Structural Insights into Regulation of *Vibrio* Virulence Gene Networks

14

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Abstract

One of the best studied aspects of pathogenic Vibrios are the virulence cascades that lead to the production of virulence factors and, ultimately, clinical outcomes. In this chapter, we will examine the regulation of *Vibrio* virulence gene networks from a structural and biochemical perspective. We will discuss the recent research into the numerous proteins that contribute to regulating virulence in Vibrio *spp* such as quorum sensing regulator HapR, the transcription factors AphA and AphB, or the virulence regulators ToxR and ToxT. We highlight how insights gained from these studies are already illuminating the basic molecular mechanisms by which the virulence cascade of pathogenic Vibrios unfold and contend that understanding how protein interactions contribute to the host–pathogen communications will enable the development of new antivirulence compounds that can effectively target these pathogens.

Keywords

Vibrio · Virulence regulation · Transcription factors · Protein structure

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14.1 Introduction

Enteric bacterial pathogens of the *Vibrio* spp. need to be able to properly regulate genetic networks to survive the harsh intestinal environment, colonize the host, produce virulence factors, and, in some cases, return to the external environment, e.g. (Almagro-Moreno et al. 2015a). These processes are regulated by a set of conserved transcription factors that respond by sensing environmental changes, such as oxygen level and pH, by binding directly to small molecular regulators, or via quorum sensing (Mey et al. 2012; Midgett et al. 2017; Rutherford et al. 2011; Li et al. 2016a; Lowden et al. 2010; Kovacikova et al. 2010). The complex regulatory networks have evolved to be temporally and spatially regulated in order to optimize virulence gene expression. One of the most well characterized regulatory systems of this type is from pandemic *Vibrio cholerae,* whose ingestion results in the diarrheal disease, cholera, for which much microbiological and structural information is known (Almagro-Moreno et al. 2015a; Clemens et al. 2017). In this chapter, we will review the protein regulators for which atomic structures are known, highlighting their structural features and what is known about their mechanism of regulation and activity, as well as outstanding questions related to their structure and function.

Induction of virulence in *V. cholerae* is controlled by a complicated regulatory cascade

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involving a number of transcription factors from diverse families (Fig. 14.1a). We will describe what is known about the structure and function of these proteins in the order they appear in this network, starting with HapR, which is expressed at high cell densities and is involved in quorum sensing (De Silva et al. 2007; Ball et al. 2017). At high cell density HapR has two main functions, inhibition of *aphA* expression, which subsequently reduces virulence gene expression, and induction of dispersal from biofilms (Zhu and Mekalanos 2003; Finkelstein et al. 1991; Kovacikova and Skorupski 2002). AphA, a member of the PadR family of transcription factors (De Silva et al. 2005), together with AphB, a LysR family transcription factors (Taylor et al. 2012; Kovacikova and Skorupski 1999), activates *tcpPH* expression (Kovacikova and Skorupski 1999; Skorupski and Taylor 1999). TcpP and TcpH are members of the ToxRS family of regulators, which consist of a transmembrane transcription factor and an integral membrane periplasmic binding partner. TcpPH, along with ToxRS, activates expression of the AraC family transcription factor ToxT (Lowden et al. 2010; Miller et al. 1987, 1989; Hase and Mekalanos 1998; Krukonis et al. 2000; Higgins et al. 1992; Matson et al. 2007). ToxT, the master regulator in *V. cholerae*, directly activates expression of the two main virulence factors, the toxin coregulated pilus (TCP) and cholera toxin (CT) (Matson et al. 2007).

Additionally, outside of this regulatory cascade, a virulence pathway in non-O1/O139 *V. cholerae* and *V. parahaemolyticus* that leads to the expression of the type 3 secretion system 2 (T3SS2). In this pathway (Fig. 14.1b), ToxRS works with another transmembrane transcription factor pair, VtrAC, in a bile dependent manner to activate the transcription of *vtrB,* a transmembrane transcription factor that activates transcription of the T3SS2 (Li et al. 2016a; Kodama et al. 2010; Gotoh et al. 2010; Hubbard et al. 2016; Miller et al. 2016). Through a combination of structural, biochemical, microbiological, and genetic information, we can start to understand and appreciate the exquisite dance of interactions and conformational changes that must occur for these transcription factors proteins to work together to respond to the environment and regulate virulence gene expression. Of course, such understanding also provides a foundation for manipulating their activity and designing inhibitors specifically targeting enteric pathogens and not the commensals around them, which could reduce the negative effects of antibiotic resistance (Cegelski et al. 2008).

14.2 HapR and Homologs Are Quorum Sensing Transcription Factors

HapR is part of the quorum sensing cascade in various *Vibrio* spp. where it is also known as SmcR (*V. vulnificus*) and LuxR (*V. harveyi*), which can all cross complement each other in their respective strains (Ball et al. 2017). They belong to the very large family of TetR transcription factors (Cuthbertson and Nodwell 2013). However, unlike many other members of the TetR family which are regulated only by directly binding small molecules, in *Vibrios* these proteins are primarily regulated via the action of autoinducers on other proteins such as autoinducer 2 though the LuxPQ system and cholera autoinducer 1 through the CqsS pathway (Ball et al. 2017; Cuthbertson and Nodwell 2013). Furthermore, unlike many other TetR family members, these proteins regulate many different genes and can act as activators and repressors (Ball et al. 2017).

While not all pandemic *V. cholerae* strains have a functional HapR, for those that do HapR plays a role in bacterial dispersion from biofilms at the beginning of infection, e.g. *V. cholerae* and *V. vulnificus*, and also at the end of infection (Zhu and Mekalanos 2003; Finkelstein et al. 1991; Stutzmann and Blokesch 2016; Zhu et al. 2002; Jobling and Holmes 1997; Kim et al. 2013). In *V. cholerae*, as well as other *Vibrios,* HapR and AphA make up a quorum sensing axis, with AphA expressed at low cell densities and HapR expressed at high cell densities (Rutherford et al. 2011; Ball et al. 2017). When expressed at high cell densities, HapR binds to the *aphA* promoter,

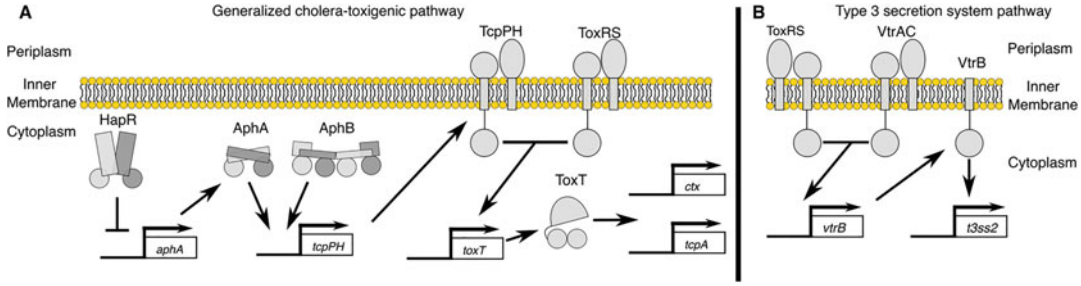

Fig. 14.1 Virulence pathway for cholera toxin and type 3 secretion system 2. (**a**) Overview of the toxigenic pathway in pandemic *V. cholerae* focusing on the proteins and genes they regulate. (**b**) The pathway for type 3 secretion system 2 expression as determined in *V. cholerae* and *V. parahaemolyticus*, again focusing on the proteins and genes they regulate

inhibiting its transcription (Ball et al. 2017; Kovacikova and Skorupski 2002). In addition to inhibiting transcription, HapR also activates transcription of several genes, including the hemagglutinin protease, which degrades the putative intestinal cell surface receptors *V. cholerae* uses to attach to cells (Ball et al. 2017; Finkelstein et al. 1991).

Analysis of *Vibrio* HapR protein family DNA binding sites has shown they bind to two motifs. Motif 1 has dyad symmetry with a variable spacer and is around 20–22 bp. While motif two is asymmetric with one half of the dyad on one side of the spacer and an incomplete dyad on the other side (Ball et al. 2017). The two motifs have generally been correlated with transcriptional repression (motif 1) and activation (motif 2) (Ball et al. 2017; Tsou et al. 2009). How these proteins interact with DNA is one question that structure has provided insight.

14.2.1 HapR Structure

The first high resolution crystal structure of a *Vibrio* HapR family member was solved in 2007, and confirmed it had an overall structure similar to other TetR family members (De Silva et al. 2007). As with other TetR family members, HapR was a homodimer, with each monomer containing 9 α-helices forming two domains, an N-terminal helix-turn-helix DNA binding domain, and a C-terminal dimerization domain containing a putative ligand binding pocket (Fig. 14.2) (De Silva et al. 2007; Cuthbertson and Nodwell 2013). The two domains are connected by a hinge region, which has been shown to be important for DNA binding (Dongre et al. 2011).

The structure contained an empty, solvent accessible pocket, suggesting that HapR is not only regulated at the expression level by the quorum sensing pathway, but also via direct binding of a small molecule ligand (De Silva et al. 2007). These features were also observed in the subsequently determined structures of *V. vulnificus* SmcR and *V. harveyi* LuxR (Kim et al. 2010; Zhang et al. 2021).

The presence of empty ligand binding pockets in these structures has naturally led to much speculation about the nature and identity of putative physiological regulatory ligands. In one study, SmcR activity was used to screen for inhibitory compounds, and 1-(5-bromothiophene-2-sulfonyl)-1H-pyrazole (qstatin) was shown to reduce the expression of elastase (Kim et al. 2018), and qstatin was also shown to inhibit homologs from other species, but not HapR (Kim et al. 2018). A structure of SmcR in complex with qstatin showed the molecule indeed bound to the previously identified pocket (De Silva et al. 2007), and that qstatin made SmcR less flexible as indicated by a decrease in the crystallographic B-factors when compared to the apo structure (Kim et al. 2018). Such ligand-induced stabilization of

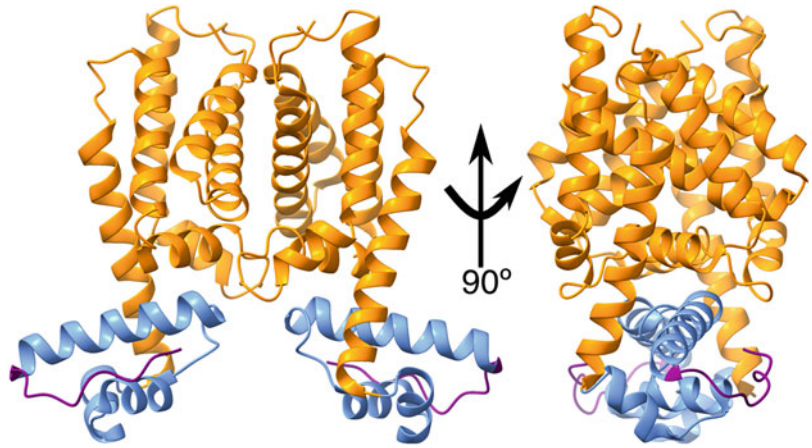

Fig. 14.2 Overview of the HapR structure from *V. cholerae* (De Silva et al. 2007). The structure of HapR dimer from *V. cholerae* with the N-terminal tails that wraps back on to the DNA binding domain in purple, the DNA binding domains in blue, and the C-terminal dimerization domains in orange

proteins is not unusual and could of course serve to lock proteins into a particular conformation.

