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

Expression, crystallization and preliminary X-ray analysis of the periplasmic stress sensory protein RseB from *Escherichia coli*

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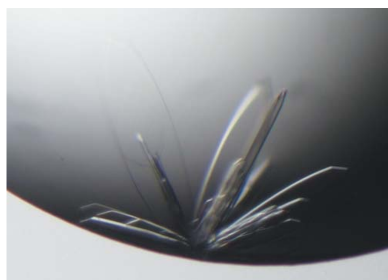
Received 19 May 2006
Accepted 18 July 2006

Sensing external stress in the bacterial periplasm and signal transduction to the cytoplasm are important functions of the CpxAR, Bae and σ^E signalling pathways. In *Escherichia coli*, the σ^E pathway can be activated through degradation of the antisigma factor RseA by DegS and YaeL. The periplasmic protein RseB plays an important role in this pathway by exerting a direct or indirect negative effect on YaeL cleavage efficiency. RseB from *E. coli*, missing the periplasmic signal sequence ($RseB_{\Delta N}$), was cloned, expressed, purified and crystallized. Crystals were obtained in two different forms belonging to space group $P42_12$ (form I) and $C222_1$ (form II) and diffracted to 2.8 and 2.4 Å resolution, respectively. In crystal form I two copies of the protein were located in the asymmetric unit according to heavy-atom analysis, while crystal form II contained three copies.



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1. Introduction

Sensing external stress can cause harmful effects on the cell envelope of Gram-negative bacteria, especially when encountering changing or unfavourable growth conditions. In *Escherichia coli*, three distinct routes of stress-response signalling across the inner membrane have evolved: the σ^E, CpxAR and the Bae pathways (reviewed by Raivio & Silhavy, 2001; Raivio, 2005; Duguay & Silhavy, 2004). All pathways can lead to increased transcription of genes encoding for proteins required for the regeneration of the cell envelope after stress exposure. Best studied is the σ^E pathway, which is known to be induced by signals originating from immature outer membrane proteins (Mecsas *et al.*, 1993; Walsh *et al.*, 2003) or from modified lipopolysaccharides (Tam & Missiakas, 2005). The central determinant in this scenario is the integrity of the anti-σ^E factor RseA, an inner membrane protein that consists of a C-terminal periplasmic domain, a single transmembrane domain and a cytoplasmic σ^E-interacting domain (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997).

The unfolded protein response towards misfolded or unfolded outer membrane proteins (OMPs) is transduced by a controlled successive degradation of the inner membrane protein RseA by the proteases DegS and YaeL (Waller & Sauer, 1996; Ades *et al.*, 1999; Kanehara *et al.*, 2001; Dartigalongue, Loferer *et al.*, 2001). DegS is attached to the periplasmic side of the inner membrane and is inactive under steady-state conditions. Induction of its proteolytic activity can be achieved by the C-termini of unfolded OMPs *in vivo*, which can be mimicked by small synthetic peptides ending with C-terminal OMP sequences *in vitro* (Walsh *et al.*, 2003). After the initial cleavage step at the periplasmic side of RseA, the anti-σ^E factor is further degraded by the integral membrane protease YaeL, which cleaves its substrates within or close to the plane of the membrane (Kanehara *et al.*, 2001; Dartigalongue, Loferer *et al.*, 2001; Brown *et al.*, 2000). A cytoplasmic fragment of RseA is generated that still binds σ^E. The destruction process of RseA is completed by the cytoplasmic ClpXP protease, which finally liberates the sigma factor (Flynn *et al.*, 2004). This proteolytic three-step process (DegS-YaeL-ClpXP) leads to an increased σ^E-dependent transcription of genes that primarily ensure the biosynthesis and assembly of outer membrane proteins and other constituents of the cell envelope to

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell.

