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Expression, crystallization and preliminary crystallographic analysis of the PAS domain of RsbP, a stress-response phosphatase from *Bacillus subtilis*

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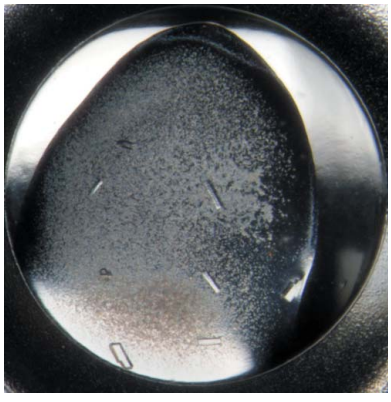

RsbP, a regulator of RNA polymerase σ^B activity in *Bacillus subtilis*, is a phosphatase containing a Per–Arnt–Sim (PAS) domain in its N-terminal region that is expected to sense energy stresses such as carbon, phosphate or oxygen starvation. Energy-stress signals are transmitted to the PAS domain and activate the C-terminal phosphatase domain of RsbP, leading to activation of the downstream anti-anti-σ^B factor RsbV. Finally, the general stress response is induced to protect the cells against further stresses. The recombinant PAS domain of RsbP was crystallized by the sitting-drop vapour-diffusion technique using 40% PEG 400 as a precipitant. The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 55.2$, $b = 71.7$, $c = 60.2$ Å, $\beta = 92.1^\circ$. Diffraction data were collected to a resolution of 1.6 Å.

1. Introduction

The bacterium *Bacillus subtilis* exploits a signal transduction mechanism to sense and respond to the deleterious effects of stresses or sudden changes in the environment. In such situations, sensed stress information is transmitted to and converges on the general stress-response transcription factor σ^B, which interacts with the apo form of RNA polymerase (Hecker & Volker, 1998). The activity of σ^B in forming the complete transcriptional complex is regulated by two distinct signalling pathways. One is responsible for the energy stress from carbon, phosphate or oxygen starvation and the other is responsible for environmental stress such as salt stress, heat shock or ethanol addition (Kang *et al.*, 1996; Benson & Haldenwang, 1993*a*; Voelker *et al.*, 1995). Each pathway is composed of a set of signal transduction proteins termed regulators of σ^B (Rsb) and connects with a phosphatase: RsbP in energy-stress signalling or RsbU in environmental stress signalling (Vijay *et al.*, 2000; Wise & Price, 1995; Voelker *et al.*, 1995). Both of them dephosphorylate a common downstream factor, the anti-anti-σ^B factor RsbV, leading to activation of σ^B by releasing it from the anti-σ^B factor RsbW by means of the partner-switching mechanism (Benson & Haldenwang, 1993*b*; Dufour & Haldenwang, 1994). As a result, σ^B induces the transcription of more than 200 general stress genes to exclude stresses or to repair the damage (Petersohn *et al.*, 1999; Price *et al.*, 2001).

RsbP is an essential protein to sense energy limitation in conjunction with the α/β hydrolase RsbQ that is co-expressed with RsbP from the *rsbQP* operon (Vijay *et al.*, 2000; Brody *et al.*, 2001). The amino-acid sequence of RsbP indicates that it is comprised of three domains: a Per–Arnt–Sim (PAS) domain, a coiled-coil domain and a PPM/PP2C Ser/Thr phosphatase domain. The C-terminal PPM/PP2C domain with ∼210 amino-acid residues is homologous in sequence to two other environmental stress signalling Rsb proteins: RsbU and RsbX (Yang *et al.*, 1996). However, the N-terminal PAS domain, which comprises ∼110 amino-acid residues, has not been observed in the sequences of any other Rsb protein. PAS domains sense changes in redox potential, oxygen tension or light intensity in a wide variety of proteins through binding of cofactors within their hydrophobic cores and protein–protein interactions (Taylor & Zhulin, 1999). Our previous crystallographic study of RsbQ indicated that it has specificity for an unknown small hydrophobic compound whose reaction

product is expected to be a signal molecule for RsbP (Kaneko *et al.*, 2005). Thus, the PAS domain of RsbP (RsbP-PAS) is a potential candidate for a regulatory sensor that initiates a switch in regulating the phosphatase activity of RsbP.