While qstatin was clearly inhibitory, its effects on SmcR DNA binding varied widely. For promoters which SmcR activates expression, including *vvpE,* there was almost no change in the affinity of SmcR to the promoter binding site. In contrast, for promoters that are repressed by SmcR, qstatin binding reduced the affinity for the promoter by six to eight fold (Kim et al. 2018). A stronger SmcR inhibitor that was identified from the same class of compounds also did not disrupt DNA binding to the SmcR activated *vvpE* promoter (Newman et al. 2021a). This suggests these inhibitors disrupt different processes depending on whether the transcription is activated or repressed by protein binding.

14.2.2 HapR DNA Binding

The ability of HapR and its homologs bind promoters and either repress or activate cognate gene expression is fascinating. Because they can bind to promoters of different lengths, it is thought they must be somewhat flexible in how they bind DNA (Newman et al. 2021b), and this characteristic is observed by crystal structures of SmcR in which the DNA binding domains are observed in narrow or wide conformations, which has also been confirmed by solution studies using small angle X-ray scattering (SAXS) (Newman et al. 2021b). Furthermore, in a natural HapR variant, HapRv2, where a small and flexible glycine is replaced by a less flexible and negatively charged aspartic acid, SAXS analysis produced a model in which the DNA binding domains were in an orientation unable to bind DNA (Dongre et al. 2011). However, a crystal structure of HapRv2 mutant protein determined in the absence of DNA showed the protein adopts a fold indistinguishable from the non-variant structure, suggesting DNA binding was blocked not by a conformational change but rather by a clash of the aspartic acid side chain with phosphates on the DNA backbone (Cruite et al. 2018).

The structures of *V. alginolyticus* LuxR bound to DNA from promoters it activates and represses were solved recently (Zhang et al. 2021). Both structures contained 21 bp of DNA and were similar in overall fold. Interestingly, the structure of the complex between LuxR and the activating promoter indicated that interactions with the protein extended beyond what was predicted to be the end of the shorter motif 2 (Zhang et al. 2021), raising the question of the nature of the observed lower binding affinity.

One possible explanation for this is the difference in interactions of the N-terminal tail of the protein, which forms contacts with the minor groove of the DNA. When bound to the repressed DNA, both N-terminal tails of the LuxR dimer are well ordered, while only one is visible in the activated DNA bound structure (Zhang et al. 2021). These contacts appear to be critical for function, as mutations in Arg9 and Arg11

severely disrupt DNA binding (Kim et al. 2010; Zhang et al. 2021). Another clue comes from the crystallographic B-factors, which for the LuxR-activated promoter structure are higher than that of the LuxR-repressed DNA structure This indicates increased flexibility of one complex in comparison to the other, which could be indicative of weaker affinity despite a similarly sized binding interface (Zhang et al. 2021). Regardless of the ultimate explanation of the disparate affinities, the observation that the structures are essentially the same when LuxR is bound to promoters it activates or represses is an important step in elucidating the detailed atomic interactions that must govern the strength of protein-DNA binding.

While the overall structures of LuxR bound to activated or repressed promoters do not significantly differ, a comparison of LuxR-DNA bound structures with the apo structure of *V. vulnificus* SmcR showed several significant changes these proteins undergo to bind DNA (Zhang et al. 2021). First, the DNA binding domains are drawn closer together by a several angstroms and are rotated with respect to each other. This movement generates significant rearrangements in the C-terminal domain, where new contacts are formed. In particular, Glu 124 moves 5 Å to form an amino acid cluster involving Arg60, Glu124, Arg122, and Glu116 (Zhang et al. 2021). Furthermore, An alignment of LuxR bound to an activated promoter (Zhang et al. 2021) with apo HapR (De Silva et al. 2007) also revealed binding DNA decreases the distance between helix 6 in the two monomers, suggesting that a compound which wedges the helices open would block the ability of these proteins to bind DNA (Fig. 14.3a, b).

Another interesting structural feature illuminated by the LuxR-DNA complex structures are differences in the proposed ligand binding domain. In the LuxR-DNA structures, while both subunits have a pocket (Zhang et al. 2021), the pockets are not connected by a solvent accessible tunnel, as observed in the apo HapR and SmcR structures (De Silva et al. 2007; Kim et al. 2010; Zhang et al. 2021). In addition, the pocket is mostly closed off from bulk solvent in the DNA bound structures, suggesting a potential ligand would be trapped inside, or unable to bind, until the protein releases from the DNA (Zhang et al. 2021) (Fig. 14.3c, d).

The HapR family of *Vibrio* proteins act in the quorum sensing pathway and therefore have garnered interest as antivirulence targets. However, targeting them is complicated as the role they play in pathogenesis differs at various stages of the process. In the beginning stages of infection, when dissemination is a problem, an inhibitor would be desired. However, in the late stages of infection, activating the proteins would be necessary to inhibit the virulence pathway. In any case, at this time the most promising approach is to use the available structural data to computationally screen for small molecule inhibitors that target the binding pocket to block the conformational changes necessary for DNA binding.

Among the outstanding questions related to the structure and function of HapR-like proteins, such as the identity of the physiological regulatory ligand and the temporal and functional effects it has on the pathway. Additionally, how does quorum sensing and ligand binding work together to regulate these proteins. And finally, a comprehensive understanding must also explain the details of how inhibitor binding modulates the binding affinity for some DNA sequences, but not others.

14.3 AphAB

AphA and AphB are two transcription factors from different families that are conserved in the *Vibrio* family. AphA is thought of belonging to a quorum sensing axis with HapR, where AphA is expressed at low cell densities and repressed by HapR at high cell densities (Rutherford et al. 2011; Kovacikova and Skorupski 2002). AphB is an environmental sensor that responds to changes in pH and oxygen (Kovacikova et al.

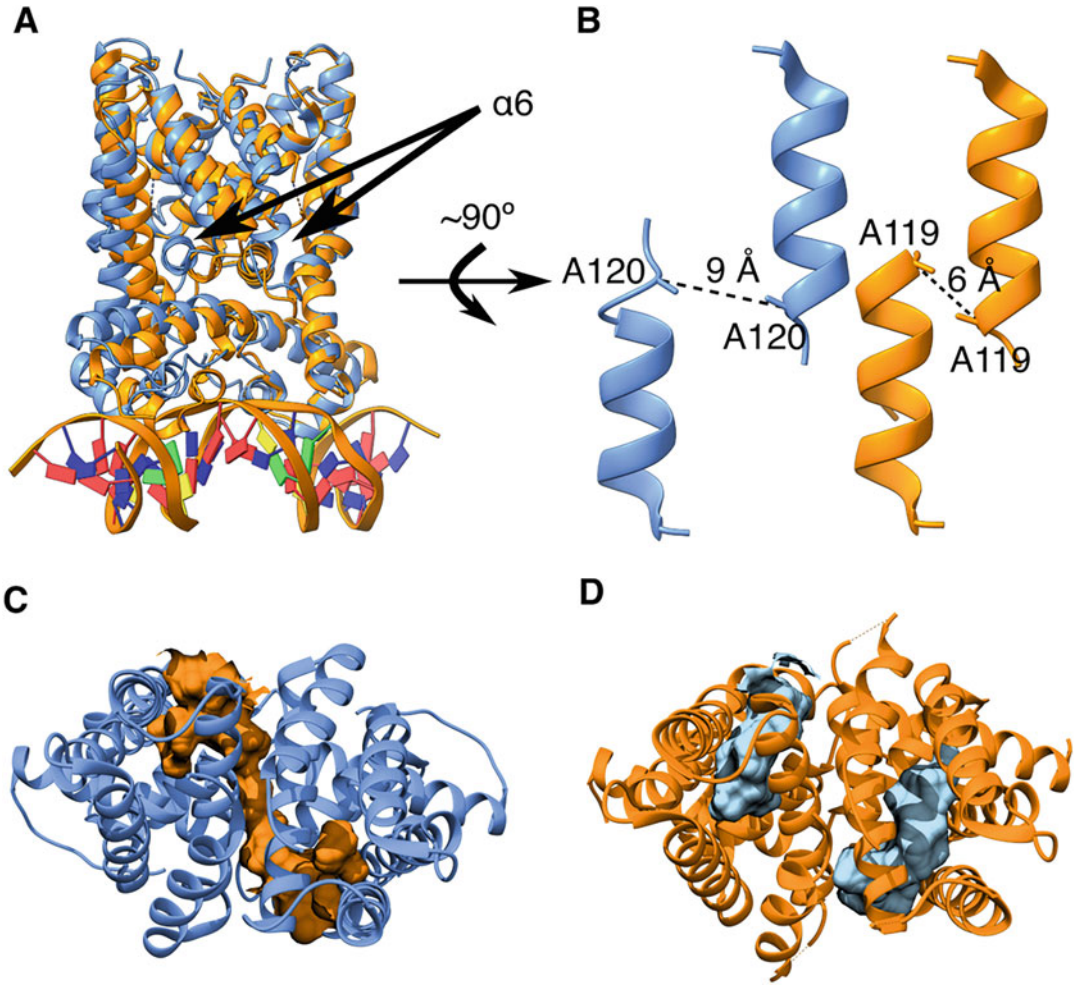

Fig. 14.3 Comparison of LuxR bound to DNA (Zhang et al. 2021) and the HapR structure (De Silva et al. 2007). (**a**) Overlay of HapR (De Silva et al. 2007) in blue and LuxR bound to DNA (Zhang et al. 2021) in orange. The proteins are viewed from at an angle to emphasize the amount of movement that is necessary for the protein to undergo to bind to DNA. Arrows point to α6 for both the HapR and LuxR-DNA structures. (**b**) α6 undergoes significant movement from the unbound to DNA bound states. In blue is the α6 from the HapR (De Silva et al. 2007) structure and in orange is α6 from the LuxR-DNA structure (Zhang et al. 2021). This close approach is facilitated by the annotated alanines as the Cα distance between the monomers in the HapR apo structure is 9.4 Å and between the same residues in the LuxR-DNA bound structures is 6.1 Å as annotated in (**c**). The proposed HapR (De Silva et al. 2007) binding pocket as determined by Castp. The protein is in blue and the pocket is shown in orange and is continuous between the subunits. (**d**) The proposed binding pockets as determined by Castp of the LuxR-DNA structure (Zhang et al. 2021). The protein is in orange and the pockets are in blue

2010; Rhee et al. 2006; Liu et al. 2011). While both these proteins are global regulators of gene expression, they have been coopted to regulate virulence gene expression in several *Vibrio spp*. (Kovacikova and Skorupski 1999; Skorupski and Taylor 1999; Jeong and Choi 2008; Gao et al. 2017; Lim et al. 2014). In some *V. cholerae* strains, AphA and AphB work cooperatively to increase the transcription of *tcpPH* under virulence inducing conditions, which suggests they respond to an activation signal (Kovacikova and Skorupski 1999, 2000; Skorupski and Taylor 1999). It is hypothesized that AphB recruits AphA to the DNA as AphA mutants that can no

longer bind DNA have their activity rescued in the presence of AphB (Kovacikova et al. 2004). While these proteins can increase expression of virulence genes through *tcpPH* transcriptional augmentation, El Tor strains can carry a mutation in the promoter of *tcpPH* that reduces AphB binding and these strains remain virulent (Kovacikova and Skorupski 2000; Kovacikova et al. 2004).

