Conformational Barrier of CheY3 and Inability of CheY4 to Bind FliM Control the Flagellar Motor Action in *Vibrio cholerae*

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Abstract

*Vibrio cholerae* contains multiple copies of chemotaxis response regulator (*Vc*CheY1–*Vc*CheY4) whose functions are elusive yet. Although previous studies suggested that only *Vc*CheY3 directly switches the flagellar rotation, the involvement of *Vc*CheY4 in chemotaxis could not be ruled out. None of these studies, however, focused on the structure, mechanism of activation or molecular basis of FliM binding of the *Vc*CheYs. From the crystal structures of Ca²⁺ and Mg²⁺ bound *Vc*CheY3 we proposed the presence of a conformational barrier composed of the hydrophobic packing of W61, M88 and V106 and a unique hydrogen bond between T90 and Q97 in *Vc*CheY3. Lesser fluorescence quenching and higher $K_m$ value of *Vc*CheY3, compared to its mutants *Vc*CheY3-Q97A and *Vc*CheY3-Q97A/E100A supported our proposition. Furthermore, aforesaid biochemical data, in conjunction with the structure of *Vc*CheY3-Q97A, indicated that the coupling of T90 and Q97 restricts the movement of T90 toward the active site reducing the stabilization of the bound phosphate and effectively promoting autodephosphorylation of *Vc*CheY3. The structure of BeF₃⁻-activated *Vc*CheY3 insisted us to argue that elevated temperature and/or adequacy of phosphate pool might break the barrier of the free-state *Vc*CheY3 and the conformational changes, required for FliM binding, occur upon phosphorylation. Structure of *Vc*CheY4 has been solved in the free and sulfated states. *Vc*CheY₄$_{sulf}$, containing a bound sulfate at the active site, appears to be more compact and stable with a longer $\alpha$ helix, shorter $\beta$4$\alpha$ loop and hydrogen bond between T82 and the sulfate compared to *Vc*CheY₄$_{free}$. While pull down assay of *Vc*CheYs with VcFliM$_{NM}$ showed that only activated *Vc*CheY3 can interact with VcFliM$_{NM}$ and *Vc*CheY4 cannot, a knowledge based docking explained the molecular mechanism of the interactions between *Vc*CheY3 and VcFliM and identified the limitations of *Vc*CheY4 to interact with VcFliM even in its phosphorylated state.

Introduction

*Vibrio cholerae*, the highly motile gram-negative bacterial pathogen that causes cholera, uses chemotaxis and motility to travel to its preferred intestinal niche to colonize [1]. Extensive studies on chemotaxis of *Escherichia coli* or *Salmonella typhimurium* showed that the ligand induced conformational change in methyl accepting chemotaxis protein (MCP) is sensed by the CheA–CheW complex eventually resulting autophosphorylation of the kinase CheA. Autophosphorylated CheA then donates phosphate to the response regulator CheY. Phosphorylated CheY interacts with the flagellar motor protein FliM and influence the direction of flagellar rotation from counter clock wise (CCW) to clock wise (CW) [2,3]. CCW rotation results smooth swimming and CW rotation causes the cell to tumble [4]. Because of the presence of a single polar flagellum, *V. cholerae* does not tumble as such but reverses direction briefly, allowing the bacterium to randomly reorient itself and swim in a new direction.

The genomes of a large number of bacterial species, including *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Rhodobacter sphaeroides*, *Myxococcus xanthus*, *Borrelia burgdorferi*, and *Tessinia pectis*, encode for multiple paralogues of the various chemotaxis genes and chemotaxis in these bacteria is more complex [5,6]. A recent genomic and bioinformatic analysis of over 450 bacteria indicates that more than 50% of the chemotaxis gene homologs have more than one copy of chemotaxis genes [5] and these genes are involved not only in flagellum-mediated chemotaxis but also in type-4 pilus-based motility [7,8], polysaccharide biosynthesis associated with pilus-based gliding motility [9] and flagellar morphogenesis [10]. In many cases, however, genetic analysis has not been successful in deciphering the function of these chemotaxis gene homologs [5,11].

The genome sequence of *V. cholerae* has three sets of Che protein and 45 MCP-like proteins [12]. Each set of che genes forms clusters where che cluster I (located on chromosome I) contains cheY1, cheA1, cheY2, cheR1, cheB1 and the putative gene cheW1; cluster II of chromosome I contains cheY1, cheB2, cheA2, cheZ and cheY3, while cluster III of chromosome II contains cheB3, cheD, cheR3, cheW2, cheV3, cheA3 and cheY4.

