overexpression Vectors and Mutagenesis

The gene of OmpA171 was extracted *via* PCR (*Pfu* polymerase, Stratagene, Heidelberg, Germany) from plasmid pRD87/171.[15] The upstream primer 5'-GGCATCCCATATGGCTCCGAAAGATAACACCTG-3' replaced the signal sequence by a start codon (underlined) and had an *Nde*I restriction site, whereas the downstream primer 5'-CGGGATCCTCAACCAAAACGGTAGGAAACACC-3'

Abbreviations: C_8E_4, *n*-octyltetraoxyethylene; C_8POE, *n*-octylpolyoxyethylene; IPTG, isopropyl-β-D-thiogalactopyranoside; OmpA, outer membrane protein A from *Escherichia coli*; OmpA171, residues 1 through 171 of OmpA forming the putative membrane domain; OmpA171d, double point mutant of OmpA171; OmpA171t, triple point mutant of OmpA171; OmpX, outer membrane protein X from *E. coli*; OmpXs, single point mutant of OmpX.

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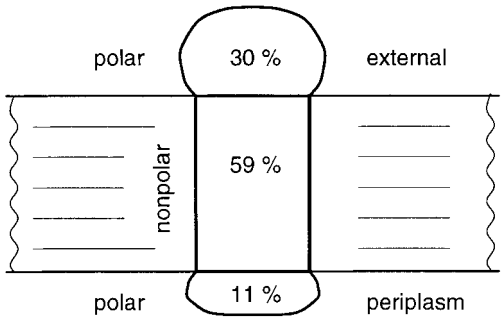

Fig. 1. Sketch of the shape of the OmpA171 model proposing an 8-stranded β-barrel with loops extending to both sides of the membrane.[13] The membrane is indicated, the percentages give the predicted masses in the three regions.

had a *Bam*HI site. Using these sites, the gene was ligated into the production vector pET3b (Novagene) to give pET3b-OmpA171.

The gene of OmpX was amplified from genomic DNA of *E. coli* K12 *via* PCR with *Pfu* polymerase. The upstream primer 5'-CCCGGAATTCCATATGGCGACTTCTACTGTA-ACTGG-3' replaced the signal sequence by a start codon (underlined) and contained *Eco*RI and *Nde*I sites for cloning into pET3b (to give pET3b-OmpX) or into M13mp19. The downstream primer 5'-ACGGGATCCT-TAGAAGCGGTAACCAACAC-3' had a *Bam*HI site.

For mutagenesis the gene of OmpA171 was taken out of pET3b-171 with *Xba*I and *Bam*HI and cloned into vector M13mp18 (Boehringer-Mannheim, Mannheim, Germany). The gene of OmpX was introduced into M13mp19 (Boehringer-Mannheim) using *Eco*RI and *Bam*HI. The mutations were performed with the phosphorothioate method (Amersham, Braunschweig, Germany).[25] DNA was sequenced with a blotter (GATC-1500) using Thermo Sequenase (Amersham).

Protein Mass Production Into Inclusion Bodies

E. coli BL21(DE3) harboring either pET3b-OmpA171 or pET3b-OmpX was grown at 37°C in 250 mL LB-medium with 100 µg/mL ampicillin, induced at OD_{578} 0.8 with 1 mM IPTG, further cultivated for 5 h and harvested by centrifugation. The pellet was suspended in 30 mL buffer-P and sonicated at 4°C. All further handling was in buffer-P. The produced inclusion bodies were separated by centrifugation (90 min, 4,300*g), homogenized in 2% (w/v) Triton X-100 (Fluka), centrifuged again, dissolved in 30 mL 6 M guanidinium-HCl, incubated for 2 h at 37°C (200 rpm, Centromat, Braun, Melsungen, Germany), and diluted into 150 mL 5% (w/v) n-octylpolyoxyetheylene (C_8POE, Bachem) with 0.6 M L-arginine. This solution was 2 times dialyzed for 24 h against 1 L 1% (w/v) C_8POE, loaded on a 70 mL Source-Q ion exchange column (Pharmacia) equilibrated with 0.6% (w/v) C_8E_4 and washed with 140 mL of the same buffer. For elution we used a 0–150 mM NaCl gradient.

