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Crystallization and preliminary crystallographic analysis of the kinase-recruitment domain of the PP2C-type phosphatase RsbU

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The general stress response of *Bacillus subtilis* provides a protective resistance to a variety of pressures. The key molecule is a subunit of RNA polymerase, σ^B, which confers promoter specificity and is regulated by two signalling modules. Each module comprises protein kinases and phosphatases and 'switch' protein substrates for the kinase and phosphatase. The phosphorylation state of the switch molecules indirectly controls the activity of σ^B. The binding of the kinase RsbT to the phosphatase RsbU stimulates its enzymatic activity towards its substrate, phosphorylated RsbV. To understand how these enzymes interact, thus regulating transcription, crystallization of the kinase-recruitment domain of RsbU in a form suitable for high-resolution structure determination is reported.

1. Introduction

Variations in environmental factors, such as reduced aeration, extremes of temperature and the availability of essential nutrients, restrict microbial growth. As a result, bacteria spend much of their life cycle in stationary phase (Msadek, 1999). The requirement for acclimatization to the environment has forced bacteria to develop a series of complex adaptive responses to stress. One of the initial responses to stress of some Gram-positive bacteria, including *Bacillus subtilis*, is to synthesize a large and diverse family of σ^B-dependent general stress proteins (Hecker & Volker, 2001). σ^B is an alternative RNA polymerase subunit conferring promoter specificity and thus directing gene expression in a defined manner.

σ^B is kept under strict control by the gene products found in the *sigB* operon, which may be functionally divided into 'upstream' and a 'downstream' modules. Each module comprises a serine/threonine protein kinase, a phosphoserine/phosphothreonine protein phosphatase and a 'switch' protein substrate for the kinase and phosphatase (Yang *et al.*, 1996). The phosphorylation state of the switch molecule indirectly controls the activity of σ^B.

In the downstream module, RsbW forms a protein–protein complex with σ^B, thus acting as an anti-sigma factor, but can also act as an ATP-dependent serine/threonine kinase, the substrate for which is the switch protein RsbV (Dufour & Haldenwang, 1994). RsbV has been termed an anti-anti-sigma factor. RsbP is a PP2C-type protein phosphatase, which also encodes a PAS sensory domain, whose substrate is the phosphorylated form of RsbV, RsbV-P (Vijay *et al.*, 2000). The following model has been derived predominantly by genetic studies (*e.g.* Kang *et al.*, 1998; Voelker *et al.*, 1996), aspects of which have more recently been confirmed by biochemical approaches (Delumeau *et al.*, 2002). During normal growth, RsbW utilizes the high ATP concentration to phosphorylate RsbV. RsbW has a lower affinity for RsbV-P than RsbV: thus, in the presence of RsbV-P, RsbW forms a protein–protein complex with σ^B, of the nature $RsbW_2$–σ^B_1, and inactivates it. Under conditions that diminish the ATP concentration, the kinase activity of RsbW is decreased in comparison to the phosphatase activity of RsbU. The ratio of RsbV:RsbV-P rises, RsbW binds preferentially to RsbV and relinquishes σ^B, with the result that σ^B is activated and general stress proteins are synthesized. The activity of σ^B is thus controlled by the phosphorylation state of RsbV.

In some respects, the RsbU–RsbS–RsbT components of the upstream module function similarly to their downstream RsbP–RsbV–RsbW homologues. In the presence of RsbS-P, the RsbT kinase from the upstream module forms a protein–protein complex with the RsbU PP2C-type phosphatase of the downstream module, stimulating its phosphatase activity towards RsbV-P (Kang *et al.*, 1998). The product of this reaction, RsbV, liberates σ^B from its inactive complex with RsbW and induces expression of the general stress proteins. Thus, RsbT links the upstream and downstream modules by binding to the N-terminal domain of RsbU. In order to understand the response of *B. subtilis* to a host of environmental stresses, it is necessary to have a molecular understanding of the control mechanisms that exert their influence on σ^B; thus, we report here the crystallization of

the N-terminal RsbT kinase-recruitment domain of RsbU in a form suitable for high-resolution structural analysis.

