A disulfide constrains the ToxR periplasmic domain structure, altering its interactions with ToxS and bile-salts

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ToxR is a transmembrane transcription factor that, together with its integral membrane periplasmic binding partner ToxS, is conserved across the Vibrionaceae family. In some pathogenic Vibrios, including V. parahaemolyticus and V. cholerae, ToxR is required for bile resistance and virulence, and ToxS achieves this in part by ensuring formation of an intra-chain disulfide bond in the C-terminal periplasmic domain of ToxR (dbToxRp). In this study, biochemical analysis showed dbToxRp to have a higher affinity for the ToxS periplasmic domain than the non-disulfide bonded conformation. Analysis of our dbToxRp crystal structure showed this is due to disulfide bond stabilization. Furthermore, dbToxRp is structurally homologous to the V. parahaemolyticus VtrA periplasmic domain. These results highlight the critical structural role of disulfide bond in ToxR and along with VtrA define a domain fold involved in environmental sensing conserved across the Vibrionaceae family.

ToxR is the founding member of a group of transmembrane transcription factors1 and is conserved throughout the gram-negative Vibrionaceae family of marine bacteria2–5. Several Vibrio species use ToxR to mediate bile resistance6, and a few pathogenic species have co-opted ToxR for virulence induction6,7. Vibrio parahaemolyticus ToxR is important for colonization and virulence in mouse models7, where it induces type III secretion system expression by working with VtrA to activate expression of VtrB7. ToxS, an integral membrane periplasmic binding partner of ToxR, is also important for V. parahaemolyticus colonization in a mouse model7. In V. cholerae, ToxR is required for human colonization8, most likely through induction of bile resistance5 and, in conjunction with TcpPH, virulence gene expression9.

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ToxR regulates many genes in response to stimuli, including ompT and ompU10,11. ToxR directly inhibits ompT expression12 and activates ompU transcription13, leading to a change in the outer-membrane porin composition14. ToxR is thought to activate virulence by augmenting TcpP activity, leading to toxT transcription15,16 and subsequent expression of cholera toxin and the toxin co-regulated pilus17,18. Given the importance of ToxR and ToxS for both bile resistance6 and pathogenesis in the Vibrio family7,8, understanding how they interact with each other and respond to the environment is critical for understanding the disease processes and providing insight into how related proteins function in other pathogenic bacteria19,20.

Previously, experiments in minimal media have shown V. cholerae ToxR activity can be regulated by the amino acid mixture of N, R, E and S (NRES)20, or bile salts20,21. The NRES mixture led to an increase in ToxR and subsequent OmpU expression20,22. In contrast, bile salts stimulated ToxR activity without increasing protein amounts20,22. This demonstrates ToxR activity can be regulated by two different mechanisms, one dependent on the Var/Csr system to increase ToxR mRNA and protein expression22, and a second to mobilize existing ToxR to become transcriptionally competent21,22. We hypothesize this potentially occurs via bile salts interacting with the ToxR periplasmic domain.

While over-expression of ToxR can lead to ToxR regulon transcription, under physiological conditions ToxR requires ToxS, an integral membrane protein with a periplasmic domain, for full activity23. In addition, ToxS protects ToxR from degradation in conditions of stationary growth and alkaline pH24,25. Furthermore, ToxS is known to affect the formation of a disulfide bond in the ToxR periplasmic domain26,27. Investigations into the contribution of cysteines C236 and C293 on V. cholerae ToxR function showed single cysteine mutants were inactive28.
However, as this paper was being prepared a report was published showing when ToxR C293S was overexpressed it formed a disulfide linked homo-dimer active in porin regulation in a ΔdegP background. Whereas a double mutant had decreased function in porin regulation, but no defects in virulence gene expression. Moreover, the double mutant was shown to be more susceptible to degradation than wild-type ToxR, even in the stabilizing presence of ToxS. In wild type ToxR, these cysteines are known to form either an intra-chain disulfide bond or a disulfide linked homodimer, which is present when toxS is deleted in V. cholerae. Since ΔtoxS strains have defects in ToxR activity, it follows that the intra-chain disulfide bonded, monomeric form of the ToxR periplasmic domain is the physiological active conformation.

