Complexation of the nickel and cobalt transcriptional regulator RcnR with DNA

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RcnR is a transcription factor that regulates the homeostasis of cobalt and nickel in bacterial cells. *Escherichia coli* RcnR was crystallized with DNA that encompasses the DNA-binding site. X-ray diffraction data were collected to 2.9 Å resolution. The crystal belonged to space group *P*6₁22 or *P*6₅22, with unit-cell parameters $a = b = 73.59$, $c = 157.66$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$.

1. Introduction

Metal homeostasis is important for the survival of bacterial cells. In 2006, a new family of predominantly bacterial metal regulators, the CsoR/RcnR family, was discovered that act as transcription factors (Liu *et al.*, 2007; Iwig *et al.*, 2006). The metal-regulatory CsoR/RcnR proteins bind to a promoter that blocks the transcription of a metal-efflux pump downstream (Higgins & Giedroc, 2014). When the metal availability increases to a threshold level, cognate metal ions bind to the CsoR/RcnR protein, reducing the affinity for DNA and thus enabling transcription of the metal-efflux pump.

Biochemical studies of several members of this family, including CsoR, a copper regulator (Chang *et al.*, 2014; Liu *et al.*, 2007), RcnR, a nickel/cobalt regulator (Iwig *et al.*, 2008; Higgins *et al.*, 2012; Carr *et al.*, 2017; Huang *et al.*, 2018; Huang & Maroney, 2019), and FrmR, a formaldehyde regulator (Osman *et al.*, 2016; Denby *et al.*, 2016), have previously been conducted. Crystal structures of CsoR and FrmR are available in both apo and effector-bound forms (Chang *et al.*, 2014; Dwarakanath *et al.*, 2012; Liu *et al.*, 2007; Porto *et al.*, 2015; Sakamoto *et al.*, 2010; Denby *et al.*, 2016; Osman *et al.*, 2016).

Escherichia coli RcnR is a homotetramer of 40 kDa. Although RcnR binds multiple metals, only the binding of the cognate metals nickel(II) and cobalt(II) inhibits DNA binding (Higgins *et al.*, 2012). The dissociation of RcnR enables the transcription of *rcnA* (encoding a nickel- and cobalt-efflux pump), *rcnB* (encoding an associated periplasmic protein) and the *rcnR* gene itself (Blaha *et al.*, 2011; Blériot *et al.*, 2011; Iwig *et al.*, 2006). Using DNA footprinting, a previous study demonstrated that RcnR binds specifically to DNA sites containing the palindromic sequence 5'-TACT-G₆-N-AGTA-3' (Iwig & Chivers, 2009). This site is repeated in tandem within the intergenic DNA between the *rcnA* and *rcnR* genes. Isothermal titration calorimetry demonstrated that each site binds an RcnR tetramer. DNA footprinting also indicated that RcnR binds nonspecifically to the intergenic DNA flanking the operator sites, leading to DNA wrapping (Iwig & Chivers, 2009). Mass spectrometry coupled with hydrogen-deuterium

Table 1
Macromolecule-production information.

Source organism	<i>E. coli</i>
DNA source	<i>E. coli</i> strain MG1655 genomic DNA
Forward primer	CTATGTCATATGTCTCATACAATCCGTGAT AAACAG
Reverse primer	GTCATACTCGAGTTATTGATATATGAATC CAGCAC
Cloning vector	pET-22b (<i>ampR</i>)
Expression vector	pET-22b
Expression host	<i>E. coli</i> DL41 pLysS
Complete amino-acid sequence of the construct produced	SHTIRDQKLKARASKIQGQVVALKKMLDE PHECAAVLQQIAAIRGAVNGLMREVIKG HLTEHIVHQDELKREEDLDVVLKVLDS YIK

exchange identified Arg14 and Lys17 of the protein as key to DNA binding (Huang *et al.*, 2018).

