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

Crystallization and preliminary X-ray analysis of alginate importer from *Sphingomonas* sp. A1

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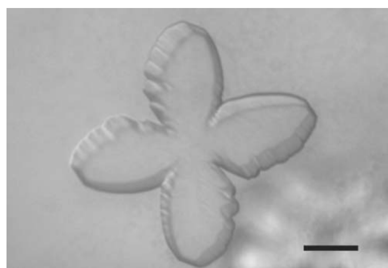
Received 15 November 2011
Accepted 16 January 2012

Sphingomonas sp. A1 directly incorporates alginate polysaccharides through a 'superchannel' comprising a pit on the cell surface, alginate-binding proteins in the periplasm and an ABC transporter (alginate importer) in the inner membrane. Alginate importer, consisting of four subunits, AlgM1, AlgM2 and two molecules of AlgS, was crystallized in the presence of the binding protein AlgQ2. Preliminary X-ray analysis showed that the crystal diffracted to 3.3 Å resolution and belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 72.5$, $b = 136.8$, $c = 273.3$ Å, suggesting the presence of one complex in the asymmetric unit.

1. Introduction

Sphingomonas sp. A1 (referred to in the following as strain A1), a Gram-negative bacterium isolated from soil, directly incorporates polysaccharides such as alginate (Momma *et al.*, 1999; Murata *et al.*, 2008). Alginate (average molecular weight 26 000) is produced by brown seaweeds and certain bacteria and is composed of β-D-mannuronate and α-L-guluronate. High-molecular-weight alginates are introduced into the cytoplasm through a 'superchannel' in strain A1 (Momma *et al.*, 1999) and are degraded by cytoplasmic alginate lyases (Yoon *et al.*, 2000; Hashimoto *et al.*, 2000). The superchannel consists of a pit on the cell surface (Hisano *et al.*, 1995), an ABC transporter (alginate importer) within the inner membrane (Momma *et al.*, 2000) and periplasmic alginate-binding proteins (AlgQ1 and AlgQ2) that mediate the transfer of alginate from the pit to the alginate importer (Momma *et al.*, 2005). Similar to other bacterial ABC transporters (Locher, 2009), the alginate importer in strain A1 is composed of two transmembrane domains (a heterodimer of AlgM1 and AlgM2) and two nucleotide-binding domains (a homodimer of AlgS). In general, microbes depolymerize polysaccharides and other macromolecules using extracellular degrading enzymes and incorporate the degraded products through their membrane. However, the pit-dependent alginate importer in strain A1 is unique in that it imports nondegraded native macromolecules. Therefore, studies of the structure and function of alginate importer in strain A1 will provide new insights into macromolecular import in bacteria. Here, we report the crystallization and preliminary X-ray crystallographic studies of recombinant alginate importer with and without a binding protein.



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2. Materials and methods

2.1. Purification of alginate importer

Four variants of alginate importer, AlgM1(d0)M2(H10)SS(WT), AlgM1(d0)M2(H10)SS(E160Q), AlgM1(d24)M2(H10)SS(WT) and AlgM1(d24)M2(H10)SS(E160Q), were constructed and expressed in *Escherichia coli* BL21 (DE3) Gold (Itoh *et al.*, 2009). Ten histidine tags were inserted into the C-terminus of the AlgM2 subunit of all of the importer variants. In the third and fourth variants, 24 N-terminal residues from the AlgM1 subunit were deleted by genetic engineering. In the second and fourth variants, Glu160 of the AlgS

subunit, which is required for ATPase activity, was replaced by Gln. Proteins were purified according to the method reported by Itoh *et al.* (2009). In brief, the cell extract was ultracentrifuged at 100 000*g* and 277 K for 60 min. The pelleted membranes were solubilized using 1% *n*-dodecyl-β-D-maltopyranoside (DDM; Nacalai Tesque) and purified by affinity (immobilized metal-ion adsorption column) and size-exclusion chromatography (gel-filtration column) (Fig. 1). Although the recombinant *E. coli* cell membranes were initially solubilized with DDM, several detergents were tested for crystallization. Detergent exchange was performed during protein purification at the affinity-chromatography step. Purified alginate importer was resolved in standard buffer (20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 10 m*M* $MgCl_2$, 10% glycerol) with detergent (at twice the critical micelle concentration) and was concentrated to a final concentration of 10 mg ml^{-1} for crystallization.