While the intra-chain disulfide bonded conformation of ToxR is certainly the predominant form, ToxR lacking a disulfide bond has been hypothesized to be present in vivo based on two prior studies. First, the periplasmic domain of ToxR and TcpP can form a disulfide linked heterodimer, which can only occur if the two cysteines in ToxR are free to form a bond. Second, bile salts induce disulfide stress by inhibiting the activity of DsbA, the chaperone responsible for inducing disulfide bond formation. Prior work in our laboratory showed the non-disulfide bonded ToxR periplasmic domain was destabilized by bile salts, however, the salts increased binding to the ToxS periplasmic domain (ToxSp). This led us to hypothesize the ToxR periplasmic domain has evolved to become destabilized by bile salts, leading to stronger ToxS binding and increased activity of ToxR.

To characterize and visualize the differences between the disulfide bonded and non-disulfide bonded forms of ToxR we expressed and purified the disulfide bonded conformer of the ToxR periplasmic domains (dbToxRp) from V. cholerae and V. vulnificus for biochemical and structural studies. The dbToxRp was able to bind ToxSp, and was destabilized by bile salts, although, unlike the non-disulfide bonded ToxR periplasmic domain (ToxRp), bile salts did not increase the binding of dbToxRp to ToxSp. The structure of dbToxRp showed the domain is globular composed of 2 α-helices, linked via the disulfide bond, and a β-sheets composed of 5 β-strands. Furthermore, we found that dbToxRp is structurally homologous to the periplasmic domain of VtrA. VtrA is a transmembrane transcription factor that shares similarities with ToxR as both have an OmpR DNA binding domain and a periplasmic domain that is involved in bile sensing.

Overall, these results demonstrate the important role of ToxR disulfide bond formation in the interaction with ToxS. This represents a first step in understanding the structure function relationship for virulence induction and bile resistance, paving the way for a better understanding of the biochemical nature of ToxR activation as well as related proteins.

Results

ToxR containing a disulfide bond is folded and active. The V. cholerae disulfide ToxR periplasmic domain (Vc-dbToxRp) construct was confirmed to be structurally and functionally similar to the previously analyzed ToxRp by comparing NMR1H,15N HSQC's, determining if chenodeoxycholate (CDC) and cholate could domain (Vc-dbToxRp) construct was confirmed to be structurally and functionally similar to the previously analyzed ToxRp构

Crystal structures reveal the structural role of the ToxR periplasmic domain disulfide bond. Efforts to solve the structure of the V. cholerae dbToxRp were unsuccessful necessitating the need to screen ToxR periplasmic domains from several Vibrio sp. to find a suitable target for crystallization. The dbToxRp from V. vulnificus, which shares 55.2% identity to the V. cholerae ToxR periplasmic domain, readily crystallized. The crystal structure of V. vulnificus intra-chain disulfide ToxR periplasmic domain (Vv-dbToxRp) was solved by molecular replacement using a selenomethionine (SeMet) labeled structure to 1.25 Å (Statistics are shown in Table S1). The SeMet and native structures were aligned in Chimera using MatchMaker with an RMSD of 0.32 Å over the backbone of all 91 resolved residues (Fig. 3a). The two structures are essentially the same we focused on analyzing the native structure. The disulfide bonded ToxR periplasmic domain consists of 5 β-strands and 2 α-helices forming an α/3 β fold. Topologically the domain starts with the first two anti-parallel β-strands followed...
by the first $\alpha$-helix. This is followed by the last 3 $\beta$-strands, and the structure terminates with the second $\alpha$-helix which forms a disulfide with $\alpha_1$. The 5 $\beta$-strands are arranged in a $\beta$-sheet with one side packed against the two $\alpha$-helices, through hydrophobic interactions (Fig. 3b). The other side of the sheet is solvent exposed. Interactions of note include a Pi-Pi stacking interaction between the Tyr236 from $\alpha_1$ and Tyr263 from $\beta_4$ (Fig. 3c left panel) in the hydrophobic core of the protein. A sulfate ion was found in the structure, stabilized by the side chain of Arg203 and the amide nitrogens of Leu222 and Gln223 (Fig. 3c middle panel). Finally, the disulfide bond between Cys232 and Cys289 covalently attaches $\alpha_2$ to $\alpha_1$ stabilizing the structure (Fig. 3c left panel). The structure is well ordered, as typified by the clearly visible electron density for the disulfide bond in a composite omit map of the structure (Fig. 3d first panel). Interestingly, the density of the $\alpha_2$-$\beta_5$ loop (residues 281–284) was poorly defined (Fig. 3d middle panel). In addition, the $\alpha_2$-$\beta_5$ loop has the highest B-factors in the structure (Fig. 3d last panel). Furthermore, standardization (Z-score) of the $C_\alpha$ B-factors showed the 281–284 $C_\alpha$ Z-scores are in the top 5% of the distribution (Fig S1). Taken together this indicates the loop is flexible. Therefore, we hypothesize a major role of the disulfide bond is to stabilize the structure, and importantly the ToxS binding region, by constraining this flexible loop as well as the relatively short helix, $\alpha_2$, which follows it.

