STRUCTURAL BIOLOGY
COMMUNICATIONS

ISSN 2053-230X

Structure of the response regulator ChrA in the haem-sensing two-component system of *Corynebacterium diphtheriae*

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Received 19 March 2015

Accepted 21 May 2015

Edited by I. Tanaka, Hokkaido University, Japan

Keywords: transcription factor; X-ray crystallography; haem; helix–turn–helix; phosphorylation.**PDB reference:** ChrA, 4yn8**Supporting information:** this article has supporting information at journals.iucr.org/f

ChrA is a response regulator (RR) in the two-component system involved in regulating the degradation and transport of haem (Fe–porphyrin) in the pathogen *Corynebacterium diphtheriae*. Here, the crystal structure of full-length ChrA is described at a resolution of 1.8 Å. ChrA consists of an N-terminal regulatory domain, a long linker region and a C-terminal DNA-binding domain. A structural comparison of ChrA with other RRs revealed substantial differences in the relative orientation of the two domains and the conformation of the linker region. The structural flexibility of the linker could be an important feature in rearrangement of the domain orientation to create a dimerization interface to bind DNA during haem-sensing signal transduction.

1. Introduction

Two-component signal transduction systems (TCSs) are ubiquitous in bacteria, fungi and plants. These systems can sense a variety of environmental changes in stimuli such as osmolarity, redox state, envelope stress, concentrations of metal ions *etc.* in order to adapt the cells to new environments by regulating the expression of their related genes (Stock *et al.*, 2000). In response to stimuli, the sensor histidine kinase (HK) in a TCS uses ATP to autophosphorylate its conserved His residue, and the phosphoryl group is then transferred to a conserved Asp residue in a cognate response regulator (RR). The phosphorylated RR is dimerized and then can bind to DNA as a transcriptional factor to promote its target gene.

In the present study, we focused on the RR of a haem-sensing TCS in the Gram-positive bacterium *Corynebacterium diphtheriae*, which causes respiratory disease. *C. diphtheriae* has two proteins in its haem-sensing TCS, ChrS and ChrA, which are responsible for iron uptake and utilization (Schmitt, 1999), since the pathogen acquires iron from the haem (Fe–porphyrin compound) in the haemoglobin of a host for the expression of the diphtheria toxin as well as for cell survival (Litwin & Calderwood, 1993; Schmitt, 1997; Schmitt & Holmes, 1991; Wandersman & Delepelaire, 2004). ChrS is a sensor HK (Ito *et al.*, 2009) and ChrA is a cognate RR. The activated ChrA specifically binds to either the *hmuO* or *hrtAB* promoter regions to promote the transcription of the genes that encode the haem oxygenase and the ABC-type haem exporter, respectively (Bibb *et al.*, 2005; Burgos & Schmitt, 2012).

Sequence analysis of ChrA shows that it belongs to the NarL/FixL subfamily of RRs consisting of N-terminal regulatory and C-terminal DNA-binding domains (Burgos & Schmitt, 2012). Several full-length crystal structures of NarL/FixJ family members have been determined and showed conserved folds in each domain, but with various relative

orientations of the two domains (Baikalov *et al.*, 1996; Leonard *et al.*, 2013; Milani *et al.*, 2005; Park *et al.*, 2013). The C-terminal DNA-binding domain contains a single helix–turn–helix (HTH) motif in the middle of a four-helix bundle (Baikalov *et al.*, 1996). The crystal structures of phosphorylated and unphosphorylated forms of the regulatory domain of FixJ (FixJn), which is involved in regulating the nitrogen-fixation gene, revealed the structural basis for the conformational change of the residues of the regulatory domain during phosphorylation (PDB entries 1dck and 1d5w; Birck *et al.*, 1999). In addition, the structure of the DNA-bound form of the C-terminal domain of NarL revealed details of the DNA-recognition and dimerization interface (PDB entry 1je8; Gao & Stock, 2009; Maris *et al.*, 2002). Despite these revelations on the structures of isolated domains, structural details of how the phosphorylation of an N-terminal regulatory domain regulates the behaviour of the C-terminal DNA-binding effector domain remain unclear.