OmpX was expressed and purified like OmpA171. It ran through the Source-Q column and was therefore concentrated from the flow-through. As checked by SDS-PAGE and electrospray mass spectrometry, the resulting OmpX was pure.

Crystallization and X-ray Data Collection

For crystallization, the OmpA171 and OmpX solutions were concentrated to about 40 mg/mL and dialyzed against 0.6% (w/v) C_8E_4 in water. Crystals were obtained at 20°C with the hanging drop method after diluting to 20 mg/mL, mixing in a 1:1 ratio with the precipitant and mounting as 5 µL drops above the reservoir. Initial conditions were established with the usual screening methods (Hampton Research, Laguna Hills, CA).

Diffraction data were collected using a wire detector (Siemens, Karlsruhe, Germany, X1000) or an image plate (MARresearch) with rotating anode generators (Rigaku, Tokyo, Japan, RU200B and RU2HC). The data were processed with programs XDS[26] or MOSFLM.[27] Synchrotron data were collected at beamline X11 at EMBL/DESY-Hamburg. These data were processed with programs DENZO[28] or MOSFLM[27] and SCALA.[29]

RESULTS

Conventional Crystals

As previous attempts to produce X-ray-grade crystals of the whole OmpA protein had failed in our and in other[18] hands, we decided to produce the putative membrane domain OmpA171 (residues 1 through 171) by export into the outer membrane. OmpA171 was isolated from the outer membrane, purified and crystallized (Table I). The crystals were extremely thin, diffracted anisotropically with high mosaicity to about 3 Å resolution and could not be reproduced readily. Lack of suitable crystals prevented a structure analysis. Similar results were obtained with OmpX (Table II). The crystals were also extremely thin, diffracted to medium resolution with high mosaicity, and could not be reproduced.

Large Scale Production Into Inclusion Bodies

We then turned to mass production of OmpA171 and OmpX into inclusion bodies. For this purpose, the signal sequences were removed and the genes were inserted into the high yield expression vector pET3b. As shown for OmpA171 in Figure 2, the expressed protein went mostly into inclusion bodies, from which it was (re)natured and purified as has been exemplified earlier with a porin.[30] This type of (re)naturation differs appreciably from previous experiments that produced OmpA in the outer membrane, extracted it therefrom, denatured it in urea and renatured it.[31] The OmpA171 yield (150 mg per liter culture) was 20 times the yield for the conventional isolation from the outer membrane. At the end, however, the yield was only 10 times higher because we used only the purest chromatographic fractions. The expression and purification of OmpX showed similar results.

Because outer membrane proteins are very stable towards unfolding in SDS-containing buffers, successful (re)naturation could be monitored by SDS-PAGE (Fig. 2). OmpA samples which have been boiled for 10 min in SDS,

TABLE I. Crystal Statistics for OmpA Derivatives

	OmpA171	OmpA171d	OmpA171t
Mutation	—	F23L/Q34K	F23L/Q34K/K107Y
Crystal thickness (μm)	5*	5‡	≥100†
Space group	$P2_12_12$	$P2_12_12$	$C2$
Unit cell (Å)			
a	52.9	53.9	70.0
b	57.2	57.0	78.0
c	58.1	121.2	50.8 (β = 91.7°)
Number of molecules in the asymmetric unit	1	2	1
Packing, V_M (Å^3/Da)¶	2.3	2.5	3.7
Diffraction limit (Å)	3.2	2.9	≤2.0#
X-ray source	Cuk$_\alpha$	X11	Cu K$_\alpha$
Data set limit (Å)	—	3.2	2.9
Observations	—	5758	27268
Unique reflections	—	1721	5770
Completeness (%)	—	96	95
R_{sym} (%)§	—	8.1	4.8

*Crystals grew with 1.4 M ammonium sulfate, 3.8% (v/v) 2-propanol, 0.5% (w/v) C_8E_4, 1.8% (w/v) hexyldimethylaminoxide, 0.1 M ammonium acetate pH 4.6 as precipitant.