Presently no structure of RcnR has been solved, and there is no structure available of any CsoR/RcnR family member bound to DNA. Therefore, the details of how these proteins interact with DNA and the conformational change that occurs between the DNA-bound and the metal-bound states is still unknown. In this study, we present the crystallization of an RcnR–DNA complex and report an analysis of the X-ray diffraction data to 2.9 Å resolution.

2. Materials and methods

2.1. Macromolecule production

The plasmid containing a DNA insert to encode *E. coli* RcnR has been described in previous work (Iwig *et al.*, 2008). The plasmid was transformed into DL41 pLysS cells. The cells were cultured in LB medium containing 34 µg ml^{−1} chloramphenicol and 75 µg ml^{−1} ampicillin. The cells were first cultured at 37°C and 220 rev min^{−1} until the OD₆₀₀ reached 0.6–0.8. Following this, 200 mg l^{−1} IPTG was added to induce expression, and the cells were cultured at 20°C overnight. The cells were harvested by centrifugation at 5500g for 20 min and then resuspended in buffer consisting of 300 mM NaCl, 10 mM EDTA, 2 mM TCEP, 10% glycerol, 20 mM HEPES pH 7.0.

The cells were lysed by freeze–thaw at least three times using a water bath at 37°C. 0.17 mg l^{−1} DNase A and one tablet of protease inhibitor per litre of culture was added following cell lysis. The lysed solution was then centrifuged at 15 000g for 30 min to remove cell debris. The supernatant was collected and filtered through a 0.22 µm filter prior to being loaded onto a 5 ml HiTrap Heparin column (GE Healthcare) equilibrated with 300 mM NaCl, 10 mM EDTA, 2 mM TCEP, 10% glycerol, 20 mM HEPES pH 7.0. The column was then washed with 25 ml loading buffer and eluted with 1000 mM NaCl, 10 mM EDTA, 2 mM TCEP, 10% glycerol, 20 mM HEPES pH 7.0. The eluted fraction was then loaded onto a 120 ml SD75 size-exclusion column (GE Healthcare), with a capacity of 1.5 ml per column. The fraction containing RcnR eluted at approximately 60 ml. The eluted fraction was then concentrated and loaded onto a 5 ml HiTrap SP Sepharose column (GE Healthcare) equilibrated with 300 mM NaCl, 10 mM EDTA, 2 mM TCEP, 10% glycerol, 20 mM HEPES pH

Table 2
Crystallization.

Method	Hanging-drop vapor diffusion
Plate type	VDX plates (Hampton Research)
Temperature (K)	277
Protein/DNA concentration	15 mg ml ^{−1} RcnR, 10 mM dsDNA (1 RcnR:1.15 dsDNA)
Buffer composition of protein–DNA solution	150 mM NaCl, 10 mM EDTA, 2 mM TCEP, 10% glycerol, 20 mM HEPES pH 7.0
Composition of reservoir solution	21–27% PEG 400, 0.1 M MES pH 6.5
Volume and ratio of drop	1:1 µl
Volume of reservoir (ml)	0.5

7.0. After washing with 25 ml loading buffer, the fraction containing RcnR was eluted with 500 mM NaCl, 10 mM EDTA, 2 mM TCEP, 10% glycerol, 20 mM HEPES pH 7.0. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

To prepare the RcnR–DNA complex, a 10 mM double-stranded DNA (dsDNA) solution was prepared. The sequence of the DNA used was forward strand, 5′-ATCTACTGGG-GGGTAGTATC-3′; reverse strand, 5′-TGATACTACCCCC-CAGTAGA-3′. The DNA was added to 15 mg ml^{−1} RcnR in 300 mM NaCl, 10 mM EDTA, 2 mM TCEP, 10% glycerol, 20 mM HEPES pH 7.0; the final ratio of tetrameric RcnR (the oligomeric form of RcnR expected to bind DNA based on biochemical studies) to dsDNA was 1:1.15. After mixing, the solution was buffer-exchanged into a solution consisting of 150 mM NaCl, 10 mM EDTA, 2 mM TCEP, 10% glycerol, 20 mM HEPES pH 7.0 in order to decrease the salt concentration.

