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Crystallization of the GTP-dependent transcriptional regulator CodY from *Bacillus subtilis*

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CodY is a GTP sensor that represses transcription of early stationary phase and sporulation genes in *Bacillus subtilis*. As nutrients become limiting, GTP levels fall and CodY-mediated repression is relieved. Crystals of CodY have been grown in the presence and absence of GTP from sodium citrate buffered solutions containing lithium sulfate and diffraction data have been collected extending to 3.5 Å spacing.

1. Introduction

Nutrient limitation and entry into stationary phase are associated with profound changes in gene expression in *Bacillus subtilis*. A network of global regulatory proteins senses the deteriorating cellular environment and the depletion of the cell's metabolic reserves and determines which of a remarkable repertoire of adaptive responses, ranging in severity from the secretion of extracellular macromolecule-degrading enzymes to the formation of a resistant spore, is most suited to survival (Sonenshein, 2000). The specific intracellular signals to which these regulators respond have proved hard to unravel. One of the better understood of these regulators is CodY, which represses during rapid growth a host of genes whose products allow adaptation to nutrient depletion. Interestingly, CodY is activated by GTP (Ratnayake-Lecamwasam *et al.*, 2001), whose intracellular concentration drops as cells pass from rapid growth to stationary phase (Lopez *et al.*, 1981).

CodY was discovered in *B. subtilis* as a factor required for repression of the dipeptide permease (*dpp*) operon during growth on nutrient-rich (glucose/amino acids) media (Slack *et al.*, 1993; Ratnayake-Lecamwasam *et al.*, 2001). CodY also mediates amino-acid repression of genes coding for enzymes involved in amino-acid utilization, Krebs cycle and urea degradation (Fisher *et al.*, 1996; Débarbouillé *et al.*, 1999; Wray *et al.*, 1997; H. J. Kim, C. Jourlin-Castelli, S. I. Kim, K. Tachikawa & A. L. Sonenshein, in preparation). The CodY regulon also includes genes involved in chemotaxis, competence and sporulation (Mirel *et al.*, 2000; Serror & Sonenshein, 1996*b*; Ratnayake-Lecamwasam *et al.*, 2001; Inaoka & Ochi, 2002). In *Lactococcus lactis*, CodY mediates nutritional regulation of genes encoding extracellular and intracellular proteases together with the oligopeptide permease (Guédon *et al.*, 2001). The identification of CodY as a GTP sensor followed from the observation that the effects of decoyinine, a GMP synthetase inhibitor and inducer of stationary-phase genes such as *dpp*, are dependent on CodY (Ratnayake-Lecamwasam *et al.*, 2001). In the presence of GTP in the millimolar concentration range (*i.e.* at concentrations typical in rapidly growing cells), CodY binds to the *dpp* promoter and inhibits transcription *in vitro* (Serror & Sonenshein, 1996*a*; Ratnayake-Lecamwasam *et al.*, 2001). At the lower concentration of GTP found in stationary phase cells (about 400 μM), CodY has a lower affinity for its recognition sequence at various promoter sites on the DNA and *dpp* and other target genes become derepressed.

CodY is a 259-residue polypeptide containing a helix–turn–helix motif for binding to DNA. It also has motifs characteristic of small GTPases, but CodY has a much lower affinity for GTP (K_d in the millimolar range) and there is no evidence that CodY is a GTPase. As CodY is otherwise not related to proteins of known structure, we have sought crystals of CodY to explore the structural basis of GTP regulation of stationary-phase gene expression in *B. subtilis*.

2. Expression and purification

CodY with a C-terminal His_6 tag was expressed from an arabinose-inducible promoter in *Escherichia coli*. To construct plasmid pKT1, the *B. subtilis codY* gene was amplified from chromosomal DNA using as primers oKT1 (5′-GGAGCTCAGGAGGATTATTTATCATGG-3′) and oKT2 (5′-ACGCATGCTTAATGATGATGATGATGATGAGATTTTAGATTTTCT-3′). Primer oKT1 included a *Sac*I site and a sequence corresponding to the ribosome-binding site and start codon region of *codY*. Primer oKT2 encoded the penultimate five

residues of CodY, six histidine residues, a stop codon and an *Sph*I site. The PCR product was digested with *Sac*I and *Sph*I and ligated to pBAD30 (Guzman *et al.*, 1995) that had been cleaved with the same enzymes. A transformant of *E. coli* KS272 strain (Strauch & Beckwith, 1988) proved to express CodY in high amounts after exposure to arabinose.

Cells were grown in LB medium supplemented with ampicillin at 310 K to an A_{600} of 0.6. Expression of recombinant CodY-His_6 was induced by the addition of arabinose to a final concentration of 0.2%. 6–8 h later the cells were harvested by centrifugation and resuspended in ice-cold 20 m*M* Na_2HPO_4 pH 7.5, 0.5 *M* NaCl, 1 m*M* AEBSF (buffer *A*) and 10 m*M* imidazole. The cells were frozen and thawed and then lysed by sonication using an MSE Soniprep 150 ultrasonic disintegrator.