‡Crystals grew with 15% (w/v) PEG-400, 15% (w/v) glycerol, 0.2 M $CaCl_2$, 0.1 M HEPES pH 7.1 as precipitant. The data set was collected after directly cooling the crystals to 100 K.

†Crystals grew with 12% (w/v) PEG-8000, 10% (v/v) methylpentanediol and 25 mM KH_2PO_4 pH 5.1 as precipitant. The crystals could be cooled to 100 K after adding further 15% (v/v) methylpentanediol. The data set refers to room temperature.

¶The crystals of OmpA171d have the same habit as those of OmpA171 and differ merely by a factor of two in their c-axes, indicating that they follow the same packing scheme. Since OmpA171 must have one molecule in the asymmetric unit, OmpA171d has two of them. The V_M value of OmpA171t crystals would be extremely low for two molecules in the asymmetric unit. Most likely there is only one molecule bringing V_M in the usual range for membrane proteins.

#Determined at 100 K using beamline ID13 at the ESRF/Grenoble.

§$R_{sym} = \Sigma_h\Sigma_i|I_{hi} - \langle I_h\rangle|/\Sigma_h\Sigma_i I_{hi}$ where h stands for the unique reflections and i counts through symmetry related reflections.

TABLE II. Crystal Statistics for OmpX

	OmpX	OmpXs
Mutation	—	H100N
Crystal thickness (μm)	5*	≥100‡
Space group	$P2_12_12$	R32
Unit cell (Å)		
a	25.4	82.6
b	64.1	82.6
c	88.3	206.9 (γ = 120°)
Number of molecules in the asymmetric unit	1	1
Packing, V_M (Å^3/Da)†	2.2	4.1
Diffraction limit (Å)	3.3	≤2.0¶
X-ray source	Cu K$_\alpha$	Cu K$_\alpha$
Data set limit (Å)	3.4	2.7
Observations	23450	57127
Unique reflections	2207	7248
Completeness (%)	99	94
R_{sym} (%)#	17.4	6.7

*Crystals grew with 58% (v/v) methylpentanediol and 0.1 M acetate pH 4.6 as precipitant. All data refer to room temperature.

‡Crystals grew with 30% (v/v) 2-propanol, 20% (v/v) glycerol, 0.2 M $CaCl_2$, 0.1 M acetate pH 4.6 as precipitant. The data set was collected after cooling the crystals directly to 100 K.

†The crystal of OmpX must have one molecule in the asymmetric unit, whereas the crystals of OmpXs could have two per asymmetric unit with an exceptionally low solvent content. Most likely, however, OmpXs contains one molecule in the asymmetric unit with a V_M value that is common for membrane proteins.

¶Determined at 100 K using the beamline X11 of the EMBL outstation at DESY/Hamburg.

#See Table I.

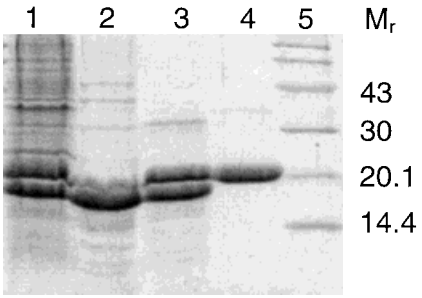

Fig. 2. SDS-PAGE for OmpA171t mass production. By boiling and not boiling in SDS, (re)natured OmpA171t could be distinguished from unfolded forms. Lane 1, cell extract; lane 2, inclusion bodies; lane 3, purified OmpA171t after (re)naturation boiled for 10 min in 3% SDS; lane 4, same as lane 3 but not boiled; lane 5, mass markers.

migrate with an $^{app}M_r$ different from that of unboiled samples.[32] The same is true for the membrane domain OmpA171[15] which migrates at an $^{app}M_r$ of 18,500 when boiled and at 22,000 when not. Accordingly, the band at 22,000 corresponds to (re)natured OmpA171. OmpX behaved similarly.