Initial crystallization screening of the RcnR–DNA complex was performed in the Nanolitre Crystallization Facility at the University of Minnesota by sitting-drop vapor diffusion using a Rigaku CrystalMation system. The initial hit consisted of 30% PEG 400, 0.1 M MES pH 6.5. The optimized crystallization conditions used to generate diffraction-quality crystals were 21–27% PEG 400, 0.1 M MES pH 6.5. The crystals were grown in VDX plates (Hampton Research) by hanging-drop vapor diffusion, combining RcnR–DNA complex solution and well solution in a ratio of 1:1 µl. The crystallization was incubated at 4°C. Crystallization information is summarized in Table 2.

To confirm that both RcnR protein and DNA were present in the crystal, 15 crystals were looped, washed ten times in well solution plus 10% PEG 400 and then dissolved in water. Under these conditions RcnR dissociates from the DNA, enabling the length of DNA in the crystals to be assessed in the absence of bound protein. SDS–PAGE and agarose gel electrophoresis analyses of this solution were then performed.

2.3. Data collection and processing

The crystals were soaked into a cryo-solution consisting of well solution plus 10% PEG 400 and flash-cooled in liquid nitrogen. The diffraction data were collected with an EIGER

16M detector (Dectris) on beamline 23-ID-B at the Advanced Photon Source (APS), Argonne National Laboratory, Argonne, Illinois, USA. The diffraction data were processed by *XDS* (Kabsch, 2010*a,b*) and subsequent data analyses were performed with *CCP4* (Winn *et al.*, 2011) and *Phenix* (Liebschner *et al.*, 2019). The solvent content was calculated using the 2013 kernal estimator method and parameters in *MATTPROB* (Kantardjieff & Rupp, 2003, Matthews, 1968; Weichenberger & Rupp, 2014). Data-collection and processing statistics are summarized in Table 3.

3. Results and discussion

3.1. Crystallization

Previous biochemical studies revealed that the core binding sequence for RcnR is 5'-TACT-G₆-N-AGTA-3' bound by a single RcnR tetramer (Iwig & Chivers, 2009). In order to find the optimal length for crystallization, we screened different DNA constructs from 15 to 23 bp derived from the natural RcnR-binding sequences found in *E. coli* with either a blunt end or an overhang. The best diffraction-quality crystals were obtained using a 20 bp dsDNA construct with an adenine overhang at the 5' end of the forward strand and a thymine overhang at the 5' end of the reverse strand, corresponding to the *E. coli* DNA sequence −75 to −56 and −55 to −74 upstream of the *rcnA* ATG, respectively (encompassing binding site 1; Iwig & Chivers, 2009).

The final crystallization conditions used to generate diffraction-quality crystals used a well solution composed of 21–27% PEG 400, 0.1 M MES pH 6.5. The crystal quality was poor when crystals were grown at 19°C, with clustered crystals appearing after ~6 h. Using a temperature of 4°C successfully slowed down crystal growth and resulted in single diffraction-

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline 23-ID-B, APS
Wavelength (Å)	1.03321
Temperature (K)	100
Detector	EIGER 16M detector
Crystal-to-detector distance (mm)	364.0
Rotation range per image (°)	0.55
Total rotation range (°)	67.1
Exposure time per image (s)	1.1
Space group	<i>P</i> 6 ₂ 22 or <i>P</i> 6 ₅ 22
<i>a</i> , <i>b</i> , <i>c</i> (Å)	73.59, 73.59, 157.66
α , β , γ (°)	90, 90, 120
Mosaicity (°)	0.24
Resolution range (Å)	29.54–2.92 (3.09–2.92)
Total No. of reflections	44201 (6557)
No. of unique reflections	5972 (907)
Completeness (%)	99.2 (96.7)
Multiplicity	7.4 (7.2)
$\langle I/\sigma(I) \rangle$	20.6 (2.5)
<i>R</i> _{meas}	0.050 (0.610)
Overall <i>B</i> factor from Wilson plot (Å ²)	86.24†

† As can be seen from Fig. 1(*d*), there are ice rings that give a spike in the Wilson plot, leading to a high overall *B* factor.

quality crystals (Fig. 1*a*). The crystals appeared after ~1 day and grew to full size in two weeks at 4°C.

