**Crystal structures of DntR Inducer Binding Domains
in complex with salicylate offer insights into the
activation of LysR-type transcriptional regulators**

Laurence Devesse^{a,b,†}, Irina Smirnova^{c,d,†}, Rosa Lönneborg^d, Ulrike Kapp^a,
Peter Brzezinski^d, Gordon A. Leonard^{a,*} and Cyril Dian^{a,#,*}

^aStructural Biology Group, European Synchrotron Radiation Facility, 6 rue Jules Horowitz, 38043 Grenoble, France. ^bDepartment of Biology and Biochemistry, University of Bath, Claverton Down, Bath, BA2 7AY, UK, ^cA.N. Belozersky Institute of Physical-Chemical Biology, Moscow State University, Moscow 119899, Russia. ^dDepartment of Biochemistry and Biophysics, Arrhenius Laboratories for Natural Sciences, Stockholm University, SE-106 91 Stockholm, Sweden.

*Correspondence:

Dr. Gordon Leonard: Tel: +33(0)476882394; Fax: +33(0)476882904; e-mail: leonard@esrf.fr; Dr.
Cyril Dian: Tel: +33(0)438789566; Fax: +33(0)438785494; e-mail: Cyril.Dian@ibs.fr

Running Title: Structures of activated DntR IBDs

[†] These authors contributed equally to this work. [#] Present address: Institut de Biologie Structurale Jean-Pierre Ebel, UMR 5075 CEA-CNRS-Université Joseph Fourier, 41 Jules Horowitz, 38027 Grenoble cedex 1.

Summary

Activation of LysR type transcription factors (LTTRs) is thought to result from conformational changes that occur when inducer molecules bind to their Inducer Binding Domains (IBDs). However, the exact nature of these changes remains to be fully elucidated. We present the crystal structures of two truncated constructs of the LTTR DntR in their *apo*- forms and in complex with its natural induce. These provide a clearer picture of conformational changes that occur in LTTR IBDs and offer insights into the mechanism of activation of LTTRs. Two of the crystal structures shows that DntR IBDs can bind up to two inducer molecules. The full extent of conformational changes is achieved only when inducer molecules are bound in both binding sites identified. Point mutations disrupting the putative secondary binding site produce DntR variants with a reduced response to salicylate in a whole cell system, suggesting that this site is functionally relevant.

Highlights

- DntR IBDs adopt open (inactive) or closed (active) conformations
- Fully closed conformation only seen when two inducer molecules are bound
- Phe90 is crucial for DntR activation
- Active LTTR homotetramers likely to adopt an expanded configuration

Introduction

LysR-type transcriptional regulators (LTTRs) are the largest family of transcription factors in prokaryotes, representing 16% of the overall repertoire of transcription regulators. Operons controlled by LTTRs are usually related to basic metabolic pathways, although some family members are implicated in stress-response and virulence factor expression (reviewed in Tropel & van der Meer, 2004; Maddocks & Oyston, 2008). LTTRs are around 300 amino acids in length and comprise an N-terminal DNA-binding domain (DBD) of approximately 70 residues and a C-terminal inducer-binding domain (IBD) of about 200 residues. LTTR DBDs are relatively well conserved winged helix-turn-helix (w-HTH) domains and are connected to the IBD by a mostly helical linker region. LTTR IBDs show greater amino acid sequence divergence but are structurally conserved, consisting of two Rossmann-like fold sub domains (RD1, RD2) separated by a hinge region. LTTRs usually require the binding of a small molecule (inducer) to activate transcription. Typically, inducers are intermediates in the degradation pathway regulated by the LTTR. A number of mutagenesis and structural investigations have shown these to bind in a cleft, the inducer binding cavity (IBC), located at the interface between RD1 and RD2.

LTTRs usually form functional oligomers, most commonly homotetramers, which bind to DNA in promoter regions (Tropel & van der Meer, 2004). In general, there are three DNA binding sites: a high affinity Recognition Binding Site (RBS), required for LTTR binding onto the DNA, and two low affinity Activation Binding Sites (ABS' and ABS'') essential for transcription regulation. In the absence of bound inducers, LTTRs bind simultaneously the RBS and ABS' sites causing the DNA to bend. Upon inducer binding, one binding site is shifted from ABS' to the upstream ABS'', probably via a “sliding dimer” mechanism (Porrúa *et al.*, 2007). This results in a relaxation of DNA bending, thus allowing RNA-polymerase binding and the consequent activation of transcription.

Elucidation of the molecular mechanism of transcriptional activation by LTTRs has been hindered by a paucity of structural information. While the crystal structures of several full-length

LTTRs are available (CbnR, Muraoka *et al.*, 2003a,b; TsaR, Monferrer *et al.*, 2010; ArgP, Zhou *et al.*, 2010; CrgA, Sainsbury *et al.*, 2009; PA01 and PA0477 from *Pseudomonas aeruginosa*, unpublished, PDB codes 3FZV and 2ESN respectively; BenM, Ruangprasert *et al.*, 2010) none of these are in complex with DNA or co-crystallised with a natural inducer. Nevertheless, these studies show that LTTR monomers can adopt two major conformations: 'compact' and 'extended'. In compact monomers the helix linking the IBD and DBD folds back over the surface of the IBD. In the extended conformation, the linker helix protrudes, to a greater or lesser degree, away from the IBD. Full length LTTRs form dimers as a result of the association of the linker helices and DBDs of two LTTR monomers.

When full-length LTTRs associate as homotetramers (CbnR, TsaR, ArgP, PA01, PA0477, BenM) two of the dimers described above associate to form a V-shaped entity comprising a tetrameric IBD core flanked at either end by a dimeric WHTH DNA binding region.. The tetrameric core is made up of two IBD dimers where, in each dimer, the two monomers are arranged in a head-to-tail fashion. Interestingly, variations are found within the core regions of different full length LTTRs homotetramers. In the crystal structures of CbnR (Muraoka *et al.*, 2003), BenM (Ruangprasert *et al.*, 2010) and inducer-free DntR (Smirnova *et al.*, 2004) the tetrameric core adopts a compressed conformation in which the RD2s of the IBDs at the tetramer interface interact. In contrast, in TsaR (Monferrer *et al.*, 2010) and ArgP (Zhou *et al.*, 2010) the IBDs at the tetramer interface do not interact and these assume a more open expanded conformation (Supplemental Figure 1).

The functional roles (if any) of the compressed and expanded tetramer conformations observed is unclear. It was suggested, based on an analysis of the crystal structures of the IBDs of BenM and CatM (Ezezika *et al.*, 2007) that activated LTTR homotetramers adopt a compressed tetramer configuration. However, in contrast, Monferrer and colleagues (Monferrer *et al.*, 2010) proposed that compressed tetramers represent the inactive form of LTTRs and that, upon inducer

binding and thus activation, LTTRs adopt an expanded conformation. However, crystal structures of inducer-bound and inducer-free crystal structures of TsaR on which this latter hypothesis was partly based reveal no significant conformational differences and the idea that inducer-binding provokes a change from a compressed to an expanded conformation in LTTR tetramers which, in turn, leads to transcriptional activation is thus not yet proven. Indeed, that the crystal structures of inducer-free full-length BenM and those of two full-length BenM mutants that activate transcription in the absence of inducer molecules are essentially identical (Ruangprasert *et al.*, 2010), suggests that conformational changes may play only a minor role in LTTR activation.

The LTTR DntR regulates the first steps in the oxidative degradation of 2,4-dinitrotoluene (2,4-DNT) by *Burkholderia* sp. strain DNT (Spanggord *et al.*, 1991; Suen & Spain 1992) and *Burkholderia cepacia* R34 (Nishino *et al.*, 2000; Johnson *et al.* 2002). For this reason, DntR from *Burkholderia* sp. strain DNT coupled to the reporter gene, *gfp* (encoding Green Fluorescent Protein, GFP), was used to develop a prototype biosensor for 2,4-DNT (Ng & Forsman 2000). This prototype biosensor had only a very limited response to 2,4-DNT but was very responsive to salicylic acid, an intermediate compound in the degradation of naphthalene in several species of soil bacteria that express LTTRs closely related to DntR (Schell, 1985; Jones *et al.*, 2003; Lessner *et al.*, 2003). Subsequent studies (Smirnova *et al.*, 2004) showed that DntR binds salicylate with a dissociation constant (K_d) of $\sim 4 \mu\text{M}$. No K_d for the binding of 2,4-DNT could be estimated at inducer concentrations of up to $120 \mu\text{M}$. Thus, if the prototype biosensor is to be useful in the detection of 2,4-DNT either a different LTTR, with specific recognition of 2,4-DNT, should be used or the inducer binding specificity of DntR must be modified. We have chosen the latter approach (Smirnova *et al.*, 2004; Lönneborg *et al.*, 2006) which would be facilitated by knowledge of the three-dimensional structure of DntR in complex with its natural inducer.

