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

*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 $P_{2_1}2_12_1$, 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-mannurionate 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.

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 000g 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 mM Tris−HCl pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 10% glycerol) with detergent (at twice the critical micelle concentration) and was concentrated to a final concentration of 10 mg ml⁻¹ 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 mM unsaturated trimannuronate (d3M), 4 mM adenosine triphosphate (ATP), 4 mM MgCl₂ 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...
Table 1  
Data-collection statistics.

<table>
<thead>
<tr>
<th></th>
<th>Alginate importer</th>
<th>Alginate importer–AlgQ2</th>
</tr>
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<tr>
<td>Wavelength (Å)</td>
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<td>1.0</td>
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<tr>
<td>Resolution range (Å)</td>
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<td>50.00–3.30 (3.36–3.30)</td>
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<td>Space group</td>
<td>P1</td>
<td>P2_2_2_1</td>
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<tr>
<td>Unit-cell parameters</td>
<td>a = 108.5 Å, b = 141.5 Å, c = 154.3 Å</td>
<td>a = 72.5 Å, b = 136.8 Å, c = 273.3 Å</td>
</tr>
<tr>
<td>Mosaicity (°)</td>
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<td>193489</td>
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<tr>
<td>Unique reflections</td>
<td>13251</td>
<td>40462</td>
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<tr>
<td>Completeness (%)</td>
<td>97.7% (92.8%)</td>
<td>96.5% (93.8%)</td>
</tr>
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</table>

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. 2a) were grown using several solutions containing 20–22% polyethylene glycol (PEG) 3000 or PEG 4000 and 100 mM 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 mM ADA–NaOH pH 6.6, 150 mM NaCl, 16 mM 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-propanesulfonate (CHAPS). The crystal belonged to the triclinic space group P1, with unit-cell parameters a = 108.5 Å, b = 141.5 Å, 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_M value (Matthews, 1968) of 2.42 Å³ 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. 2b–2d) 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.

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 mM d3M, 4 mM ATP, 5.4 mM MgCl₂, 16 mM CHAPS, 140 mM non-detergent sulfobetaine (NDSB) 256 (Anatrace), 0.042% Cymal-6, 14 mM Tris–HCl pH 8.0, 70 mM NaCl, 7% glycerol] was mixed with an equal volume of reservoir solution [22% PEG 4000, 50 mM ADA–NaOH pH 6.6, 134 mM tricine–NaOH pH 8.4, 100 mM NaCl]. Preliminary characterization of the alginate importer–AlgQ2 crystal indicated an orthorhombic system (space group P2_2_2_1), with unit-cell parameters a = 72.5 Å, b = 136.8 Å, 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_M value of 3.23 Å³ 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 CCP4 (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.

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References