14.3.1 AphA Structure

AphA is a member of the PadR family of environmental sensors (Rutherford et al. 2011; Kovacikova and Skorupski 2002; De Silva et al. 2005; Barthelmebs et al. 2000) and was one of the first of this family to have its structure determined (De Silva et al. 2005). These proteins function as a dimer composed of two monomers, with each monomers consisting of an N-terminal winged helix-turn-helix domain and an extended C-terminal dimerization domain composed of three helices (5–7). Rather than forming a standard 4-helix bundle, helices 6–7 from one monomer interact with those from an adjacent monomer in an antiparallel fashion, forming a relatively flat sheet of 4 helices (De Silva et al. 2005). Overall, the structure resembles a bridge with the pillars being the DNA binding domains and the top being the C-terminal helices (Fig. 14.4a).

The structure of PadR bound to ligand and PadR bound to DNA have been solved providing additional insight into the function and regulation of these proteins (Park et al. 2017a). Although no pocket was identified in AphA, in PadR, binds phenolic acids in between the N-terminal and C-terminal domain, and it seems likely AphA could also undergo conformation changes in this region to bind ligand (Fig. 14.4b) (Park et al. 2017a).

The structure of PadR bound to DNA shows PadR binds in the major groove in a diagonal relative to the DNA helix axis (Park et al. 2017a). Furthermore, comparing the PadR-DNA structure to the apo AphA structure shows the protein must undergo a conformational to contact DNA (Fig. 14.4c). These observations suggest AphA would bind AphB somewhere along its long axis most likely along the extended C-terminal dimerization domain (Fig. 14.4c).

14.3.2 AphB Structure

AphB belongs to the LysR-type transcriptional regulator family, which is the largest family of transcription factors in bacteria and examples are also found in archaea and eukaryotes (Taylor et al. 2012; Kovacikova and Skorupski 1999; Maddocks and Oyston 2008). LysR proteins are involved in a diverse set of processes, usually responding to environmental or metabolic cues, usually via ligand binding to the regulatory domain, although some are thought to respond directly to redox changes (Maddocks and Oyston 2008; Jo et al. 2019). AphB activity has been shown to increase under conditions of acidic pH and anaerobic conditions (Kovacikova et al. 2010; Taylor et al. 2012).

The structure of AphB was determined in 2012 and showed that it formed a tetramer, but has the two-fold symmetry of a dimer of dimers (Fig. 14.5) (Taylor et al. 2012). Each monomer consists of a helix-turn-helix DNA binding domain, a helical dimerization domain, and a C-terminal regulatory domain. The regulatory domain consists of two lobes, RD-I and RD-II, which formed a clamshell-like structure with a proposed binding pocket in the middle (Taylor et al. 2012). Each dimer is composed of two monomers, one in a compact conformation, and another in an extended conformation, which dimerize via antiparallel interactions of the helical domains, forming an L shape (Taylor et al. 2012). Two of the L-shaped dimers associate via their regulatory domains to form a tetramer through a two-fold symmetry rotation (Fig. 14.5a). This produces a complex with four DNA binding sites, two inner binding sites, from the compact monomers, and two outer DNA binding sites, from the extended monomers. Interestingly, the DNA binding helices are positioned such that they are too close to fit into major grooves without significant rearrangement (Taylor et al. 2012).

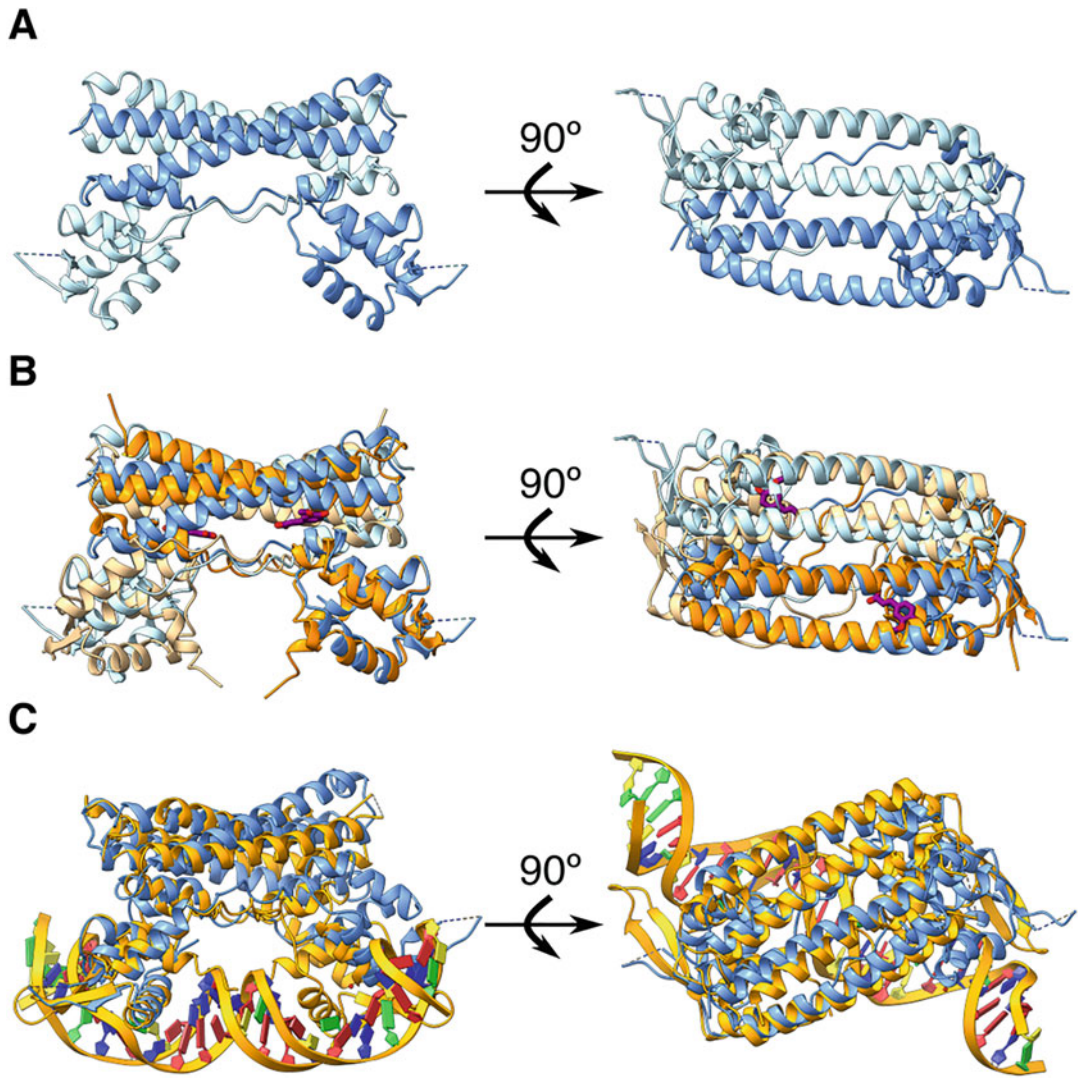

Fig. 14.4 AphA overview and comparison to PadR bound to ferulic acid or DNA. (**a**) AphA (De Silva et al. 2005) in blue with side and top views. The two chains are in different shades for visibility. (**b**) AphA (De Silva et al. 2005), in blue, aligned with PadR bound to ferulic acid (Park et al. 2017a). The protein is in orange and the ferulic acid between the N- and C-terminal domain is colored purple. (**c**) AphA (De Silva et al. 2005) in blue aligned with PadR bound to DNA (Park et al. 2017a) in orange

Mutations in the regulatory domain have been shown to increase AphB activity by making it insensitive to response to alkaline pH and/or anaerobic conditions (Taylor et al. 2012). The crystal structure of one of these mutants, N100E, highlights some of the structural changes AphB likely undergoes upon activation (Fig. 14.5a). In N100E, while the compact and extended monomer conformations still form a dimer, the tetramer no longer showed two-fold symmetry, and the DNA binding domains became spaced further apart and therefore more able to accommodate DNA binding (Fig. 14.6) (Taylor et al. 2012). This suggests a model in which ligand binding activates AphB via a conformational change in the regulatory domain that is passed on to the DNA binding domains, separating them to allow DNA binding.

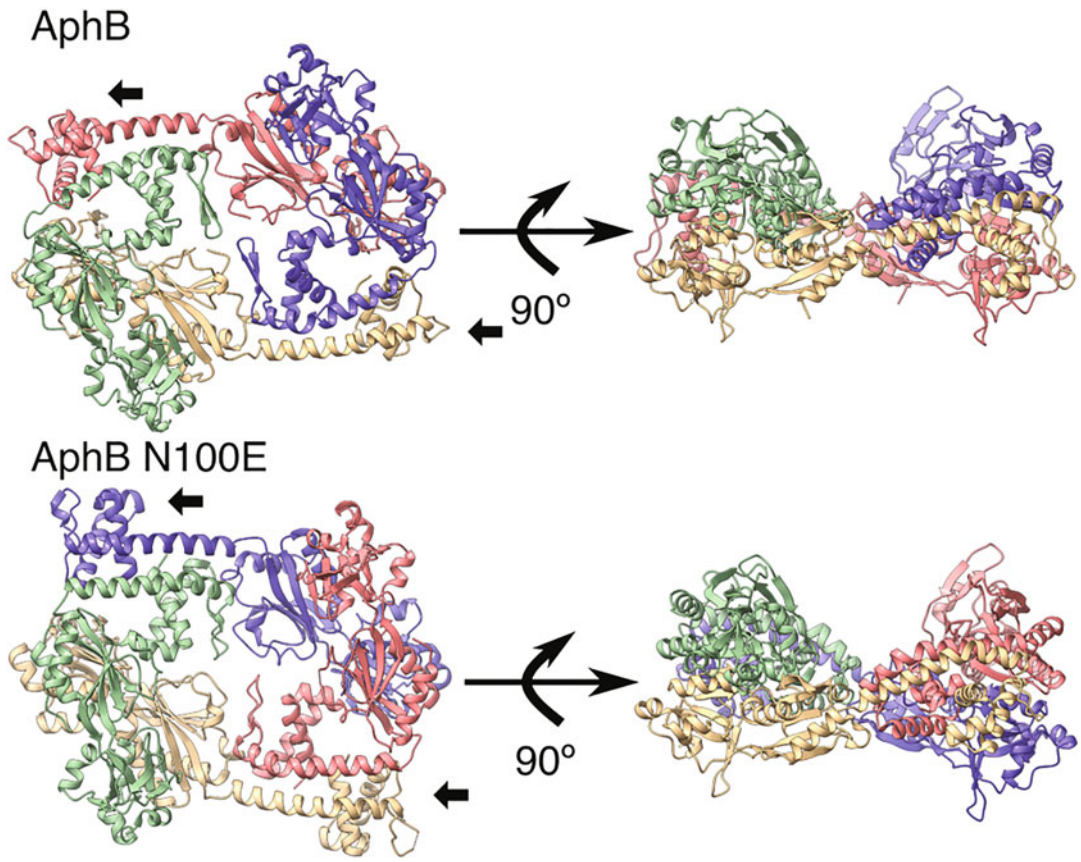

Fig. 14.5 AphB and AphBN100E structures (Taylor et al. 2012). Top is the structure of AphB wildtype and bottom is the structure of AphBN100E. The complexes are colored by chain. The black arrows point to the extended monomers DNA binding domain. Note in AphBN100E how they are flexed away from the core structure