Our efforts to modify the inducer binding specificity of DntR were hampered by an inability to obtain crystals of full-length DntR in complex with salicylate. Recently, however, we have

produced two truncated versions of DntR (Δ N90-DntR, residues 90 – 301 of DntR; Δ N80-DntR, residues 80 – 301) and have obtained crystal structures of both constructs in the *apo*- form and in complex with salicylate. The complexes represent the first crystals of an IBD of an LTTR obtained by co-crystallisation with a natural inducer/ligand.

The results of our crystal structure analyses allow an experimental determination of the mode of interaction between salicylate and DntR. They also provide, for the first time, a full picture of the conformational changes that can occur in the IBDs of LTTRs upon inducer binding. The Δ N90-DntR-salicylate crystal structures also suggest that the IBDs of DntR can bind up to two salicylate moieties, one in the primary DntR IBC, the second near the N-terminus of the IBD and that the full extent of conformational changes in the IBD is achieved only when salicylate molecules are bound at both sites. We examine the functional and structural relevance of the secondary inducer binding site and, based on the conformational changes we observe upon inducer molecule binding, present evidence that upon activation LTTR tetramers adopt an expanded, and not compressed, conformation.

Results

Inducer binding to Δ N80- and Δ N90-DntR.

Δ N90-DntR comprises residues 90-301 of full length DntR, contains a F90M mutation and consists of residues making up the IBD of DntR. Δ N80-DntR comprises the IBD plus 10 residues of the linker region and contains a L80M mutation. As expected, the IBD folds into the two sub-domains, RD1 (residues 90-166 and 274-301) and RD2 (residues 171-269), linked by a hinge region (residues 167-170 and 270-273) (Figure 1A). In all the crystal structures described here IBD monomers associate as head-to-tail homodimers very similar in quaternary structure to that previously reported for the IBDs of LTTRs (Tyrell *et al.*, 1997; Choi *et al.*, 2001; Ezezika *et al.*, 2007; Sainsbury *et al.*, 2010; Quade *et al.*, 2011) (Figure 1B).

As expected (Smirnova *et al.*, 2004), the IBC of DntR is a cavity located at the RD1/RD2 interface (Figure 2A, 2B). The aromatic ring of salicylate is enclosed in a pocket formed primarily by the side chains of aromatic and hydrophobic residues (I106, G107, Y110, F111, G152, F167, R248, I273). Hydrophilic interactions near the mouth of the pocket provide binding specificity: hydrogen bonds are formed between the hydroxyl group of the inducer and Nε2 of H169 and the O1' atom of the salicylate carboxyl group and Nε2 of H206, while water-mediated interactions between the inducer molecule and the main chain atoms of T104, L153 (both amide hydrogen) and L151 (carbonyl oxygen) further stabilise protein-ligand interactions. All of the residues listed above were predicted to be involved in salicylate binding by DntR in previous modelling studies (Smirnova *et al.*, 2004) which were used as a basis for the design point mutations that broaden the inducer specificity of DntR (Lönneborg *et al.*, 2007). However, these modeling studies did not predict the exact nature of salicylate-DntR interactions. The interactions elucidated in the crystal structures described here will thus provide an unambiguous template for the modification of DntR in order to improve its response to 2,4-DNT.

Unexpectedly, our studies reveal a second salicylate binding site in the ΔN90-DntR-salicylate crystal structures (Figure 3A). This site is present in crystal form I and in the two monomers making up the A/B dimer in crystal form II. This second site is found in a cleft (the F90 cleft), close to the N-terminus of the ΔN90-DntR construct, formed by the displacement of the side chain of residue M90 (F90 in full-length DntR) from the hydrophobic pocket in which it is normally found. The cleft is formed by the side chains of M90 (crystal form I only), P92, S95, R97, F99, I126, P285, G286 and W289 (Figure 3B). As the cleft normally accommodates the side chain of a phenylalanine residue, it is ideally shaped to allow the binding of other single-ring aromatic moieties such as salicylate (Figure 2B). The pocket is not as specific for salicylate as is the DntR IBC and we observe different modes of salicylate binding in this region in crystal forms I and II, respectively (Figures 3C, 3D). Nevertheless, hydrophilic interactions (notably direct hydrogen bonds between the ligand oxygen atoms and S95

and a solvent-mediated interaction with the side chain of D284) provide some degree of specificity. This secondary site was seen for none of the molecules in the a.u. of the crystal structure of Δ N80-DntR-salicylate.

Conformational changes upon inducer binding.

Superimposing the crystal structures of *apo*- and inducer bound Δ N80- and Δ N90-DntR onto the crystal structure of the IBD of full-length DntR in the absence of its natural inducer molecule (PDB code, 1UTB; Smirnova *et al.*, 2004) using RD1 as a reference, reveals two major IBD conformations: *open* and *closed* (grey or blue in Figure 4A, Table 1). The closed conformation is seen only for Δ N90-DntR and occurs only when inducer molecules are bound in *both* the primary and secondary sites described above.

The transition from open to closed IBD conformations involves the rotation of RD2 towards RD1 during which the β 8- α 8 and β 9- α 9 loops (RD2) move closer to the β 4- α 6 loop of RD1 (see secondary structure labelling in Figure 1A). When salicylate is bound in the IBC of an open DntR IBD conformer water molecules link the inducer molecule to the surface of the IBD. In the closed conformation the water molecules are expelled from the RD1-RD2 interface, a hydrogen bond is formed between the carbonyl oxygen of L153 (RD1) and the amide hydrogen of G205 (RD2) and the salicylate moiety becomes completely enfolded within the IBD (Figure 4B). Analysis of the domain motions using the DynDom Protein Domain Motion Analysis server (Hayward & Berendsen, 1998; Table 1) confirms that the fully closed conformation, involving a RD2 domain rotation of 21°, occurs only when both salicylate-binding sites are occupied.

The conformations described above are reminiscent of those reported for the IBD of BenM (Ezezika *et al.*, 2007). Here also, the largest IBD conformational changes occur when both co-inducers are bound. However, for BenM the changes observed are relatively small (the largest RD2 domain rotation is $\sim 10^\circ$), presumably because the inducer molecules were introduced into crystals of

the IBDs of BenM via soaking experiments and room for conformational changes was limited by crystal packing (see Dickerson *et al.*, 1994; Danley, 2006 for a discussion of this). The crystal structures describe here should suffer less from such limitations the complexes were obtained by co-crystallisation. The domain motions observed are thus more likely to accurately reflect the full extent of conformational changes that occur in the IBDs of LTTRs upon inducer binding and thus activation.

Analysis of salicylate binding with isothermal calorimetry and protein intrinsic fluorescence quenching

The secondary salicylate binding site identified is not observed in the crystal structure of Δ N80-DntR-salicylate complex reported here. To assess whether the second site also occurs in full-length DntR (wt-DntR) Isothermal Calorimetry (ITC) was used to measure the binding of salicylate to wt-DntR, Δ N80-DntR and Δ N90-DntR. In each case, the binding isotherms obtained (Supplemental Figure 5A, Table 2) are consistent with the binding of only one inducer molecule, presumably in the IBC, with a $K_d \sim 10 \mu\text{M}$ depending on the construct (Table 2). Similar results were obtained for Δ N90-DntR construct by monitoring intrinsic fluorescence quenching upon inducer binding (Supplemental Figure 5B). The implications of this will be discussed below.

Experiments using whole-cell systems

In order to clarify if the apparent secondary site has a functional role, experiments using a whole-cell system containing a pQEwdntRHis6: $P_{\text{DNT}}:gfp$ plasmid (see Lönneborg *et al.*, 2007 for details) were performed. In this system, inducer binding to DntR, and subsequent transcriptional activation, leads to the expression of GFP. The resulting fluorescence is monitored by flow cytometry.

