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Crystallisation of the *Bacillus subtilis* sporulation inhibitor SinR, complexed with its antagonist, SinI

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Abstract The transcription factor SinR, a pleiotropic regulator of late growth processes in *Bacillus subtilis*, has been crystallised as a complex with its antagonist SinI, in a form suitable for structural analysis. The SinI:SinR crystals diffract X-rays generated from a rotating copper anode source to 2.3 Å spacing and a complete native dataset has been collected to this resolution limit. The space group of the crystals is $P3_121$ (or its enantiomorph $P3_221$) with cell dimensions $a = b = 60.76$ Å, $c = 87.79$ Å. Assuming that there is a single SinI:SinR heterodimer in the asymmetric unit, the crystals have a V_m of 2.53 Å$^3 \cdot$ Da^{-1}.

Key words: Bacillus subtilis; Sporulation; X-ray diffraction

1. Introduction

Upon encountering nutrient deprivation, *Bacillus subtilis* initiates alternate responses that enable the bacterium to overcome the hostile environmental conditions. These responses include the production of antibiotics, the synthesis and secretion of extracellular degradative enzymes, the development of competence and motility, and ultimately the formation of a spore, which can lie dormant until more favourable environmental conditions are restored [1]. These profound alterations in metabolism and morphology are controlled by an elaborate molecular circuitry that comprises many components and is responsive to a variety of intracellular and extracellular stimuli.

Among the control elements that determine which of the alternative pathways should be followed, are the proteins of the *sin* sporulation inhibition) locus – SinR, a 14 kDa tetrameric DNA binding protein, and SinI, a 6 kDa antagonist of SinR [2–5]. SinR is dual-function regulatory protein whose activity is required for the development of competence and motility but is inhibitory to sporulation and exoprotease production. Although little is known about how SinR regulates competence and motility, the mechanism by which it exerts its negative effects on sporulation and exoprotease production is better understood. SinR overproduction leads to diminished expression of the key sporulation genes *spo0A*,, *spoIIA*, *spoIIE* and *spoIIG* as well as of the gene encoding subtilisin, *aprE* [3,4,6]. For *spo0A*, *spoIIA* and *aprE*, direct binding of SinR to upstream promoter regions has been demonstrated. The locations of the SinR binding sites with respect to the transcription start site varies among these promoters, however, suggesting that different mechanisms of repression may operate.

For entry into sporulation, the activity of SinR must be switched off. This is achieved by the action of the SinI protein which down-regulates SinR activity through the formation of a SinI:SinR protein–protein complex [5]. In its complex with SinI, the SinR tetramer is disrupted and SinR is no longer capable of binding to DNA, and the repression of *spo0A* and the stage II sporulation genes is relieved. Expression of SinI itself is positively regulated by the binding of the phosphorylated form of Spo0A protein to the *sinI* promoter [6]. Therefore, the *sin* operon is likely to be a major control factor for entry into stage II sporulation.

SinI and SinR share sequence similarities over a 39 amino acid residue segment which spans residues 2–40 of SinI and residues 65–104 of SinR. It has been proposed that these residues form the multimerisation surfaces of the two proteins [5]. The N-terminal region of SinR, which contains a putative helix–turn–helix motif, is proposed to form the DNA binding surface [2]. In order to examine these possibilities, we have crystallised the SinI:SinR complex with the aim of solving its three-dimensional structure by X-ray crystallography. The preparation and characterisation of these crystals is described herein.