As the physiological ligand for AphB is unknown, effort has been put into identifying the nature of ligand, as well as finding potential inhibitors. To that end, our laboratory performed a virtual screen centered around the ligand binding pocket (Fig. 14.7a) (Taylor et al. 2012; Privett et al. 2017). A screen identified several potential ligands that were experimentally tested, and one was unexpectedly found to increase AphB activity. Furthermore, in silico modeling using AutoDock showed the ligand was unlikely to bind in the putative pocket, but rather binds in a secondary pocket between the dimer interface of the two regulatory domains, defined by K103, R104, and R224 (Fig. 14.7b) (Privett et al. 2017). A subsequent study found that K103 is acetylated in stationary phase, corroborating the importance of this secondary pocket for AphB function (Jers et al. 2018).

In another study, a screen identified a small molecule inhibitor, ribavirin, that presumably bound in the regulatory domain pocket as it was unable to bind the constitutively active AphB N100E mutant (Mandal et al. 2016). Ribavirin was able to inhibit in vitro production of virulence factors and was also able to inhibit *V. cholerae* colonization in mouse models (Mandal et al. 2016). The authors of this study noted that other LysR proteins have homologous residues within their binding pockets, suggesting ribavirin could inhibit other LysR family members, and subsequently ribavirin has been shown to inhibit

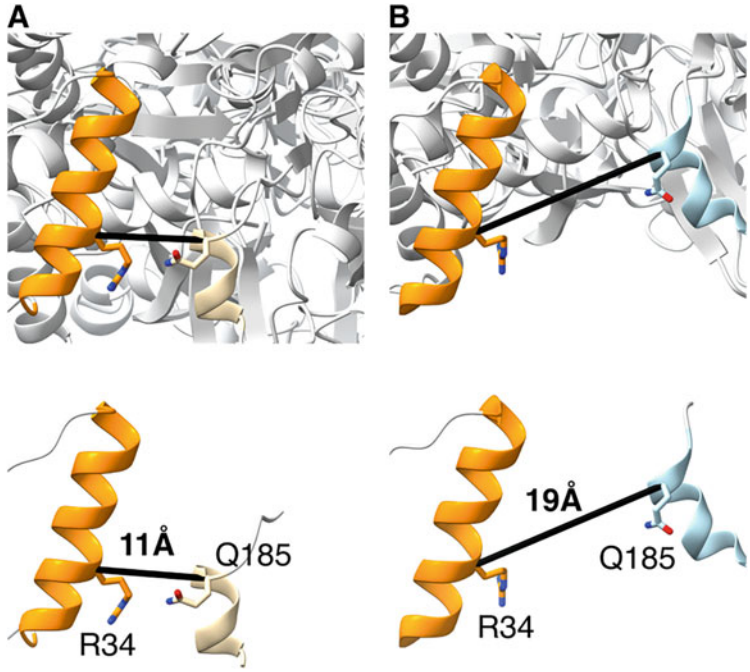

Fig. 14.6 Flexing of the extended monomers DNA binding domain from the core of the protein. The DNA binding helix, in orange, from congruent AphB, and AphBN100E (Taylor et al. 2012) extended monomers were aligned. The distance from R34 on the DNA binding helix to Q185 from the partner regulatory domain was measured for each complex. (**a**) AphB protein with the DNA binding helix in orange and the helix with Q185 in tan with the rest of the protein in light gray. Top, the helices are shown with the rest of the protein and bottom only the helices are present for clarity. (**b**) Is the same view for AphBN100E with the DNA binding helix in orange, and the helix with Q185 in blue. Top is the helices with the rest of the protein in gray and bottom are the helices by themselves. The distance measured for each complex is shown and labeled

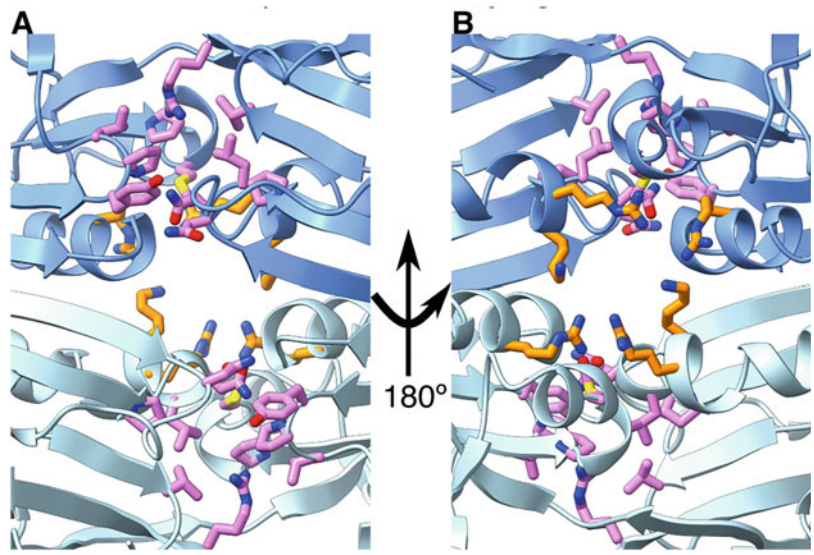

Fig. 14.7 The different binding pockets in the *V. cholerae* AphB regulatory domain dimer (Taylor et al. 2012). The regulatory domain dimer is in blue with the chains in different shades. (**a**) The ligand binding pocket identified in each regulatory domain is shown with the amino side chains making up the pocket in violet. (**b**) The second pocket between the regulatory domain dimer shown with the side chains in orange

colonization of *Salmonella typhi* and enteropathogenic *E. coli* in mouse models, presumably through AphB homologs (Mandal et al. 2016).

In addition to *V. cholerae*, AphB has been implicated in *V. vulnificus* acid tolerance and indirectly in promoting pathogenesis (Rhee et al. 2006; Jeong and Choi 2008; Elgaml and Miyoshi 2017). To determine if there were changes in the regulatory domain in response to oxidative changes, the regulatory domain of *V. vulnificus* AphB (VvAphB-RD) was solved in the presence of various peroxides. VvAphB-RD formed a dimer that was structurally similar to that seen in the *V. cholerae* AphB full length structure, and the pocket formed by dimerization of the regulatory domains was preserved (Park et al. 2017b). The VvAphB-RD was incubated with peroxides to determine if C227 was involved in redox sensing, as proposed previously, by being converted to cysteine-sulfenic acid (Liu et al. 2011; Conte and Carroll 2013). While no changes in C227 were detected upon peroxide treatment, when the protein was incubated with cumene hydroperoxide electron density was observed in the secondary binding pocket described above (Privett et al. 2017; Park et al. 2017b). This further suggests the pocket formed by regulatory domain dimerization is important for modulating AphB activity.

There are two main outstanding questions related to AphA and AphB function. One is the mechanism by which AphB responds to low pH and anaerobic conditions. While it has been suggested that C227 is involved in sensing low oxygen levels (Liu et al. 2011), subsequent studies have been unable to replicate that finding (Taylor et al. 2012; Park et al. 2017b). A second is that because many LysR family proteins are activated by ligand binding, and as small molecules have been shown to influence AphB activity, it is reasonable to presume AphB has a physiological regulatory ligand or ligands that interact via one or both of the sites that have been identified in the regulatory domain or between the regulatory domain dimers (Fig. 14.7) (Taylor et al. 2012; Maddocks and Oyston 2008; Privett et al. 2017). Another major question is how AhpB and AphA interact with each other and with DNA (Kovacikova and Skorupski 1999, 2001). Modeling suggests there must be considerable distortion of either the DNA or proteins for both AphA and AphB to bind the promoter DNA determined by DNaseI footprinting (Kovacikova and Skorupski 2001). Finally, given AphA and AphB work together in *V. cholerae*, it is likely that other PadR-LysR protein pairs will be identified that work together to regulate transcription in other bacteria.

14.4 ToxRS

ToxR and ToxS are the founding members of the ToxR family of transmembrane transcription factors that work in concert with integral membrane periplasmic binding partners. Conserved across the Vibrionaceae, ToxR is responsible for adapting the bacteria to environmental stressors, such as bile salts, antimicrobial peptides, and acidic conditions (Miller et al. 1989; Provenzano et al. 2000; Mathur and Waldor 2004). ToxS binds to ToxR, leading to full transcriptional activation, and protects ToxR from protease degradation. In certain conditions, ToxS is required for ToxR activity (Mey et al. 2012; Midgett et al. 2017; Almagro-Moreno et al. 2015b), and *toxS* mutants are less competitive than wildtype in infant mouse models (Pearson et al. 1990). In addition to their role in environmental stress response, these proteins have also been coopted into regulating virulence in some species, including *V. parahaemolyticus* and *V. cholerae* (Hubbard et al. 2016; Herrington et al. 1988; Whitaker et al. 2012).

ToxR is essential for *V. cholerae* to transition from the aquatic environment to being pathogenic in the human intestine (Herrington et al. 1988). ToxR augments the activity of another transmembrane transcription factor, TcpP, at the *toxT* promoter (Hase and Mekalanos 1998; Krukonis et al. 2000; Krukonis and DiRita 2003; Morgan et al. 2011). ToxT then goes on to activate expression of the toxin coregulate pilus (TCP) and cholera toxin (CT), the two major *V. cholerae* virulence factors responsible for cell attachment and diarrhea (Almagro-Moreno et al. 2015a; Matson et al. 2007).

In *V. parahaemolyticus*, ToxR is required for colonization in various animal models (Hubbard et al. 2016; Whitaker et al. 2012), in part this is due to ToxR activating expression of the T3SS2. The secretion system is activated in a bile dependent manner requiring ToxR to augment the activity of VtrA, which then leads to expression of the transmembrane transcription factor VtrB (Kodama et al. 2010; Gotoh et al. 2010; Hubbard et al. 2016). VtrB subsequently activates the transcription of the genes encoding the T3SS2 (Kodama et al. 2010; Gotoh et al. 2010). Interestingly, *V. cholerae* also encodes these VtrA and VtrB, and in non-O1/O139 strains they are involved in the expression of a type three secretion system (Miller et al. 2016; Alam et al. 2010).