Experiments were performed using wt-DntR and three mutants (S95A, R97C, F90A) holding

mutations in the proposed secondary inducer-binding site. Two mutations (R97C, F90A) drastically reduced the response to salicylate. The effect of the S95A mutation, while still significant, was less prominent (Figure 5A). Experiments were also performed using different compounds (2,4-DNT, 2-nitrotoluene, 4-nitrotoluene, 2-hydroxytoluene, 2-nitrobenzoic acid, 4-nitrobenzoic acid, benzoic acid) either separately or in combination with salicylate. As expected (Lönneborg *et al.*, 2007) none of these compounds alone induced any significant whole-cell response (data not shown). However, addition of a mixture of salicylate and 4-nitrobenzoate (4-NB) resulted in an increased, synergistic transcriptional response for wt-DntR and the mutants R97C and S95A (Figure 5B-D). 4-NB is an environmental pollutant, which can serve as a sole carbon and energy source for soil bacteria and is an intermediate in, and an inducer of, the 4-nitrotoluene degradative pathway in *Pseudomonas* sp. strain 4NT (Haigler & Spain, 1993). Nitrobenzoates, including 4-NB, are also chemoattractants for *Pseudomonas* strains (Parales, 2004).

Discussion

ITC and intrinsic fluorescence quenching do not provide evidence of a second salicylate binding site for the Δ N90-DntR construct, despite crystallographic evidence that there is one. This implies a relatively low K_d for this site and could be an indication of the non-specific binding of salicylate to the Δ N90-DntR construct due both to the nature of the construct and the high concentration of the ligand in the crystallisation mixtures (see Experimental Procedures). However, a relatively low binding affinity for a secondary site is also observed for inducer binding to the IBD of BenM. Here, the K_d for the co-inducer benzoate, which does not bind in the BenM IBC, is 1.1 mM (Ezezika *et al.*, 2007; Clark *et al.*, 2004). Nevertheless, that we do not see evidence for a secondary inducer binding site in the crystal structure of the Δ 80-DntR-salicylate complex suggests that caution should be exercised before definitively assigning this secondary site a functional role. There are,

though, many reasons to believe that the secondary site identified may be relevant to the functionality of full-length DntR.

DntR is not the first LTTR in which two effector binding sites have been identified. BenM responds synergistically to two different effector molecules, benzoate and *cis,cis*-muconate. While BenM-mediated transcription is activated in the presence of either benzoate or *cis,cis*-muconate on their own, full transcriptional activation only occurs in the presence of *both* effector molecules (Clark *et al.*, 2004). Furthermore, the full extent of conformational changes observed for the BenM IBD upon inducer binding is only observed when both effector-binding sites are occupied (Ezezika *et al.*, 2007). For DntR we observe a fully closed conformation of the IBD only when both salicylate sites identified are occupied. The behaviour, at least structurally, of DntR IBDs is thus similar to that of BenM IBDs.

In wt-DntR, F90 is part of a flexible region between the IBD and the linker helix. The complete inability of a DntR F90A mutant to activate transcription in the presence of salicylate (Figure 5A) suggests that interactions involving this region, which adopts different conformations in extended and compact LTTR monomers (Figure 6A), are crucial for transcription activation. In the crystal structures of the open (i.e. inactive) DntR IBDs the side chain of F90 (or its equivalent in the Δ N90-DntR construct) is exposed to solvent (Figure 3B). As this hydrophobic side chain is held in the cleft only by relatively weak van der Waals forces, it is easy to imagine that during a change in the conformation of DntR this could flip out of the cleft. Indeed, such a situation is seen when comparing the structures of compact and extended monomers of TsaR (Monferrer *et al.*, 2010). In the compact form of TsaR the residue W90 occupies a solvent-accessible cleft in a similar position to that in which F90 is found in open DntR IBDs (Figure 6B). In extended monomers the linker helix is longer, reaching all the way to the IBD. Here (Figure 6A, 6B), the side chain of W90 is removed from the cleft and the residue stabilises the extension of the linker helix. A similar situation occurs in compact and extended monomers of CbnR (Muraoka *et al.*, 2003) where the homologous residue is

V90. Thus, in DntR, F90 may form part of, and help to stabilise, the linker helix in activated extended conformers. In doing so, its side chain will vacate the cleft in which it is found in inactive DntR monomers leaving it available for the binding of other molecules with a suitable structure (i.e. salicylate, Figure 6B).

Crystal structures show that the direction of the linker helices relative to LTTR IBDs varies in both extended (Monferrer *et al.*, 2010) and compact (Ruangprasert *et al.*, 2010) monomers. This suggests that in solution the linker helices (and thus the positions of the DBDs) are mobile. In DntR this could mean that the F90 cleft could become available for ligand binding even in compact monomers (Figure 6B). In DntR, occupation of vacant F90 cleft by an inducer molecule may therefore help to define preferred orientations of the linker helices in both compact and extended monomers and thus to stabilise the active conformation of DntR.

Experiments using the whole-cell system show that mutations targeting two of the residues that line the putative second inducer binding site result in a lower response of DntR to salicylate (Figure 5A). The very reduced response of a R97C mutant can be explained by the fact that in wt-DntR this residue forms a salt-bridge with D284. Disruption of this interaction could have serious consequences on the ability of DntR to adopt its active conformation. In the case of the more moderately reduced response of the S95A mutant, one possible explanation is that salicylate binding in the secondary site is important for DntR functionality and that, as for BenM, both primary and secondary inducer binding sites should be fully occupied for DntR to become fully active and that the S95A mutation, in line with the interactions seen the Δ N90-DntR-salicylate crystal structures, makes the putative second binding less receptive to salicylate.

Given the apparent low specificity of the putative secondary binding site for salicylate, experiments monitoring the *in vivo* response of pQEwtdntRHis6:PDNT:*gfp*-containing whole cells response to different molecules in combination with salicylate were designed to investigate whether molecules other than salicylate might bind in this site. The synergistic transcriptional response

observed to a combination of salicylate and 4-NB (Figure 5B) suggests that such binding could be a possibility. However, the degree of synergism observed (~ 1.5 for wt-DntR, ~ 1.7 for S95A-DntR, ~ 1.3 for R97C-DntR) varies little between wt-DntR and mutants changing the characteristics of the F90 cleft. This suggests that 4-NB might not bind in the secondary site but rather in a third, as yet unidentified location in DntR. This intriguing possibility clearly requires further investigation.

Transcription activation by DntR

The crystal structures of inducer-free and inducer-bound BenM IBDs led Ezezika and colleagues to propose a mechanism for transcription activation of LTTRs in which ligand-induced conformational changes the IBDs are transmitted, resulting in the dimeric DBDs formed in an LTTR homotetramer moving closer together (Ezezika *et al.*, 2007). This allows the DBDs to bind to different sets of promoter sites, thus providing the relaxation of DNA bending required for the activation of transcription. In their model, the active BenM homotetramer is in compressed configuration and is stabilised by interactions between so-called contact helices of the RD2 domains of BenM IBDs at the tetramer interface.

The concept that the transmission of ligand-induced conformational changes is involved in LTTR activation was supported by Monferrer and colleagues (Monferrer *et al.*, 2010). However, in their crystal structures TsaR tetramers adopt an expanded conformation stabilised by association of the linker helices and DBDs and not by interactions between RD2 domains of IBDs. It was thus suggested that the compressed conformation of LTTR homotetramers observed by Ezezika *et al.* represented the inactive form of LTTRs while the expanded configuration is the active form. Given that both these hypotheses were made on the basis of crystal structures showing either small (BenM) or no (TsaR) conformational changes upon inducer binding, of TsaR, it is still unclear which of these two proposals is more likely to be correct.