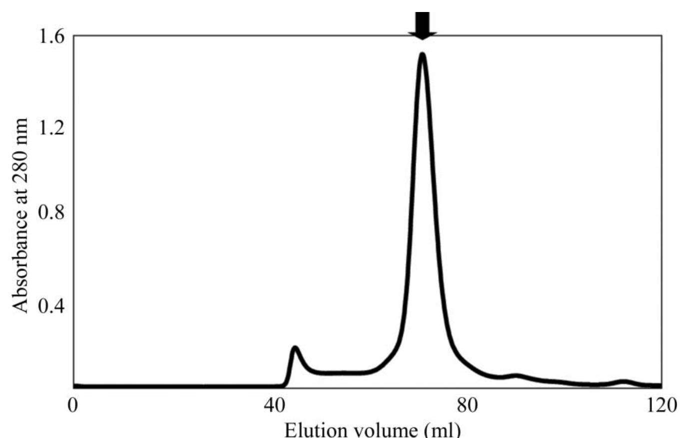

Figure 1
Gel-filtration chromatogram for the final stage of purification of the protein [AlgM1(d24)M2(H10)SS(E160Q)] using Cymal-6. Superdex 200 (GE Healthcare) was used. The elution peak indicated by an arrow was collected for crystallization.

2.2. Crystallization and X-ray analysis

Alginate importer was crystallized in the presence and the absence of AlgQ2 using sitting-drop vapour diffusion in a 96-well plate. The alginate importer crystal was prepared using 1 µl purified alginate importer (7 mg ml^{-1}) in standard buffer containing detergent mixed with an equal volume of reservoir solution. The alginate importer–AlgQ2 complex was crystallized using a sample solution consisting of 7 mg ml^{-1} importer, 3 mg ml^{-1} AlgQ2, 1 m*M* unsaturated trimannuronate (d3M), 4 m*M* adenosine triphosphate (ATP), 4 m*M* $MgCl_2$ in standard buffer containing detergent. Initial screenings for crystallization conditions were performed using commercial crystallization kits from Molecular Dimensions and Hampton Research. Crystallization conditions were further refined using a 24-well or a 96-well plate. Single crystals were soaked in a cryoprotectant solution containing 20% glycerol and then cooled in a stream of cold nitrogen gas. The diffraction patterns were then examined on beamlines BL38B1, BL41XU and BL44XU at SPring-8.

X-ray diffraction data for the alginate importer crystal were collected using an MX225HE CCD detector (Rayonix) on beamline

