Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Expression, purification, crystallization and preliminary X-ray analysis of *Pseudomonas fluorescens* AlgK

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Received 22 March 2007
Accepted 4 April 2007

AlgK is an outer-membrane lipoprotein involved in the biosynthesis of alginate in Pseudomonads and *Azotobacter vinelandii*. A recombinant form of *Pseudomonas fluorescens* AlgK with a C-terminal polyhistidine affinity tag has been expressed and purified from the periplasm of *Escherichia coli* cells and diffraction-quality crystals of AlgK have been grown using the hanging-drop vapour-diffusion method. The crystals grow as flat plates with unit-cell parameters $a = 79.09$, $b = 107.85$, $c = 119.15$ Å, $\beta = 96.97^\circ$. The crystals exhibit the symmetry of space group $P2_1$ and diffract to a minimum d-spacing of 2.5 Å at Station X29 of the National Synchrotron Light Source, Brookhaven National Laboratory. On the basis of the Matthews coefficient ($V_M = 2.53$ Å^3 Da^{-1}), four protein molecules are estimated to be present in the asymmetric unit.

1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes chronic and debilitating biofilm infections in the lungs of cystic fibrosis (CF) patients (Lyczak *et al.*, 2002). After initial colonization, conditions in the CF lung select for bacterial cells that secrete copious amounts of alginate (Boucher *et al.*, 1997; Lam *et al.*, 1980; Martin *et al.*, 1993). Alginate is a key factor in the establishment of *P. aeruginosa* biofilm infections: it is a major matrix component of the biofilm, it protects bacterial cells from the host's immune response and is involved in biofilm adherence (Govan & Deretic, 1996). Alginate is a negatively charged polysaccharide consisting of an unbranched chain of $\beta(1\rightarrow4)$-linked β-D-mannuronic acid (M) and its C5 epimer α-L-guluronic acid (G). Alginate synthesized by Pseudomonads contains blocks of polymannuronate (-M-M-M-M-) interspersed with mixed mannuronate–guluronate blocks (-G-M-M-G-). Mannuronate residues in the polymer are also selectively acetylated at either or both their O2 and O3 hydroxyl positions (Gacesa, 1998; Sherbrock-Cox *et al.*, 1984; Skjak-Braek *et al.*, 1986).

In bacteria, alginate production begins with the synthesis of an activated sugar-nucleotide precursor, GDP-mannuronate, in the cytoplasm. The steps involved in the production of this sugar precursor have been well characterized (Jain & Ohman, 2004). Less clear is how GDP-mannuronate is polymerized, modified in the periplasm and transported across the cell envelope. Ten proteins located in the *algD* operon (Ohman & Chakrabarty, 1981; Ohman *et al.*, 1985) have been implicated in these processes. Alg8, which has sequence similarity to glycosyltransferases, is the mannuronyl-transferase responsible for polymerization of the GDP-mannuronic acid (Remminghorst & Rehm, 2006*b*). Alg8 is also hypothesized to be involved in the export of the polymer to the periplasm, where individual mannuronate residues within the polymer are either epimerized at the C5 position to guluronate by the polymer-level epimerase AlgG (Chitnis & Ohman, 1990; Franklin *et al.*, 1994) or acetylated at the O2 and/or O3 hydroxyls (Gacesa, 1998; Sherbrock-Cox *et al.*, 1984; Skjak-Braek *et al.*, 1986) by the concerted action of AlgI, AlgF and AlgJ (Franklin & Ohman, 1993, 1996). Alg44, AlgK and AlgX are essential for alginate polymerization (Jain & Ohman, 1998; Maharaj *et al.*, 1993; Monday & Schiller, 1996; Remminghorst &

Rehm, 2006*a*; Robles-Price *et al.*, 2004). Alg44 is believed to play a role in the regulation of polymerization (Remminghorst & Rehm, 2006*a*), while AlgK (Jain & Ohman, 1998) and AlgX (Robles-Price *et al.*, 2004) have been suggested to help protect the nascent polymer from the action of AlgL. AlgL is an alginate lyase (Schiller *et al.*, 1993) which appears to have a dual role in polymerization and the degradation of the polymer within the periplasm (Albrecht & Schiller, 2005; Bakkevig *et al.*, 2005). The alginate polymer is hypothesized to be exported through the outer membrane by AlgE, a putative β-barrel secretin (Rehm *et al.*, 1994). Recent data have also shown that alginate polymerization requires the presence of protein components in both the inner and outer membranes (Remminghorst & Rehm, 2006*b*), an observation that lends support to the hypothesis that these proteins form a multi-protein complex that mediates the polymerization, modification and secretion of alginate.