To confirm that the crystals contained both RcnR and DNA, crystals were washed and dissolved. SDS-PAGE confirmed the presence of RcnR (Fig. 1*b*) and an agarose gel confirmed the presence of DNA (Fig. 1*c*).

3.2. Data processing and analysis

A diffraction image is shown in Fig. 1(*d*). Data processing indicated that the lattice was primitive and belonged to the Laue group *6/mmm*, with unit-cell parameters *a* = *b* = 73.59,

Figure 1

(*a*) Crystals of the RcnR–DNA complex. Average crystal dimensions were 120 × 60 × 60 μm. (*b*) 12% SDS–PAGE gel stained with Coomassie Blue. Lane 1, RcnR only; lane 2, RcnR + DNA mixture; lane 3, RcnR from dissolved crystals. The 20 kDa band corresponds to a small amount of RcnR dimer. (*c*) 5% agarose gel stained with ethidium bromide. The left lane is a 10 bp DNA ladder and the right lane is from dissolved crystals. (*d*) Diffraction image collected from an RcnR–DNA complex crystal. The outer ice ring lies at a resolution of 3.3 Å.

$c = 157.66 \text{ \AA}$, $\alpha = \beta = 90$, $\gamma = 120^\circ$ (Table 3). The presence of a $6_{1/5}$ screw axis was evident based on systematic absences. As such, the space group is either $P6_122$ or $P6_522$.

Previous biochemical studies demonstrated that RcnR binds DNA as a tetramer (Iwig & Chivers, 2009). Surprisingly, Matthews coefficient calculations support either one RcnR monomer with one dsDNA (solvent content 58%) or one RcnR dimer with one dsDNA (solvent content 39%). For comparison, the previously identified 1:1 relationship between an RcnR tetramer and the dsDNA core-binding sequence would give a solvent content of <1% (Iwig & Chivers, 2009). However, a recent native mass-spectrometry study that used a 24-mer dsDNA demonstrated that even at a 1:1 ratio of DNA to RcnR tetramer, there was a population of 2:1 DNA:RcnR tetramer present (Huang *et al.*, 2018). The 20 bp DNA construct sequence used in crystallization is contained within the 24-mer DNA used by Huang and coworkers, and the ratio of DNA to RcnR tetramer in the crystallization was 1.15:1. As such, it appears likely that the 2:1 DNA:RcnR tetramer observed by native mass spectrometry has been selectively crystallized in this study, with the asymmetric unit containing one DNA molecule and half an RcnR tetramer, with the tetramer generated by a crystallographic twofold axis. However, we cannot rule out that the crystals contain a non-physiological dimeric form of RcnR bound to the DNA, but this seems unlikely. The known crystal structures of the other protein family members CsoR (Chang *et al.*, 2014; Dwarakanath *et al.*, 2012; Liu *et al.*, 2007; Porto *et al.*, 2015; Sakamoto *et al.*, 2010) and FrmR (Denby *et al.*, 2016; Osman *et al.*, 2016) clearly show that the tetramer is a dimer of dimers. Studies of CsoR/RcnR family members suggest that the DNA

binds across the dimer–dimer interface of the disk-shaped tetramer, requiring the presence of all four monomers (Fig. 2; Chang *et al.*, 2011, 2014; Tan *et al.*, 2014). As such, it seems unlikely that a dimer would be an effective DNA binder, particularly as the most extensive monomer–monomer interactions that would represent the most likely dimeric assembly are those that define the disk thickness, not the disk face (Liu *et al.*, 2007; Sakamoto *et al.*, 2010).