The soluble lysis fraction was loaded onto a nickel-chelation column equilibrated in buffer *A* containing 10 m*M* imidazole. After washing, the column was developed with a 10–500 m*M* imidazole gradient in buffer *A*. CodY fractions were pooled and concentrated by ultrafiltration in an Amicon cell and loaded onto a Superdex 200 (120 ml, Pharmacia) gel-filtration column equilibrated in 20 m*M* Tris–HCl buffer pH 7.5. This two-step purification procedure yielded 100 mg of pure protein suitable for crystallization trials from 2 l of shaking culture. CodY was concentrated to 25–30 mg ml^{-1} in either 20 m*M* Tris–HCl pH 7.5 or 20 m*M* HEPES pH 7.5 and stored as frozen aliquots at 193 K.

3. Crystallization

Dynamic light-scattering measurements were used to establish suitable buffers and additives for use in crystallization. In particular, we noticed that the apparent dimer–tetramer distribution observed on gel filtration was altered to a monodisperse solution of dimers upon addition of GTP. Crystallization experiments were set up at 291 K using the hanging-drop vapour-diffusion method exploiting a variety of screening kits (Brzozowski & Walton, 2001; Jancarik & Kim, 1991). Large single crystals (1.0 × 0.5 × 0.3 mm) of CodY were grown over several days from hanging drops consisting of 1 µl of 10–20 mg ml^{-1} protein and an equal volume of 0.8–1.1 *M* sodium citrate solutions in the pH range 5.6–6.5. The size and the diffraction quality of these crystals were improved by varying the concentration of the precipitant and by using additives including nucleotides and sugars (Figs. 1*a*–1*d*). These *C*-centred monoclinic crystals diffracted to 3.5 Å spacing at room temperature using Cu $K\alpha$ radiation from a rotating-anode generator. The unit cell was large (a = 319, b = 115, c = 170 Å, $\beta = 112^\circ$) and the intensity of the scattering fell sharply after a few exposures. If the solvent content is in the 40–60% range, there will be between 16 and 24 molecules in the asymmetric unit and the Matthews coefficient will be 2.1–3.1 $Å^3\ Da^{-1}$. Attempts to freeze these crystals led to weaker diffraction, increased mosaicity and/or increased anisotropy in the diffraction pattern. Interestingly, when drops containing these crystals were transferred and re-equilibrated over a second reservoir solution containing higher concentrations of sodium citrate, we observed a doubling in the *c* dimension.

More promising crystals grew from drops consisting of 1 µl of 10–20 mg ml^{-1} CodY and an equal volume of 0.05–0.1 *M* sodium citrate pH 5.6 and 1.4 *M* lithium sulfate. The space group and the quality of these crystals

