Crystallization and preliminary X-ray analysis of outer membrane phospholipase A from *Escherichia coli*

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Abstract The outer membrane phospholipase A (OMPLA) of *Escherichia coli* is one of the few integral outer membrane proteins displaying enzymatic activity. It is encoded as a mature protein of 269 amino acids preceded by a signal sequence of 20 amino acids. There is no sequence homology with water-soluble lipases and phospholipases. Crystals of the mature enzyme were obtained at 22°C from 24–28% (v/v) 2-methyl-2,4-pentanediol in Bis-Tris buffer, pH 5.9–6.0, with 1 mM calcium chloride and 1.5% (w/v) β-octylglucoside. They have the symmetry of the trigonal spacegroup $P3_121$ (or $P3_221$) with cell dimensions of $a = b = 79.6$ Å and $c = 102.8$ Å ($\alpha = \beta = 90°$, $\gamma = 120°$). Native crystals diffract to a resolution of 2.6 Å.

Key words: Outer membrane phospholipase; Membrane protein crystallization; X-Ray crystallography; *E. coli*

1. Introduction

Most bacterial outer membrane proteins are involved in the transport of nutrients and ions by forming pores or receptors. The outer membrane phospholipase A (OMPLA; also designated PldA protein, after its structural gene *pldA*, or detergent-resistant phospholipase A) of *Escherichia coli* is one of the few enzymes present in the outer membrane. The exact function of OMPLA is unknown. It displays a Ca^{2+}-dependent hydrolytic activity towards both phospholipids and lipids [1–3]. High activity is induced when the outer membrane is damaged, e.g. by phage-induced lysis [4] or temperature shock [5]. It is obvious that the *E. coli* OMPLA and all other membrane phospholipases, embedded in their own substrate, require a very strict regulatory mechanism for their enzymatic activity to prevent unwanted lysis of the cell envelope. In normally growing cells OMPLA seems to be dormant and *E. coli* mutants missing the *pldA* gene are normally viable under laboratory conditions, which suggests that the protein has no essential role [6]. Nevertheless, OMPLA might be important for growth of the bacteria in their natural environment since it occurs widespread and is well-conserved among *Enterobacteriaceae*, including pathogenic species [6].

The *pldA* genes from several *Enterobacteriaceae* have been cloned [5–7]. OMPLAs show no sequence homology to water-soluble (phospho)lipases and do not contain any cysteine residues. The *E. coli* gene codes for a 30-kDa mature protein of 269 amino acid residues preceded by a signal sequence of 20 amino acid residues. The signal sequence is essential for translocation of the protein across the inner membrane, after which OMPLA folds and inserts into the outer membrane. The mature OMPLAs of *E. coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Proteus vulgaris* are highly homologous, with 79% of the amino acid residues being identical in all four proteins [6]. Like porins, OMPLA lacks long hydrophobic segments which could span the outer membrane in an α-helix conformation [6,7]. A topology model has been proposed [6] that predicts a β-barrel structure analogous to the porins for which the structure has been solved by X-ray diffraction [8–10]. Circular dichroism spectroscopy indeed revealed a very high content of β-strands in *E. coli* OMPLA [11]. To establish the details of the topology and the reaction and activation mechanism of OMPLA, knowledge of the three-dimensional structure is essential.

Crystallization of membrane proteins is a great challenge, and not many successful crystallizations have been published, let alone successful structure determinations. Although in the past crystals of OMPLA from *E. coli* have been reported [12] a structure determination was not possible because of high anisotropy in the resolution of the diffraction pattern. Since then the overexpression and purification of lipopolysaccharide-free OMPLA from *E. coli* have been substantially improved [11], making it worth to reinvestigate the crystallization protocol. Here we report the successful crystallization of OMPLA from *E. coli*. Determination of the crystal structure will provide insight into the catalytic mechanism, calcium dependence and substrate specificity of this lipolytic enzyme. Data on the regulation of OMPLA activity can be of general interest to the understanding of the regulatory mechanisms in or at biological membranes.

2. Materials and methods

2.1. Protein purification

Recombinant OMPLA from *E. coli* without its signal sequence was overexpressed and purified as described [11]. As a result of the cloning strategy the protein has an N-terminal extension of six amino acid residues (Ala-Arg-Ile-Arg-Ala-Pro). The protein accumulated in inclusion bodies, but could be refolded in vitro efficiently in the presence of 0.65% Triton X-100 (Serva). After purification to homogeneity by ion-

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Abbreviations: Bis-Tris, bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane; MPD, 2-methyl-2,4-pentanediol; OMPLA, outer membrane phospholipase A (EC 3.1.1.32); PEG 400, polyethyleneglycol 400; V_M, Matthews coefficient.

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exchange chromatography, lipopolysaccharide-free OMPLA was concentrated in a Centricon-10 microconcentrator (Amicon) and subsequently dialyzed for one week at 4°C against a buffer containing 10 mM sodium succinate at pH 6.0, 1 mM sodium azide and 1.0% (w/v) β-octylglucoside (Boehringer). The protein solution used for crystallization contained pure OMPLA at a concentration of 8 mg/ml and the concentration of the detergent was adjusted to 1.5% (w/v).