2. Materials and methods

2.1. Preparation of the SinI:SinR complex

All chemicals, bacterial strains and plasmid vectors were obtained from commercial suppliers. The genes encoding SinI and SinR were cloned into pET22b and pET21d vectors [7] respectively for high level expression in *E. coli* strain BL21 (DE3). The purification of SinR and SinI was based on protocols described previously [3,5]: Q-Sepharose column chromatography was used in place of DEAE-Sepharose for the purification of SinI and the purification of SinR included S-Sepharose ion-exchange chromatography prior to further purification on Heparin-Sepharose. The complex was prepared by mixing equimolar amounts of the two purified Sin proteins in a buffer of 50 mM 2-[*N*-morpholino]ethanesulphonic acid (MES), pH 6.0, 250 mM NaCl. The complex was subsequently resolved from the uncomplexed species by ion-exchange chromatography on Pharmacia Mono-S resin, using a gradient of 250 mM NaCl to 1 M NaCl in 50 mM MES buffer, pH 6.0. More detailed characterisation of the SinI:SinR complex, a heterodimer, will be described elsewhere. For crystallisation experiments, the complex was desalted and concentrated to 10 mg $\cdot$ ml^{-1}, in 10 mM MES buffer, pH 6.0.

2.2. Crystallisation and data collection

Crystallisation conditions were established by a 'sparse matrix' approach to the hanging drop method of vapour diffusion [8]. Briefly, 2 μl of concentrated protein was mixed with an equal volume of well solution and suspended on a siliconised glass coverslip over 1 ml of well solution. Crystals were grown at 18°C, using 300 mM zinc acetate as the precipitant in 50 mM MES buffer pH 6.0, containing 1–4% monomethylether polyethyleneglycol 2000 and 200 mM sodium chloride.

A single crystal was transferred directly into mother liquor that contained 27% ethylene glycol as a cryoprotective agent and then almost immediately mounted in a small loop of fine rayon fibre and flash frozen in a stream of N_2 at 120 K in [9]. Complete native data were collected from a single crystal on an R-AXIS IIC imaging plate detec-

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Fig. 1. A photomicrograph of crystals of the SinI:SinR complex. The approximate dimensions of these crystals are 0.4 mm × 0.1 mm × 0.1 mm.

tor, using CuK_α radiation generated by a Rigaku RU-200 generator, operating at 50 kV and 100 mA. A series of 60 consecutive images were recorded with exposure times of 1 hour per frame as the crystal was rotated about an axis approximately parallel to c* with an oscillation angle of 2° per exposure. Data were processed with DENZO [10] and scaled and reduced with the CCP4 programs, ROTAVATA and AGROVATA [11].

3. Results and discussion

Crystals with a rod-like morphology appear after 2 days and grow to maximum dimensions of 0.6 mm × 0.15 mm × 0.15 mm over a period of about a week (Fig. 1). Analysis of washed and dissolved crystals by polyacrylamide gel electrophoresis under denaturing and non-denaturing conditions reveals the presence of both proteins in the same relative proportions as observed in the SinI:SinR complex in solution (Fig. 2a,b).

Crystals exposed to synchrotron radiation at room temperature diffract strongly at first, but they are sensitive to radiation and lose their capacity to diffract after 2 exposures. Native data were therefore collected from a crystal that had been flash-frozen. Analysis of the diffraction data, which extends to d_{min} = 2.3 Å, shows that the crystals display trigonal symmetry with unit cell parameters of $a = b = 60.76$ Å, $c = 87.79$ Å. Inspection of the axial reflections reveals the presence of one two-fold axis and one three-fold screw axis indicating that the crystals are in the trigonal space group $P3_121$ (or its enantiomorph $P3_221$). 55 790 intensity measurements were recorded of 8 746 unique reflections, a mean redundancy of 6.4. The native data are 99.9% (99.5%) complete in the resolution range 20 Å–2.3 Å, with an R_{sym} of 0.061 (0.169) where R_{sym} is defined as:

$$\Sigma|I - < I >| / \Sigma I$$

and where I is the intensity for an observation of a multiply recorded reflection. 88.1% (72.0%) of the data have $I/\sigma(I)$ of greater than 3 standard deviations above the mean where values quoted in brackets refer to the highest resolution shell (2.42 Å–2.3 Å). Assuming that there is a single SinI:SinR heterodimer (M_r of 18 480) in the asymmetric unit, the V_m of these crystals is 2.53 $Å^3 \cdot Da^{-1}$, and the solvent content is 51%, which is within the observed range for protein crystals [12]. In light of the distinct sequence similarity between SinI and SinR, Patterson self-rotation functions were calculated. However, these do not reveal the presence of non-crystallographic symmetry elements. A search for heavy-metal derivatives of the SinI:SinR complex is now underway.