	Crystal form I	Crystal form II
Space group	$P42_12$	$C222_1$
Unit-cell parameters (Å)	a = 164.3, b = 164.3, c = 81.5	a = 98.6, b = 200.7, c = 109.7
Beamline	PXII-X10SA (SLS)	ID23-EH1 (ESRF)
Resolution (Å)	20–2.8 (2.9–2.8)	20–2.4 (2.5–2.4)
Wavelength (Å)	0.9790	0.9762
Oscillation angle (°)	1	0.5
Unique reflections	28941 (4291)	40893 (4373)
Completeness (%)	97.9 (92.8)	95.2 (89.4)
Redundancy	12.4 (12.0)	3.7 (3.8)
Average $I/\sigma(I)$	18.8 (2.4)	12.6 (2.4)
$R_{\rm meas}$†	8.7 (50.9)	11.1 (57.7)

† As defined by Diederichs & Karplus (1997).

restore the outer membrane integrity (Rouviere *et al.*, 1995; Dartigalongue, Missiakas *et al.*, 2001; Rezuchova *et al.*, 2003; Rhodius, 2006).

Recently, it was demonstrated that the overall stability of the RseA–σ^E complex is significantly influenced by the presence of RseB, a periplasmic protein of 33 kDa molecular weight. RseB is known to directly interact with the periplasmic part of RseA (De Las Penas *et al.*, 1997) and thus presumably exerts a direct or indirect negative effect on YaeL cleavage efficiency (Ades *et al.*, 1999; Grigorova *et al.*, 2004). Complex formation of RseB and RseA increases RseA stability against otherwise uncontrolled YaeL activity without prior DegS cleavage of RseA. Consequently, when RseB is titrated away from the complex by stress signals, *e.g.* misfolded aggregates of MalE31 (Collinet *et al.*, 2000), YaeL attains the ability to degrade RseA. Thereby, RseB may act as a sensor, in addition to DegS, and makes the pathway sensitive to extracytoplasmic stress signals other than immature OMPs (Grigorova *et al.*, 2004).

Structures of key players of the periplasmic stress response have been reported. Recently, two groups solved the structure of DegS (Wilken *et al.*, 2004; Zeth, 2004). Additionally, structures are available of the cytoplasmic domain of the anti-σ^E factor RseA in complex with σ^E and of a cytoplasmic fragment of RseA bound to the ClpXP adaptor protein SspB (Campbell *et al.*, 2003; Levchenko *et al.*, 2005). RseB does not show obvious homology to proteins deposited in the PDB. Determination of the RseB structure may support to assign its exact function in the extracytoplasmic stress response, particularly regarding the stabilizing effect on RseA and its putative role in sensing incoming stress signals.

We are currently aiming to determine the three-dimensional X-ray structure of $\text{RseB}_{\Delta\text{N}}$ using the MIR approach and extend our efforts to the crystallization of RseB and RseA complexes.

2. Material and methods

2.1. Cloning, expression and purification

The gene encoding the N-terminally truncated version of RseB missing the first 22 residues (*rseB*$_{\Delta N}$) was amplified by PCR from a plasmid with full length *RSEB* (M. Grininger, unpublished results) using the primers *rseb-SS* (5′-CGGGCATATGTCATTAGTGACA-GGTAGCC-3′) and *rseB_C-term* (5′-CGCGGCCACGAGTCATT-GCGCTGCCCCGAAC-3′) and subcloned into the pET-22b(+) vector (Novagen). The recombinant plasmid was transformed into *E. coli* strain BL21 DE3 (Stratagene) and cells were selected on agar plates containing 100 µg ml^{-1} ampicillin. Single colonies were chosen for further inoculation first in Luria–Bertani broth and were subsequently cultivated in Terrific Broth medium with 100 µg ml^{-1} ampicillin. For C-terminally His$_6$-tagged $\text{RseB}_{\Delta\text{N}}$, bacteria were grown at 310 K to an OD_{600} of 0.7 and subsequently cooled to 293 K for expression. Expression was induced by the addition of 0.5 m*M* isopropyl β-D-thiogalactopyranoside (IPTG) and cells were allowed to grow for 4 h. Cells from a 9 l culture were harvested and resuspended in buffer *A* containing 20 m*M* Tris–HCl pH 7.4, 300 m*M* NaCl, 5 m*M* imidazole and 10%(*v*/*v*) glycerol and broken using a French Press. The solution was centrifuged at 40 000*g* for 1 h at 277 K to remove insoluble material and unbroken cells. The supernatant was batch-incubated by end-over-end rotation with Ni–NTA matrix (Ni–NTA Fastflow, Qiagen, Hilden) for 30 min at room temperature. The matrix was extensively washed with buffer *A* and nonspecific binders were eluted with a stepwise gradient of buffer *A* containing 10, 15, 20 and 32 m*M* imidazole. RseB was then eluted with buffer *A* containing 500 m*M* imidazole. The eluate was dialysed against buffer *B* (20 m*M* Tris–HCl pH 7 and 10%(*v*/*v*) glycerol, centrifuged at 40 000*g* for 30 min and applied to anion-exchange chromatography (MonoQ HR5/5, Amersham) using buffer *B*. Pure RseB protein appeared in the flowthrough and was concentrated to 1 or 5 mg ml^{-1} for crystallization trials.