2.2. Crystallization

Crystallization was carried out by the hanging-drop vapour diffusion technique at 22°C. Drops were prepared by mixing 3 μl of protein solution with 2 μl of the reservoir solution. All reservoir solutions contained calcium chloride as an additive. Based on results obtained by Gros et al. [12] initial crystallization experiments were performed with 15–30% (v/v) 2-methyl-2,4-pentanediol (MPD; Fluka). The pH was screened over a range from 5.5 to 7.0 and the effect of 1, 2, 3 and 10 mM calcium chloride examined. Subsequently the high melting-point isomer of heptane-1,2,3-triol (Oxyl), polyethyleneglycol 400 (PEG 400; Merck) and sodium chloride were tested as additives.

2.3. X-Ray diffraction experiments

All glassware used for mounting of the crystals was siliconized. X-Ray studies were done in house at 18°C on a FAST area detector or on a MacScience image plate system connected to an Elliot GX-21 rotating anode, with crystals mounted in thin walled glass capillary tubes. Cryogenic data collection using flash-frozen crystals was carried out in house on a FAST area detector as well. FAST data were processed using the program MADNES [13], image plate data using DENZO (Z. Otwinowski, Yale University). Merging of data was done with programs from the BIOMOL package (Protein Crystallography Group, University of Groningen).

3. Results and discussion

Recently, the overexpression and purification of lipopolysaccharide-free OMPLA from *E. coli* have been substantially improved [11] and the crystallization protocol [12] has been reinvestigated. This allowed for reproducible formation of well-ordered three-dimensional crystals amenable to X-ray structural analysis.

The conditions used so far to crystallize membrane proteins employed either PEG or high salt concentrations as crystallizing agents [8,9,14–20]. OMPLA from *E. coli* is the first membrane protein crystallized using an organic solvent such as MPD as crystallizing agent. Hexagonal rod-like crystals appear within 1–4 weeks of equilibrium and reach a typical size of $0.25 \times 0.25 \times 0.60$ mm^3 after 3 weeks. The best crystals are obtained at 22°C with reservoir solutions containing 24–28% (v/v) MPD, 100 mM bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (Bis-Tris; Sigma) at pH 5.9 or 6.0 and 1 mM calcium chloride. All reservoir solutions contain calcium chloride as an additive, because of its necessity for the activity of the protein. The concentration of calcium chloride has a distinct effect on the crystallization behaviour of OMPLA. We observe that in the presence of 2, 3 and 10 mM calcium chloride only needle-like microcrystals appear. The addition of sodium chloride or heptane-1,2,3-triol is not beneficial for crystallization, whereas the addition of 1%, 2% or 3% (v/v) of PEG 400 increases the number of crystals about two- fold without affecting their dimensions. The crystals are stable for at least one month in a solution containing a 1% or 2% (v/v) higher MPD concentration than the reservoir solution to which the drop had been equilibrated, 100 mM Bis-Tris at pH 6.0, 0 or 3% (v/v) PEG 400 and 1 mM calcium chloride.

At 18°C the crystals are resistant to radiation damage for at least 30 h in the X-ray beam generated by the rotating anode. The crystals belong to the trigonal spacegroup $P3_121$ (or $P3_221$) with unit cell dimensions of $a = b = 79.6$ Å and $c = 102.8$ Å ($\alpha = \beta = 90°$, $\gamma = 120°$). The presence of 1% or 3% PEG 400 during crystallization has no influence on the cell dimensions. The spacegroup contains six asymmetric units per unit cell. Given the monomer molecular weight of 30 kDa, a packing with one molecule per asymmetric unit is most likely since this corresponds to a Matthews coefficient (V_M) [21] of 3.2 Å^3/Da and 61% solvent content. This agrees well with values obtained for other membrane proteins [8,9,15–19], ranging from a V_M of 2.5 Å^3/Da (51% solvent content) for crystals of a light-harvesting complex [19] to a V_M of 5.2 Å^3/Da (76% solvent content) for crystals of maltoporin from *E. coli* [16].

Several native datasets have been collected in-house at 18°C from crystals formed both in the absence and in the presence of 1% or 3% (v/v) PEG 400 during crystallization. The best datasets are 95% complete to 2.9 and 2.8 Å resolution, repectively. So far, cryogenic data collection with a crystal directly taken from the stabilizing mother liquor has been performed once and seems to yield a data set of a slightly higher resolution with cell dimensions shrunken to $a = b = 78.5$ Å and $c = 101.6$ Å ($\alpha = \beta = 90°$, $\gamma = 120°$).

Determination of the crystal structure of OMPLA from *E. coli* should provide insight into its catalytic mechanism, calcium dependence and substrate specificity, and into the topology of this intriguing membrane protein as well. With the procedures now established, crystals of OMPLA from *E. coli*, amenable to X-ray structure analysis, can be obtained reproducibly. A search for heavy-atom derivatives is in progress.

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