In the present study, we report the full-length structure of ChrA at 1.8 Å resolution in an unphosphorylated state. The structure showed domain orientations and a linker conformation that differed from those of other members of the NarL/FixJ family. Structural comparisons of phosphorylation sites and interdomain interactions provided further insight into the activation mechanisms of ChrA on domain rearrangement and conformational change in the linker region, which would create an interface for dimerization and DNA binding.

2. Materials and methods

2.1. Expression and purification of ChrA

The ChrA gene was isolated from a *C. diphtheriae* C7(–) genome that was provided by Dr Iwaki (National Institute of Infectious Diseases, Japan) and was then ligated into modified pGEX6P-1. An expression plasmid (pChrA-C7) was introduced into *Escherichia coli* strain BL21(DE3)pLysS and the transformed cells were spread onto an LB agar plate with 50 mg ml^{−1} ampicillin and 34 mg ml^{−1} chloramphenicol. A single colony was inoculated into the LB medium and grown *via* a rotary shaker at 37°C overnight. The cells were transferred into 1 l Terrific Broth and grown at 25°C. The expression of ChrA was induced using isopropyl β-D-1-thiogalactopyranoside and the cells were further grown overnight. The cultured cells were harvested by centrifugation (8000g, 15 min, 4°C) and stored at −80°C. The cell pellet was resuspended in lysis buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 5 mM EDTA), disrupted by sonication and centrifuged at 40 000 rev min^{−1} (Hitachi P45AT rotor) for 30 min at 4°C. The supernatant was purified using a GStrap FF column and an ÄKTA system (GE Healthcare). The column was washed with wash buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA) followed by elution buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 10 mM reduced glutathione). The eluted sample was digested by PreScission protease (GE Healthcare) during dialysis in dialysis buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.01% (v/v) Tween-20] both with and

Table 1

X-ray data-collection and refinement statistics for ChrA.

Values in parentheses are for the highest resolution shell.

Data collection	
X-ray source	BL41XU, SPring-8
Wavelength (Å)	1.0000
Resolution (Å)	30.0–1.80 (1.86–1.80)
Space group	<i>I</i> ₄ 22
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 84.88, <i>c</i> = 169.32
<i>R</i> _{merge} [†] (%)	9.6 (41.8)
<i>I</i> / <i>σ</i> (<i>I</i>)	30.0 (7.8)
No. of observed reflections	404586
No. of unique reflections	28694
Multiplicity	14.1 (14.3)
Completeness (%)	98.1 (97.0)
Wilson <i>B</i> factor (Å ²)	14.5
Structure refinement	
<i>R</i> _{free} / <i>R</i> _{work} [‡] (%)	20.2/17.6
Root-mean-square deviations	
Bonds (Å)	0.017
Angles (°)	1.76
Mean <i>B</i> factor (Å ²)	
Overall	16.9
All atoms	17.0
Protein	15.6
Waters	26.3
Ramachandran plot	
Favoured (%)	99.0
Allowed (%)	1.0
Disallowed (%)	0

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity for reflection hkl and $\langle I(hkl) \rangle$ is the average intensity over symmetry-related and equivalent reflections. [‡] $R_{\text{work}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$, where F_{obs} and F_{calc} are the observed and calculated structure-factor amplitudes for reflection hkl . R_{work} was calculated for 95% of reflections used for structure refinement. R_{free} was calculated for the remaining 5% of reflections that were randomly selected and excluded from refinement.

without Tween-20. The dialyzed sample was loaded onto the GStrap column and the flowthrough fraction was collected and concentrated with Centriprep YM-10 (Merck Millipore) to a concentration of 10 mg ml^{−1}. The purified protein contained residues 2–199 of ChrA and five extra residues (Gly-Pro-Leu-Gly-Ser) of a cloning artifact instead of the initial methionine at the N-terminus of ChrA.

2.2. Analytical size-exclusion chromatography

ChrA solution (10 mg ml^{−1}) was analyzed using a Superdex 200 5/150 GL column (GE Healthcare) at a flow rate of 0.1 ml min^{−1} using an injection volume of 15 µl in buffer consisting of 25 mM Tris–HCl pH 7.8, 150 mM NaCl. The column was calibrated using gel-filtration standards from Bio-Rad. The apparent molecular weight was determined using the elution volume of the protein and the calculated calibration curve.