Mutant Crystals

Using electrospray mass spectroscopy the M_r of OmpA171 was determined as 18843 ± 3, which was 32 Da lower than expected and prompted us to resequence the DNA. This revealed the two mutations F23L and Q34K presumably introduced by PCR. The expected M_r of the double mutant is 18,841 and agrees with the observed value. Consequently, the protein was renamed OmpA171d. OmpA171d formed very thin crystals with a packing that was a distorted version of the previous crystals, and showed similarly unfavorable diffraction properties. Still, a data set could be collected (Table I).

The OmpA171d mutations were at the borderline between the membrane and the external region of the model[13] (Fig. 1). Experiencing already two mutations, we added third ones in the external region. First, we tried to combine the modifications with the introduction of cysteines for a structure analysis by multiple isomorphous replacement and produced mutants T21C, M61C, T88C, T117C, and S120C in the predicted β-strand connections

L1, L2, T2, L3, and L3, respectively. None of them improved the crystal quality.

Then we abandoned the cysteine concept and removed the charged and presumably mobile Lys107 in the predicted β-strand connection L3 by an aspartate for relaxing a conceivable charge repulsion, but observed no improvement. Furthermore, we exchanged Lys107 for a tyrosine in order to introduce a less mobile, more bulky, but still adhesive side chain into this position. This semi-directed second attempt resulted in a triple mutant (OmpA171t, Table I) that crystallized reproducibly with sizes of about $1{,}000 \times 200 \times 100\ \mu m^3$, or more rarely as cubes with edge lengths up to 500 µm (Fig. 3). These crystals showed low radiation damage at room temperature, diffracted isotropically to high resolution and could be cooled to 100 K. A data set was collected (Table I). Since the conventional crystal production failed also with OmpX, we introduced mutations into the polar regions of the OmpX model.[21,22] (Fig. 1). Again, we first followed the cysteine concept, hoping to achieve simultaneously good crystals as well as phasing opportunities. Thus we produced mutants M21C, S35C, S49C, and S134C in the predicted β-strand connections L1, T1, L2, and L4, respectively, but had no success. Then we tried more direct mutations and exchanged His100 of L3, which is charged at the crystal pH, for the uncharged shorter side chain of asparagine. This mutant formed reproducibly crystals with sizes up to $300 \times 300 \times 200\ \mu m^3$ (Fig. 4) that diffracted isotropically to high resolution (Table II). A data set was collected.

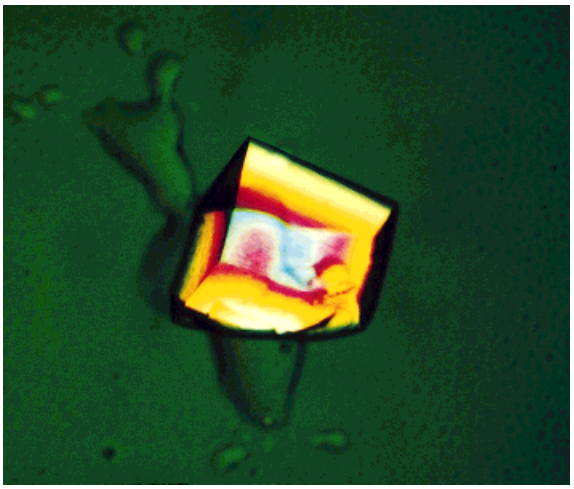

Fig. 3. Crystal of OmpA171t, the (re)natured putative membrane domain of OmpA, diffracting X-rays isotropically beyond 2 Å resolution. The edge length is 500 µm. The protein contained a semi-directed mutation.