Structural studies of ToxR and ToxS seek to understand the mechanism by which the protein pair responds to environmental signals, including bile, as well as the manner in which ToxS activates and stabilizes ToxR, as well as protecting it from protease degradation.

14.4.1 ToxR Structure

ToxR is 34 kDa transmembrane transcription factor that has a winged helix-turn-helix (wHTH) DNA binding domain followed by a variable linker connecting to the transmembrane domain, and a C-terminal periplasmic domain (Miller et al. 1987; DiRita and Mekalanos 1991). Because of the inherent difficulties involved with solving transmembrane protein structures, initial ToxR structural studies have focused on individual soluble domains, and recent works have provided insight into some of the questions surrounding ToxR function (Midgett et al. 2020; Gubensäk et al. 2021a, b).

14.4.2 DNA Binding Domain

The ToxR DNA binding domain is homologous to the OmpR family of winged helix-turn-helix (wHTH) transcription factors (Miller et al. 1987; Aravind et al. 2005). These domains consist of a β-sheet domain that is followed by a helix-turn-helix domain (HTH) with a two β-strand wing following the third helix (Martínez-Hackert and Stock 1997; Sadotra et al. 2021; Blanco et al. 2002; Schlundt et al. 2017). Structures of ToxR homologs PhoB and OmpR bound to DNA show third helix and wing domain make the contacts with DNA. The third helix binds the major groove of the recognition site, and the wing domain binds in the minor groove (Sadotra et al. 2021; Blanco et al. 2002; Schlundt et al. 2017). Both OmpR and PhoB make head to tail contacts on the DNA, suggesting that in vivo these domains can form a curved filament like structure as proposed by Blanco et al. (2002). This is important as ToxR is known to bind stretches of DNA that can be over 100 bp long suggesting that up to 10 copies of ToxR can bind (Krukonis et al. 2000; Crawford et al. 1998; Li et al. 2000). Furthermore, ToxR DNA binding domain is thought to bind to TcpP through interactions with its wing domain (Morgan et al. 2019; Crawford et al. 2003).

Recently an NMR structure of the DNA binding domain of ToxR was solved, showing it forms a wHTH domain. Similar to the CadC DNA binding domain, the C-terminal end formed an extra strand in the β-sheet domain (Gubensäk et al. 2021b) (Fig. 14.8a). The structure also suggests that to bind DNA helix 3 must lengthen as seen in OmpR and the OmpR-DNA structures (Fig. 14.8c) (Sadotra et al. 2021).

The ability of the ToxR-DBD to bind DNA was assessed by NMR. While the binding was weak, in the μM range, the ToxR-DBD had the highest affinity to the *toxT* promoter being almost 100 fold better than the binding affinity to the *ompU* and *ompT* promoters. This was interpreted as a consequence of ToxR having to capture the promoter for TcpP to bind to activate *toxT* transcription (Gubensäk et al. 2021b). If this is the case a similar mechanism should play out at the *vtrB/vttRB* promoter (Hubbard et al. 2016; Miller et al. 2016). It is interesting that the ToxR-DBD exhibits the highest affinity to a promoter that it does not directly activate. In addition, there are two caveats that point the way to future studies. The first is the ToxR-DBD is isolated from the full-length protein and there could be there are other determinants to DNA binding. The second

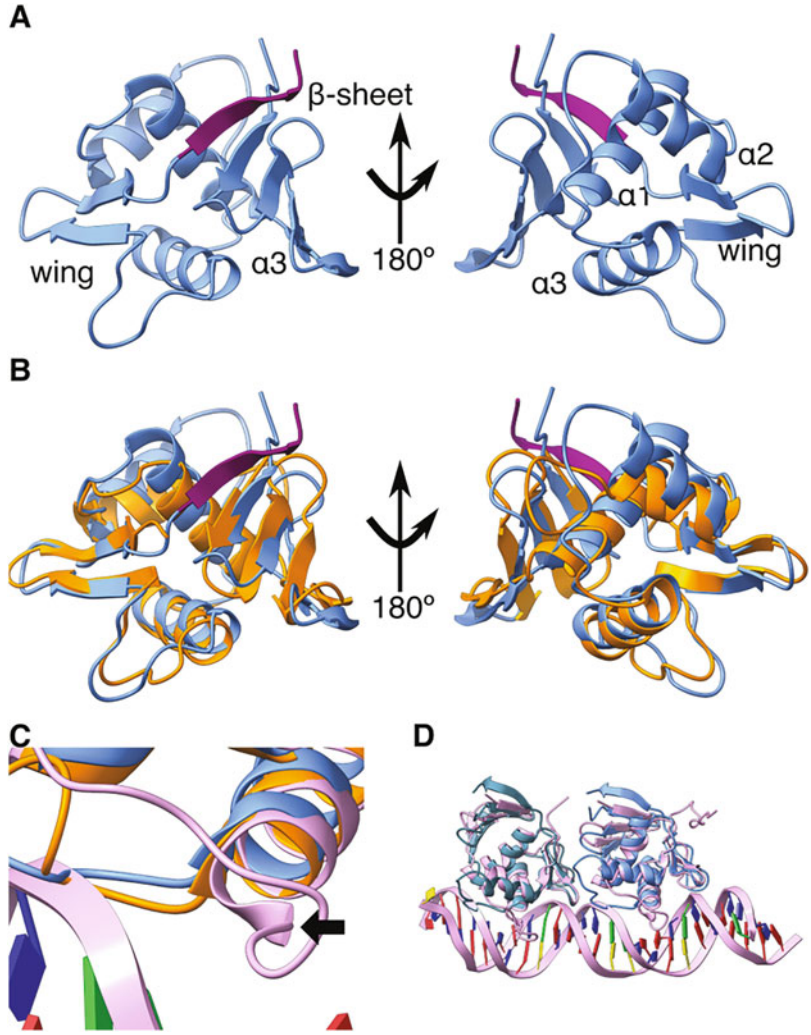

Fig. 14.8 The NMR structure of the ToxR DNA binding domain. (**a**) In blue is the DNA binding domain of ToxR as determined by NMR (Gubensäk et al. 2021b), with the helices, wing, and β-sheet domain annotated. The extra β-strand is in purple. (**b**) Is an overlay of the ToxR (Gubensäk et al. 2021b), in blue, and the OmpR (Sadotra et al. 2021), in orange, DNA binding domains. Note α3 is about the same length in both structures. (**c**) Comparison of the ToxR (Gubensäk et al. 2021b), in blue, OmpR, in orange, and OmpR-DNA (Sadotra et al. 2021), in plum, DNA binding domains. The arrow points to the extension of α3 in the OmpR DNA bound structure, not present in OmpR alone or the ToxR DBD

is the DNA fragments were minimal binding domains and there are likely to be avidity effects with longer pieces of DNA.

It has been suggested that the DBDs of ToxR and TcpP interact using their wing domains (Krukonis and DiRita 2003; Morgan et al. 2019; Crawford et al. 2003). This view is supported by structures of OmpR, PhoB, and RstA bound to DNA in a head to tail fashion (Sadotra et al. 2021; Blanco et al. 2002; Li et al. 2014). However, because mutating residues in the wing domain can also impact DNA binding, it is difficult to distinguish changes in DNA binding from those involving protein–protein interactions. Arguing against direct contact, NMR experiments failed to observe direct interactions between the ToxR-DBD and the TcpP-DBD (Gubensäk et al. 2021b). Additional biochemical and structural studies are required to determine how ToxR and TcpP DBDs interact at the ToxT promoter.

14.4.3 Periplasmic Domain Structure

The role the ToxR periplasmic domain plays in activating ToxR remains unclear (Midgett et al. 2017, 2020; Lembke et al. 2020). The observation that ToxR activity increases in the presence of bile salts independent of an increase in protein expression levels has led to the hypothesis that the

ToxR periplasmic domain acts as a direct sensor of bile salts (Mey et al. 2012; Midgett et al. 2017). This idea is supported by the observation that the interaction of the ToxR periplasmic domain with ToxS is increased in the presence of bile salts even though the salts destabilize the ToxR periplasmic domain (Midgett et al. 2017). This led to hypothesis that destabilization of the ToxR periplasmic domain leads to increased binding to ToxS allowing for ToxR activation (Midgett et al. 2017). In addition to bile salts modulating the interaction between ToxR and ToxS, the ToxR periplasmic domain has two cysteines that primarily form an intrachain disulfide bond that increases the affinity of the ToxR periplasmic domain to ToxS (Midgett et al. 2020). While this would suggest ToxR and ToxS remain in contact throughout the infection cycle, there is a proposed model where ToxR is activated by forming disulfide linked homodimers, independent of ToxS (Lembke et al. 2020).

A thorough understanding of how the ToxR periplasmic domain changes conformation during activation could lead to the development of ToxR inhibitors which would block the ToxR-ToxS periplasmic domain interaction, leading to premature ToxR proteolysis. To visualize this interface, two structures of the ToxR periplasmic domain have been solved, one by X-ray crystallography using the *V. vulnificus* ToxR periplasmic domain, and the other by NMR using the *V. cholerae* ToxR periplasmic domain (Midgett et al. 2020; Gubensäk et al. 2021a) (Fig. 14.9a). The ToxR periplasmic domain structure from *V. vulnificus* consists of 5 β-strands and 2 α-helices. The 5 β-strands are arranged in a β-sheet with one face of the sheet facing the solvent and the other face packed against the two helices, which are connected by a disulfide bond. Interestingly, the loop connecting the last beta strand to the second helix (β5-α2 loop) is disordered, suggesting a role of the disulfide bond is to constrain the loop, α2, and the last β-strand (Midgett et al. 2020).

The structure of the *V. cholerae* ToxR periplasmic domain has a similar fold, though the last β-strand and α-helix are now part of a flexible C-terminal loop that was modeled in to wrap around the globular domain in two different directions to form the disulfide bond with the cysteine in helix 1 (Fig. 14.9b) (Gubensäk et al. 2021b). The exposed disulfide bond in these NMR structures would appear to be targets for DsbC cleavage to allow the protein to refold in a more stable configuration.

Both studies found that the ToxR periplasmic domain is a monomer in solution in both the oxidized and reduced forms (Midgett et al. 2020; Gubensäk et al. 2021a). Therefore, it seems likely that ToxR does not form dimers, even when in the active state. This helps to clarify a point of confusion, as previous microbiological and molecular studies have presented contradictory results involving dimerization, depending on the protein fusion construct and expression strains used (DiRita and Mekalanos 1991; Ottemann and Mekalanos 1995; Dziejman and Mekalanos 1994; Dziejman et al. 1999; Lembke et al. 2018; Kolmar et al. 1995). It is likely that other interactions drive ToxR proteins to come into proximity with each other, which the previous experiments were mimicking.