Despite its importance in energy-stress signal transduction, how RsbP responds to stress signals and how its phosphatase domain is activated in the protein are poorly understood, primarily because of a lack of structural information. Determination of the tertiary structure of RsbP-PAS would help in understanding these mechanisms. In this article, we present the expression, crystallization and preliminary X-ray studies of RsbP-PAS.

2. Methods and results

2.1. Protein expression and purification

The full-length *rsbP* gene was cloned from a single colony of *B. subtilis* strain 168 by the colony-PCR method using two oligonucleotide primers (forward, 5′-GGTGGTCATATGGACAAACAATTGAATGATGCACCA-3′; reverse, 5′-GGTGGTGTCGACTTTTACATCAACTAATATAAAACATTCGTC-3′). The *rsbP-PAS* sequence corresponding to the Met1–Pro108 region was then subcloned into the *Nde*I and *Sal*I restriction-enzyme sites of pET-21c (Novagen Inc.), generating an expression plasmid in which the *rsbP-PAS* gene was expressed under the control of the T7 RNA polymerase promoter with a 6×His tag at the C-terminus. The resultant pET-rsbP-PAS plasmid was used to transform the host strain *Escherichia coli* BL21 (DE3) (Novagen). The transformants were grown in LB medium (Sigma–Aldrich) containing 50 µg ml^{-1} ampicillin at 310 K to an optical density of 0.6 at 600 nm. After induction with 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside, growth was continued for a further 14 h at 291 K and the cells were harvested by centrifugation. The harvested cells were washed and then resuspended after an initial freeze–thaw step in lysis buffer (50 m*M* potassium phosphate pH 7.0, 200 m*M* sodium chloride, 10 m*M* imidazole). The suspension was sonicated after treatment with 0.5 mg ml^{-1} lysozyme and 50 µg ml^{-1} DNase I for 30 min at 277 K. The solution was centrifuged at 12 500*g* for 1 h and the cell debris was discarded. The supernatant was loaded onto an Ni^{2+}-chelating affinity column (GE Healthcare). The column was washed with ten bed volumes of lysis buffer and two further bed volumes of lysis buffer with 50 m*M* imidazole. The protein was eluted with lysis buffer containing 200 m*M* imidazole. The fractions were combined and dialyzed against 20 m*M* potassium phosphate pH 7.0. The protein was further purified using Mono-Q (GE Healthcare) with a linear gradient of 0.0–0.2 *M* NaCl in 20 m*M* phosphate pH 7.0. The purified RsbP-PAS gave a single band on the SDS–PAGE profile. Prior to crystallization, the sample buffer was exchanged with 20 m*M* phosphate pH 7.0 and concentrated by centrifugation in a Centriprep filter (Amicon Inc.) to a concentration of 30–35 mg ml^{-1}. The purified RsbP-PAS is composed of 123 amino acids with a molecular mass of 14 kDa, which included nine additional amino acids (VDKLAAALE) as a cloning artifact at the C-terminus before the 6×His tag.

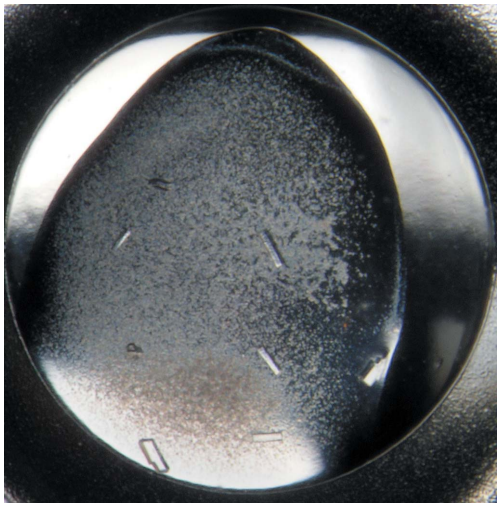

Figure 1
Crystals of recombinant RsbP-PAS. The maximum dimensions of the crystals are approximately 0.1 × 0.02 × 0.02 mm.

