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Received 5 March 2013

Accepted 19 April 2013

Purification, crystallization and preliminary X-ray analysis of the effector domain of AlsR, an LysR-type transcriptional regulator from *Bacillus subtilis*

AlsR from *Bacillus subtilis*, a member of the LysR-type transcriptional regulator (LTTR) family, regulates the transcription of the *alsSD* operon encoding enzymes involved in acetoin biosynthesis. LTTRs represent the largest known family of transcriptional regulators in bacteria. In this study, AlsR_{82–302}S100A, representing the effector domain, was produced in *Escherichia coli*, purified and crystallized using the sitting-drop vapour-diffusion method in the presence of 2.1 M DL-malic acid pH 7.0 at 293 K. The crystals belonged to space group C2, with unit-cell parameters $a = 142.91$, $b = 74.96$, $c = 94.39$ Å, $\beta = 110.543^\circ$. X-ray data extending to a resolution of 2.6 Å were collected.

1. Introduction

Bacillus subtilis forms acetoin under anaerobic fermentative growth conditions and as a product of the aerobic carbon-overflow metabolism. This reaction requires the enzymes acetolactate synthase (AlsS) and acetolactate decarboxylase (AlsD) encoded by the *alsSD* operon. The *alsSD* promoter was found to be activated in response to acetate accumulation during fermentative growth or the aerobic stationary growth phase and low pH (Renna *et al.*, 1993). The transcriptional regulator AlsR was found to be essential for *alsS–lacZ* reporter gene expression under all growth conditions tested. AlsR belongs to the family of LysR-type transcriptional regulators (LTTRs; Schell, 1993).

The LTTRs were first described by Henikoff *et al.* (1988). Members of the LTTRs are widely distributed within the bacterial and archaeal kingdoms. They are involved in the regulation of metabolic functions such as amino-acid synthesis, sugar catabolism, antibiotic resistance, aromatic compound degradation and virulence (Schell, 1993; Clark *et al.*, 2004; Maddocks & Oyston, 2008; Quade *et al.*, 2011). LTTRs typically have a molecular weight of between 30 and 36 kDa and consist of two domains: an N-terminal DNA-binding domain (~65 residues) with a winged-helix–turn–helix motif and a C-terminal regulatory domain (~200 residues) for effector binding and oligomerization. These two domains are connected via a large linker helix (~30 residues) which is involved in oligomerization of the proteins. LTTRs have been shown to adopt a wide range of oligomerization states, such as dimers (Zhou *et al.*, 2010), tetramers (Muraoka *et al.*, 2003; Monferrer *et al.*, 2010) and even octamers (Sainsbury *et al.*, 2009).

LTTRs bind at promoter sequences composed of a high-affinity regulatory binding site (RBS) with a conserved palindromic sequence T-N₁₁-A and a lower affinity activator binding site (ABS) (Schell, 1993). Binding of the regulator at both sites and its oligomerization controls promoter activity. They can activate or repress the expression of target genes depending on the position of the LTTR box relative to the transcriptional start site (Maddocks & Oyston, 2008). Binding of LTTRs to target promoters is modulated by effectors. Effector binding at the C-terminal effector-binding domain (EBD) results in conformational changes that in turn control the affinity of the N-terminal DNA-binding domain for its target promoter (Schell, 1993). For AlsR, the metabolite acetate or an acidic pH value have been postulated to be the inducing agents.

Structural studies of full-length LTTRs have been hampered owing to their low solubility and their tendency to aggregate (Ezezika *et al.*, 2007; Smirnova *et al.*, 2004). To date, only a few full-length LTTRs have been crystallized. Some effector-domain structures have been described, but only the analyses of BenM and DntR resulted in structures which contained the effector (Ezezika *et al.*, 2007; Devesse *et al.*, 2011). The structure of the effector domain of BenM, one of the closest structural relatives of AlsR, with 36% amino-acid sequence identity, was first solved. The full-length structure of BenM was subsequently determined (Ruangprasert *et al.*, 2010).

In previous studies, amino acids of *B. subtilis* AlsR located in the potential co-inducer binding site were mutated to analyse the functional role of AlsR. A mutation at position 100 from serine to alanine resulted in a complete loss of transcriptional activation *in vivo* and *in vitro*. Moreover, the binding ability of AlsR(S100A) was altered. Binding of the wild-type AlsR protein to an *alsS* promoter fragment containing the RBS and the ABS resulted in the formation of three complexes (complexes I, II and III) that were retarded in electrophoretic mobility shift assays (EMSA). In contrast, the transcription-incompetent AlsR(S100A) mutant failed to form the slowest migrating complex III, indicating its importance for the formation of the higher ordered transcription-competent complex at the DNA (Frädrih *et al.*, 2012).