**VtrA and ToxR periplasmic domains are structural and functional homologues.** The structure of the Vv-dbToxRp was similar to the previously published structure of the VtrA periplasmic domain from *V. parahaemolyticus* [34]. VtrA, like ToxR, is a transmembrane transcription factor with a winged helix-turn-helix domain and a C-terminal periplasmic domain involved in virulence induction in response to bile salts [32,33], potentially by sensing them with its binding partner VtrC [34]. However, to take an unbiased approach to find ToxR periplasmic domain structural homologs we performed a DALI search [36]. The search returned a number of proteins with various functions generally unrelated to the function of ToxR. Interestingly, VtrA was listed as the 118th result that matched the dbToxRp. To gain more insight we curated the list to remove potential duplicated proteins, assessed the cellular location of the proteins (extracellular, cytoplasmic, periplasmic, etc.), and determined if any contained disulfide bonds for the top 50 proteins, in which VtrA ranks 38. Only 4 other periplasmic proteins were identified, and 8 extracellular proteins. Out of those only 5 had disulfide bonds, and none of the disulfide bonds are in comparable areas as the dbToxRp bond (Fig S2). Given the above results and the low percent identity of the hits returned from the DALI server we further examined the structures using Chimera [34]. The pdb files of the structures up to the VtrA periplasmic domain were downloaded from the PDB and aligned to the Vv-dbToxRp structure using MatchMaker [35], with the secondary structure weighted at 95%. Interestingly, MatchMaker [35] aligned the
VtrA periplasmic domain to dbToxRp with the lowest all atom RMSD of 5.5 Å (Table S2), suggesting despite the placement of VtrA in the DALI search the VtrA and ToxR periplasmic domains are structural homologs. Given the structural homology between the domains, we examined the alignment of the two structures in Chimera, using the default parameters, as well as assessed topological plots produced by Pro-origami. The alignment and plots showed the domains have the same topology, with all secondary structure elements in similar positions. Both have the N-terminal two β-strands followed by the first helix, then the next three β-strands completing the β-sheet, and finally terminating with the second helix (Fig. 4). The main difference between the two structures is the position of the last β-strand and α-helix. In VtrA the last helix is longer pushing the last β-strand further away from the core structure (Fig. 4b middle panel). Also, as the periplasmic domain of VtrA does not contain cysteines (Fig. 4b last panel) there can be no stabilizing disulfide bond. However, based on the clear topological, structural, and functional similarities we propose the periplasmic domains of ToxR and VtrA represent a domain family utilized in sensing the extra-cellular environment.