Table 1
Diffraction data statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	SPring-8 BL38B1
Detector	R-AXIS V
Wavelength (Å)	1.0000
Resolution (Å)	50–1.60 (1.66–1.60)
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 55.2, b = 71.7, c = 60.2, β = 92.1
Redundancy	4.5 (4.4)
No. of observations	276352
No. of unique reflections	61886
Completeness (%)	100 (100)
R_{merge} (%)†	2.9 (45.0)
$I/\sigma(I)$	19.16 (4.25)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the average of $I(hkl)$.

2.2. Crystallization

Initial crystallization conditions for RsbP-PAS were screened using the sitting-drop vapour-diffusion method at 277 and 293 K with the commercially available sparse-matrix screening kits Crystal Screen, Crystal Screen II (Hampton Research), Wizard I, Wizard II, EBS Cryo I and EBS Cryo II (Emerald BioStructures). 1 µl protein solution was mixed with an equal volume of reservoir solution and equilibrated against 70 µl reservoir solution. Several small monoclinic crystals appeared from EBS Cryo I condition No. 6 and Wizard II condition No. 12. Well diffracting crystals were obtained using a reservoir consisting of 40% PEG 600, 100 m*M* sodium cacodylate pH 6.5 and 200 m*M* lithium sulfate and grew to maximum dimensions of approximately 0.10 × 0.02 × 0.02 mm in a week (Fig. 1).

2.3. X-ray diffraction study

X-ray diffraction data were collected from RsbP-PAS crystals on beamline BL38B1 at SPring-8 using a Rigaku R-AXIS V imaging-plate detector. The crystals were flash-cooled under a cold N_2 gas stream at 100 K without first being transferred to a cryoprotectant solution. Data collection was performed at a wavelength of 1.0 Å with a total oscillation range of 220°. Each diffraction image was taken with an oscillation angle of 1.0° and an exposure time of 5.0 s. The crystal-to-detector distance was set to 180 mm. The data were integrated and scaled using the *HKL*-2000 program package (Otwinowski & Minor, 1997).

3. Results

Single crystals of recombinant RsbP-PAS were obtained using the sparse-matrix method and were suitable for X-ray analysis. X-ray diffraction data for RsbP-PAS were collected to a resolution of 1.6 Å

from flash-cooled crystals. The diffraction data sets were indexed with a primitive monoclinic lattice, with unit-cell parameters a = 55.2, b = 71.7, c = 60.2 Å, β = 92.1°. Systematic absences revealed that the crystals belonged to space group $P2_1$. Crystallographic data and processing statistics are summarized in Table 1. The number of molecules in the asymmetric unit was estimated by the Matthews probability (Kantardjieff & Rupp, 2003). The highest probability of 0.81 was obtained for four molecules in the asymmetric unit and the Matthews coefficient was estimated to be 2.14 Å^3 Da^{-1}, corresponding to a solvent content of 42%. A self-rotation function was calculated with κ angles of 90°, 120° and 180° to examine for the presence of noncrystallographic fourfold, threefold and twofold axes, respectively. Significant peaks only appeared on the κ = 180° section. The crystallographic 2_1 screw axis was observed as the expected strong peak along the b axis. In addition, other strong peaks also appeared on the a^* and c axes and perpendicular to the b axis. These results indicate a tetrameric assembly with 222 point-group symmetry in the asymmetric unit. Alternatively, there may be an arrangement of dimers with twofolds that lie almost parallel to the a^* and c axes.

The structure of RsbP-PAS is expected to maintain a conserved PAS-domain fold. However, molecular-replacement trials using several PAS-domain structures failed. We have prepared and crystallized a selenomethionine derivative of RsbP-PAS and Se-SAD phasing and model building are currently in progress.

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