2. Materials and methods

2.1. Limited proteolysis of AlsR

Chymotrypsin treatment of purified AlsR protein was carried out in AlsR buffer [100 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% (v/v) glycerol]. The AlsR protein concentration was adjusted to 1 mg ml⁻¹ and the enzyme concentration to 20 µg ml⁻¹. Samples were incubated at room temperature and the reaction was stopped at different time points by boiling for 10 min at 363 K in SDS sample buffer. Reaction products were separated using 15% SDS-PAGE stained with Coomassie Blue. N-terminal sequencing of an AlsR fragment with a molecular weight of 26 000 ± 3000 by the Edman degradation method indicated the presence of a fragment starting at residue 82. MALDI-TOF mass-spectrometric analyses determined a molecular mass of 25 015 Da for the C-terminal AlsR protein fragment (Fig. 1).

2.2. Cloning, expression and purification

The corresponding *alsR*₈₂₋₃₀₂S100A gene was generated *via* PCR amplification using the primer pair EH336 (5'-TCCCCCGGGG-

CACAGCGGACGGCCCGC-3') and EH236 (5'-GCGAGCTCTCATGTACCTGCATCACTC-3') with pHRBalsRS100A as a template. The PCR product was digested with *Xma*I and *Sac*I and subsequently ligated into vector pET52bTrx carrying an N-terminal thioredoxin-Strep (Trx-Strep) tag and an HRV-3C protease-cleavage site (Frädrih *et al.*, 2012).

For production and purification of the *B. subtilis* AlsR₈₂₋₃₀₂S100A protein, a 6 l culture of *Escherichia coli* BL21(DE3) cells containing pET52bTrx-AlsR₈₂₋₃₀₂S100A was grown at 310 K in LB medium with the addition of appropriate antibiotics. At an optical density of 0.8 at 578 nm, expression of *alsR* was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by a temperature shift to 298 K. The Trx-Strep-AlsR₈₂₋₃₀₂ S100A fusion protein was expressed overnight. The cells were harvested by centrifugation at 3000g and resuspended in washing buffer (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM dithiothreitol, 1 mM EDTA). Cells were disrupted *via* passage through a French Press at 132.4 MPa and were centrifuged for 1.5 h at 27 000g to remove cell debris. The clear supernatant was applied onto two 6 ml Strep-Tactin columns (IBA, Göttingen, Germany) equilibrated with washing buffer. The columns were washed with at least ten column volumes of washing buffer and the protein was eluted with three column volumes of elution buffer [washing buffer with the addition of 10% (v/v) glycerol,

Figure 2

Purification of *B. subtilis* AlsR *via* gel-permeation chromatography. (a) The chromatography of AlsR₈₂₋₃₀₂S100A resulted in three distinct peaks. The first peak contained aggregated AlsR₈₂₋₃₀₂S100A protein. In the second peak AlsR₈₂₋₃₀₂S100A eluted with a molecular weight of 44 000 ± 5000, indicating dimeric organization of the native protein. The third peak corresponded to elution of the Trx-Strep tag. (b) SDS-PAGE analysis of column fractions 6–16 revealed the presence of AlsR₈₂₋₃₀₂S100A in fractions 9–11 and of the Trx-Strep tag in fractions 12–13. PageRuler Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, USA) was used as molecular-weight standards.

Figure 1
Limited proteolysis of *B. subtilis* AlsR. A Coomassie Blue-stained gel of limited proteolysis of AlsR with chymotrypsin is shown. The arrows indicate the sizes of full-length AlsR and the stable fragments.

10 mM NDSB-195 (Merck, Darmstadt, Germany) and 2.5 mM desthiobiotin]. The purification was monitored by separation of the Trx-Strep-AlsR_{82–302} S100A fusion protein on an SDS–PAGE gel and visualization by Coomassie Blue R250 staining. HRV-3C protease (Merck, Darmstadt, Germany) was used to remove the Trx-Strep tag. Cleavage was performed according to the manufacturer's instructions at 277 K and was monitored *via* SDS–PAGE. Subsequently, the His-tagged HRV-3C protease was removed *via* affinity chromatography on an Ni–IDA column (Machery & Nagel, Düren, Germany). The protein solution was concentrated using Vivaspin 15 10 kDa

Figure 3
Crystal of AlsR_{82–302}S100A protein obtained using 2.1 M DL-malic acid pH 7.0.

Figure 4
Stereographic projection of a *MOLREP* (Vagin & Teplyakov, 2010) self-rotation function calculated in a search for twofold rotation ($\chi = 180^\circ$). Strong contour lines indicate intersections of rotation axes. Lines of latitude as well as lines of longitude are 10° apart. The twofold axis along *y* (red) corresponds to the symmetry of space group *C2*. Two strong NCS axes (purple and blue) are perpendicular to each other and to a third axis which coincides with the crystallographic symmetry. Together, they indicate the presence of D_2 point-group symmetry in the asymmetric unit, which is likely to arise from a dimer of dimers of AlsR_{82–302}S100A. The weaker axes (cyan) might indicate pseudo-twofold symmetries that relate the two domains of AlsR to each other.

Table 1

Data-collection statistics for AlsR_{82–302}S100A.

Values in parentheses are for the outermost resolution shell.