14.5 VtrAC

VtrA and VtrC are another transmembrane transcription factor, integral membrane periplasmic binding partner pair, like ToxRS, they are also conserved across the *Vibrio* family (Li et al. 2016a; Alam et al. 2010). VtrA is a structural homolog of ToxR and also responds to bile salts (Li et al. 2016a; Gotoh et al. 2010; Midgett et al. 2020). VtrC stabilizes VtrA, and both proteins are required for bile salt induction of the type 3 secretion system 2 expression (T3SS2) in *V. parahaemolyticus,* which is required for cytotoxicity (Li et al. 2016a; Kodama et al. 2010; Gotoh et al. 2010; Hubbard et al. 2016; Miller et al. 2016). However, regulation of expression of the secretion system is indirect, as VtrAC along with ToxR activates the expression of VtrB, a transmembrane transcription factor without a periplasmic domain, which activates transcription of the T3SS2 (Li et al. 2016a; Kodama et al. 2010; Gotoh et al. 2010; Hubbard et al. 2016; Miller et al. 2016; Alam et al. 2010). Interestingly, unlike ToxR, VtrAC is selective to which bile salts it responds. VtrAC has been shown to

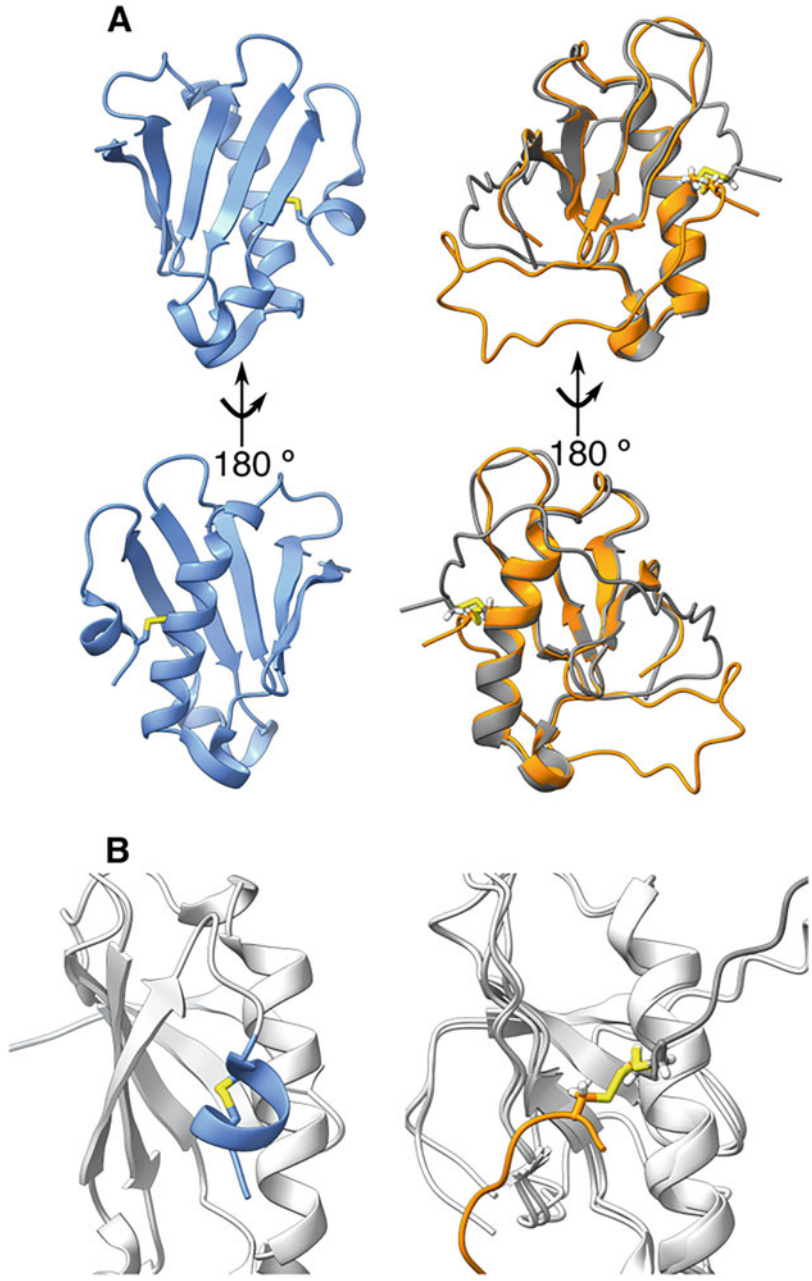

Fig. 14.9 The structure of the ToxR periplasmic domain from *V. vulnificus* and *V. cholerae* determined by X-ray crystallography (Midgett et al. 2020) and NMR (Gubensäk et al. 2021a), respectively. (**a**) On the right in blue is the X-ray structure of the ToxR periplasmic domain (Midgett et al. 2020) from *V. vulnificus*. On the left, in orange and brown, are two of the NMR calculated structures of the ToxR periplasmic domain (Gubensäk et al. 2021a) from *V. cholerae*. Note that the C-terminal portion of this structure wraps around the protein from both directions. (**b**) Detail of the disulfide bond and the 7 C-terminal residues in each structure. In the X-ray structure, in blue, the C-terminal residues form a helix which helps shield the disulfide bond from the environment (Midgett et al. 2020). While the NMR structures (Gubensäk et al. 2021a), in orange and brown, have the disulfide exposed to the environment

respond most strongly to glycol- and taurodeoxycholate, then by deoxycholate, then glycol- and taurochenodeoxycholate, and finally the conjugated cholate salts (Li et al. 2016a; Gotoh et al. 2010). VtrAC activity is not induced by the unconjugated primary bile salts, which do activate ToxR (Midgett et al. 2017; Gotoh et al. 2010).

14.5.1 VtrA Periplasmic Domain Structure

The structure of VtrA in complex with VtrC was determined in 2016 (Li et al. 2016a). Interestingly, the VtrC periplasmic domain could not be expressed without the VtrA periplasmic domain, and the domains formed an obligate heterodimer. VtrC forms an 8-strand β-barrel that extends into

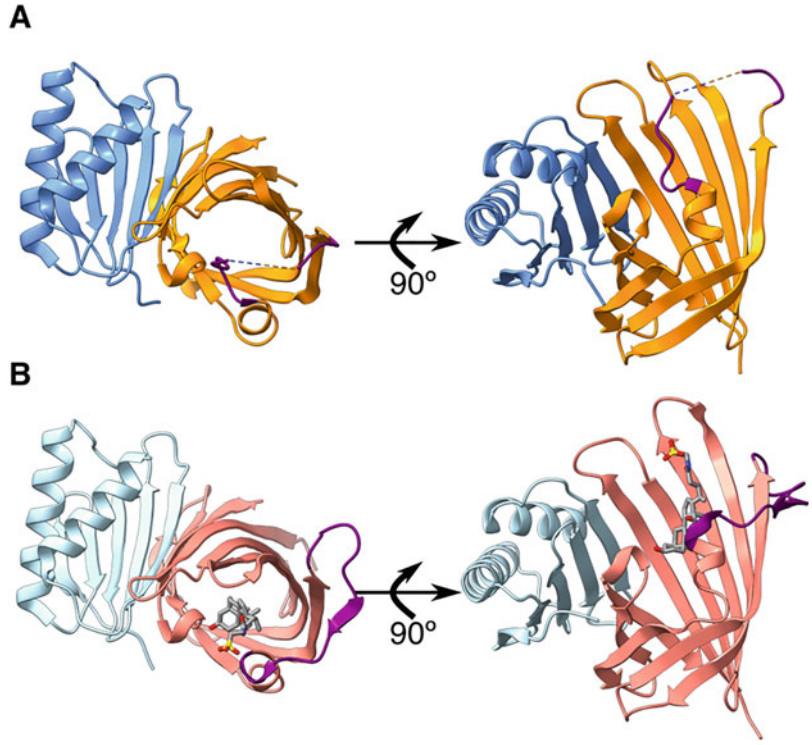

Fig. 14.10 The structures of the apo-VtrAC and VtrAC-taurodeoxycholate bound periplasmic domains. (**a**) The apo-VtrAC periplasmic domain structure. VtrA is in blue and VtrC is in orange (Li et al. 2016a). (**b**) The VtrAC-taurodeoxycholate (TDC) bound periplasmic domain structures (Li et al. 2016a). VtrA is in light blue, VtrC is in coral, and the TDC is colored by element and shown in sticks. The residues (110–123) that are displaced in the TDC bound structure are colored in purple in both (**a**, **b**)

the last β-strand of the VtrA β-sheet. One side of the 5 stranded VtrA β-sheet interacts with VtrC and the other side with the two VtrA helices (Fig. 14.10) (Li et al. 2016a). Unlike the ToxR periplasmic domain, in which the two helices are held together with a disulfide bond, the two helices in VtrA are held together with non-covalent interactions (Li et al. 2016a; Midgett et al. 2020). VtrA and ToxR are clearly structural homologs despite the lack of sequence homology (Fig. 14.11) (Midgett et al. 2020), and a DALI search failed to find proteins with similar folds, indicating these periplasmic domains are part of a new family involved in environmental sensing (Li et al. 2016a; Midgett et al. 2020; Holm and Laakso 2016).

14.5.2 VtrC Periplasmic Domain Structure

A DALI search suggests VtrC is a member of the lipocalin family and therefore might bind a hydrophobic ligand, such as bile salts (Li et al. 2016a; Holm and Laakso 2016), and the structure of VtrAC in complex with bound taurodeoxycholate (TDC) was subsequently solved (Li et al. 2016a). Overall, the apo and ligand bound structures are similar, expect for a loop moves from the center of the β-barrel to the side of the barrel (residues 110–123), opening a pocket to bind TDC (Fig. 14.10b) (Li et al. 2016a). Despite the observed different activities of VtrAC for different bile salts (Gotoh et al. 2010), the structures do not provide much insight into this discrimination. For instance, deoxycholate and cholate only differ by cholate having a hydroxyl on C7 (Fig. 14.12b). Although the structure appears to be capable of accommodating such a difference, VtrAC is partially activated by the conjugated cholates and does not respond to cholate (Gotoh et al. 2010). In addition, the role of ligand conjugation in binding to VtrC is not clear, as the taurine conjugate does not make any contacts with VtrC (Fig. 14.12c). Given VtrAC is preferentially activated by conjugated bile salts (Gotoh et al. 2010) means there is more research to be done to understand how VtrAC discriminates between conjugated and unconjugated bile salts.