2.3. Crystallization, X-ray data collection and structure determination

The crystallization of purified ChrA was screened using commercially available screening kits. A hit condition, No. 20 from Crystal Screen 2 (Hampton Research), was optimized to 0.1 M MES pH 6.5, 1.6–1.7 M magnesium sulfate using the sitting-drop vapour-diffusion method at 4°C. The crystals belonged to space group *I*₄22, with unit-cell parameters

$a = b = 84.88$, $c = 169.30$ Å. The crystals were cryoprotected by transferring them into a buffer consisting of the reservoir solution with an additional 30% glycerol. X-ray diffraction data were collected on BL41XU at SPring-8, Japan. The diffraction images were collected at 1 Å wavelength as a total of 170 images in rotation steps of 1°. All data were integrated and scaled using the *HKL-2000* program suite (Otwinowski & Minor, 1997). The initial phase was obtained *via* the molecular-replacement (MR) method. The coordinates of the NarL receiver domain from *Mycobacterium tuberculosis* (PDB entry 3eul; Schnell *et al.*, 2008) gave the best results as a model in an automatic search for homologous models using

BALBES (Long *et al.*, 2008). The MR phase was improved by *DM* (Cowtan, 1994) and was then used to build the model using *ARP/wARP* (Langer *et al.*, 2008). The model was manually rebuilt by *Coot* (Emsley & Cowtan, 2004) and refined by *REFMAC5* (Murshudov *et al.*, 2011) using the 1.8 Å resolution data. The statistical results of the X-ray data collection and structure refinement are shown in Table 1.

3. Results

3.1. Overall structure of ChrA

Full-length ChrA crystallized in space group $I4_122$ with one monomer in the asymmetric unit. The packing arrangement in the crystal implied no possibility of dimer formation of ChrA when using our crystallization conditions. Size-exclusion chromatography also suggested that ChrA is a monomer in solution (Fig. 1). ChrA consists of two domains: an N-terminal regulatory domain (ChrAn) consisting of residues 1–116, which contains the conserved Asp (Asp50) as the phospho-acceptor residue, and a C-terminal DNA-binding domain (ChrAc) consisting of residues 141–199 as an effector domain (Fig. 2). The two domains are connected by a long linker (117–140) that contains a short β -strand (β_6 ; 119–120) followed by a helix (α_6 ; 122–131) and a loop of nine residues (132–140). The β_6 strand in the N-terminal portion of the linker region interacts with the β_5 strand of the regulatory domain through hydrogen bonds to join the β -sheet in the core of the regulatory domain. The α_6 helix in the linker also makes contact with the regulatory domain through hydrophobic and hydrophilic interactions.

The most characteristic feature observed in our ChrA structure was the linearly extended structure of the loop in the C-terminal portion of the linker region, which is characterized by an absence of interactions between the regulatory and the DNA-binding domains. Indeed, the unique structural characteristics of ChrA are clearly manifested in structural comparisons with the overall structures of other structurally characterized full-length RRs of NarL/FixJ family members (Fig. 3): in *Escherichia coli* NarL is involved in nitrate/nitrite signal transduction (39% sequence identity; PDB entry 1rnl; Baikalov *et al.*, 1996), in *Staphylococcus aureus* VraR is involved in vancomycin resistance-associated responses (34% sequence identity; PDB entry 4gvp; Leonard *et al.*, 2013), in *Streptococcus pneumoniae* spr1814 is involved in the gene regulation of antibiotic transporters (26% sequence identity; PDB entry 4hye; Park *et al.*, 2013) and in *Pseudomonas fluorescens* StyR is involved in styrene catabolism (34% sequence identity; PDB entry 1yio; Milani *et al.*, 2005). Each of the regulatory and DNA-binding domains in the RRs of this family shows topologically similar structures (see below), but the relative orientations of the two domains and the conformations of the linker regions differ substantially among them. For example, NarL and VraR have an L-shaped linker which consequently creates a widely extended interface between the two domains. In spr1814 the relative orientation of the two domains and the conformation of the N-terminal portion of