dbToxRp lacks a dimerization interface. The periplasmic domain of ToxR has been proposed to mediate dimerization, which is thought to be essential for activity. Prior studies replacing the periplasmic domain of ToxR with proteins known to dimerize, resulted in active constructs. Therefore, we assessed the ability of the purified disulfide bonded ToxR periplasmic domain to form dimers in-vitro and in the crystal. To assess the ability of the domain to dimerize in solution we performed analytical ultracentrifugation. The domain sedimented as a single species with a molecular weight of 11.8 kDa and 12.7 kDa, consistent with a monomer (Fig S3) of predicted
molecular weight of 11.5 kDa. In the crystal the *V. vulnificus* dbToxRp made two contacts whose biological relevance were assessed using PISA. Both contacts were determined to be due to crystal packing (Fig S4). Therefore, we conclude dbToxRp is most likely monomeric.

### Discussion

Here we have biochemically characterized and solved the structure of the ToxR periplasmic domain with an intra-chain disulfide bond. This conformation was found to be similar to the non-disulfide conformation as confirmed by NMR, indicating both constructs consist of a stable core fold. In addition, the disulfide conformer is
destabilized by bile salts, similar to previously published results\(^2\). However, in contrast to ToxRp\(^2\), bile salts had no effect on the binding of the dbToxRp conformer to ToxSp. Furthermore, dbToxRp bound ToxSp about 2–4 fold more in our pull down assay than the non-disulfide bond conformer, suggesting the disulfide bond stabilizes the ToxS binding interface.

The dbToxRp structure is clearly homologous to the structure of the VtrA periplasmic domain\(^1\). Furthermore, given VtrA and ToxR share functional similarities we can hypothesize how ToxR and ToxS interact based on what is known about VtrA. Previously, the VtrA periplasmic domain was crystallized with its binding partner the VtrC periplasmic domain. The structure showed the proteins interact in two ways, via parallel beta strand hydrogen bonds between the $\beta_5$ strand of the VtrA sheet and the most N-terminal $\beta$-strand of the VtrC beta-barrel (arrow 1 in Fig. 5a left panel), as well as $\beta$-sandwich like interaction between the VtrA $\beta$-sheet and the C-terminal region of the VtrC $\beta$-barrel (arrow two in Fig. 5a left panel)\(^4\). Based on this, we predict ToxS is a structural homolog of VtrC and the ToxR periplasmic domain will bind ToxS in a similar manner, as shown by a structural alignment of dbToxRp with VtrAC (Fig. 5a right panel). Despite these structural similarities there are differences between these two regulatory pairs. First, VtrA requires VtrC for expression\(^4\) whereas ToxR can be expressed by itself\(^2\,20,21,23,26\). Second, ToxR depends on the formation of an intra-chain disulfide bond to properly fold while the VtrA periplasmic domain is cysteine free. It would be interesting to determine if the ToxR periplasmic domain could be stabilized without a disulfide bond and what effect that would have on ToxR function.

By combining our results with insight gleaned from the VtrAC structure, we can begin to explain how ToxR and ToxS might interact. At least in vitro, ToxR can exist in three forms, a monomer with an intra-chain disulfide bond, a disulfide-free monomer, and a (likely non-physiological) disulfide bonded homodimer. Disulfide formation in the ToxR monomer apparently constrains the $\beta 5$-$\alpha 2$ loop and the adjacent $\alpha 2$ helix. This, based on structural homology with VtrAC, stabilizes the binding interface between ToxR and ToxS. Such an increase in stability is consistent with observations regarding the degradation of the double cysteine mutant in Lembke et al. 2018\(^2\). Interestingly it has been observed that ToxS enhances ToxR intra-chain disulfide bond formation\(^2\). How this occurs in the context of the above results is unexplored.