To further investigate symmetry within the crystal, a native Patterson map was examined. Along the w direction there are three consecutive peaks separated by an interval of approximately 0.07 (11 $\text{\AA}$; Fig. 3*a*), with the third peak being twice the height and possibly composed of two close peaks overlaid. The distance between Patterson peaks is consistent with the spacing of nearest-neighbor α -helices in the RcnR/CsoR family, which are separated by distances of 10–12 $\text{\AA}$ (Fig. 3*b*). Therefore, this feature may be an operator corresponding to the translation of multiple parallel α -helices in the RcnR structure, a similar pattern to those arising from β -strands within the β -helix domain of maltose *O*-acetyltransferase (Lo Leggio *et al.*, 2001). If this is correct, then the Patterson peaks suggest that the α -helices lie perpendicular to the c axis within the crystal.

Despite this information, phasing by molecular replacement (MR) is challenging for a number of reasons. The tetrameric RcnR protein in the crystals has a molecular weight of 40.5 kDa and the dsDNA used in crystallization has a molecular weight of 12.5 kDa. As half a tetramer is present per dsDNA, this gives a weight ratio of 62% protein to 38% dsDNA within the crystal. This makes it difficult to distinguish between MR solutions based on one component alone. Despite *E. coli* RcnR having a sequence identity of 48% to the known crystal structure of *Salmonella enterica* serovar Typhimurium apo FrmR (PDB entry 5lcy), correctly placing a model based on the Patterson is nontrivial (Osman *et al.*, 2016). Structural evidence suggests that the helices within the tetramer can change their relative orientation (Denby *et al.*, 2016; Porto *et al.*, 2015). Thus, obtaining clear solutions involving small all-helical monomers where DNA binding may cause additional structural changes is challenging. Previous studies suggest that RcnR recognizes a transition between A-type and B-type DNA in which the central G tract has A-form DNA character and the flanking inverted repeats contain B-form DNA (Iwig & Chivers, 2009). Therefore, obtaining a good dsDNA model for MR and generating clear phasing solutions are also difficult. Hydroxyl-radical footprinting demonstrates that RcnR binds to one face of the DNA interacting with the minor groove in the TACT repeats and the major groove of the G tract. Finally, the 2.9 $\text{\AA}$ resolution of the data adds to the challenge of MR phasing.

As such, we are pursuing *ab initio* phasing using anomalous diffraction. *E. coli* RcnR monomers have two methionine residues in a total number of 89 residues, so the incorporation of selenomethionine into the protein (provided that the methionine side chains are ordered) should provide sufficient signal to phase. In addition, we are combining this with dsDNA in which three DNA nucleotides of each strand are

Figure 2
Structure of *Streptomyces lividans* apo CsoR (PDB entry 4adz; Dwarakanath *et al.*, 2012) colored by monomer. Interactions between the red and blue (or green and yellow) monomers are more extensive than those between the red/blue and green/yellow dimers. Areas of metal ion binding in family members are indicated by broken circles. dsDNA is proposed to bind across the face of the tetramer in a northwest to southwest orientation based on electrostatic surface potentials (traversing from the N-termini of the red/yellow dimer at the front or the blue/green dimer behind; Chang *et al.*, 2014; Denby *et al.*, 2016; Dwarakanath *et al.*, 2012; Osman *et al.*, 2016; Porto *et al.*, 2015). This figure was generated using PyMOL v.1.8.05 (Schrödinger).

Figure 3

(a) Native Patterson map section ($u = 0$). Contours are drawn at 1σ . Peaks are labeled and their distances to the origin are shown in Å. (b) A slice through the homotetrameric structure of the *E. coli* formaldehyde-sensing transcriptional repressor FrmR showing the arrangement of helices (PDB entry 5lbm; Denby *et al.*, 2016). FrmR is the closest homolog of RcnR with a known structure. Each monomer is individually colored. Example FrmR helix–helix distances are shown in Å, demonstrating the spatial relationship between pairs of helices and correspondence to the RcnR native Patterson peaks. The equivalent of a dimer is expected in the asymmetric unit. This figure was generated using PyMOL v.1.8.05 (Schrödinger).

substituted with bromine derivatives. Multiple constructs with different sets of substituted nucleotides are being synthesized to find constructs that minimize interfering effects on DNA conformation that affect RcnR binding and crystal packing.

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