AlgK is an outer-membrane (OM) lipoprotein required for the production of long chains of alginate polymer (Jain *et al.*, 2003; Jain & Ohman, 1998). The amino-acid sequence of AlgK provides little information on the potential role(s) it may play in alginate secretion; however, it does reveal four putative Sel1-like repeats (SLR). The SLR motif is very similar to the tetratricopeptide (TPR) repeat motif (Blatch & Lassle, 1999; D'Andrea & Regan, 2003) and is generally found in adaptor proteins involved in the assembly of large multi-protein complexes (Mittl & Schneider-Brachert, 2007). Structural studies on AlgK have been undertaken to confirm the presence and to further characterize the putative SLR motifs and to shed light on the role of AlgK in alginate biosynthesis. The crystallization of AlgK and the preliminary X-ray analysis are presented.

2. Materials and methods

2.1. Cloning and expression

The nucleotide sequence for the AlgK gene from *P. fluorescens* Pf-5 (ATCC BAA-477) obtained from the UniProtKB/TrEMBL database was used to design gene-specific primers. The amino-acid sequences of AlgK proteins from *P. fluorescens* Pf-5, *P. aeruginosa* PAO1, *P. putida* (strain KT2440) and *P. syringae* pv. Tomato (strain DC3000) were aligned using the multiple sequence-alignment program *T-Coffee* (EMBnet Switzerland; Notredame *et al.*, 2000) and used to determine the signal peptidase II cleavage site of AlgK from *P. fluorescens*. The *algK* gene encoding the mature protein without its signal sequence (*SS*) was amplified using the genomic DNA of *P. fluorescens* WCS 374r as a template and the following *algK*-specific primers (forward, GTCCATGGCTGGCCTGCCGGACCAGCG; reverse, GGCCTCGAGTAGGGATTCCTCGCCGTCTTCTTC). The amplification product was digested with *Nco*I and *Xho*I and cloned into the pET26a expression vector (Novagen). This expression plasmid (pET26a-ΔSS-C1M-AlgK) encodes a modified version of the AlgK protein in which its native signal sequence has been replaced by the *pelB* signal sequence to ensure transport of the recombinant protein into the periplasm of the host cells. Additional modifications included the mutation of the N-terminal cysteine of the mature protein to methionine (C1M) to preclude lipidation and the addition of an eight-residue noncleavable C-terminal His_6 tag to facilitate purification. After secretion into the periplasm, the protein contains 452 residues including the His tag and is predicted to have a molecular weight of 49 561 kDa (Gasteiger *et al.*, 2005).

Escherichia coli BL21 CodonPlus (DE3) cells (Stratagene) transformed with the AlgK expression vector were grown in 1 l Luria–Bertani broth (LB) at 310 K until the OD_{600} of the cells attained 0.6, at which point AlgK expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.6 m*M*. After induction, the cells were grown for 15 h at 298 K and were subsequently harvested by centrifugation at 5500 rev min^{-1} for 10 min. The pellet was cooled on ice for 5 min prior to purification.

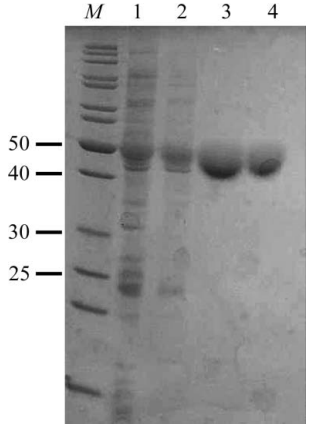


Figure 1
SDS–PAGE analysis of AlgK during purification. Lane *M*, molecular-weight markers (kDa); lane 1, crude cell lysates after induction; lane 2, periplasmic extract; lane 3, purified AlgK after Ni-affinity column chromatography; lane 4, AlgK after gel-filtration chromatography.