The structure of the dbToxRp provides a potential explanation to why breaking the disulfide bond interferes with ToxS binding and how bile salts can modulate the interaction. In the absence of intra-chain disulfide bond formation, ToxR is destabilized and it is unlikely the short $\alpha 2$ helix would form. Additionally, $\alpha 2$ might assume an alternate conformation in which it blocks interaction with ToxS. Given bile salts increase the ToxRp-ToxSp interaction suggests bile salts disrupt whatever alternate structure $\alpha 2$ forms in the absence of the disulfide bond, thereby allowing ToxS binding. In dbToxRp, the loop and $\alpha 2$ are already in a conformation that stabilizes the interface, rather than blocking it, and bile salts have no effect on ToxS binding.

This leaves open the question of how ToxS augments ToxR activity. ToxR is thought to be active as a dimer and its periplasmic domain has been presumed to mediate dimerization\(^38-41\). While it has been proposed that
ToxR cytoplasmic domains may interact in the presence of DNA29,43, when the periplasmic domain of ToxR was replaced by MalE, which does not dimerize, ToxR activity decreased, indicating the periplasmic domain mediates dimerization39,40. Our biochemical analysis indicates the domain is a monomer in solution and structural analysis failed to find a dimer interface, arguing for other determinants of dimerization. Given our data, and the stipulation the work in this report has been done with purified domains, we propose ToxS is the most likely candidate for this, and the disulfide bond dependent interaction of ToxR with ToxS results in formation of a dimer of ToxR and ToxS dimers, forming a complex competent for ToxR activation. Future structural studies aimed at determining the structures of ToxSp as well as that of the complex between dbToxRp and ToxSp should clarify if this is the mechanism for dimerization.

Alternately, given ToxR binds DNA, DNA could very well provide the impetus for ToxR oligomerization. In such a model ToxR binds DNA, and weak protein-protein interactions could promote dimerization or higher order oligomers, as seen in experiments with VtrA44. Yet another possibility is DNA organizes ToxR in the membrane without specific interactions between domains. Determining which might be the case will require reconstruction of full-length ToxR in a membrane and in the presence of DNA.

While the work described here has been performed with isolated domains and require experiments with full-length proteins for confirmation, our results suggest a model of bile salt mediated ToxR activation, in which ToxR is a monomer in the membrane, binding to ToxS. The non-disulfide bonded conformation has a low affinity for ToxS. The interaction between the two proteins increases in the presence of bile salts, potentially by the destabilization of steric clashes. This would lead to increased ToxR binding to DNA, allowing for ToxR-ToxR interactions, ensuring ToxR activity in the face of bile salt induced disulfide stress45. Alternately, ToxR without a disulfide bond is the conformation that responds to bile salts. However, a double cysteine to serine mutant showed reduced porin regulation in the face of osmotic stress, although the mutant was still able to regulate virulence production27. When ToxR contains an intra-chain disulfide bond, its affinity for ToxS is increased and unaffected by bile salts. Given this form of ToxR is the most abundant, we believe it is involved in bile sensing. When the domain is destabilized by bile salts, this signal is transmitted across the membrane, increasing the ability of ToxR to bind DNA. This effect is augmented by interaction with ToxS, which remains bound to ToxR. In this model, the action of ToxS with DNA allows for the dimerization of ToxR (Fig. 5b). Recently, Lembke et. al. 2020 has suggested the ToxR periplasmic domains may have weak protein-protein interactions that are stabilized by ToxS. Then in the