The crystal structures we describe here show that ligand induced conformational changes to

LTTR IBDs can be much larger than previously observed. Interestingly, when the crystal structures of open and fully closed DntR IBDs were superimposed (using RD1 as a reference) on the IBDs observed in crystal structures of TsaR (Monferrer *et al.*, 2010) it is clear that the latter adopt a fully closed conformation (Figure 4C). We have previously determined the crystal structure of full-length DntR in an open IBD conformation (Smirnova *et al.*, 2004). Here, the homotetramers observed adopt a compressed configuration. We tried to model the active form of DntR by aligning the fully-closed conformation of Δ N90-DntR with the IBDs in this crystal structure using the RD1s at the tetramer interface as a reference. This model results in a steric clash between contact helices from the RD2 subdomains at the tetramer interface (Figure 6C). This suggests that the closed IBD conformation adopted by DntR IBDs upon ligand binding would force some reorganisation of the DntR homotetramer. This observation, and the fact that the crystal structures of TsaR clearly show this LTTR with IBDs in the active conformation, strongly supports the mechanism proposed by Monferrer and colleagues, where activation of LTTRs involves the switching between compressed (inactive) and expanded (active) homotetramer conformations upon the binding of an inducer molecule.

Conclusions

The crystal structures described here have allowed an experimental determination of the mode of interaction between salicylate and DntR and have provided a fuller picture of the conformational changes that can occur in LTTR IBDs upon inducer molecule binding. The fully closed conformations we observe strongly support the proposed mechanism (Monferrer *et al.*, 2010) in which LTTR activation involves a switching between compressed (inactive) and expanded (active) homotetramer conformations upon the binding of an inducer molecule.

The Δ N90-DntR-salicylate crystal structures presented here show that Δ N90-DntR can bind up to two salicylate moieties: one in the primary DntR IBC, one in a cleft (the F90 cleft) the extreme

N-terminus of the Δ N90-DntR IBD. Analysis of the crystal structures of TsaR (Monferrer *et al.*, 2010) and CbnR (Muraoka *et al.*, 2003) suggest that the F90 cleft will be available for inducer molecule binding in extended conformers of full length DntR and imply a role for F90 in the stabilisation of DntR monomers adopting this conformation.

The functional relevance of the secondary site identified is supported by mutagenesis results and in our crystal structures the full extent of conformational changes to the DntR IBD is observed only when inducer molecules are bound in both the binding sites identified. This implies that the maximum activation of transcription by DntR will only be triggered when both effector binding sites available in the IBD are occupied.

Materials

Cloning, expression, purification

Δ N90-DntR: The Δ N90-DntR (M90 - A301) fragment of the DntR gene from *Burkholderia sp.* *DNT* was PCR amplified from the vector pLCN60.9 (Ng & Forsman, 2000) using the forward primer TATTATCAAACCATGGACCCATTTGCCAGCACGCGCACC containing a NcoI site and the reverse primer TATCATTATCAGATCTTGCTTCAGAGAAAAGCTCGAC containing a BglII site. The amplicon was ligated into the cloning ATG vector pQE60 (QIAGEN) and transformed by electroporation to *E. coli* M15[pREP4] cells. Protein expression and purification was carried out using two slightly different protocols: **Protocol A:** Bacteria were grown and Δ N90-DntR-His₆ purified as previously described for full length DntR (Smirnova *et al.*, 2004). As expected, the retention volume of Δ N90-DntR-His₆ during column chromatography was changed compared to that of the full-length protein. Pure protein fractions were pooled and dialysed against a buffer consisting of either (1) 0.4 M NaCl; 20 mM HEPES-NaOH (pH 7.7); 2 mM MgSO₄; 17% glycerol; 1 mM DTT or (2) 0.3 M NaCl; 50 mM NaH₂PO₄-NaOH (pH 7.5); 2 mM MgSO₄; 17% glycerol; 1 mM DTT and

concentrated to ~15 mg/ml. **Protocol B:** 2 l of cells were grown aerobically at 37°C in Luria-Bertani broth supplemented with 100 µl/ml ampicillin and 25 µl/ml kanamycin. When OD₆₀₀ reached 0.5 cells were cooled to 20°C and expression induced overnight by the addition of 1 mM IPTG. Cells were harvested by centrifugation and resuspended in 30 ml of a buffer containing 0.3 M NaCl, 25 mM NaH₂PO₄-NaOH (pH 8.0), 5 mM imidazole, DNase I, lysozyme, Complete EDTA-free Protease Inhibitor tablets (Roche) then frozen. For purification, thawed cells were sonicated for 10 minutes on ice, then centrifuged to clarify the cell lysate. The supernatant was loaded onto a 5 ml His-Trap column (GE Healthcare) equilibrated with 5 mM imidazole, 0.3 M NaCl and 25 mM NaH₂PO₄-NaOH (pH 8.0). An imidazole gradient of 5 mM to 500 mM in buffer was run on the column. ΔN90-DntR-His₆ eluted at an imidazole concentration of ~250 mM. Pure protein fractions were pooled, dialysed into a buffer comprising 0.3 M NaCl, 2 mM MgSO₄, 1mM DTT, 5% glycerol and 20 mM NaH₂PO₄-NaOH (pH 7.5) and concentrated to 30 mg/ml. **ΔN80-DntR:** The ΔN80-DntR (M80 - A301) fragment of the DntR gene from *Burkholderia sp. DNT* was PCR amplified from the vector pLCN60.9 (Ng, 2000) using the primers (forward) TTCCATGGAGACGGCACTGACGACCC and TTCCATGGACCCATTTGCCAGCACG (reverse). As for ΔN90-DntR, the amplicon was ligated into the pQE60 vector and transformed into *E.coli* M15[pREP4] cells. Expression and purification of ΔN80-DntR-His₆ was carried out using protocol B described above.

Crystallisation

apo-ΔN80-DntR: 1 µl of the protein stock (0.2 M NaCl; 2 mM MgSO₄; 10% glycerol; 20mM NaH₂PO₄-NaOH pH 8; 1mM DTT; 12 mg/ml ΔN90-DntR-His₆) was mixed with 1 µl of reservoir solution (0.2 M Na/K Tartrate, 0.1 M Tris pH 8.0; PEG 8000 33%) and equilibrated at 20° C against 1 ml of the reservoir solution. Long thin needle-shaped crystals appeared after 1 week. For the collection of diffraction data these were flash-cooled at liquid nitrogen temperature in mother liquor supplemented with 20% glycerol. **ΔN80-DntR-salicylate:** 1 µl protein stock (0.3 M NaCl; 20mM

NaH₂PO₄-NaOH pH 8; 20 mM Na salicylate; 2 mM MgSO₄; 5% glycerol; 1mM DTT; 6-10 mg/ml ΔN80-DntR-His₆) was mixed with 1 μl of reservoir solution (0.1M bis-tris pH 6.5, 0.2M MgCl₂, 25% PEG 3350) and at equilibrated against 1 ml of the reservoir solution at 20° C. Long thin plate-shaped crystals grew after 1 week. Before X-ray data collection crystals were flash cryo-cooled after cryoprotection in mother liquor supplemented with 20% glycerol and 20mM Na salicylate. ***apo-ΔN90-DntR***: 1 μl of the protein stock (0.4 M NaCl; 20 mM HEPES-NaOH pH 7.7; 2 mM MgSO₄; 17% glycerol; 1.2 - 1.5% N-octyl-β-D-glucopyranoside (OG), 10-12 mg/ml ΔN90-DntR-His₆) was mixed with 1 μl of reservoir solution (0.2 M Na/K Tartrate, 0.1 M Tris pH 8.0-8.5; PEG 8000 28-30%) and equilibrated at 18°C against 1 ml of the reservoir solution. Clumps of long, fragile, very thin, needle-shaped crystals appeared after 4-5 days. The clumps were broken and small crystal fragments scooped into nylon loops along with some crystallisation mother liquor. The crystals were then flash-cryo-cooled at liquid nitrogen temperature without further cryo-protection. ***ΔN90-DntR-salicylate, crystal form I***: Crystallisation was performed as described for *apo-ΔN90-DntR* with the exception that the protein stock comprised 0.3 M NaCl; 0.05 M NaH₂PO₄-NaOH pH 7.5; 50 mM Na salicylate; 2 mM MgSO₄; 17% glycerol; 1.2-1.5% OG, 12 mg/ml ΔN90-DntR-His₆. Large hexagonal bi-pyramid-shaped crystals appeared after 7-9 days. For X-ray data collection, these were flash cryo-cooled after cryoprotection in 0.2 M Na/K Tartrate, 0.1 M Tris pH 8.0; 50 mM Na salicylate; 28% PEG 8000; 10% glycerol. ***ΔN90-DntR-salicylate, crystal form II***: Crystallisation stock comprised a 1:1 mixture of protein stock (protocol B, above) and a buffer containing 400 mM NaCl, 20 mM HEPES pH 7.9, 2 mM MgSO₄, 20% glycerol, 1 mM DTT, 13 μl 0.5 M Na salicylate in 25 mM HEPES pH 7.9 and 13 μl OG. 4 μl of this was mixed in a 1:1 ratio with a reservoir solution (31% PEG 8000, 0.1 M Tris HCl (pH 8.0) and 0.2 M Na/K tartrate) and equilibrated at room temperature against 1 ml of the reservoir solution. Large, plate-shaped crystals grew in two weeks. For X-ray data collection, crystals were flash cryo-cooled without further cryoprotection.