2. Materials and methods

2.1. Domain analysis

To determine the domain boundaries, purified RsbU protein was incubated with trypsin in a ratio of 2000:1 at room temperature in a buffer of 20 m*M* Tris–HCl pH 7.5, 10 m*M* $MgCl_2$, 100 m*M* NaCl, 1 m*M* DTT. Proteolysis was quenched after 30 min by the addition of the protease inhibitor PMSF to a final concentration of 1 m*M* and freezing at 193 K. The two domains of the digested protein were separated by SDS–PAGE before peptide-sequencing analysis was performed using an Applied Biosystems 'Procise' 494A liquid-pulse peptide sequencer.

2.2. Molecular cloning, expression and purification of N-RsbU

For high-level protein production, the N-terminal domain of RsbU, *N-rsbU*, was subcloned by PCR methods into the T7-RNA polymerase-based expression vector (Studier & Moffatt, 1986) pET15b (Novagen) and expressed in *Escherichia coli* strain BL21 (DE3). Double-stranded plasmid DNA was prepared and sequenced on both strands to ensure that no PCR-derived mutations had occurred. Cell cultures were grown in YT media containing 100 mg ml^{-1} ampicillin at 310 K. At an optical density of 0.6 at 600 nm, protein expression was induced by the addition of IPTG to a final concentration of 1 m*M*. After 3 h further growth at 303 K, cells were harvested by centrifugation and the cell pellet frozen at 193 K until use.

Cells were resuspended in 50 ml of 50 m*M* Tris–HCl buffer pH 7.4 containing 5 m*M* EDTA, 2 m*M* DTT and one EDTA-free 'Complete' protease-inhibitor cocktail tablet (Hoffman–La Roche) before disruption by sonication. The cell extract was clarified by centrifugation at 19 000 rev min^{-1} for 60 min before loading onto a DEAE Sepharose column (Amersham Biosciences) pre-equilibrated in buffer *A* (50 m*M* Tris–HCl pH 7.4, 2 m*M* EDTA, 1 m*M* DTT). N-RsbU was eluted with a linear gradient of 0–1 *M* NaCl in buffer *A*. Fractions containing N-RsbU were pooled, concentrated and further purified by hydrophobic interaction chromatography with phenyl Sepharose, size-exclusion chromatography using Superdex 75 and finally MonoQ high-resolution ion-exchange chromatography. The fractions containing N-RsbU were pooled, concentrated and dialysed into a buffer of 10 m*M* Tris–HCl pH 7.4 for crystallization.

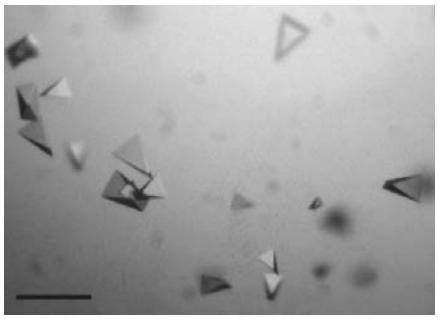

Figure 1
Photomicrograph of typical crystals of N-RsbU. The bar represents 0.3 mm.

2.3. Crystallization of N-RsbU and X-ray analysis

The crystallization conditions for N-RsbU were established by sparse-matrix screening (Molecular Dimensions Ltd) and sitting-drop vapour diffusion. An equal volume of well solution was mixed with 1 µl of N-RsbU concentrated to 10 mg ml^{-1} and placed in the central 'cup' of a 24-well sitting-drop crystallization tray at 291 K. For X-ray data collection, a single crystal was transferred directly into mother liquor supplemented with 30% glycerol to act as a cryoprotectant. The crystal was mounted in a small loop of fine rayon fibre and flash-cooled directly in liquid nitrogen. For X-ray data collection, the crystal was transferred to a stream of nitrogen gas at 100 K (Oxford Cryosystems) with the aid of an extended arc (Oxford Cryosystems). Diffraction data were integrated and scaled using *MOSFLM* (Leslie, 1987) and *SCALA* (Collaborative Computational Project, Number 4, 1994), respectively, and all further data analyses were performed using programs from the *CCP*4 suite.