Figure 5. Models of potential interactions between ToxR and ToxS periplasmic domain using the VtrAC structure, and how ToxR and ToxS interact to induce virulence as well as bile resistance. (a) The left panel shows the VtrAC (5kev) (VtrA orange, VtrC in gray) structure with arrows pointing to the interfaces between the proteins. Arrow 1 points to the VtrA β5-strand that extends the VtrC β-sheet, and arrow 2 points to VtrA β-sandwich interaction with VtrC. In the right panel the Vv-dbToxRp structure (blue) was aligned with VtrA in the VtrAC structure. VtrC (gray) is presented as a stand in for ToxS. The numbered arrows point to the β5 strand (1) and the potential β-sheet interface (2). (b) A model of the potential interactions between ToxR, ToxS, and bile salts. ToxR is in blue with the helices depicted as circles and numbered, ToxS is in yellow, and DNA is represented by an orange and blue helix. The non-disulfide bonded form of ToxR, with only helix1, interacts weakly with ToxS (1). However, bile salts increase the interaction of the non-disulfide bonded ToxR to ToxS, leading to ToxR binding DNA allowing for ToxR periplasmic domain dimerization and activation (2). In addition, ToxS favors the formation of the ToxR intra-chain periplasmic disulfide bond, which increases the affinity between ToxR and ToxS (3). In this case bile salts destabilize the periplasmic domain, which remains bound to ToxS. The destabilization is transmitted across the membrane to the DNA binding domain allowing for transcriptional activity, augmented by ToxS (4). Again, ToxR binding to ToxS and DNA allows for ToxR periplasmic domain interactions.
presence of bile salts and DNA the ToxR-ToxS interaction peaks and this allows DsbC to form ToxR disulfide linked homodimers, through Cys236, leading to transcriptional activity that does not rely on ToxS29. However, they were unable to demonstrate chromosomally expressed wild type ToxR formed disulfide linked dimers in the presence of bile salts. Furthermore, they did not demonstrate ToxR-ToxS periplasmic domain interactions without disulfide bonds. In addition, our data suggests the dsToxR:p binds ToxS with a higher affinity than the non-disulfide bonded form, unaffected by bile salts. Consequently, there are two competing models for how ToxR is activated by bile salts. First, the model from Lembke et al. 2020, where bile salts induce a disulfide bond in the ToxR periplasmic domain, activating transcription that is independent of ToxS29. Second, the model put forward in this report, where bile salt induced destabilization is transmitted across the membrane, allowing ToxR to bind DNA augmented by the bound ToxS. As more studies are performed, by us and others, it will be interesting to see what model emerges to explain bile salt induced ToxR activation.

Materials and Methods
Cloning of ToxR periplasmic domains. The sequences of the ToxR periplasmic domains from V. vulnificus, V. parahaemolyticus, V. fischeri, V. harveyi, and Photobacterium profundum were identified by using the ToxR periplasmic sequence from V. cholerae (T199–E294). The six ToxR periplasmic domains were codon optimized and synthesized, from 5’ to 3’, with a NcoI site, a 6xHis N-terminal tag followed by the coding sequence, a BamHI site, all flanked by primer sites to amplify the constructs. The constructs were PCR amplified, cut with the appropriate restriction enzymes, and the digested constructs were purified using a PCR clean up kit (Qiagen). The pET16b plasmid was digested with the same restriction enzymes, treated with CIP (NEB), and purified using a PCR clean up kit (Qiagen). The inserts were ligated into the plasmid using the Quick Ligase (NEB) and the reaction was used to transform DH5α. Colonies from the transformations were selected to colony PCR to determine if the plasmids contained an insert of the appropriate size. Then selected colonies were cultured overnight for mini-preps following manufacture instructions (Qiagen). The resulting plasmids were sequence verified using a primer for the T7 promoter.

Expression and purification. The plasmids with the different ToxR periplasmic domains were used to transform Shuffler T7 Express cells (NEB). The strains were double selected to produce stable expression strains46. After double selection the strains were checked for production of soluble protein, by performing a scaled down purification. The ToxR periplasmic domain from V. vulnificus was found to produce the greatest amount of soluble protein. Production cultures of V. vulnificus and V. cholerae ToxR periplasmic domains were started by picking a colony from a freshly streaked plate incubated overnight at 37°C, and inoculating 2 ml of ZYP47 supplements with 0.8% glucose (ZYP-0.8 G) with 200 µg/ml of carbenicillin, and incubated overnight at 30°C. The next morning the culture was used to inoculate Terrific Broth Modified (Fisher Scientific) media supplemented with 2 mM MgSO4 and 200 µg/ml carbenicillin at a ratio of 1:25, then grown to OD600 of 1–1.2. The cultures were centrifuged at 600 xg for 10 minutes, at 25°C, with the brake turned off. The cells were resuspended in an equal volume of M9 media with 100 µg/ml carbenicillin and incubated for 1 h at 37°C. The cultures were induced with 1 mM IPTG and incubated overnight at 25°C with loose covers to allow gas exchange.