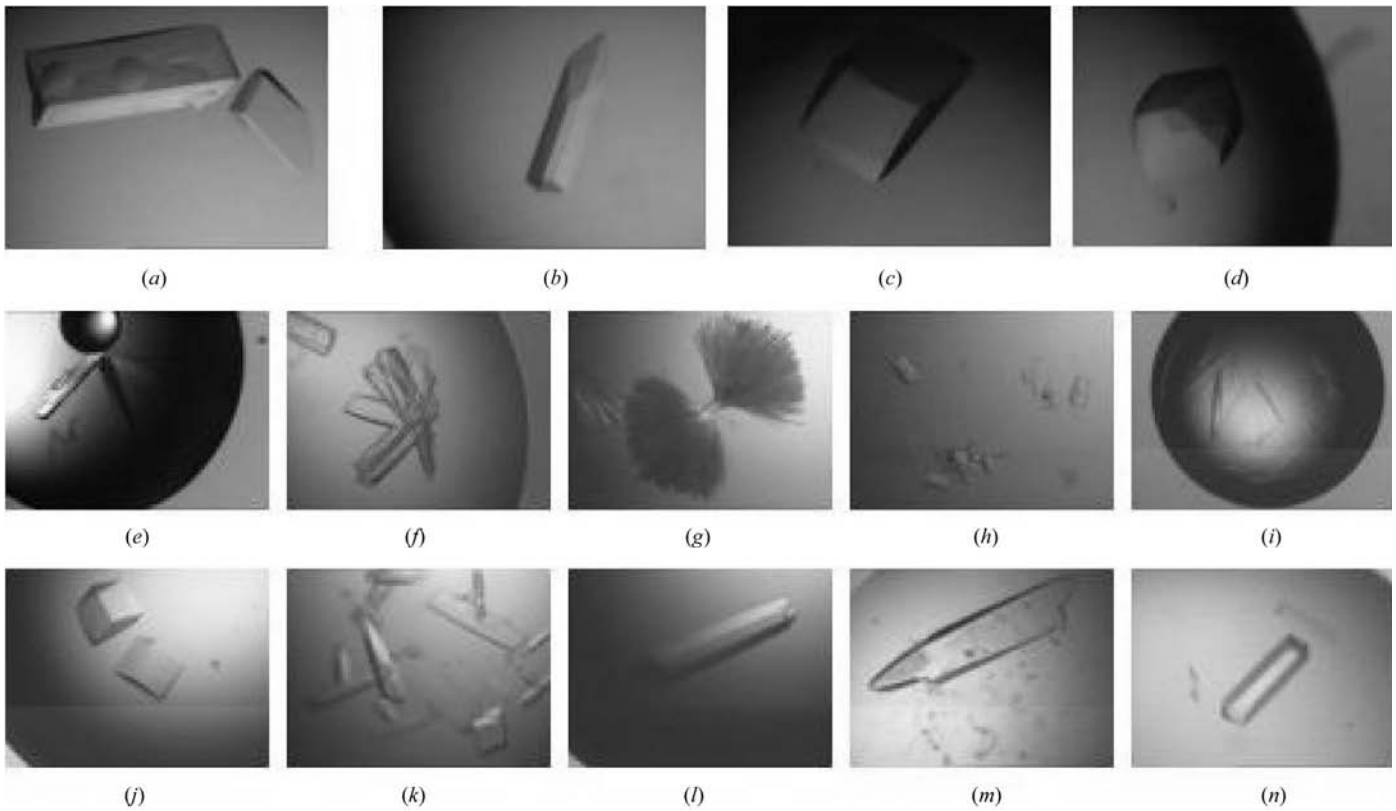

Figure 1
Crystals of CodY grown (*a*–*d*) from sodium citrate pH 5.6–6.5 solutions containing (*a*) 2 m*M* AMP-PNP, (*b*) 2 m*M* GTP, (*c*) 100 m*M* glucose and (*d*) 100 m*M* maltose; (*e*–*n*) from sodium citrate pH 5.6, Li_2SO_4 solutions containing the following additives (*e*) none, (*f*) 2 m*M* AMP-PNP, (*g*) 2% PEG 8000, (*h*) 5–10% glycerol, (*i*) 2 m*M* GTP and 10 m*M* valine, (*j*) 2 m*M* GTP and 10 m*M* isoleucine, (*k*) 5% dioxan, (*l*) 50 m*M* non-detergent sulfabetaine (NDSB), (*m*) 5% dioxan and 50–100 m*M* NDSB, (*n*) 5% dioxan, 50 m*M* NDSB and 5 m*M* GTP. The panel width in (*e*) and (*i*) corresponds to ∼4.5 mm and in (*a*)–(*d*), (*f*)–(*h*) and (*j*)--(*n*) it corresponds to ∼1.5 mm.

in terms of their visual appearance (Figs. 1*e*–1*n*) and the extent of their X-ray diffraction was again dependent on the presence of ligands and additives.

4. Data collection

Crystals grown from sodium citrate/lithium sulfate were transferred to solutions containing mother liquor supplemented with 25% glycerol and flash-frozen in liquid N_2. The crystals were exposed to Cu $K\alpha$ X-ray radiation generated by a Rigaku RU-200 rotating-anode generator and the diffraction pattern was recorded on a MAR Research image plate. Indexing of the diffraction patterns revealed a range of unit-cell parameters, some of them very large indeed.

From one of the crystals grown in the presence of 3% dioxan, 50 m*M* non-detergent sulfobetaine (NDSB) and 5 m*M* GTP (Fig. 1*n*), a complete three-dimensional data set to 3.5 Å spacing was obtained on beamline ID14-2 at the ESRF (λ = 0.933 Å) with a CCD ADSC Quantum 4 detector. The data were processed using *MOSFLM* (Collaborative Computational Project, Number 4, 1994). The crystals belong to space group $P4_122$ or its enantiomorph, with unit-cell parameters $a = b = 111.7$, $c = 119.4$ Å. The data set, containing 9934 unique reflections in the resolution range 25–3.5 Å, has an overall redundancy of 6.6 (4.6), figures in parentheses representing the 3.5–3.7 Å shell. The data are 99.0% (95.2%) complete with a overall $R_{\rm merge}$ (on intensities) of 0.074 (0.765); the mean $I/\sigma(I)$ is 5.9 (1.0). Although the $R_{\rm merge}$ value in the highest resolution shell is large, these data were included because the redundancy in this shell is high. If it is assumed that there are two protomers in the asymmetric unit, the solvent content is 61.5% and the Matthews coefficient is 3.2 $\text{Å}^3\ \text{Da}^{-1}$.

5. Discussion

The CodY crystals we have at present are suitable for structural analysis and higher resolution data sets are a priority. The dependence of the crystal morphology on the presence or absence of GTP suggests that we have crystals of both the unliganded and GTP-liganded forms. The influence of valine and isoleucine on the crystallization outcomes is also interesting in view of the observation that these amino acids stimulate binding of *B. subtilis* CodY to target DNA sites (R. Shivers & A. L. Sonenshein, unpublished work).

The absence of sequence similarity to proteins of known structure indicates that the structure of CodY will not be solved by molecular-replacement methods. We are therefore searching for heavy-atom derivatives to enable structure solution either by isomorphous replacement or anomalous dispersion methods. Structures derived from existing crystals should illuminate the basis of specificity for the GTP ligand and the nature of the conformational changes induced by the binding of the nucleotide. The goal is to understand how GTP binding alters the interactions of CodY with DNA, studies that will require crystals of complexes between CodY and DNA.

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