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Crystallization and preliminary X-ray crystallographic studies of DesR, a thermosensing response regulator in a two-component signalling system from *Streptococcus pneumoniae*

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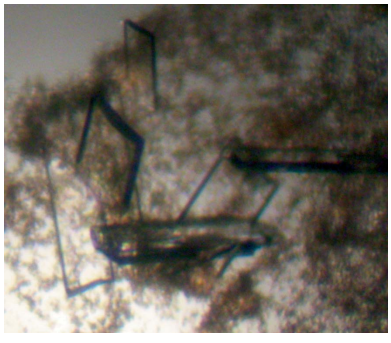
The response regulator DesR, which activates the transcription of the *des* gene by binding to a regulatory region, is essential for controlling the fluidity of membrane phospholipids. DesR from *Streptococcus pneumoniae* was overexpressed in *Escherichia coli*. The protein was purified and crystallized for structural analysis. Diffraction data were collected to 1.7 Å resolution using synchrotron radiation and the crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 46.91, b = 71.38, c = 117.73 Å. Assuming the presence of a dimer in the asymmetric unit, this corresponds to a V_M of 2.21 Å^3 Da^{-1}.

1. Introduction

All organisms must interact with their environment to survive. Their life is a continuous balancing act, maintaining constant internal conditions in response to the continuously changing external environment. Two-component signal transduction systems provide a fundamental stimulus–response coupling mechanism that requires organisms to sense and respond to variations in many different environmental conditions. Typical two-component systems consist of a membrane-bound signal-sensing protein histidine kinase (HK) and a cytosolic cognate response regulator (RR) protein (Hoch, 2000). In the presence of extracellular stimuli, the activation of a sensor histidine kinase leads to ATP-dependent autophosphorylation in the cytoplasmic domain followed by transfer of the phosphoryl group to a cognate response regulator. Phosphorylated response regulators typically function as transcriptional factors by binding to promoter regions to activate specific gene expression (West & Stock, 2001).

The transcription of the *des* gene increases in response to a decrease in temperature in some bacteria, including *Bacillus subtilis* (Aguilar *et al.*, 2001) and cyanobacteria (Suzuki *et al.*, 2000). The *des* gene product, Δ5-acyl lipid desaturase (Δ5-Des), catalyzes the introduction of a *cis* double bond at the Δ5 position of existing saturated fatty acids. The resulting increase in unsaturated fatty-acid content causes membrane fluidity to return to its original state or close to it, with concurrent restoration of normal cellular activity at the lower temperature (Diaz *et al.*, 2002; Mansilla & de Mendoza, 2005). The molecular mechanism of cold induction of unsaturated fatty-acid biosynthesis and transcriptional control of the *des* gene, termed the Des pathway, is a two-component signal transduction system. This regulatory system, which has recently been studied in *B. subtilis*, is composed of a membrane-associated histidine kinase DesK and a transcriptional regulator DesR, which strictly regulates transcription of the *des* gene (Aguilar *et al.*, 2001; Cybulski *et al.*, 2002). Upon cold shock, DesK senses a decrease in membrane lipid fluidity and is autophosphorylated at a conserved histidine residue in the cytoplasmic C-terminal domain. Phosphorylated DesK is able to phosphorylate DesR on a specific aspartic acid residue (Albanesi *et al.*, 2004). DesR, like other response regulators, is made up of two domains: a conserved N-terminal regulatory domain containing a conserved aspartic acid residue and a C-terminal effector domain which can bind to DNA. Phosphorylation of an aspartic acid residue in the regulatory domain leads to conformational changes of DesR. Phosphorylated DesR is able to bind to the regulatory region up-



stream of the *des* gene promoter and then activates the *des* gene (Cybulski *et al.*, 2004). When the *des* gene is induced, synthesis of the Δ5-Des protein which inserts double bonds into the acyl chains of membrane lipids is activated and this protein regulates the membrane lipid fluidity.