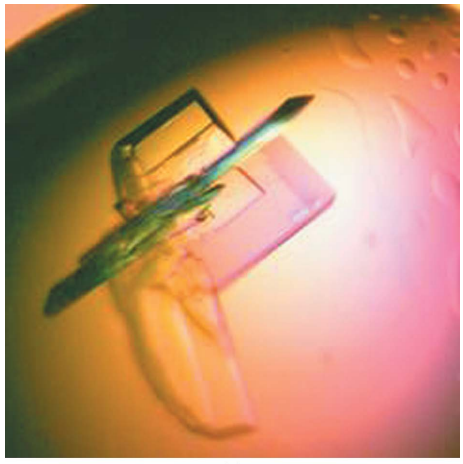
2.2. Purification

The first step of the purification protocol involved the release of the periplasmic contents by osmotic shock. Briefly, the cell pellet from 1 l bacterial culture was washed with 100 ml buffer *A* (10 m*M* Tris–HCl pH 7.5, 30 m*M* NaCl) and resuspended in 50 ml buffer *B* [30 m*M* Tris–HCl pH 7.5, 0.1 m*M* EDTA, 20%(*w*/*v*) sucrose, 1 m*M* phenylmethylsulfonyl fluoride (PMSF)] and mixed at 281 K for 10 min. Cells were then harvested by centrifugation for 20 min at 11 300*g* and the resulting pellet was resuspended in 75 ml buffer *C* [0.1 m*M* $MgCl_2$, 1 m*M* PMSF, one tablet of Roche Complete protease-inhibitor cocktail (EDTA-free) and 15 m*M* β-mercaptoethanol] and mixed for 1 h at 281 K. The cells were subsequently centrifuged for 20 min at 8000 rev min^{-1}, after which the supernatant was collected and centrifuged further for 30 min at 20 400*g*. The final supernatant (*i.e.* the periplasmic contents) was dialyzed overnight against 4 l buffer *D* (20 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 10 m*M* imidazole) at 281 K. The protein was purified by mixing the periplasmic contents with 10 ml Ni–NTA agarose (Qiagen) pre-equilibrated with buffer *D* for 1 h at 281 K. The protein-bound Ni–NTA resin was washed four times with 35 ml buffer *E* (20 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 20 m*M* imidazole, 1 m*M* PMSF, 5 m*M* β-mercaptoethanol, half a tablet of Roche Complete protease-inhibitor cocktail) using the batch method. After packing the resin in a column, the His-tagged protein was eluted with 30 ml buffer *F* (20 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 150 m*M* imidazole, 1 m*M* PMSF, 5 m*M* β-mercaptoethanol, half a tablet of Roche Complete protease-inhibitor cocktail). The eluted protein was collected in 1–1.5 ml fractions. Coomassie Plus Protein Assay Reagent (Pierce) was used to identify the fractions containing the protein. The eluted protein at 5–10 mg ml^{-1} in buffer *F* was further purified and buffer-exchanged into buffer *G* (20 m*M* Tris–HCl pH 8.5, 20 m*M* NaCl, 0.2 m*M* EDTA, 5 m*M* β-mercaptoethanol) by size-exclusion chromatography using a Superdex 200 HR 10/30 gel-filtration column

(Pharmacia). SDS–PAGE was used to confirm the purity of the protein after each step of purification (Fig. 1).

2.3. Crystallization

The purified protein was concentrated to 30 mg ml^{-1} in buffer *G* and screened for crystallization conditions using commonly available commercial screens. The hits obtained shared two common features: all the crystals grew at a neutral pH (6.5–7.5) using polyethylene glycol (PEG) as the precipitant. The molecular weight of the PEG ranged from 4K to 20K. The best-looking and best-diffracting crystals were obtained from condition No. 33 [10%(*w*/*v*) PEG 6000, 0.1 *M* MES pH 6.0] of the pH Clear I crystallization screen (Qiagen) using the hanging-drop vapour-diffusion method (Fig. 2*a*). Crystals took approximately 3–6 weeks to grow. The 6 µl drop consisted of a 1:1 mixture of protein and well solution and was equilibrated over 1 ml well solution. The trays were incubated at 293 K. The crystals grew as flat plates in clumps and were very fragile; acupuncture needles were used to carefully pry apart the crystals and manipulate them. A detergent screen (Hampton Research) showed that the direct addition of 0.4 µl 5.6 m*M* CYMAL-6 to a 3.6 µl drop containing a 1:1 ratio of protein to mother liquor produced more singular crystals (Fig. 2*b*). Drops containing detergent were hung above wells containing 0.6 ml well solution without detergent.



(*a*)

(*b*)

Figure 2
Crystals of AlgK. (*a*) The crystals of the AlgK protein are characteristically clumped. The dimensions of the largest crystals are approximately 0.5 × 0.11 × 0.35 mm. (*b*) Crystals of AlgK after addition of 0.56 m*M* CYMAL-6 detergent. The dimensions of these crystals were similar to or slightly smaller than the crystals grown without detergent.