3. Results and discussion

3.1. Proteolysis of intact RsbU

Analysis of the amino-acid sequence of RsbU predicts that the C-terminal catalytic domain adopts the same fold as PP2C-type phosphatases (Das *et al.*, 1996). In RsbU, approximately 220 amino acids comprise the catalytic domain and 110 residues describe the N-terminal domain. The aspartic acid residues in the active site of human PP2C phosphatase that coordinate one of the two bound manganese ions, Asp38, Asp60, Asp239 and Asp282, are all conserved in RsbU (Asp137, Asp154, Asp274, Asp324). Limited proteolytic digestion of intact RsbU yields two fragments of approximate molecular weight 25 and 12 kDa. Six cycles of amino-acid sequencing of the larger of these two fragments, expected to be the C-terminal catalytic domain of RsbU, gave the sequences LGTKVP (16.5 pmol), VPQEEA (11.3 pmol) and GTKVPQ (6.4 pmol). These sequences indicate that trypsin had cleaved immediately after residues Leu111, Lys115 and Leu112, generating N- and C-terminal fragments with approximate masses of 13 300 and 25 300, respectively, in agreement with those observed by SDS–PAGE. The phosphatase activity of the proteolytically derived C-terminal fragment of RsbU towards RsbV-P is no longer regulated by the N-terminal domain and cannot be stimulated by the presence of RsbT (data not shown). Sequence alignment of RsbU with the RsbX phosphatase, which encodes solely a PP2C-type domain, reveals that trypsin cleavage occurs in RsbU at positions which align closely to the first few amino acids of RsbX; indeed, Leu111 of RsbU aligns with Met1 of RsbX. Based on these results, fragments of *rsbU* which encode the N-terminal (residues 1–112) or C-terminal (118–335) domains of RsbU were amplified by PCR and subcloned into pET15b for high-level protein expression in suitable strains of *E. coli*). Proteins were purified to homogeneity as judged by electrophoretic methods.

3.2. Crystallographic analysis of N-RsbU diffraction

An extensive screen led to crystals of N-RsbU with pyramidal morphology which appeared after 6 d from 12% PEG 20 000 buffered with 100 m*M* MES–NaOH pH 6.5, growing to maximum dimensions of 0.3 × 0.3 × 0.3 mm over a further 8 d (Fig. 1. Complete native data were collected from a single crystal, in low- and high-resolution passes, on beamline ID14-EH2 of the ESRF, Grenoble, France. It is apparent from the diffraction data that reflections along 00*l* of the order $[2n + 1]$ (where n is an integer) were systematically absent, establishing that the crystals belong to space group $C222_1$, with unit-cell parameters a = 42.6, b = 45.3, c = 77.4 Å. These diffraction data were 100% (100%) complete in the resolution range 30–1.6 Å, with an R_{sym} of 0.071 (0.163) and $I/\sigma(I)$ = 4.4 (4.0), where values in parentheses refer to the highest resolution shell, 1.69–1.60 Å. A total of 48 906 observations were reduced to 10 180 unique

reflections. With a single molecule of N-RsbU in the asymmetric unit of 13 080 Da, the Matthews coefficient is 1.43 Å^3 Da^{-1}, corresponding to a solvent content of 14% (Matthews, 1968) and suggestive of extremely tight packing within the crystal.

In the absence of any meaningful sequence homology between N-RsbU and any other protein of known structure, we have prepared selenomethionine-substituted protein in preparation for a multiple-wavelength anomalous dispersion experiment to determine the crystallographic phases for N-RsbU. We hope that the structure of N-RsbU, in combination with our continuing biochemical and genetic experiments, will provide details on a molecular level as to how the activity of a phosphatase of *Bacillus* is regulated by a kinase in a signalling network.

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