2.2. Crystallization and X-ray crystallographic analysis

Crystallizations were performed by the hanging-drop vapour-diffusion method using commercially available crystal screens from Hampton Research with 1.2 µl protein solution and 0.6 µl reservoir solution. Drops were equilibrated against 500 µl reservoir solution. Derivative crystals were prepared using commercially available Pt salts (Hampton Research). Prior to data collection, $\text{RseB}_{\Delta\text{N}}$ and derivative crystals were directly frozen in liquid nitrogen. Data were collected at beamline ID23-EH1 of the ESRF synchrotron-radiation source (European Synchrotron Radiation Facility, Grenoble, France) and beamline PXII-X10SA at the SLS (Swiss Light Source, Villigen, Switzerland) at 100 K (see Table 1). Diffraction patterns were recorded on 225 mm MAR CCD (PXII-X10SA) and ADSC Q315 detectors (ID23-EH1). Diffraction intensities were integrated using *XDS* and scaled and merged using *XSCALE* (Kabsch, 1988).

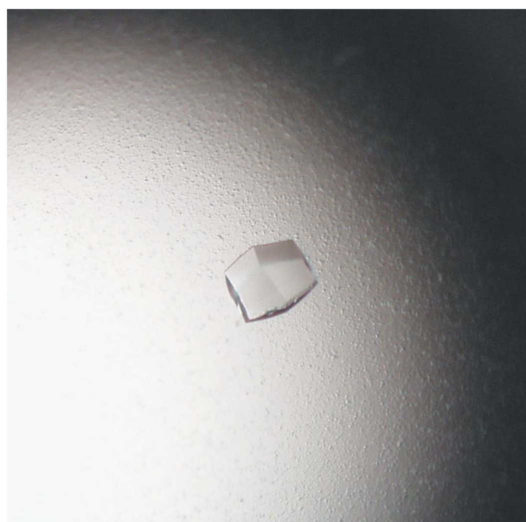

Figure 1
Tetragonal crystal of RseB from *E. coli* with dimensions of 0.25 × 0.25 × 0.05 mm crystallized in space group $P42_12$ (crystal form I).

3. Results and discussion

We initially started to overexpress full-length protein RseB from *E. coli* including the N-terminal periplasmic signal sequence. However, the yield of the C-terminally His_6-tagged wild-type protein was not sufficient for structural studies and we assumed that overexpression of an RseA-binding protein might lead to a self- and downregulating signal in the periplasmic space. This supports the idea that overexpression of RseB results in an increased stability of the RseA–σ^E complex and furthermore indicates the importance of the maintenance of a distinct cellular ratio of these proteins (Collinet *et al.*, 2000). In contrast, for example, full-length DegS, which is inactive under normal growth conditions, was strongly overexpressed in the periplasm (Grininger *et al.*, 2004). In order to direct RseB to the cytoplasm, we deleted the N-terminal signal sequence and cloned two N-terminally truncated versions of RseB using two different vector systems, yielding N- or C-terminally His-tagged proteins. We purified $RseB_{\Delta N}$ *via* a two-step procedure. Interestingly, only the C-terminally tagged construct yielded crystals. In contrast to previous observations where RseB behaved as a monomer (Missiakas *et al.*, 1997), we observed by size-exclusion chromatography that RseB is predominantly dimeric (data not shown).

Figure 2
Crystals of RseB in orthorhombic space group $C222_1$ with dimensions of 0.2 × 0.8 × 0.02 mm (crystal form II).