So far, the molecular characterization of all four CheYs of *V. cholerae* (namely, VcCheY1–VcCheY4) is restricted to a few in vitro
studies where some of the chemotaxis related genes are found to be involved in the virulence of V. Cholerae [13–15] [3]. Attempts to identify the V. cholerae cheY responsible for the flagellar motion showed that a deletion of cheY impairs chemotaxis [1] while insertional disruption and duplication of the cheY gene result in decreased and increased motility respectively [13]. Swarming assay and assessment of the swimming behaviour indicated that only VcCheY3 directly switches flagellar rotation, although this study could not rule out the involvement of VcCheY4 in the motor action [14]. Later, Banerjee and Chaudhuri (2009) showed that inactivation of cheY or cheH generates a less motile and less adherent mutant [15]. Sequence analysis of VcCheYs indicate that only 17% residues are identical among them which comprise the residues involved in binding of the divalent metal ion and stabilization of the phosphorylated intermediate (Figure 1a). This implies that the basic machinery for the phosphorylation is intact for all four VcCheYs. Available literature, however, suggest that deletion of the cheY1 and cheY2 genes does not cause any defect in chemotaxis [14] and motility or adherence remains unaffected for the insertional mutants of these genes does not cause any defect in chemotaxis [14] and motility or adherence remains unaffected for the insertional mutants of cheY1 or cheY2 [15]. All these observations point to the fact that VcCheY3 and VcCheY4 are the key response regulators to control chemotaxis in V. cholerae.

Structures of CheY from different bacterial sources suggest that although all of these response regulators possess an overall β/α/β fold, small differences in the amino acid sequence or point mutations lead to the subtle conformational variations that make each of these proteins unique in terms of their function [16–18]. Also, T87I and T87I/Y106W mutants of EcCheY were found to be phosphorylatable although these mutants were unable to generate clockwise rotation of the flagella [19]. In addition, both of these mutants had ~5-fold lower autodephosphorylation rates and the mutants were completely resistant to CheZ activity, indicating that an isoleucyl side-chain at position 87 renders EcCheY unable to perform its chemotactic functions [20].

VcCheY3 bears only 37% sequence identity with that of VcCheY4 (Figure 1a) and so far, nothing is known about the structure, mechanism of activation or molecular basis of Flim binding for these two key response regulators, implicated in chemotaxis and virulence of V. cholerae. Here we report, the structures of VcCheY3 in Ca²⁺ and Mg²⁺ bound states, BeF³⁻ activated VcCheY3 (VcCheY3-BeF³⁻) and of the mutant VcCheY3-Q97A. Our structural observations identified a unique conformational barrier in VcCheY3 that controls its phosphorylation event. Implication of this barrier is established by fluorescence spectroscopic study on VcCheY3 and its mutants VcCheY3-Q97A, VcCheY3-Q97A/E100A and VcCheY3-D60A, comparison of their emission values and pull down assay with Flim. We have also reported the structures of VcCheY4 in free and sulfate bound states here and comparison of these structures helped us to argue that VcCheY4 has a strong tendency to be phosphorylated and the phosphorylated state would be more stable compared to its free state. While our pull down assay showed that only activated VcCheY3 can interact with Flim and VcCheY4 cannot, structure based docking explained the molecular mechanism of the interactions between VcCheY3 and Flim and identified the structural limitations of VcCheY4 to interact with Flim even in its phosphorylated state.

Materials and Methods
Cloning, Overexpression and Purification
VcCheY3 and VcCheY4 were purified according to the previously described protocols [21,22]. Briefly, the genes encoding VcCheY3 and VcCheY4 were amplified from V. cholerae O395 genomic DNA and cloned into pET28a⁺ vector. After transformation, cells were grown at 37°C until the optical density at 600 nm (OD₆₀₀) reached 0.4 to 0.6. Protein expression was induced by the addition of IPTG (isopropyl-D-thiogalactopyranoside) to a final concentration of 0.1 mM. The cells were harvested by centrifugation and the resuspended pellet was lysed by sonication in presence of PMSF. The cell lysate was then centrifuged (12000 g for 50 mins) at 4°C. The 6xHis tagged protein was isolated from the supernatant using Ni²⁺-NTA affinity chromatography (Qiagen) and were eluted with lysis buffer containing 150 mM imidazole. The eluted fractions were checked by 15% SDS-PAGE, pooled and dialyzed overnight against the thrombin cleavage buffer (0.05 M Tris–HCl pH 7.5, 250 mM NaCl) and the 6xHis tag was cleaved with 1 U thrombin by overnight incubation at 4°C. The proteins were further purified by gel filtration chromatography using a Sephacryl S-100 (GE-Healthcare) column (78×1.4 cm) pre-equilibrated with thrombin cleavage buffer containing 0.02% sodium azide at 4°C.