Temperature (K)	100
Wavelength (Å)	0.85
Oscillation range (°)	0.1
Space group	<i>C2</i>
Unit-cell parameters	
<i>a</i> (Å)	142.91
<i>b</i> (Å)	74.96
<i>c</i> (Å)	94.39
β (°)	110.543
Resolution (Å)	20.0–2.6 (2.67–2.60)
Observed reflections	98028 (5634)
Unique reflections	28684 (1988)
Multiplicity	3.42 (2.83)
Data completeness	0.99 (0.939)
$R_{\text{merge}}^\dagger$	0.109 (0.409)
Average $I/\sigma(I)$	8.88 (2.23)

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual measurement and $\langle I(hkl) \rangle$ is the corresponding mean value.

molecular-weight cutoff devices (Sartorius, Göttingen, Germany). Gel-permeation chromatography on a Superdex 75 HR 10/30 column (Amersham Biosciences, Piscataway, USA) pre-equilibrated with GPC buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 10% (v/v) glycerol] was used as the final purification step. Separation was performed at a flow rate of 0.5 ml min^{−1} at 290 K. The following protein standards were used to calibrate the column: albumin (molecular weight 67 000), carboanhydrase (29 000) and lysozyme (14 000). AlsR_{82–302}S100A eluted at 10.30 ml, which corresponds to a molecular weight of 44 000 ± 5000 and suggests that AlsR_{82–302}S100A forms a dimer (Fig. 2). The AlsR_{82–302}S100A fractions were pooled based on purity and were subsequently concentrated to 11 mg ml^{−1}. The AlsR protein concentration was determined using a 2-D Quant kit (Amersham Biosciences, Piscataway, USA) according to the manufacturer's instructions. The purified AlsR protein was stored at 277 K.

2.3. Crystallization of AlsR_{82–302}S100A

Crystallization of AlsR_{82–302}S100A was performed at 293 K in 96-well Intelli-Plates (Art Robbins Instruments, Sunnyvale, USA) using the sitting-drop vapour-diffusion method. Initial crystallization screening was performed using commercially available crystallization kits from Qiagen (Hilden, Germany). The plates were set up by mixing 0.2 µl AlsR_{82–302}S100A protein solution (8 mg ml^{−1}) with 0.2 µl crystallization solution using a HoneyBee crystallization robot (Zinsser Analytic GmbH, Frankfurt, Germany). A small rod-like crystal was noticed in The Classics II Suite condition B11 (2.1 M DL-malic acid pH 7.0; Fig. 3). The crystal reached its final length of approximately 0.26 mm after 13 d. The crystal was flash-cooled in liquid nitrogen prior to data collection.

2.4. Data collection and analysis

X-ray diffraction data were collected from a single crystal on beamline ID23-1 of the European Synchrotron Radiation Facility (ESRF), Grenoble, France equipped with a PILATUS 6M detector. The oscillation range per image was 0.1° and a complete data set was recorded over a total of 180°. Data were processed in space group *C2* to a high-resolution limit of 2.6 Å using the *XDS* package (Kabsch, 2010). Complete data-collection and processing statistics are shown in Table 1.

3. Results and discussion

Limited proteolysis of AlsR resulted in a protein fragment of approximately 26 kDa starting at residue 82 and extending into the C-terminal effector-binding domain (Fig. 1). The mutated effector domain of AlsR, AlsR_{82–302}S100A, was successfully produced in *E. coli* with a Trx-Strep tag and purified *via* affinity chromatography and gel-permeation chromatography (Fig. 2). The AlsR_{82–302}S100A protein was crystallized using the sitting-drop vapour-diffusion method. After 13 d, diffracting crystals with a length of 0.26 mm were obtained (Fig. 3). A data set was collected from a single crystal on the ID23-1 beamline of the ESRF and resulted in 99% completeness to 2.6 Å resolution. The crystals belonged to the monoclinic space group C2, with unit-cell parameters $a = 142.91$, $b = 74.39$, $c = 94.39$ Å, $\beta = 110.54^\circ$. Calculation of the Matthews parameter (Matthews, 1968) suggested the presence of four AlsR_{82–302}S100A molecules in the asymmetric unit ($V_M = 2.37$ Å³ Da^{−1}; solvent content = 0.4822).

The *MOLREP* self-rotation function (Vagin & Teplyakov, 2010) revealed three perpendicular twofold rotation axes, one of which coincides with the crystallographic axis. This indicated the arrangement of four AlsR_{82–302}S100A molecules in the asymmetric unit with D_2 point-group symmetry (Fig. 4). From this result, we deduced a dimer-of-dimers oligomeric composition of AlsR in the crystal. This form was also found for BenM, the closest relative of AlsR within the LTTR family (Clark *et al.*, 2004). The obtained data provide the basis for the thorough understanding of a central regulator of fermentation in the Gram-positive model bacterium *B. subtilis* at the atomic level.

We thank Wolf-Dieter Schubert for helpful discussions. This study was funded by grants from the Deutsche Forschungsgemeinschaft (Ha3456-1/3) and Fonds der Chemischen Industrie.

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