For SeMet labeling the frozen culture was restreaked onto a plate containing 200 µg/ml of carbenicillin and incubated overnight at 37°C. In the evening a single colony was used to inoculate 2 ml ZYP-0.8 G media with 200 µg/ml of carbenicillin and incubated at 37°C. In the evening the culture was used to inoculate M9 media with 100 µg/ml carbenicillin at 1:12.5 ratio. The culture was grown overnight at 30°C. The next morning the culture was used to inoculate M9 media at a ratio of 1:20. The culture was incubated at 37°C till an OD600 of 0.5. Then 25 mg/L of Lysine, phenylalanine, and threonine; 12.5 mg/L of isoleucine, leucine, and valine; and 15 mg/L of selenomethionine were added to the culture. The culture was incubated for 15 minutes then induced with 1 mM IPTG and incubated at 25°C with a loose cover allowing air exchange overnight.

For 15N and 13C labeling the cultures were started from a single fresh colony in ZYP-0.8 G with 200 µg/ml of carbenicillin overnight at 30°C. The cultures were used to inoculate TB media at a ratio of 1:250 and incubated at 37°C till an OD600 of 2. The cultures were centrifuged at 600 xg at 25°C for 20 minutes with the brake turned off. The supernatant was discarded and the cells were resuspended in an equal volume of M9 media with 100 µg/ml carbenicillin, 3 g/L 15N-NH4Cl, and 10 g/L of U-13C-glucose. The cultures were incubated at 37°C for 1 h then induced with 1 mM IPTG and incubated at 25°C with a loose cover allowing air exchange overnight.

To purify the protein the cells were harvested by centrifugation at 4500 xg, at 4°C. The cells were resuspended in lysis buffer (wash buffer (20 mM HEPES pH 7.4, 20 mM imidazole, 200 mM NaCl), A Complete tablet EDTA free (Roche), 3 mM MgSO4, 1.5 mM EDTA). The cells were lysed with three passes through a French press. The lysate was centrifuged at 100,000 xg for 45 minutes. The resulting supernatant was filtered using a 0.45 µm filter before purification. The protein was captured using a His-Trap column (GE Healthcare). The column was washed with 10 CV’s of wash buffer, 10 CV’s of high salt wash buffer (20 mM HEPES pH 7.4, 20 mM imidazole, 1 M NaCl), 10 CV’s of wash buffer, followed by 9 CV’s of 9% elution buffer (20 mM HEPES pH 7.4, 500 mM imidazole, 200 mM NaCl). The protein was eluted using a gradient to 100% elution buffer over 4 CV’s with a 2 CV hold at 100%. The relevant fractions were pooled and flowed over an equilibrated thio-propyl sepharose 6B (GE Healthcare) column in order to bind protein with free cysteines. The flow through was collected and concentrated to about 2 ml. The final purification was performed using a S75 16/600 (GE Healthcare) column with the gel filtration buffer (20 mM HEPES pH 7.4, 200 mM NaCl for crystallization and biochemistry, or 20 mM KPO4 pH 7.4, 200 mM NaCl for NMR). The relevant fractions were pooled and concentrated as required.

To obtain the non-disulfide bonded form of the ToxR periplasmic domain the thio-propyl column was washed with 2 CV’s of wash buffer, then with 3 CV’s of wash buffer with 100 mM DTT. Two more CV’s of wash buffer with DTT were added to the column and incubated overnight at 16°C. The following morning the protein was eluted and concentrated to about 2 ml for gel filtration as above.
Velocity analytical ultracentrifugation. Analytical ultracentrifugation was carried out using Beckman
ProteomeLab XL-A and an AN-60 rotor. The protein was diluted to an absorbance between 0.3–0.4 in 20 mM
HEPES pH 7.4, 200 mM NaCl, 100 µM EDTA. The rotor was run overnight at 30,000 rpm with concentration
monitored at 280 nm. The data was processed using SEDNTERP. The presented sedimentation coefficients and molecular weights are calculated from the major sedimentation peaks representing over 90% of the signal.