In order to understand the molecular mechanism of the two-component signal transduction system called the Des pathway, which regulates the level of unsaturated fatty acids, in turn controlling the membrane fluidity, we report the overexpression, crystallization and preliminary X-ray crystallographic analysis of the DesR response regulator from *Streptococcus pneumoniae* as a first step towards protein structure determination for the Des pathway.

2. Materials and methods

2.1. Cloning, protein expression and purification

The full-length *desR* gene (UniProtKB/TrEMBL accession No. Q8DNC2) was amplified from *S. pneumoniae* genomic DNA by polymerase chain reaction (PCR) using the forward primer 5′-GG-AATTC**CATATG**AAAGTATTAGTCGCAGAAGATCAAAGT-3′ and the reverse primer 3′-ACA**CAGCTG**TCATAACCAACCAGAT-TCTTTTGCG-5′ (*Nde*I and *Xho*I restriction sites are indicated in bold). The PCR product was digested with *Nde*I and *Xho*I restriction endonucleases and ligated to digested pET-28a(+) (Novagen). The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) strain and the cells were grown at 310 K in Luria–Bertani medium supplemented with kanamycin (50 µg ml^{-1}). The transformants were grown in LB medium at 310 K until they reached an optical density of 0.45 at 600 nm and expression was then induced with 0.2 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were grown for 12 h at 291 K after induction and were then harvested by centrifugation at 5000*g* for 30 min at 277 K. The pelleted cells were suspended in buffer *A* (20 m*M* Tris–HCl pH 7.9, 500 m*M* NaCl, 5 m*M* imidazole) and lysed by sonication. The crude lysate was centrifuged at 20 000*g* for 1 h at 277 K. The supernatant was loaded onto an Ni^{2+}-chelated HiTrap chelating HP column (GE Healthcare, USA) equilibrated with buffer *A*. The protein was eluted with a linear gradient of buffer *A* containing 1 *M* imidazole. The final purification step was performed by gel filtration on a HiLoad 16/60 Superdex 200 column (GE Healthcare, USA) that had previously been equilibrated with buffer *B* (20 m*M* Tris–HCl pH 7.9, 200 m*M* NaCl, 5 m*M* $MgCl_2$, 2 m*M* DTT, 10% glycerol). The DesR protein was eluted as a peak corresponding to a molecular mass of 45 kDa, suggesting that DesR exists as a dimer (data not shown). The purified proteins were concentrated to 18 mg ml^{-1} using an Amicon Ultra-15 ultrafiltration device (Millipore, USA). The protein concentration was determined by the method of Bradford with bovine serum albumin as a standard.

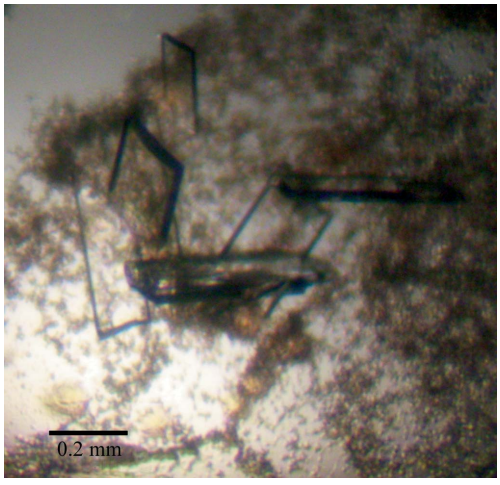


Figure 1
Crystals of DesR protein from *S. pneumoniae*. The crystal size was 0.2 × 0.3 × 0.05 mm.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 46.91, b = 71.38, c = 117.73
Resolution range (Å)	50–1.7 (1.76–1.70)
Total reflections	340515
Unique reflections	42035 (2901)
Redundancy	8.1 (2.9)
Completeness (%)	94.7 (67)
$R_{\rm merge}$† (%)	6.5 (29.2)
$\langle I/\sigma(I)\rangle$	25 (1.9)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl, $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl.