The gene encoding Flim was amplified from V. cholerae O395 genomic DNA and cloned into pET21b⁺ vector with a C-terminal 6xHis-tag to get optimal expression level and solubility. The Flim protein was purified by growing cells in LB media to an optical density 0.6-0.8 at 600 nm and induced with 1 mM IPTG. The cells were harvested after induction at 37°C for 5 h. Cell pellet was resuspended in lysis buffer containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 1 mM PMSF and 10 mg lysozyme and lysed by sonication. After centrifugation (14000 g, for 45 mins and at 4°C) Flim was purified with C-terminal 6xHis-tag was isolated from the supernatant by using Ni²⁺-NTA agarose (Qiagen) and the protein was eluted with lysis buffer containing 200 mM Imidazole. After checking in 12% SDS-PAGE the eluted fractions were dialyzed against the lysis buffer.

Mutagenesis
VcCheY3-D60A, VcCheY3-Q97A and VcCheY3-Q97A/E100A were prepared by two-step PCR and verified by commercial sequencing. All the mutant proteins were purified using the same protocol described for the wild type protein.

Flim-CheY Interaction through Nickel Pull-down Assay
50 μl of Ni²⁺-NTA slurry (Qiagen) was washed three times with binding buffer containing 10 mM imidazole, 150 mM NaCl, 5 mM MgCl₂, 0.15% Tween 20 and 50 mM Tris-Cl (pH 7.5) and the resin was then incubated with 0.1 ml purified Flim protein in a concentration of 0.2 mg/ml at 25°C for 20 mins with gentle shaking. The beads were then washed for three times with the binding buffer before adding VcCheY3, VcCheY3-Q97A, VcCheY3-Q97A/E100A, VcCheY3-D60A or VcCheY4. For activation, respective protein was pre-incubated for 20 mins with BeF₃⁻ (100 mM). The mixture was then added in the Flim binding buffer containing Ni²⁺-NTA resin maintaining 1:1 molar ratio and incubated for another 10 mins at 25°C. The beads were washed three times with the buffer and then resuspended in 25 μl of 4×SDS-PAGE gel loading buffer and were subjected to SDS-PAGE analysis and Coomassie blue staining.

Fluorescence Spectroscopy
Fluorescence measurement was carried out using a spectrophotometer, Hitachi F-7000. Fluorescence was measured at an excitation wavelength of 295 nm and an emission wavelength of 340 nm with slit widths of 2.5 nm and 5 nm for excitation and emission, respectively. All reactions were carried out at 25°C. Equilibrium titrations of VcCheY3, VcCheY3-Q97A/E100A, VcCheY3 and VcCheY4 were measured by fluorescence spectroscopic study on O395 genomic DNA and cloned into pET28a⁺ vector. After transformation, cells were grown at 37°C until the optical density at 600 nm (OD₆₀₀) reached 0.4 to 0.6. Protein expression was induced by the addition of IPTG (isopropyl-D-thiogalactopyranoside) to a final concentration of 0.1 mM. The cells were harvested by centrifugation and the resuspended pellet was lysed by sonication in presence of PMSF.
VcCheY3-Q97A and VcCheY3-D60A were carried out with acetyl phosphate (acP) and beryllium fluoride (BeF\(_3\)). The reactions in presence of acP were performed in a buffer containing 20 mM sodium phosphate (pH 7.5), 50 mM NaCl, and 2 mM MgCl\(_2\) and the same were 50 mM Tris-Cl (pH 7.5), 150 mM NaCl and 5 mM MgCl\(_2\) for BeF\(_3\). For all proteins the final concentrations were 1 mM, BeF\(_3\) concentrations varies from 0 to 400 mM and the concentrations varies from 0 to 6 mM for acP. The fluorescence values were corrected for dilution. \(K_{in}\) was determined as described previously by Lukat et al [1992] [23]. Acetyl phosphate and BeF\(_3\) concentrations were plotted versus \((Io - I)/I\) and \(I_{inf}\), where \(I_0\) is initial fluorescence intensity, \(I\) is the intensity at the corresponding acetyl phosphate concentration, and \(I_{inf}\) is the intensity at the saturating concentration. From the plot, the reciprocal of the slope of the line corresponds to the \(K_{in}\) value.

According to proposed reaction scheme [23,24], shown as follows, \(K_{in} = K_s \cdot k^2/k^3\).

\[
\text{CheY} + R \rightarrow P \xrightarrow{K_s} \text{CheY} / R \rightarrow P \xrightarrow{k^2} \text{CheY} \sim \\
\text{P} \xrightarrow{k^3} \text{CheY} + P_i
\]

Where \(K_s\) is the equilibrium dissociation constant between CheY and acetyl phosphate (the phosphor-donor, R→P) and \(k^2\) and \(k^3\) are the phosphorylation and dephosphorylation rate constants, respectively.