Obviously, the crystallization problems with OmpA171 and OmpX had been solved in a perfect manner. It is remarkable that in both cases the disordered crystals had packing densities in the range commonly observed with water-soluble proteins, whereas most likely, the well-ordered crystals showed packing densities in the usual range for membrane proteins (Tables I and II).

DISCUSSION

Reviewing our experiences, we suggest that good membrane protein crystals are so rare because they require polar packing contacts, as demonstrated by all known high-resolution crystal packings of this protein type. Finding a packing with suitable polar contacts is particularly difficult, because there is only a relatively small polar surface fraction which is concentrated at two poles (Fig. 1). Such a geometry excludes a large part of all possible crystal packing schemes, diminishing the chances for

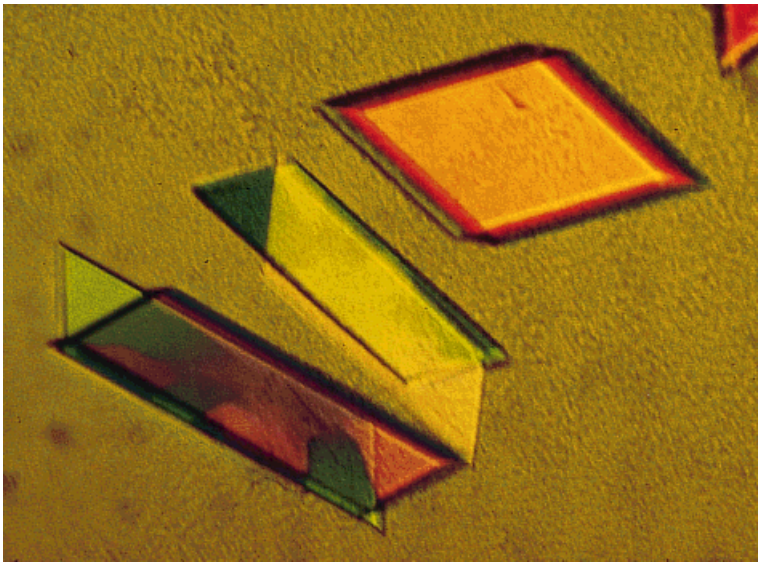

Fig. 4. Crystal of OmpXs, a semi-directed (re)natured OmpX mutant diffracting X-rays isotropically beyond 2.0 Å resolution. The edge length of the diamond is 300 µm.

crystallization. For increasing these chances, one either has to enlarge the polar surface as it has been done by attaching F_v fragments,[33] or one has to change the surface itself like we have done it here. For both OmpA171 and OmpX, the models indicated which amino acid residues are in the two polar regions at both sides of the membrane. We adopted these hypotheses and mutated in these regions in a semi-directed manner. In OmpA171, success came after exchanging a positively charged lysine for a tyrosine that is known to bind to various types of counter surfaces. In the same manner, a dramatic improvement of OmpX crystal quality came after removing a positively charged histidine. For most membrane proteins of interest, predicted models of similar quality are available and can be exploited in the same way.

A second crucial point is the production of sufficient homogeneous material for crystallization. Membrane proteins are usually inserted into the membrane of the producing organism, the storing capacity of which is very limited. An alternative path is the cytosolic mass production into inclusion bodies with subsequent (re)naturing using detergents, which avoids any lipid contamination. Somewhat unexpectedly it could be demonstrated that a membrane protein could be (re)natured to the exact conformation of the wild-type version.[30] Since crystallization of (re)natured material has now been demonstrated for three membrane proteins, we propose this as an approach to be tested for all membrane proteins. It should be noted, however, that we have sacrificed half of the produced OmpA171t and OmpXs during purification in order to achieve maximum homogeneity.

CONCLUSION

We propose mutagenesis and cytosolic mass production for the crystallization of membrane proteins. The mutations should follow the available models and concentrate on the two putative polar surfaces. We propose to start by changing charges. In our experiments only a few mutations were necessary for obtaining perfect X-ray-grade crystals of two different (only 22% sequence identity) membrane proteins, indicating that this method may be applicable for a broad range of membrane proteins.

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