X-ray data collection and processing

Diffraction data (Table 3) were collected at 100 K on ESRF beamlines ID23-1 (Nurizzo *et al.*, 2006), ID23-2 (Flot *et al.*, 2010) or ID29 and integrated using either MOSFLM (Leslie, 2006) or XDS (Kabsch, 2010). In the cases where XDS was used, intensities were converted to mtz format using POINTLESS (Evans, 2005), and symmetry-related intensities merged using SCALA (Evans, 2005). For MOSFLM-integrated data SCALA was used both for the scaling and merging of intensities. In all cases structure factors were derived using TRUNCATE (French & Wilson, 1978). For Δ N80-DntR-salicylate diffraction data were collected using a helical protocol (Flot *et al.*, 2010). For *apo*- Δ N90-DntR the $5 \times 7 \mu\text{m}^2$ focal spot of ID23-2 was used to isolate two positions in a crystal of approximate dimensions $40 \times 10 \times 5 \mu\text{m}^3$. A complete diffraction data set was compiled by combining partial datasets obtained at the two positions. For Δ N90-DntR-salicylate, crystal form I two data sets were collected from the same crystal: one for structure solution, the other for refinement.

Structure solution, model building, refinement

apo*- Δ N80-DntR:** RD1 and RD2 of the IBD of subunit A from the crystal structure of acetate-bound full length DntR (PDB code: 1UTB) were used as search models for molecular replacement (MR) calculations using the program PHASER (McCoy *et al.*, 2007). Refinement of the resulting model was carried out using rounds of manual rebuilding using COOT (Emsley *et al.*, 2010) interspersed with restrained refinement using PHENIX (Adams *et al.*, 1997). ***Δ N80-DntR-salicylate: MR calculations using PHASER were carried out with the same search models as for *apo*- Δ N80-DntR. At the end of the protocol, three complete Δ N80-DntR monomers had been placed in the a.u. For the fourth, only RD2 was correctly positioned. RD1 for this monomer was thus placed into the electron density by hand. Refinement of the model consisted of an initial round of rigid body refinement in

REFMAC5 (Murshudov *et al.*, 1997) in which, for each monomer, RD1 and RD2 were refined as separate rigid bodies. Further refinement was carried out as for *apo*- Δ N80-DntR with the exception that non-crystallographic symmetry (NCS) restraints were applied. ***apo*- Δ N90-DntR**: Residues 90 to 301 of subunit A from the crystal structure of acetate-bound full length DntR (PDB code: 1UTB) provided the search model for MR calculations using the program PHASER. Initial refinement of the molecular replacement solution consisted of a rigid body refinement step with, for each monomer, RD1 and RD2 refined as separate rigid bodies. Further refinement of the model was limited to 3 rounds of manual rebuilding using COOT interspersed with restrained refinement using REFMAC5 during which NCS restraints were applied. ***Δ N90-DntR-salicylate, crystal form I***:: This crystal structure was solved using the S-SAD technique (Dauter *et al.*, 1999). SHELXD (Schneider & Sheldrick, 2002) was used to determine the sulphur atom substructure, SHELXE (Sheldrick, 2008) to derive phase probability distributions to $d_{\min} = 2.3 \text{ \AA}$, ARP/wARP (Perrakis *et al.*, 1999) for automatic model building. The model was completed manually using COOT then used as a search model in MR calculations using PHASER against the high-resolution dataset. Refinement then consisted of rounds of manual rebuilding using COOT interspersed with restrained refinement using REFMAC5. ***Δ N90-DntR-salicylate, crystal form II***:: RD1 and RD2 of a partially refined model of Δ N90-DntR-salicylate, crystal form I provided the search model for MR calculations with PHASER. Subsequent structure refinement was carried out as for crystal form I.

A summary of refinement statistics and the composition and geometry of all final models is given in Table 3.

Intrinsic fluorescence quenching upon inducer binding

Inducer binding to Δ N90-DntR-His₆ was monitored as quenching of the protein intrinsic fluorescence (Tetin & Hazlett, 2000). Fluorescence measurements and data treatment were carried

out mainly as described before (Smirnova *et al.*, 2004). Protein fluorescence spectra (280-400 nm) were recorded at 22°C with excitation at 274 nm using Fluorolog (Horiba). Emission values at 326 nm were used in the data processing. The incubation medium consisted of 50 mM NaH₂PO₄-NaOH (pH 7.7); 0.1 M Na₂SO₄; 17% glycerol and ΔN90-DntR-His₆ concentrations were 1.5 - 2 μM. High concentrations of salicylate could not be used owing to undesirable internal filter effect. The K_d was estimated with the aid of the program SigmaPlot10 (SPSS Inc.) by a least square fit to the equation:

$$(F_0 - F) / F_0 = 0.5k(K_d + [P] + [I] - \sqrt{(K_d + [P] + [I])^2 - 4[P][I]}) \quad (1),$$

where F_0 and F are the fluorescence values before and after the addition of salicylate, respectively; k is a constant; K_d is a dissociation constant; $[P]$ and $[I]$ are molar concentrations of DntR and salicylate, respectively.

Isothermal titration calorimetry

ITC was carried out at 22°C (ΔN90-DntR, wt-DntR) or 15°C (ΔN80-DntR) using a VP-ITC Microcal calorimeter (Microcal, Northampton, MA, USA). Prior to all measurements, all proteins were freshly purified and dialysed against a buffer comprising 400 mM NaCl, 20 mM HEPES pH 7.9, 2 mM MgSO₄, 5% glycerol. For wt-DntR, 1mM DTT was also added. A 1 M Na salicylate stock solution was prepared in the same ITC buffer and was diluted sequentially to obtained 1mM and 1.5mM stock solutions. Titrations consisted of 5-7 μL aliquot injections made at time intervals of 5 min to ensure that the titration peak returned to the baseline. Data were analyzed and corrected for the heat of dilution of injectant into buffer using program Origin (v5.0) provided by the manufacturer.

Construction, growth and flow cytometric analysis of whole cell systems

The mutations R97C, F90A and S95A were introduced to the wt-DntR gene carried on the plasmid pQEwdntRHis6:P_{DNT}:*gfp* using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Introduction of the correct mutations was

confirmed by sequencing. Prior to analysis, *E.coli* DH5 α cells were transformed with the relevant plasmid. Cells were cultured in M9 medium (1 x Difco M9 minimal salts, 1 mM MgSO₄, 0.1 mM CaCl₂, 0.2% glucose, 0.7mg/ml of each amino acid) supplied with 100 μ g/ml ampicillin for maintenance of the plasmid at 30°C with shaking. Freshly transformed cells were grown overnight, then 1 ml cultures were inoculated to an OD₆₀₀ of 0.05. After 2h, salicylate was added at a 1:1000 dilution from DMSO stock-solutions with final concentrations in range of 1-1000 μ M. Cultures were also grown adding 500 μ M 4-NB with or without 500 μ M salicylate. 4h and 22h after induction, samples were diluted in PBS and analyzed by flow cytometry. A FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) was used for the analysis with the same settings each time. As a control, a culture grown with addition of only DMSO was analyzed for all DntR variants. The fold of induction is measured as the mean fluorescence for 10 000 cells grown in the presence of inducer (salicylate and/or 4-NB) divided by the mean fluorescence for 10 000 cells grown in the absence of inducer.

Acknowledgements

We thank Drs Carlo Petosa and Matthew Bowler for critical readings of the manuscript. This work was, in part, supported by grants from the Swedish Research Council.