2.2. Crystallization and X-ray analysis

Preliminary crystallization screening was performed in 96-well IntelliPlates (Art Robbins Instruments, USA) at 295 K using the sitting-drop vapour-diffusion method (0.5 µl protein solution and 0.5 µl reservoir solution equilibrated against 70 µl reservoir solution). The initial screening was set up by hand using an eLINE electronic pipette (BIOHIT). Commercial screening kits such as Crystal Screens I and II, MembFac, PEG/Ion I and II, PEGRx 1 and 2, Salt Rx, Index and Natrix from Hampton Research and Wizard I and II from Emerald BioStructures were used. Initial crystals were obtained using the PEGRx 1 screen [0.1 *M* sodium citrate tribasic dehydrate pH 5.0, 30%(*v*/*v*) Jeffamine ED-2001 pH 7.0] within a week. Crystal growth was scaled up to the hanging-drop vapour-diffusion method in 24-well VDX plates (Hampton Research, USA). About 50 optimization drops were set up; each hanging drop was prepared by mixing 1 µl protein solution and 1 µl reservoir solution [0.1 *M* sodium citrate tribasic dehydrate pH 5.0, 18%(*v*/*v*) Jeffamine ED-2001 pH 7.0] and was equilibrated over 500 µl reservoir solution. Single crystals were obtained within 3 d and used for X-ray diffraction experiments. Several cryosolvents were tested for data collection under cryogenic conditions, including glycerol, ethylene glycol, PEG 400 and MPD, but oil gave the best results. Crystals were transferred to perfluoropolyether oil PFO-X175/08 (Hampton Research, USA) and flash-frozen in a liquid-nitrogen stream. The data set was collected using an ADSC Quantum 310 CCD detector on beamline 4A at the Pohang Light Source (Pohang, South Korea). A total of 360 images were collected with an oscillation angle of 1° and 5 s exposure per frame with a crystal-to-detector distance of 250 mm. The wavelength of the synchrotron X-rays was 1.000 Å. A data set was collected to 1.7 Å resolution from a single crystal. The data set was indexed, processed and scaled using the *HKL*-2000 software package (Otwinowski & Minor, 1997).

3. Results and discussion

The gene encoding the DesR protein from *S. pneumoniae* was cloned into the pET-28a(+) vector and transformed into *E. coli* BL21 (DE3). DesR is composed of 199 amino acids with a calculated molecular weight of 22.3 kDa. The protein was overexpressed in *E. coli* and purified for structural studies. The DesR protein was shown to be a

dimer, as expected, using gel-filtration chromatography. The yield was approximately 20 mg protein from a 1 l culture. Crystals suitable for X-ray analysis were obtained with optimized crystallization conditions using 0.1 *M* sodium citrate pH 5.0 and Jeffamine ED-2001 as precipitants. The crystal grew to dimensions of 0.2 × 0.3 × 0.05 mm within 3 d (Fig. 1). The crystals diffracted to 1.7 Å resolution at Pohang Light Source synchrotron facility and belonged to the primitive orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 46.91$, $b = 71.38$, $c = 117.73$ Å. The presence of one dimer of DesR in the asymmetric unit gave a V_M value of 2.21 Å^3 Da^{-1}, with a corresponding solvent content of 44.4% (Matthews, 1968). This is consistent with the result of the gel-filtration experiment. The data-collection statistics are summarized in Table 1. All attempts to solve the structure by the molecular-replacement method using other classes of response regulators [NarL from *E. coli* (PDB code 1rnl; Baikalov *et al.*, 1996) and DosR from *Mycobacterium tuberculosis* (PDB code 3c3w; Wisedchaisri *et al.*, 2008)] as search models failed. In order to solve the phase problem by the multi-wavelength anomalous diffraction method, selenomethionine-labelled protein has been expressed, purified and crystallization experiments for the selenomethionine-substituted protein are currently under way.

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