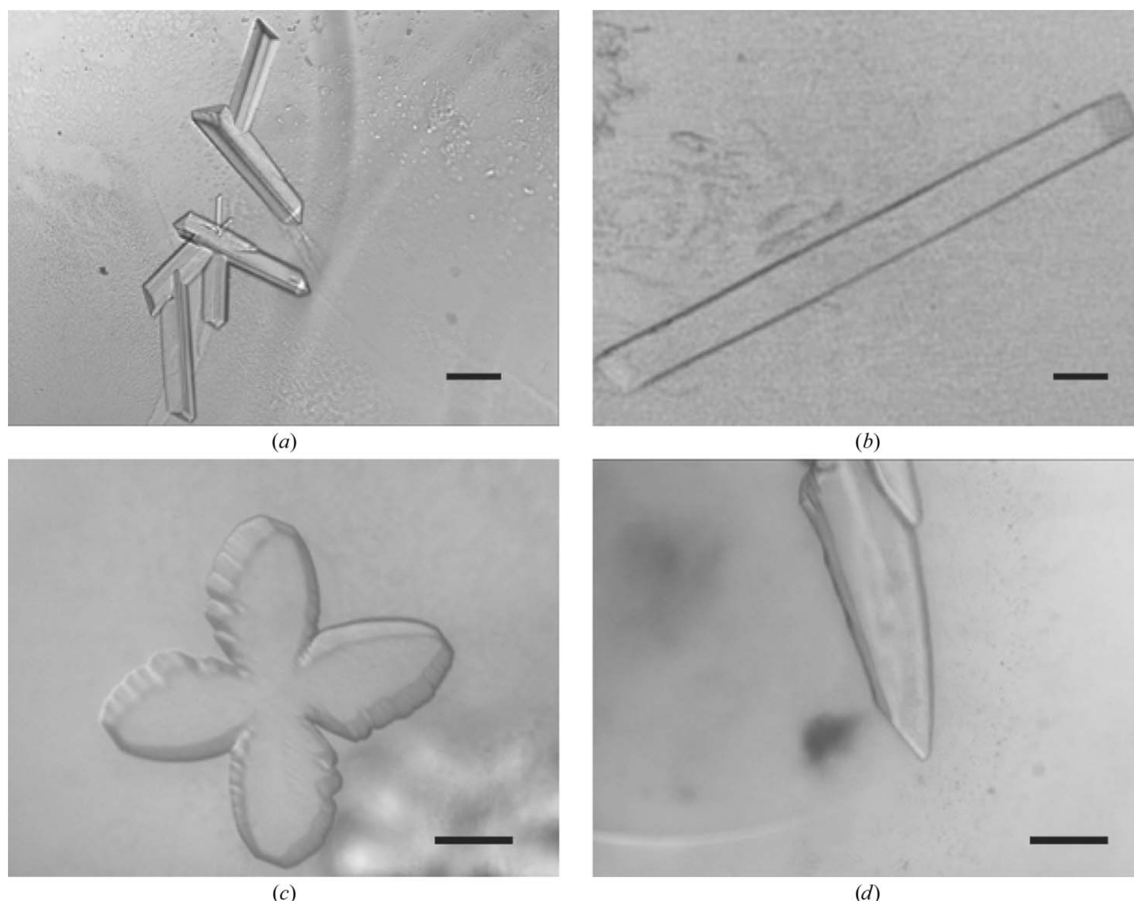

Figure 2
Crystal images. (*a*) Rod-shaped crystal of the alginate importer. (*b*, *c*, *d*) Different shaped alginate importer–AlgQ2 complex crystals. The scale bar is 0.1 mm in length.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Alginate importer	Alginate importer–AlgQ2
Wavelength (Å)	1.0	1.0
Resolution range (Å)	50.00–7.00 (7.25–7.00)	50.00–3.30 (3.36–3.30)
Space group	*P*1	$P2_12_12_1$
Unit-cell parameters (Å, °)	*a* = 108.5, *b* = 141.5, *c* = 154.3, α = 112.8, β = 93.5, γ = 90.7	*a* = 72.5, *b* = 136.8, *c* = 273.3
Mosaicity (°)	1.67	0.87
Total observations	28646	193489
Unique reflections	13251	40462
Completeness (%)	97.7 (92.8)	96.5 (93.8)
$\langle I/\sigma(I)\rangle$	20.0 (2.7)	18.7 (2.0)
$R_{\rm merge}$	0.052 (0.329)	0.055 (0.487)

Table 2
List of the molecular-replacement solutions obtained using *MOLREP*.

Search model	Solution No.	$wR_{\rm fac}$	Score
AlgQ2 (PDB entry 1kwh)	1	0.551	0.408
	2	0.554	0.408
	3	0.553	0.407
	4	0.553	0.407
	5	0.551	0.405
AlgQ2 with alginate (PDB entry 1j1n)	1	0.537	0.432
	2	0.548	0.416
	3	0.548	0.415
	4	0.548	0.414
	5	0.549	0.413

BL41XU and those for the importer–AlgQ2 complex were collected using an MX225HE CCD detector on beamline BL44XU. Diffraction data were indexed, integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

Initially, we tried to crystallize the alginate importer. Rod-shaped crystals of alginate importer (Fig. 2*a*) were grown using several solutions containing 20–22% polyethylene glycol (PEG) 3000 or PEG 4000 and 100 m*M* *N*-(2-acetamido) iminodiacetic acid (ADA) pH 6.6 with various additives such as salts and detergents. Crystals that diffracted to 7 Å resolution were obtained from AlgM1(d24)M2(H10)SS(WT) purified using 0.06% 6-cyclohexyl-1-hexyl-β-D-maltoside (Cymal-6; Anatrace) and a reservoir solution consisting of 22% PEG 3000, 100 m*M* ADA–NaOH pH 6.6, 150 m*M* NaCl, 16 m*M* 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-propanesulfonate (CHAPSO). The crystal belonged to the triclinic space group *P*1, with unit-cell parameters *a* = 108.5, *b* = 141.5, *c* = 154.3 Å, α = 112.8, β = 93.5, γ = 90.7°. Data-collection statistics for the alginate importer crystal are summarized in Table 1. Six importer molecules per asymmetric unit gave a $V_{\rm M}$ value (Matthews, 1968) of 2.42 $Å^3\ Da^{-1}$ and a solvent content of 49%.