References

- Adams, P.D., Afonine, P.V., Bunkoczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.-W., Kapral, G.J., Grosse-Kunstleve, R.W. *et al.* PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Cryst.* **D66**, 213-221.
- Choi, H., Kim, S., Mukhopadhyay, P., Cho, S., Woo, J., Storz, G., & Ryu, S.E. (2001). Structural basis of the redox switch in the OxyR transcription factor. *Cell* **105**, 103-113.

- Clark, T.J., Phillips, R.S., Bundy, B.M., Momany, C. & Neidle, E.L. (2004). Benzoate decreases the binding of cis,cis-muconate to the BenM regulator despite the synergistic effect of both compounds on transcriptional activation. *J. Bacteriol.* **186**, 1200–1204.
- Dickerson, R.E., Goodsell, D.S & Neidle, S. (1994). " . . . the tyranny of the lattice . . . " *Proc. Natl. Acad. Sci. USA* **91**, 3579-3583.
- Danley, D.E. (2006). Crystallization to obtain protein-ligand complexes for structure-aided drug design. *Acta Cryst.* **D62**, 569-575.
- Dauter, Z., Dauter, M., de La Fortelle, E., Bricogne, G. & Sheldrick, G.M. (1999). Can anomalous signal of sulfur become a tool for solving protein crystal structures? *J. Mol. Biol.* **289**, 83-92.
- Evans, P.R. (2005). Scaling and assessment of data quality, *Acta Cryst.* **D62**, 72-82.
- Emsley, P., Lohkamp, B., Scott, W.G. & Cowtan, K. (2010). Features and development of *Coot*. *Acta Cryst.* **D66**, 486-501.
- Ezezika, O.C., Haddad, S., Clark, T.J., Neidle, E.L. & Momany, C. (2007). Distinct effector-binding sites enable synergistic transcriptional activation by BenM, a LysR-type regulator. *J. Mol. Biol.* **367**, 616–629.
- Flot, D., Mairs, T., Giraud, T., Guijarro, M., Lesourd, M., Rey, V., van Brussel, D., Morawe, C., Borel, C., Hignette, O. *et al.* The ID23-2 structural biology microfocus beamline at the ESRF. *J. Synchrotron Rad.* **17**, 107-118.
- French G.S. & Wilson K.S. (1978) On the treatment of negative intensity observations. *Acta. Cryst.* **A34**, 517-525.
- Haigler, B.E. & Spain, J.C. (1993). Biodegradation of 4-nitrotoluene by *Pseudomonas sp.* strain 4NT. *Appl. Environ. Microbiol.* **59**, 2239-2243.
- Hayward, S. & Berendsen, H. J. C. (1998). Systematic Analysis of Domain Motions in Proteins from Conformational Change; New Results on Citrate Synthase and T4 Lysozyme. *Proteins, Structure, Function and Genetics* **30**, 144-.

- Johnson, G.R., Jain, R.K. & Spain, J.C. (2002). Origins of the 2,4-dinitrotoluene pathway. *Journal of Bacteriology* **184**, 4219-4232.
- Jones, S.E., Lloyd, L.J., Tan, K.K. & Buck, M. (2003). *J. Bacteriol.*, **185**, 6707-6711.
- Lessner, D.J., Parales, R.E., Narayan, S & Gibson, G.T. (2003). Expression of the nitroarene dioxygenase genes in *Comamonas sp.* strain JS765 and *Acidovorax sp.* strain JS42 is induced by multiple aromatic compounds. *J Bacteriol.* **185**, 3895–3904.
- Kabsch, W. (2010). Integration, scaling, space-group assignment and post-refinement. *Acta Cryst.* **D66**, 133-44.
- Leslie, A.G.W. (2006). The integration of macromolecular diffraction data. *Acta Cryst.* **D62**, 48-57.
- Lönneborg, R., Smirnova, I., Dian, C., Leonard, G.A. and Brzezinski, P. (2007) *In Vivo* and *in Vitro* Investigation of Transcriptional Regulation by DntR. *J. Mol. Biol.* **372**, 571-582.
- Maddocks, S.E. & Oyston, P.C. (2008). Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. *Microbiology* **154**, 3609-23.
- McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C & Read, R.J. (2007) Phaser crystallographic software. *J. Appl. Cryst.* **40**, 658-674.
- Monferrer, D., Tralau, T., Kertesz, M.A., Dix, I., Solà, M. & Usón, I. (2010). Structural studies on the full-length LysR-type regulator TsaR from *Comamonas testosteroni* T-2 reveal a novel open conformation of the tetrameric LTTR fold. *Molecular Microbiology* **75**, 1199–1214.
- Muraoka S., Okumura R., Ogawa N., Nonaka T., Miyashita K. & Senda T. (2003). Crystal structure of a full-length LysR-type transcriptional regulator, CbnR: unusual combination of two subunit forms and molecular bases for causing and changing DNA bend. *J Mol Biol.* **328**, 555-66.
- Murshudov, G.N., Vagin, A.A. & Dodson, E.J. (1997). Refinement of macromolecular structures by the maximum-likelihood method. *Acta Cryst.* **D53**, 240-255.
- Ng, L.C. & Forsman, M. (2000). Whole cell biosensor for the detection of explosives. The FOA Defence Research Establishment research report.

- Nishino, S.F., Paoli, G.C. & Spain, J.C. (2000). Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. *Appl Environ Microbiol* **66**, 2139-47.
- Nurizzo, D., Mairs, T., Guijarro, M., Rey, V., Meyer, J., Fajardo, P., Chavanne, J., Biasci, J.-C., McSweeney, S. & Mitchell, E. (2006). The ID23-1 structural biology beamline at the ESRF. *J. Synchrotron Rad.* **13**, 227-238.
- Parales, R.E. (2004). Nitrobenzoates and Aminobenzoates Are Chemoattractants for *Pseudomonas* Strains. *Appl. Environ. Microbiol.* **70**, 285-292.
- Perrakis, A., Morris, R.M. & Lamzin, V.S. (1999). Automated protein model building combined with iterative structure refinement. *Nature Struct. Biol.* **6**, 458-463.
- Porrúa, O., García-Jaramillo, M., Santero, E., and Govantes, F. (2007) The LysR-type regulator AtzR binding site: DNA sequences involved in activation, repression and cyanuric acid-dependent repositioning. *Mol Microbiol.* **66**, 410–427.
- Ruangprasert, A., Craven, S.H., Neidle, E.L. & Momany, C. (2010). Full-Length Structures of BenM and Two Variants Reveal Different Oligomerization Schemes for LysR-Type Transcriptional Regulators. *J. Mol. Biol.* **404**, 568-586.
- Sainsbury, S., Lane, L.A., Ren, J., Gilbert, R.J., Saunders, N.J., Robinson, C.V. et al. (2009). The structure of CrgA from *Neisseria meningitidis* reveals a new octameric assembly state for LysR transcriptional regulators. *Nucleic Acids Res.* **37**, 4545–4558.
- Sainsbury, S., Ren, J., Nettleship, J.E., Saunders, N.J., Stuart, D.I., & Owens R.J. (2010). The structure of a reduced form of OxyR from *Neisseria meningitidis*. *BMC Struct Biol.* **10**, 10-10.
- Schell, M.A. (1985). Transcriptional control of the *nah* and *sal* hydrocarbon-degradation operons by the *nahR* gene product. *Gene*, **36**, 301-309.
- Schneider, T.R. and Sheldrick, G.M. (2002). Substructure solution with SHELXD. *Acta Cryst.*, **D58**, 1772-1779.
- Sheldrick, G.M. (2008). A short history of SHELX. *Acta Cryst.* **A64**, 112-122.