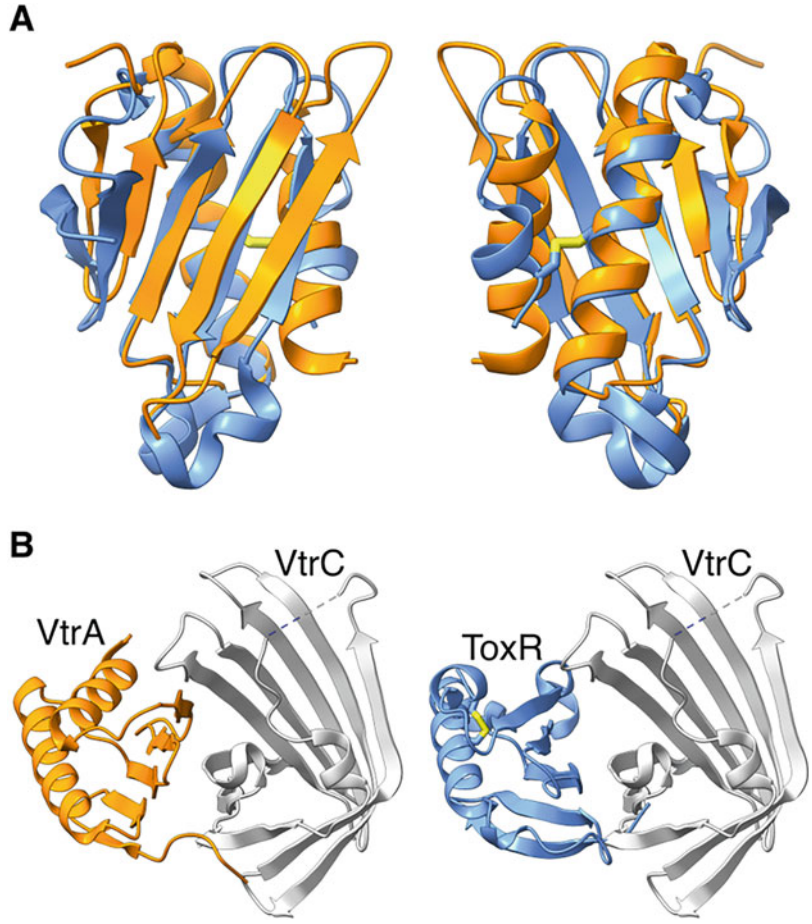

Fig. 14.11 The ToxR and VtrA homology provide a model of how ToxR may interact with ToxS. (**a**) The X-ray structure of the ToxR periplasmic domain (Midgett et al. 2020), in blue, overlaid with the VtrA periplasmic domain structure (Li et al. 2016a), in orange. (**b**) On the left is the VtrAC structure (Li et al. 2016a) with VtrA in orange and VtrC in light gray. On the right the ToxR X-ray structure (Midgett et al. 2020), in blue, was aligned with the VtrAC structure (Li et al. 2016a). For clarity only ToxR, in blue, and VtrC, in light gray, are displayed

The structures also fail to clarify how VtrC passes information about its state to VtrA, as the apo and ligand-bound structures of VtrA are essentially the same, and there are no obvious changes to either the VtrA and VtrC interfaces (Li et al. 2016a) (Fig. 14.13). The question of how ligand binding to VtrC leads to VtrA activation remains unanswered.

These structures will allow us to address fundamental questions about virulence regulation, from the atomic level to organismal level to probe host–pathogen interactions. Chief among these questions, what is the role of the periplasmic domain interfaces in activating transcriptional regulation? Given the ToxRS periplasmic domains can be separately purified makes them the ideal model to investigate the relationship of individual residues to ligand mediated interactions, virulence gene expression, and intestinal colonization in animal models. Besides using genetic methods to determine if disrupting the periplasmic domain interface can interfere with virulence, these structures can provide the basis for small molecule screening to determine if pharmacological intervention is a viable method for inhibiting virulence, not only in *Vibrio*'s but also other bacteria genera with homologous systems, e.g. PsaEF from *Yersinia pseudotuberculosis* (Yang and Isberg 1997). Furthermore, the structures of the periplasmic and DNA binding domains provide a stepping stone to determining the full-length structures to understand how information is passed through the membrane, how ToxR oligomerizes on DNA, as well as how it functions with TcpP and VtrA to regulate gene expression.

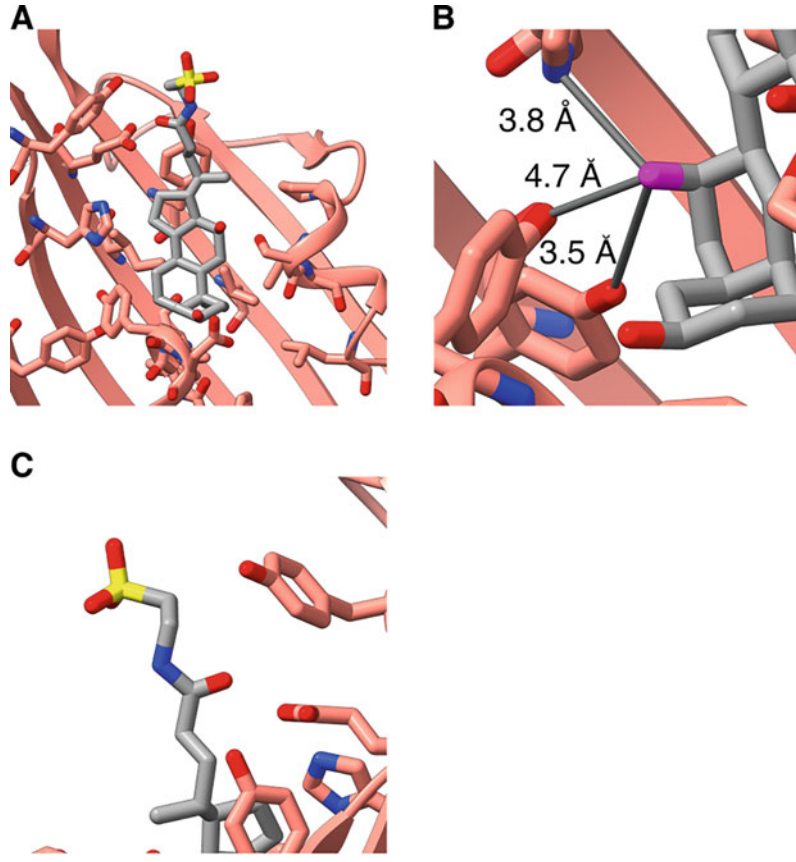

Fig. 14.12 Exploring aspects of TDC binding to VtrC. (**a**) Overview of TDC binding to VtrC (Li et al. 2016a). The side chains of VtrC amino acids within 5 Å of TDC are displayed as sticks. (**b**) Taurocholate modeled in the binding pocket by adding an oxygen, in magenta, at the C7 position of TDC. Distances from the modeled oxygen to the closest side chains are shown and labeled. (**c**) Detail showing the taurine conjugate lack of interactions

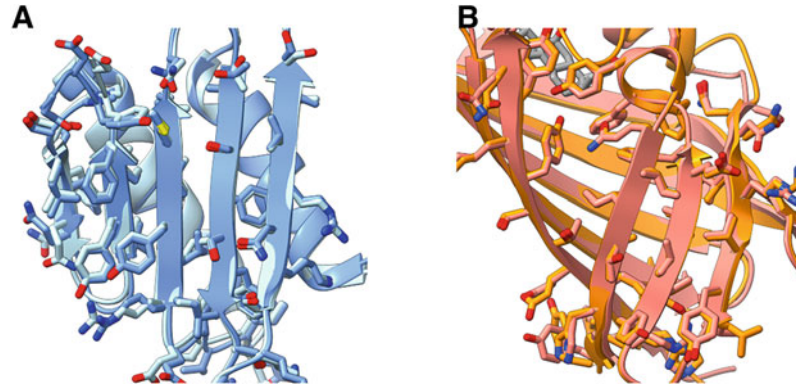

Fig. 14.13 Detail of the interfaces used by VtrA and VtrC to bind each other in the apo and TDC bound state. (**a**) Overlay of VtrA in the apo state in blue and in the TDC bound state in light blue showing the interface used to bind VtrC (Li et al. 2016a). (**b**) Overlay of VtrC in the apo state in orange and the TDC bound state in coral showing the interface VtrC uses to bind VtrA (Li et al. 2016a)

14.6 ToxT

The transcriptional activity of ToxR and TcpP is regulated by bile salts and other stressors found in the intestine, and therefore it is activated fairly early in the colonization process (Mey et al. 2012; Midgett et al. 2017; Mathur and Waldor 2004; Miller and Mekalanos 1988; Fan et al. 2014; Yang et al. 2013). However, because virulence gene expression is a metabolically costly endeavor for *V. cholerae*, it makes sense to have virulence gene expression ready, but "on hold," until the environmental conditions are optimal. This is accomplished through the master regulator, ToxT, which activates expression of the two *V. cholerae* virulence factors, the toxin coregulated pilus (TCP) and cholera toxin (CT) (Higgins et al. 1992; DiRita et al. 1991). Early studies showed that ToxT was inhibited by the unsaturated fatty acid components of bile (Chatterjee et al. 2007; Gupta and Chowdhury 1997). This suggests while ToxT is being translated it is inhibited by the unsaturated fatty acids until the bacteria reach the intestinal surface. Inhibition of ToxT activity is thought to be achieved by blocking or destabilizing dimer formation (Shakhnovich et al. 2007; Cruite et al. 2019; Childers et al. 2011; Hung et al. 2005), because ToxT binds some promoters with two identified "Toxboxes," and other in vitro assays indicate ToxT activates transcription as a dimer (Withey and DiRita 2006; Bellair and Withey 2008; Shakhnovich et al. 2007; Cruite et al. 2019; Childers et al. 2011). The structural mechanism by which ToxT is inhibited by bile components was clarified when the structure of it was determined in 2010.

14.6.1 ToxT Structure

The crystal structure of ToxT shows a typical AraC protein fold with an N-terminal regulatory domain (NTD) containing a cupin fold composed of beta-strands, and three alpha helices making up the dimerization region. The C-terminal DNA binding domain (CTD) contains seven helices and two helix-turn-helix motifs (Fig. 14.14) (Lowden et al. 2010). Fortuitously, ToxT crystallized with a fatty acid ligand bound to its regulatory domain pocket. The ligand was identified as cis-palmitoleic acid (PAM), a fairly common, 16-carbon monounsaturated fatty acid (UFA). Analysis of the structure showed the negatively charged carboxylic acid head group of PAM bridged two positively charges lysine side chains, one from the NTD and the other from the CTD (Lowden et al. 2010). The presence of the PAM in the NTD pocket appears to stabilize ToxT in a closed conformation, in which the two domains are in close contact, burying the fatty acid along with the lysine side chains, and preventing the two DNA binding helices from assuming a parallel orientation necessary for DNA binding. It is hypothesized that upon release of the PAM ligand and its negatively charged head group, charge-charge repulsion of the two lysine side chains lead to an open conformation where the two domains separate, and the DNA binding helices are freed to assume a parallel orientation. Subsequent studies demonstrated that UFAs including PAM and oleic acid inhibit ToxT DNA binding, whereas saturated fatty acids do not (Lowden et al. 2010). A number of other ToxT crystal structures were subsequent solved, and they also contained UFA (Cruite et al. 2019; Li et al. 2016b).