To overcome the poor diffraction quality and low symmetry of the above crystals, we crystallized alginate importer with the periplasmic binding protein AlgQ2. Several crystals of the alginate importer–AlgQ2 complex (Figs. 2*b*–2*d*) were grown under conditions similar to those used to obtain the alginate importer crystals. To confirm complex formation, the crystals were washed in reservoir solution and subjected to SDS–PAGE. The results clearly indicated the presence of alginate importer and AlgQ2 in the crystals (Fig. 3). Single crystals were screened by X-ray diffraction using beamlines BL38B1, BL41XU and BL44XU at SPring-8. Alginate importer–AlgQ2 complex crystals tended to diffract better than unbound alginate importer crystals.

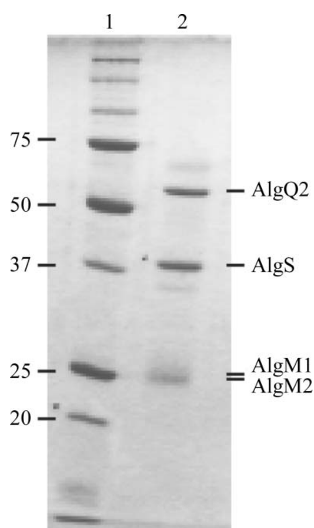

Figure 3
SDS–PAGE of an alginate importer–AlgQ2 complex crystal. Lane 1, molecular-weight standards (labelled in kDa); lane 2, proteins in the crystal.

The best crystal structure was obtained from AlgM1(d24)M2(H10)SS(E160Q) purified with 0.06% Cymal-6. 2 µl sample solution [7 mg ml^{-1} AlgM1(d24)M2(H10)SS(E160Q), 3 mg ml^{-1} AlgQ2, 1 m*M* d3M, 4 m*M* ATP, 5.4 m*M* $MgCl_2$, 16 m*M* CHAPSO, 140 m*M* nondetergent sulfobetaine (NDSB) 256 (Anatrace), 0.042% Cymal-6, 14 m*M* Tris–HCl pH 8.0, 70 m*M* NaCl, 7% glycerol] was mixed with an equal volume of reservoir solution (22% PEG 4000, 50 m*M* ADA–NaOH pH 6.6, 134 m*M* tricine–NaOH pH 8.4, 100 m*M* NaCl). Preliminary characterization of the alginate importer–AlgQ2 crystal indicated an orthorhombic system (space group $P2_12_12_1$), with unit-cell parameters *a* = 72.5, *b* = 136.8, *c* = 273.3 Å. Data-collection statistics for the alginate importer–AlgQ2 complex crystal are summarized in Table 1. One AlgM1(d24)M2(H10)SS(E160Q)–AlgQ2 complex per asymmetric unit gave a $V_{\rm M}$ value of 3.23 $Å^3\ Da^{-1}$ and a solvent content of 62%, indicating that the crystal was suitable for structural analysis.

Initial molecular-replacement trials were performed with *MOLREP* (Vagin & Teplyakov, 2010) in *CCP*4 (Winn *et al.*, 2011) using the coordinates of AlgQ2 with or without oligoalginate (PDB entries 1kwh and 1j1n; Momma *et al.*, 2002; Mishima *et al.*, 2003) as a model. A prominent solution was obtained using a model of AlgQ2 with alginate (Table 2), suggesting that AlgQ2 interacts with alginate importer in the closed conformation. However, a further search using the coordinates of the maltose transporters MalF, MalG and MalK or their complex (Oldham *et al.*, 2007) as models failed. A search for selenomethionine derivatives for phasing by the multiple-wavelength anomalous dispersion method is currently in progress.

We wish to thank Dr Seiki Baba (SPring-8 beamline BL38B1) and Dr Kazuya Hasegawa (SPring-8 beamline BL41XU) of the Japan Synchrotron Radiation Research Institute (JASRI) and Dr Eiki Yamashita (SPring-8 beamline BL44XU) of Osaka University for their help with data collection. We would also like to thank Ms Chizuru Tokunaga and Ms Ai Matsunami for their excellent technical assistance. This work was supported in part by Grants-in-Aid and the Targeted Protein Research Program from the Ministry of Education, Science and Culture of Japan (KM, BM and WH).

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