- Smirnova, I.A., Dian, C., Leonard, G.A., McSweeney, S., Birse, D. & Brzezinski, P. (2004). Development of a Bacterial Biosensor for Nitrotoluenes: The Crystal Structure of the Transcriptional Regulator DntR. *J. Mol. Biol.* **340**, 405-418.
- Spanggord, R.J., Spain, J.C., Nishino, S.F. & Mortelmans, K.E. (1991). Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. *Appl Environ Microbiol* **57**, 3200-5.
- Suen, W.-C. & Spain, J.C. (1992). Cloning and characterization of *Pseudomonas* sp. Strain DNT genes for 2,4-Dinitrotoluene Degradation. *Journal of Bacteriology* **175**, 1831-1837.
- Tetin, S.Y. & Hazlett, T.L. (2000) Optical spectroscopy in studies of antibody–hapten interactions, *Methods* **20**, 341–361.
- Tropel, D. & van der Meer, J.R. (2004). Bacterial transcriptional regulators for degradation pathways of aromatic compounds. *Microbiol Mol Biol Rev.* **68**, 474-500.
- Tyrrell, R., Verschueren, K.H., Dodson, E.J., Murshudov, G.N., Addy, C., Wilkinson, A.J. (1997). The structure of the cofactor-binding fragment of the LysR family member, CysB: a familiar fold with a surprising subunit arrangement. *Structure* **5**, 1017-1032.
- Quade, N., Dieckmann, M., Haffke, M., Heroven, A.K., Dersch, P. & Heinz, D.W. (2011). Structure of the effector-binding domain of the LysR-type transcription factor RovM from *Yersinia pseudotuberculosis*. *Acta Cryst.* **D67**, 81-90.
- Zhou X., Lou Z., Fu S., Yang A., Shen H., Li Z., Feng Y., Bartlam M., Wang H. & Rao Z. (2010). Crystal structure of ArgP from *Mycobacterium tuberculosis* confirms two distinct conformations of full-length LysR transcriptional regulators and reveals its function in DNA binding and transcriptional regulation. *J Mol Biol.* **396**, 1012-24.

Figure Legends

Figure 1: The three-dimensional structure of DntR IBD monomers (A) and homodimers (B).

The protein is shown in cartoon representation. RD1 is shown in yellow, RD2 in magenta. Amino

acids at the RD1/RD2 hinge region are shown in cyan, those at the extreme N-terminus in black. Secondary structure elements are labelled as defined for full-length LTTRs (Monferrer *et al.*, 2010). The monomer shown is subunit A from *apo*- Δ N90-DntR. The dimer is the A/E dimer from the same crystal form. Figures 1, 2, 3, 4, and 6 were created using PyMol (The PyMoL Molecular Graphics System, Schrödinger, LLC; <http://www.pymol.org/>).

Figure 2: The primary salicylate binding site. **A:** The ligand bound in the IBC is shown in sphere representation, carbon atoms in grey, oxygen atoms red. **B:** Direct and solvent mediated hydrogen bonding interactions (dashed lines), between salicylate (sticks, orange carbons) and residues in the DntR IBC. Amino acids from RD1 (green carbons) and RD2 (grey carbons) are shown as sticks. For clarity the side chains of many residues that form the hydrophobic pocket in which the aromatic ring of salicylate sits are not shown. Omit difference electron density at the 3 x rms level is shown as a blue mesh. The molecule shown is Δ N90-DntR-salicylate crystal form II molecule D. Supplemental Figures 2 and 3 show similar diagrams for all other molecules containing salicylate in the DntR IBC.

Figure 3: The secondary salicylate binding site. **A:** The salicylate molecule bound in the secondary site is shown in sphere representation, the ligand bound in the IBC as blue sticks. **B:** The secondary salicylate binding cleft occupied by F90 (top left; wt-DntR; PDB code, 1UTB), M90 (top right; *apo*-DN90DntR) and salicylate (bottom; Δ N90-DntR-salicylate, crystal form I) in the latter the position of the 'flipped out' side chain of M90 is also shown. **C:** The environment of salicylate in the secondary site in Δ N90-DntR-salicylate, crystal form II, molecule A. Salicylate and relevant protein side chains are shown in stick representation and coloured as in Figure 2B. Omit difference electron density at the 3 x rms level is shown as a blue mesh. Supplemental Figure 4 shows similar diagrams for all other molecules containing salicylate in the secondary site. **D:** The different secondary site ligand

environments seen in crystal forms I (green carbon atoms) and II (grey carbon atoms) of Δ N90-DntR-salicylate.

Figure 4: DntR IBD domain motions upon salicylate binding. **A:** Superpositions (based on RD1 only) of *apo*- Δ N90-DntR (grey cartoon), Δ N90-DntR-salicylate with inducer bound only in the IBC (magenta cartoon) and Δ N90-DntR-salicylate with the inducer bound both in the IBC and the secondary site (blue cartoon). Only when both salicylate binding sites are occupied does the DntR IBD adopt the closed conformation. **B.** The environment of the salicylate moiety in the IBCs of open and closed DntR IBDs. Colour scheme as in Figure 2B. **C:** Superpositions of the structures of open (grey cartoon), closed (blue cartoon) DntR IBDs and the IBD seen in the crystal structure of TsaR (orange, Monferrer *et al.*, 2010). The latter clearly adopts a closed conformation.

Figure 5: *In vivo* inducer response for wt-DntR and the mutants F90A, S95A and R97C. **A:** Transcription activation measured as the fold of induction 4 h after addition of salicylate (1-1000 μ M): wt-DntR (circles), S95A (squares), R97C (triangles) and F90A(diamonds). **B to D:** Transcription activation in response to 500 μ M salicylate, 500 μ M 4-nitrobenzoate (4NB) and simultaneous addition of both 500 μ M salicylate and 4NB. Data are shown for wt-DntR (B), for S95A (C) and for R97C (D).

Figure 6: A potential activation mechanism for DntR homotetramers. **A:** The extended (wheat) and compact (orange) monomers seen in the crystal structure of TsaR (Monferrer *et al.*, 2010) The side chain of W90 is shown in green spheres. **B:** Close up of extended (left) and compact (right) monomers of TsaR superposed with the crystal structure (blue cartoon, salicylate with blue carbon atoms) of Δ N90-DntR-salicylate (form I) showing how secondary site inducer binding may help to modulate extended and compact conformations of DntR. For TsaR W90 is shown as green sticks. **C:**

In the crystal structure of inducer free wt-DntR, homotetramers adopt a compressed, inactive configuration (centre). Maintaining this conformation when DntR IBDs adopt their fully closed active conformations would lead to steric clashes between RD2 contact helices at the tetramer interface (left). A more likely conformation for active DntR homotetramers is thus the open conformation observed for TsaR (right: Monferrer *et al.*, 2010).

Table 1. DynDom analysis of DntR IBD domain motions. The reference structure is the open IBD of wt-DntR with acetate bound (PDB code, 1UTB; Smirnova *et al.*, 2004). Rotation angle denotes movement of RD2 relative to its position in 1UTB. Rmsd is shown for the *whole* of the IBD and is given in order to show the variation in the open and closed conformations observed. The letters at the end of the IBD name denote different molecules in the a.u., the subscripts I and II different crystal forms. Where NCS restraints were applied in structure refinement, results are shown only for one monomer in the a.u.

Table 2. ITC analysis of salicylate binding to Δ N90DntR, Δ N90DntR and full-length DntR.

Table 3. Data processing and structure refinement statistics. Numbers in parentheses are for the highest resolution shell.

Figure 1

Figure 2

Figure 3

Figure 4

A **B** **C**

Figure 5

Figure 6

Table 1.

DntR	Inducers bound	Rotation angle (°)	rmsd C _α (Å)
<i>1UTB-A</i>		-	-
<i>apo-ΔN90DntR-A</i>		3.4	0.49
<i>apo-ΔN80DntR-A</i>		None found	0.48
<i>apo-ΔN80DntR-B</i>		None found	0.69
<i>apo-ΔN80DntR-C</i>		None found	0.50
<i>apo-ΔN80DntR-D</i>		None found	0.37
<i>apo-ΔN80DntR-E</i>		None found	0.44
<i>apo-ΔN80DntR-F</i>		None found	0.46
<i>apo-ΔN80DntR-G</i>		None found	0.43
<i>apo-ΔN80DntR-H</i>		None found	0.44
$\Delta N90DntR-Sal-C_{II}$	1	None found	0.87
$\Delta N90DntR-Sal-D_{II}$	1	None found	1.06
$\Delta N80DntR-Sal-A$	1	None Found	0.69
$\Delta N90DntR-Sal-A_I$	2	21.0	2.21
$\Delta N90DntR-Sal-A_{II}$	2	17.5	1.82
$\Delta N90DntR-Sal-B_{II}$	2	13.6	1.58

Table 2.