Table 1
Data-collection statistics.

Values in parentheses correspond to the highest resolution shell.

Wavelength (Å)	1.1
Temperature (K)	100
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 79.09, b = 107.85, c = 119.15, β = 96.97
Resolution (Å)	35–2.5 (2.59–2.50)
Total No. of reflections	508244
No. of unique reflections	68703
Redundancy	7.40 (7.46)
Completeness (%)	99.9 (99.9)
Average $I/\sigma(I)$	8.4 (2.6)
$R_{\rm merge}$† (%)	10.2 (53.3)

† $R_{\rm merge} = \sum\sum|I(k) - \langle I\rangle|/\sum\sum I(k)$, where $I(k)$ and $\langle I\rangle$ represent the diffraction-intensity values of the individual measurements and the corresponding mean values, respectively.

2.4. Data collection

Prior to data collection, the crystals were cryoprotected using a solution containing 10%(*w*/*v*) PEG 6000, 0.1 *M* MES pH 6.0, 25%(*v*/*v*) glycerol and 0.1%(*w*/*v*) hyaluronate. Hyaluronate was added to help stabilize the crystals during cryoprotection (Sugahara & Kunishima, 2006). To prevent cracking, the crystals were cryoprotected in the drop in which they were grown by sequential addition of glycerol cryoprotectant. Initially, all but a few microlitres of the mother liquor in the drop were removed and replaced with 7 µl 10%(*w*/*v*) PEG 6000, 0.1 *M* MES pH 6.0, 0.1%(*w*/*v*) hyaluronate containing 2.5%(*v*/*v*) glycerol. This washing procedure was repeated several times, increasing the glycerol concentration by 2.5% each time until a final concentration of 25%(*v*/*v*) glycerol was attained. The cryoprotected crystals were immediately flash-cooled in liquid nitrogen or in a cold stream at 100 K and either tested and/or stored. There was a considerable variation in the quality of the crystals and extensive testing of the crystals was conducted on our home source (R-AXIS IV^{++} image-plate detector using Cu $K\alpha$ X-ray radiation from an RU-H3R rotating-anode generator) in order to find suitable candidates for data collection at the National Synchrotron Light Source (NSLS). A complete set of native data was collected on Station X29 (NSLS) at 100 K. A total of 360 images of 1° $\Delta\varphi$ oscillations were collected (*i.e.* 360° of data) on an ADSC Quantum-315 detector with a 300 mm crystal-to-detector distance and an exposure time of 4 s per image. The data were integrated, reduced and scaled using *d*TREK* (Pflugrath, 1999).

3. Results

AlgK from *P. fluorescens* has been expressed and purified to homogeneity (Fig. 1). Approximately 15 mg of 98% pure AlgK was obtained per litre of cell culture. Diffraction-quality crystals have been grown and characterized (Fig. 2). The key to obtaining good-quality crystals was the inclusion of the detergent CYMAL-6. The crystals diffract to 2.5 Å and belong to space group $P2_1$, with unit-cell parameters a = 79.09, b = 107.85, c = 119.15 Å, β = 96.97°. The data-collection statistics are summarized in Table 1. On the basis of density calculations ($V_{\rm M}$ = 2.53 Å^3 Da^{-1}), we estimate that four molecules of the protein are present in the asymmetric unit (Matthews, 1968). There is no evidence in the literature that this protein is oligomeric;

however, our size-exclusion chromatography results are consistent with the protein being a dimer in solution. This suggests that two dimers are present in the asymmetric unit. We are currently in the process of determining the structure of this protein using selenomethionine incorporation and the multiple-wavelength anomalous diffraction technique (Hendrickson, 1991).

This work is supported by a research grant from the Canadian Institutes of Health Research (CIHR) to PLH. PLH and LLB are recipients of a Canada Research Chair and a CIHR New Investigator award, respectively. C-LK was funded in part by graduate scholarships from the Natural Science and Engineering Research Council of Canada, the Canadian Cystic Fibrosis Foundation, the Ontario Graduate Scholarship Program and Ontario Student Opportunities Trust Fund and the Hospital for Sick Children Foundation Student Scholarship Program. Station X29 at National Synchrotron Light Source, Brookhaven National Laboratory is supported by the United States Department of Energy.

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