Figure 1

Molecular-weight estimation of ChrA using size-exclusion chromatography. (a) Size-exclusion chromatograms of ChrA (red line) and standard proteins (black line). The ChrA solution was applied onto a Superdex 200 5/150 GL column (GE Healthcare) pre-equilibrated with a buffer consisting of 25 mM Tris–HCl pH 7.8, 150 mM NaCl. The standard proteins consisted of a mixture of thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B₁₂ (1.4 kDa). (b) The estimated molecular weight of ChrA (22 kDa) is consistent with the molecular weight based on the amino-acid sequence (21.5 kDa).

the linker region, which includes $\beta 6$ and $\alpha 6$, are very similar to those of ChrA when there is interdomain contact. In the case

of StyR, the linker region separates the two domains, while helix $\alpha 5$ is directly connected to helix $\alpha 6$ without a connecting

Figure 2

Overall structure of response regulator ChrA shown as a stereo diagram. The N-terminal regulatory domain, linker region and C-terminal DNA-binding domain are coloured purple, blue and green, respectively. The DNA-recognition helix $\alpha 9$ in the DNA-binding domain is coloured orange. The phospho-acceptor Asp50 and the bound sulfate ion in the regulatory domain are shown as stick models. The Mg^{2+} bound to Asp50 is shown as a green sphere.

Figure 3

Structural comparison of the domain orientations in the NarL/FixJ family. (a) ChrA, (b) NarL (PDB entry 1rn1), (c) VraR (PDB entry 4gvp, chain A), (d) spr1814 (PDB entry 4hye, chain A) and (e) StyR (PDB entry 1yio) are represented in the same colour scheme as in Fig. 1. The regulatory domains of each protein are aligned in the same orientation.

Figure 4
Phosphorylation site of ChrA. (a) Superposition of the regulatory domains of ChrA (purple) and FixJ (cyan). The phospho-acceptors Asp50, Lys99 and Thr77 (purple) of ChrA interact with the sulfate ion (orange). In FixJn, the phospho group linked to Asp54 (green) is stabilized by interaction with Thr82 and Lys104 (blue). (b) Structure of the phosphorylation site of the N-terminal regulatory domain of ChrA. Sulfate and Mg²⁺ ions are bound to the conserved Asp50. The sulfate ion is stabilized by interaction with Gln52, Thr77, Thr81 and Lys99. Mg²⁺ is present in an octahedral coordination geometry involving the side chains of Asp8, His10 and Asp50 and three water molecules.

loop. This irregular linking structure of StyR results in a relative domain orientation that is distinct from that of ChrA. These structural comparisons showed that our ChrA structure is uniquely able to provide novel information about the functional mechanism of the RR in a TCS.

3.2. C-terminal DNA-binding domain of ChrA (ChrAc)

ChrAc consists of 58 residues and folds into an apparent four-helix bundle ($\alpha 7$ – $\alpha 10$; Fig. 2). The interfaces between these helices consist mostly of hydrophobic residues, and $\alpha 8$ – $\alpha 9$ form a conventional helix–turn–helix (HTH) motif for DNA binding. We predicted that when the phosphorylation-activated ChrA binds to DNA as a dimer, the recognition helix in the HTH motif would be inserted into the major groove and helix $\alpha 10$ and the $\alpha 7$ – $\alpha 8$ loop region would make up the dimerization interface (Maris *et al.*, 2002). In our ChrA structure neither the dimerization interface nor the DNA-binding sites were blocked by either the regulatory domain or the linker region.

The structure of ChrAc is very similar to those of other members of the FixL/NarL family, such as NarL, VraR, spr1814 and StyR, which can be superimposed with r.m.s.d.s for C α atoms of 1.6, 0.8, 1.2 and 1.4 Å, respectively. Like ChrA, the crystal structure of StyR showed that the DNA-binding and dimerization interface of the DNA-binding domain are exposed to the solvent. In contrast, NarL and VraR exhibited access to the HTH that was sterically blocked by their N-terminal regulatory domains. The ChrAc structure observed in our ChrA crystal presumably reflects the structural characteristics before the dimerization of an RR when binding to DNA.

3.3. The N-terminal regulatory domain of ChrA (ChrAn)

ChrAn also exhibits the conserved tertiary structure of an RR with an α/β fold (Fig. 2). However, by comparison with a typical regulatory domain structure (*e.g.* FixJn; five α -helices and one β -sheet consisting of five β -strands; PDB entry 1d5w; Birck *et al.*, 1999), the helix $\alpha 2$ region of ChrA forms a loop structure rather than a helix owing to the deletion of four residues (Fig. 4a).