Differential scanning fluorometry. Differential scanning fluorometry was performed to assess the sta-

bility of the V. cholerae dbToxRp in the presence and absence of additives as described21. STATA15 was used to
analyze the data. The results are reported from three independent experiments as mean ± standard deviation.

Pull downs. Pull downs were performed as described21. Briefly, the chitin binding domain tagged ToxS peri-
plasmic domain (CBD-ToxSp) was captured on chitin beads from a lysate. After washing the beads purified ToxR
periplasmic domain with and without the disulfide bond were added to the tubes with and without 2 mM sodium
chenoedoxycholate. After the final wash SDS sample buffer was added to the beads and the tubes were boiled.
Samples were diluted and run on a gel with the results quantified and statistics determined using STATA15. The
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Nuclear magnetic resonance. 1H,15N HSQC48 were acquired at 298 K, on a 700 MHz Avance NMR spec-
trometer equipped with a 5 mm TCI cryoprobe, utilizing 16 scans and 2048 × 256 points. The ToxR periplasmic
domain without the disulfide bond was analyzed at a concentration of 580 µM in 20 mM phosphate buffer pH 6.9,
200 mM NaCl, 0.2 mM TCEP, 0.02% NaN3, 0.1 mM Pefablock and 1.7% D2O. The disulfide bonded ToxR peripla-
smic domain was analyzed in the 20 mM KPO4 pH 7.4, 200 mM NaCl with 1% D2O.

Crystallization and data processing. Crystallization was carried out in a sitting drop with 3 mg/ml of
the SeMet labeled V. pylori intra-chain disulfide bond ToxR periplasmic domain (Vv-dbToxRp) mixed 1:1
with 1.7 M ammonium sulfate, 0.1 M HEPES pH 7.5, and 0.1 M ammonium formiate. The well solution with 40%
glucose was used as the cryo-protectant. To cryo-protect the crystals the mother liquor was exchanged with the
cryo-protectant in the well. Diffraction data was collected at the NSLS2 FMX beam line set at the Se adsorption
edge for SAD data collection. The diffraction data was processed in XDS48 with a space group of P 2 1 2 1
unit cell of 39.960 40.530 49.570 90.00 90.00 90.00. The structure was solved using PHASER 52 as implemented in PHENIX 50, with the
previously solved SeMet structure as the search model. The structure was refined using PHENIX Auto-Sol and refined
using PHENIX Refine50 with COOT21 for manual model building. Chimera was used for structure visualization
and analysis24.

Native Vv-dbToxRp was crystallized by adding in a 1:1 ratio 4 mg/ml of Vv-dbToxRp to 2.0 M ammonium
sulfate, and 0.1 M sodium cacodylate pH 6.3 in a sitting drop. Crystals were cryo-protected by dragging the crys-
tal through 75% paratone N and 25% paraffin oil. Data was collected at the NSLS2 FMX beam line. The data was
processed using XDS48 with the space group P 2 1 2 1 2 with a unit cell of 39.99 40.46 50.29 90.00 90.00 90.00. The structure was solved by molecular replacement using PHASER 52 as implemented in PHENIX 50, with the
previously solved SeMet structure as the search model. The structure was refined using Refine in PHENIX50 with COOT21 for manual model building. Chimera was used for structure visualization and analysis24.

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Author contributions
F.J.K. and C.R.M. conceived the project and wrote the paper. C.R.M. also performed some of the experiments and solved the X-ray structures. R.A.S. performed experiments as directed, primarily purifying and crystallizing the periplasmic domain for X-ray crystallography. M.P. performed the N.M.R. experiments, wrote the methods for the N.M.R. experiments, and prepared the H.S.Q.C. overlay for inclusion in the figures.

Competing interests
The authors declare no competing interests.

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