14.6.2 ToxT Regulation

The model that emerged from these studies is that upon crossing the mucosal layer, the concentration of bile decreases, leading to a lower concentration of free UFA. Release of UFA from ToxT induces the open form, which is able to dimerize and bind to DNA, activating transcription of TCP and CT and inducing virulence. To date, efforts to crystallize ToxT in complex with DNA have failed, and unlike other AraC proteins with similar NTDs, ToxT did not crystallize as a dimer (Soisson et al. 1997; Shrestha et al. 2015; Midgett et al. 2021). Despite the lack of structural information on the ToxT dimer, a recent crystal structure of apo-ToxT provides some clues as to how

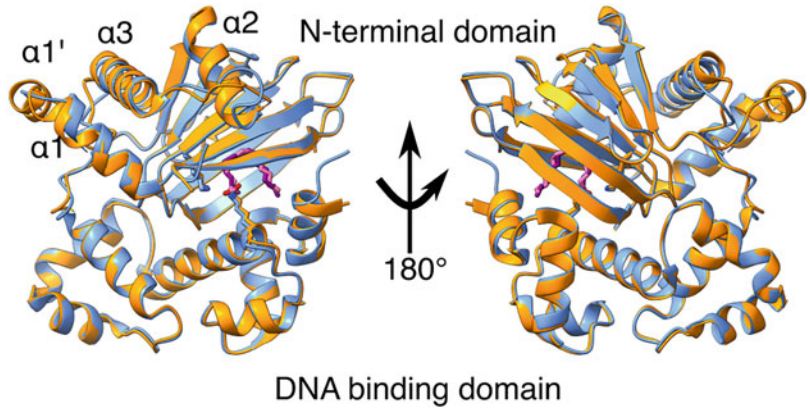

Fig. 14.14 Overlay of two structures of ToxT. Overlay of the ToxT structure from (Lowden et al. 2010) in blue and (Li et al. 2016b) in orange. Note the high degree of similarity between the structures. The bound fatty acids are shown in purple, (Lowden et al. 2010), and pink, (Li et al. 2016b). The N-terminal and DNA binding domains are labeled. The alpha helices in the N-terminal domain that are potentially involved in dimerization are labeled. Note the absence of the α1' helix in the 3GBG structure

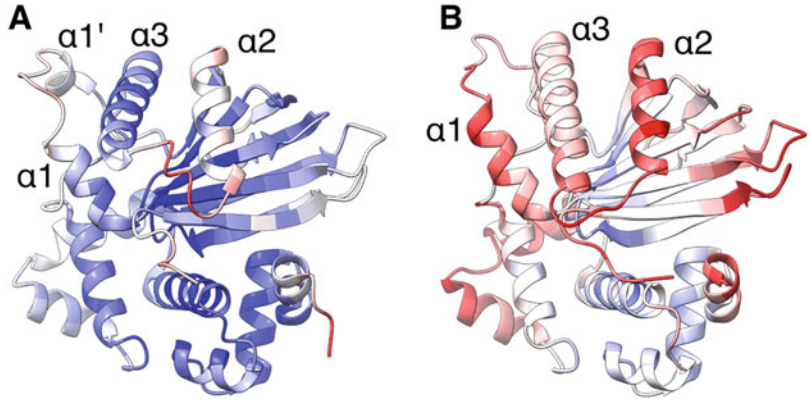

Fig. 14.15 Comparison of the dimerization helices and overall B-factors of the ToxTenvK231A UFA bound structure to the apo-ToxTenvK231A structure (Cruite et al. 2019). (**a**) UFA bound and (**b**) apo $ToxT_{env}$K231A structures were overlaid in ChimeraX and colored by B-factor. The alpha helices in the N-terminal domain are numbered. Note α1' forms behind α3 in the apo-$ToxT_{env}$K231A structure and is no longer visible in this orientation

ligand binding might influence dimerization and lead to ToxT inhibition.

Given the critical role of the two lysine side chains in stabilizing the inactive form of ToxT, it made sense to characterize ToxT variants with these side chains altered, and it was shown that removing one of the positive charges by changing the C-terminal lysine to alanine reduced sensitivity to UFA (Cruite et al. 2019). Structural analysis of this mutant identified two different forms of ToxT, one resembling the previously determined wild-type structure and containing PAM, but importantly, another form without bound ligand. While the apo form was still monomeric, analysis of the structure showed significant changes in the crystallographic B-factors, particularly in the dimerization and DNA binding regions (Fig. 14.15). The model that emerged from this

work is that ToxT is regulated by a dynamics based allosteric mechanism in which loss of ligand leads to an increase in the overall flexibility of ToxT, enabling both the dimerization and DNA binding regions to assume their active conformations (Cruite et al. 2019).

14.6.3 ToxT Inhibitors

The UFA bound to the ToxT pocket assumes a distinct U-shape with the bulk of the fatty acid chain buried in the NTD pocket (Lowden et al. 2010). Interestingly, a known ToxT inhibitor, virstatin, somewhat resembles the folded configuration of the UFA, suggesting virstatin's inhibitory mechanism is similar to that of the natural ligand (Hung et al. 2005). Based on these observations, we hypothesized that molecules mimicking the U-shaped conformation of the bound fatty acid, that were also covalently constrained would bind more strongly to ToxT as they would be "prefolded" and not have to pay the thermodynamic cost in terms of the decrease in entropy associated with a dynamic, free fatty acid folding into a single conformation in the binding pocket. We therefore designed a series of inhibitors with bicyclic 6 carbon ring systems with different degrees of saturation. All contained both a methyl group and a carboxylic head group with different chain lengths attached to the rings (Woodbrey et al. 2017). These compounds outperformed virstatin in culture, and crystal structures showed the compounds bound in the pocket displacing the fatty acid, with the carboxylic acid forming ionic bonds with the two lysine side chains that bound the fatty acid carboxylate (Fig. 14.16) (Woodbrey et al. 2017). Analysis of the crystal structures indicated the pocket could accommodate a ligand with a longer tail, and subsequent compounds were shown to be even more effective than the initial series, and outperformed virstatin in mouse models of colonization at concentrations nontoxic to the bacteria (Woodbrey et al. 2018). While the fatty acids themselves are not chiral, the bound conformations they adopt are very specific and "chiral-like." By making use of this insight with different chemical scaffolds has led to even more potent inhibitors that have demonstrated the usefulness of mimicking constrained fatty acids to develop selective inhibitors to fatty acid binding proteins (Markham et al. 2021).

Studying ToxT has led to an exciting new hypothesis that fatty acids regulate virulence inducing ToxT homologs in many enteric pathogens, as has been shown to occur in *Salmonella enterica* and enterotoxigenic *Escherichia coli* (Midgett et al. 2021; Golubeva et al. 2016; Bosire et al. 2020). Interestingly, these proteins appear to bind fatty acids slightly differently (Lowden et al. 2010; Cruite et al. 2019; Midgett et al. 2021), suggesting there is flexibility in the binding pocket. This opens an opportunity to target these proteins specifically and individually, which would help to minimize the cross reactivity of any resulting antivirulence therapeutics. Moreover, the studies involving ToxT inhibitors provide proof-of-principle that mimicking constrained fatty acid conformations is a viable method to pharmacologically manipulate protein activity and is likely applicable to other diseases.

An outstanding question in this area is how ToxT, and indeed other AraC proteins, specifically and selectively bind to DNA. ToxT is thought to bind to adjacent Tox-boxes, which would require substantial rearrangements in the N-terminal domain and DNA binding domains (Cruite et al. 2019). While SAXS studies have provided some evidence of this (Cruite et al. 2019), a high-resolution ToxT-DNA complex structure would provide much needed insight into how these AraC-family proteins respond to ligand binding to regulate transcription.

14.7 Summary

This is an exhilarating time as great progress has been in understanding the structures of many of the proteins that contribute to regulating *Vibrio spp.* virulence. The insights gained from these studies and structures are stimulating further work to illuminate the basic molecular mechanisms by which the virulence cascade unfolds. This will enable the development of atomistic models of how protein interactions contribute to the host–pathogen communications that

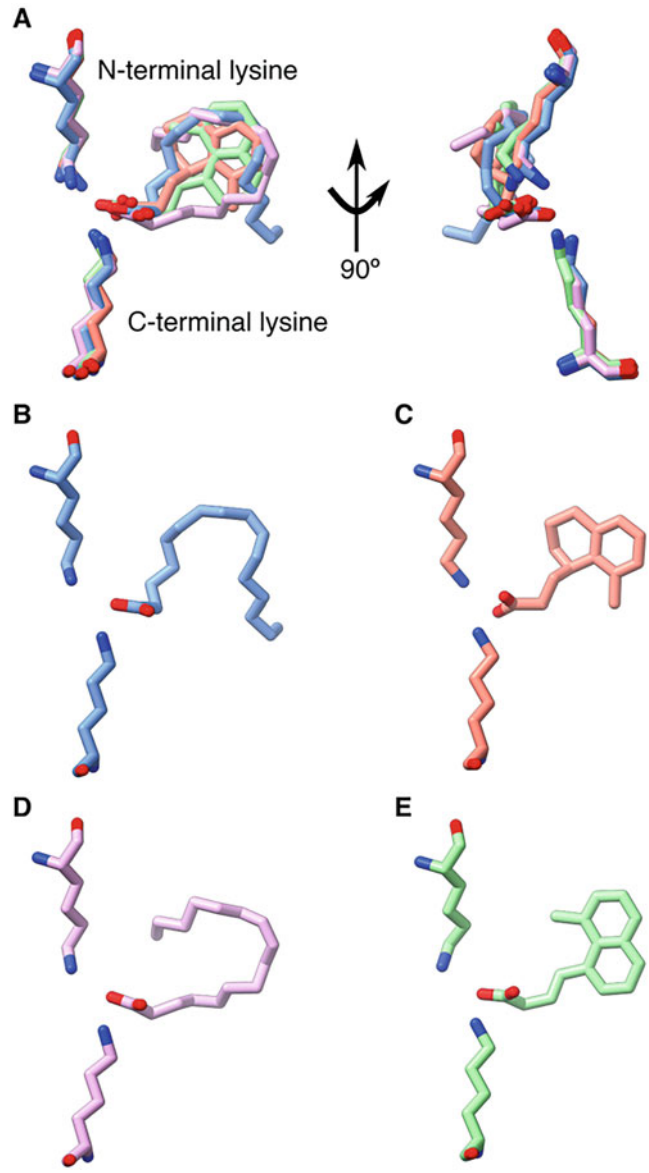

Fig. 14.16 Conformations of various ligands bound to ToxT. (**a**) Overlay of the ligands with the two lysines from the N-terminal and C-terminal domains shown. (**b**) The palmitoleic acid bound to ToxT from the (Lowden et al. 2010) structure. (**c**) Compound 5a (Woodbrey et al. 2018) bound to ToxT. (**d**) UFA bound to $ToxT_{env}$ (Cruite et al. 2019). (**e**) Compound 3b (Woodbrey et al. 2018) bound to ToxT

leads to virulence. Additionally, such understanding will enable the development of new antivirulence compounds that can specifically target these pathogens and provide a foundation to target homologous proteins in other bacterial pathogens and in other disease states.

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