	$\Delta N90DntR$	$\Delta N80DntR$	WT DntR
Analysis mode	Single binding site model	Single binding model	Single binding model
Temperature (K)	295	288	295
Stoichiometry	2.16 ± 0.010	1.38 ± 0.005	1.46 ± 0.025
K_a (M^{-1})	$0.847 \pm 0.018 \cdot 10^5$	$0.963 \pm 0.041 \cdot 10^5$	$1.26 \pm 0.122 \cdot 10^5$
K_d (μM)	11.80 ± 0.25	10.38 ± 0.17	7.93 ± 0.77
ΔH (cal.mol ⁻¹)	-5507 ± 85.82	-5352 ± 27.27	-4396 ± 96.06
ΔS (cal.mol ⁻¹ .K ⁻¹)	3.89	4.67	8.45

Table 3

	<i>apo</i> -ΔN80-DntR	ΔN80-DntR + salicylate	<i>apo</i> -ΔN90-DntR	ΔN90-DntR + Salicylate		
				Crystal form I		Crystal form II
<i>Data collection</i>				<i>Structure solution</i>	<i>Structure refinement</i>	
Beamline	ESRF ID23-2	ESRF ID23-2	ESRF ID23-2	ESRF ID23-1		ESRF ID29
Wavelength (Å)	0.873	0.873	0.873	1.82	0.976	0.976
Space group	$P2_12_12_1$	$P2_1$	$P2_12_12_1$	$P3_121$		$P2_1$
Unit cell dimensions	$a = 81.2 \text{ Å}$ $b = 111.5 \text{ Å}$ $c = 213.30 \text{ Å}$	$a = 54.19 \text{ Å}$ $b = 118.96 \text{ Å}$ $c = 80.56 \text{ Å}$ $\beta = 108.6^\circ$	$a = 79.47 \text{ Å}$ $b = 125.00 \text{ Å}$ $c = 184.84 \text{ Å}$	$a = b = 95.2 \text{ Å}$ $c = 67.4 \text{ Å}$		$a = 65.91 \text{ Å}$ $b = 82.11 \text{ Å}$ $c = 86.27 \text{ Å}$ $\beta = 111.3^\circ$
Resolution range (Å)	49.5-2.8	47.1-2.9	43.90-3.0	35.2–2.3	52.27-1.85	82.11-1.95
R_{sym} (%)	15.0 (49.6)	16.7 (57.5)	18.2 (62.2)	10.6 (35.6)	8.6 (55.2)	8.8 (62.0)
Completeness (%)	97.5 (93.6)	94.1 (88.8)	99.2 (99.8)	100.0 (100.0)	98.9 (99.8)	99.4 (99.4)
Multiplicity	4.5 (2.8)	2.6 (2.4)	3.9 (3.9)	40.9 (41.3)	4.8 (4.8)	4.3 (4.4)
$\langle I/\sigma(I) \rangle$	10.2 (2.3)	5.4 (1.7)	6.8 (2.3)	33.3 (12.8)	11.2 (2.9)	11.7 (2.7)
<i>Refined model composition</i>						
Monomers / a. u.	8	4	8		1	4
Protein residues	1701	831	1692		215	845
Water molecules	97	53	0		181	295
Salicylate	-	3	-		2	6
Glycerol	4	0	0		0	0
PEG	-	3			1	0
Wilson B -value (Å ²)	40.2	62.8	40.2		22.2	24.1
Overall B -Value (Å ²)	32.6	32.3	19.9		24.2	26.2
<i>Model quality indicators</i>						
$R_{\text{work}} / R_{\text{free}}$ (%)	22.0/24.0	24.2/28.7	19.4/26.4		18.0/21.6	20.0/25.0
Rmsd bond lengths (Å)/bond angles (°)	0.003/0.82	0.01/1.41	0.012/1.45		0.022/1.89	0.015/1.60
Estimated coordinate error (Å)	0.41	0.48	0.37		0.073	0.129
<i>Ramachandran analysis</i>						
% Favoured	98.4	94.0	95.6		98.6	100
% Allowed	1.6	4.6	4.2		0.9	0
% Disallowed	0	1.5	0.1		0.5	0
wwPDB ID code	2y84	2y7w	2y7r		2y7p	2y7k

Supplemental Information

The crystal structures of DntR Inducer Binding Domains in complex with salicylate offer mechanistic insights into the activation of LysR-type transcriptional regulators.

Laurence Devesse^{a,b,†}, Irina Smirnova^{c,d,†}, Rosa Lönneborg^d, Ulrike Kapp^a,
Peter Brzezinski^d, Gordon A. Leonard^{a,*} and Cyril Dian^{a,#,*}

Figure 1: Compressed (left) and expanded (right) LTTR homotetramers. The different subunits making up the tetramers are shown in cartoon representation and in different colours. The compressed tetramer is from the crystal structure of *apo*-CbnR (Muraoka *et al.*, 2003) the expanded tetramer is that observed in both *apo*- and inducer bound TsaR (Monferrer *et al.*, 2010).

Figure 2: The primary salicylate binding site interactions observed in the crystal structures of Δ N90-DntR in complex with salicylate. The DntR IBD is shown as a grey cartoon, the salicylate molecule bound in the IBC in a stick representation (carbon atoms orange, oxygen atoms red). Amino acids involved in DntR-salicylate interactions are shown in stick representation (carbon atoms in grey, oxygen atoms red, nitrogen atoms blue). Hydrogen bonding interactions are shown as dashed black lines. Omit difference electron density at the 3 x r.m.s. level is shown as green chicken wire.

Figure 3: Salicylate bound in the IBCs of Δ N80-DntR in the crystal structure of Δ N80-DntR in complex with salicylate. Omit difference electron density at the $2 \times$ r.m.s. level is shown as blue chicken wire for molecules A (left), B (middle), C (right) in the a.u. For salicylate bound in the IBCs of monomers B and C in the a.u. of the Δ N80-DntR-salicylate crystal structure the disposition of the inducer in the IBC is similar to that seen for Δ N90-DntR, although the relatively low resolution of the diffraction data and disorder in the $\alpha 8\beta 8$ loop (which includes H206) means that not all inducer-protein interactions are visible. For molecule A the entire $\alpha 8\beta 8$ loop is disordered. Here, the ligand in the IBC, is oriented slightly differently and is held in place only by hydrophobic interactions between the aromatic ring of the salicylate and the hydrophobic pocket described above for Δ N90-DntR. Monomer D in the a.u. of the Δ N80-DntR-salicylate crystal structure does not bind salicylate in its IBC.

Figure 4: The secondary salicylate binding site observed in the crystal structures of $\Delta N90$ -DntR in complex with salicylate. The colour scheme is as for Supplementary Figure 1. Omit difference electron density is shown at 3 x r.m.s or (crystal form II, Chain B) 2 x r.m.s. levels.

Figure 5: Biophysical analyses of salicylate binding by $\Delta N90$ -DntR, $\Delta N80$ -DntR and wt-DntR.

A: ITC profiles measured during salicylate binding by $\Delta N90$ -DntR, $\Delta N80$ -DntR and wt-DntR showing differential power (ΔP) and raw injection heats (open circles) for titrations of 5 μl of 1 mM, 1.5 mM and 1.0 mM salicylate into 33.2 μM , 42 μM and 40 μM of $\Delta N90$ -DntR, $\Delta N80$ -DntR and WT-DntR, respectively. The solid line represents a nonlinear least squares fit using a single-site binding model. $K_{d_{\text{obs}}}$ were $11.80 \pm 0.25 \mu\text{M}$, $10.38 \pm 0.17 \mu\text{M}$ and 7.93 ± 0.77 and the stoichiometry were 2.16 ± 0.01 , 1.38 ± 0.005 and 1.46 ± 0.025 for $\Delta N90$ -DntR, $\Delta N80$ -DntR and WT-DntR, respectively.

B: The quenching of the intrinsic fluorescence of $\Delta N90$ -DntR upon salicylate binding. The relative fluorescence intensity $(F_0 - F)/F_0$ is shown as a function of the sodium salicylate concentration. The line represents a least square fit to equation (1) (see Materials and Methods) with $K_d = 11 \mu\text{M}$.