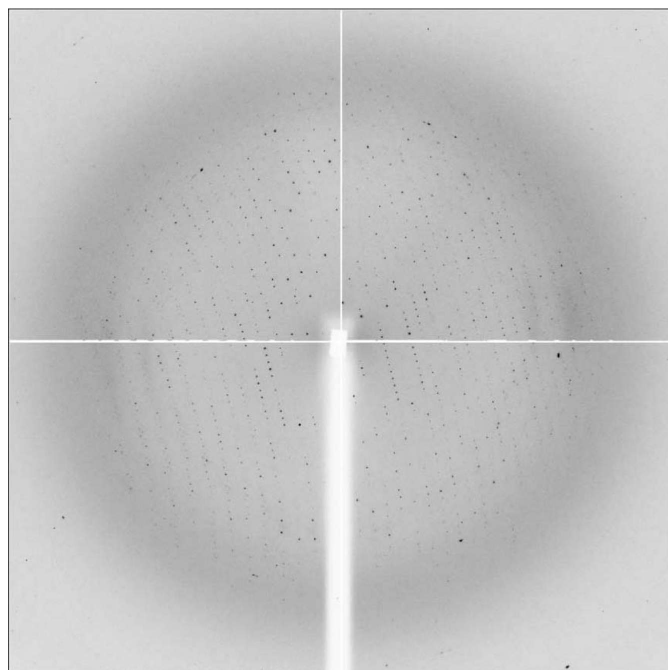

Figure 3
Diffraction image of a tetragonal crystal.

The crystal screens applied to the protein mainly yielded two different crystal forms under several conditions at low protein concentrations (1 mg ml^{-1}). Small tetragonal crystals (form I) appeared after incubation for two weeks at 291 K. These conditions were further refined using additive screens. Under optimized conditions, crystals grew in a solution containing 2.4 *M* sodium malonate pH 7, 0.3 *M* dimethylethylammonium propane sulfonate to final dimensions of 0.25 × 0.25 × 0.05 mm (see Fig. 1). Orthorhombic crystals (form II) were obtained from a solution containing 0.2 *M* magnesium chloride hexahydrate, 0.1 *M* Tris–HCl pH 8.5, 25%(*w*/*v*) polyethylene glycol 3350 and 10 m*M* L-cysteine after three weeks. These crystals grew in clusters and had a sickle-shaped appearance (see Fig. 2). Data-collection statistics of all data sets collected are summarized in Table 1. The tetragonal crystals belong to space group $P42_12$, with unit-cell parameters a = 164.3, c = 81.5 Å, α = 90°, and diffracted to 2.8 Å (see Fig. 3) resolution with an R_{meas} of 8.7% and an $I/\sigma(I)$ of 18.8. The orthorhombic crystals diffracted to 2.4 Å resolution and belong to the orthorhombic space group $C222_1$, with unit-cell parameters a = 98.6, b = 200.7, c = 109.7 Å, α = 90°, an R_{meas} of 11.1% and an $I/\sigma(I)$ of 12.64.

We set out to solve the structure by SIR or MIR techniques using a variety of Pt and Hg derivatives. The best isomorphous phases resulted from labelling of $RseB_{\Delta N}$ type I crystals soaked in reservoir solution containing 0.5 m*M* K_2PtCl_4, $K_2Pt(SCN)_6$ or $[Pt_2I_2(H_2NCH_2CH_2NH_2)_2](NO_3)_2$ with the derivative data collected at the Pt edge. From the analysis of heavy-atom site occupancies, it became obvious that only two monomers of the protein make up the asymmetric unit (Matthews coefficient of 4 Å^3 Da^{-1}; 69.3% solvent content). Crystals of form II are likely to contain three monomers in the asymmetric unit with a solvent content of 53.1% (Matthews, 1968). Phases were determined at 3.2 Å using the program *SOLVE* (Terwilliger & Berendzen, 1999) and were improved using the program *RESOLVE* (Terwilliger, 2000). Manual model building based on the *RESOLVE* result is currently in progress.

The authors would like to cordially thank the staff of the EMBL Grenoble Outstation and the ESRF for maintenance and operation of the ESRF JSBG beamlines. The help of Dr Ehmke Pohl at beamline PX2-X10SA, SLS is also greatly appreciated. The authors also thank Martin Grininger for cloning *RSEB* from genomic DNA and Professor Dieter Oesterhelt, Max Planck Institute of Biochemistry, for continuous interest and support.

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