Determination of the crystal structure of the SinI:SinR complex is a first step towards understanding the mechanism of action of this important regulatory system. It will provide a framework for exploring how SinR binds DNA and forms tetramers. In particular, we are curious to understand how SinI induces disruption of preformed SinR tetramers to form SinI:SinR heterodimers. The sequence similarities between the two proteins are intriguing, implying as they do, that interactions formed between SinR and SinI in their complex closely resemble those formed between pairs of SinR monomers in forming tetramers.

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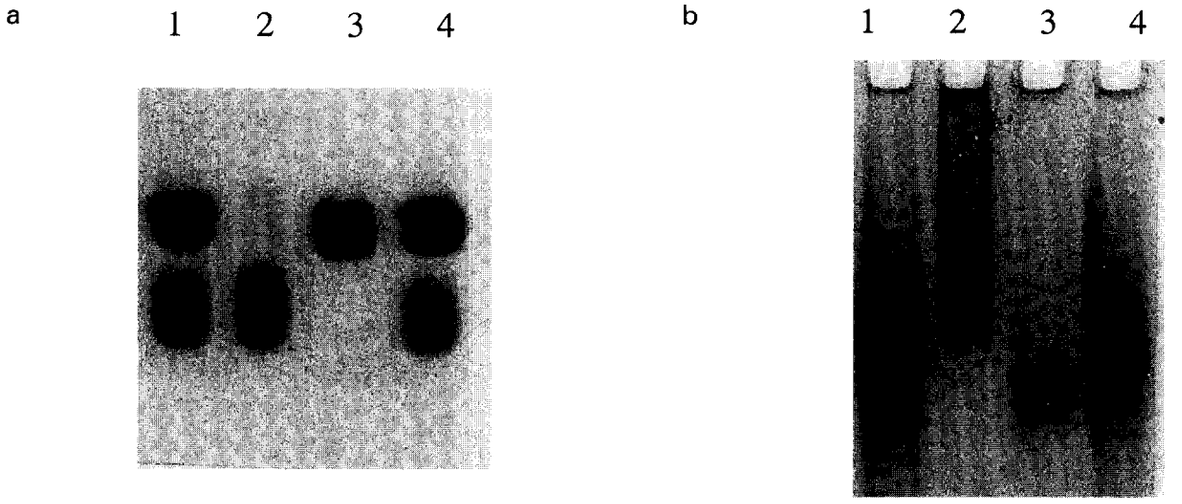

Fig. 2 (a) A 17.5% denaturing polyacrylamide gel, stained with Coomassie brilliant blue showing: lane 1, a sample of the SinI : SinR complex as purified by ion-exchange chromatography; lane 2, a sample of purified SinI protein; lane 3, a sample of purified SinR protein; and lane 4, crystals of the SinI : SinR complex that have been carefully washed to remove extraneous mother liquor and dissolved in water. The somewhat diffuse nature of the bands is in part due to the high acrylamide concentration and the relatively poor staining of SinI. (b) A 7.5% non-denaturing ('native') polyacrylamide gel, stained with Coomassie brilliant blue, showing in lane 1, crystals of the SinI : SinR complex that have been carefully washed to remove extraneous mother liquor and dissolved in water; lane 2, a sample of purified SinR protein; lane 3, a sample of purified SinI protein and lane 4, a sample of the SinI : SinR complex as purified by ion-exchange chromatography. Note the tendency of SinR (pI ~9.2) to aggregate under these native gel conditions, at pH 8.8.

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