The phosphorylation site of ChrAn, the conserved Asp50, is located at the C-terminal end of strand $\beta 3$ of the regulatory domain and is exposed to the solvent in the present structure (Fig. 4b). In our high-resolution structure of ChrA, the Asp50 carboxylate, together with one water molecule, bridges the distance (4 Å) between the sulfate ion (SO₄²⁻) and the magnesium ion (Mg²⁺). Mg²⁺ is present in an octahedral coordination geometry involving the side chains of Asp8, His10 and Asp50 and three water molecules, where Asp8 is also highly conserved as an acidic residue in the NarL/FixJ family. On the other hand, the binding of SO₄²⁻ is stabilized through interactions with four residues (Gln52, Thr77, Thr81 and Lys99), which, except for Thr81, are also highly conserved. As shown in Fig. 4(a), the SO₄²⁻-bound state of ChrAn is reminiscent of phosphorylated FixJn (Birck *et al.*, 1999). The position of SO₄²⁻ and its interaction with Thr77 and Lys99 in ChrA are similar to those observed in the phosphorylated form of FixJ, *i.e.* the position of the phosphate group (–PO₄) and its interaction with Thr82 and Lys104 (Birck *et al.*, 1999).

4. Discussion

With respect to the activation of the RR in a TCS, the phosphorylation of the conserved Asp in the regulatory domain is thought to induce the dimerization of the RR, and the DNA-

binding domain in the resultant dimerized RR binds to the target DNA to promote gene expression. This phosphorylation-triggered mechanism is possibly applicable to *C. diphtheriae* ChrA. The local structure of our ChrAn in the SO_4^{2-} -bound form apparently mimics that of the RR regulatory domain of the phosphate-bound form (Fig. 4a), but ChrA in this state is a monomer (Fig. 2). The effect of the SO_4^{2-} binding to ChrAn is probably localized and it cannot induce further conformational change for the dimerization of ChrA. The dimer formation of ChrA may be controlled by other specific interactions between phosphate groups covalently linked to Asp50 and surrounding residues, which would induce a conformational change around the dimerization interface.

Based on comparisons with the dimeric structure of phosphorylated FixJn, the $\alpha 4$ – $\beta 5$ region is considered to be a dimerization interface of the regulatory domain. On the other hand, another dimerization interface ($\alpha 1$ and $\alpha 5$) has been proposed from the recent structural analysis of beryll-fluoride-activated VraR (Leonard *et al.*, 2013). In addition, the crystal packing of spr1814 suggested another interface consisting of the $\alpha 4$ helix, the $\alpha 5$ – $\alpha 6$ loop and a portion of the linker helix $\alpha 6$ (Park *et al.*, 2013). Although the present structure of ChrA in a monomeric state cannot provide direct information on the dimerization interface, our structural data for ChrA suggest that conformational change of $\alpha 4$ – $\beta 5$ following phosphorylation of Asp50 might affect the conformation of the linker region, since $\alpha 4$ – $\beta 5$ directly interacts with it. The most flexible position in a linker can be found at Pro135-Arg136, which exhibits a higher temperature factor (average $26.5 \text{ \AA}^2$) relative to other main-chain atoms (average of $13.7 \text{ \AA}^2$). This observation suggests that this position can act as one of the hinges that enable the protein to undergo large-scale conformational changes for the dimerization and the relative orientation of domains.

In the present study, we revealed the high-resolution structure of an RR in the haem-sensor system of a *C. diphtheriae* TCS. On the basis of the structural characterization, it was suggested that dynamic motion of the two domains was essential for conversion to the active form upon activation *via* phosphorylation of the conserved Asp, and that the conformational flexibility of the linker region must play a key role in interdomain and intermolecular interactions. From this point of view, our structure of ChrA could amount to a snapshot of the interconversion of the active and inactive forms of an RR.

Acknowledgements

The experiments at SPring-8 were performed with the approval of JASRI (Proposal Nos. 2013A1365 and 2013B1206). We appreciate help from the staff at BL41XU.

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