If it is assumed that the observed quenching is a direct effect of the reduced quantum yield of phospho-CheY relative to that of CheY, the steady-state fluorescence at a given concentration of phospho-donor may be related to the steady-state fluorescence at a given concentration of phospho-donor. All experimental data points were fitted by linear fit analysis using Microsoft EXCEL and Origin 8.

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**Figure 1. Sequence alignment and overall structure comparison of CheYs.** (a) Amino acid sequences of VcCheY1–VcCheY4 are aligned with CheY6 of Rhodobacter sphaeroides, CheY of Escherichia coli and CheY1 of Helicobacter pylori. Secondary structural elements are marked and labelled at the top. At the bottom important conserved residues implicated in activation/metal binding are marked as (*) whereas other important residues are indicated as ('); as EcChey and StCheY possess 99% sequence identity only EcChey was shown in the alignment file. (b) Stereo representation showing the comparison of the overall structures of VcCheY3 (violet), StCheY (green), each in free state, with VcCheY4free (yellow); (c) Superposition of the overall structure of VcCheY4free (yellow) on VcCheY4sulf (orange) showing the significant differences in helix \(\alpha_4\) and \(\beta_4\) loop.

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Crystallization and Data Collection

Crystallization data of VcCheY3 [21] and VcCheY4 [22] have been published earlier. Briefly, crystals of VcCheY3 that grew in low-pH condition using 5% (w/v) PEG 6000 in 0.1 M Tris–HCl pH 8.0 as precipitant, belong to space group R3 and diffracted to a resolution of 1.67 Å. Crystals of VcCheY3 were also grown in the presence of Mg2+ in a similar condition which diffracted up to 2.2 Å. VcCheY4 crystals grew in AMS at two different pH conditions. In the high-pH condition, hexagonal-shaped crystals were obtained using 0.8 M ammonium sulfate, 0.1 M Bis-Tris pH 9.0, 4% glycerol as precipitant. In the low-pH condition, cube-shaped crystals were obtained using 0.8 M ammonium sulfate, 0.1 M citrate, 4% glycerol as precipitant. The low-pH and high-pH condition crystals were diffracted up to 1.67 Å and 1.9 Å with the space group R2 and P321 respectively.

Crystals of VcCheY3-Q97A mutant grew in a drop consisting of 2 μl protein (6 mg/ml) solution and an equal volume of precipitant containing 1.6 M ammonium sulfate, 0.1 M Tris, pH 8.0. Cube-shaped crystals of VcCheY3-Q97A belonging to space group R3 diffracted to a resolution of 2.4 Å. Activated VcCheY3 were prepared by mixing 20 μl of protein (6 mg/ml) solution with 5 μM of BeF2 solution and incubated for 5 minutes on ice. Crystals of activated VcCheY3 were grown in a drop contains 2 μl of above mixture and equal volume of precipitant solution consisting of 10% (w/v) PEG 6000 in 0.1 M Tris–HCl pH 8.0 and equilibrated for 7 days against 20% (w/v) PEG 6000 in 0.1 M Tris–HCl pH 8.0. Activated VcCheY3 crystals, after brief soaking in cryoprotectant solution containing 1 μM of BeF2, diffracted up to 2.1 Å with the space group R3.

For data collection, crystals were fished out from the crystallization drops using nylon loop, briefly soaked in cryoprotectant solution and flash-cooled in a stream of nitrogen (Oxford Cryosystems) at 100 K. The diffraction data sets were collected using a MAR Research image-plate detector of diameter 345 mm and Cu Kα radiation generated by a Bruker–Nonius FR591 rotating-anode generator equipped with Osmic Max Flux confocal optics and operated at 50 kV and 70 mA. Data were processed and scaled using AUTOMAR (http://www.marrresearch.com/automar/run.html). Data-collection and processing statistics are given in Table 1.

Structure Determination and Refinement

The structures of wild type VcCheY3, VcCheY4, VcCheY3-Q97A and activated VcCheY3 (VcCheY3-BeF2−) were solved by molecular replacement using MOLREP of CCP4 suite [25]. Packing considerations indicated the presence of one molecule in the asymmetric unit for all the structures. The wild type VcCheY3 structure in its Ca2+ bound form was solved by using the coordinates of the Salmonella CheY (PDBID: 2CHE) as template. The structure was refined by alternating cycles of model building and refinement using O’ of CNS [26,27] to a final Rsym and Rfree values of 20.2% and 22.9% respectively. The poly-ala model of VcCheY3 was used as search model for VcCheY4 (low pH) and the refined structure of VcCheY4 (low pH) was used as search model to determine the structure high pH CheY4 (VcCheY4high). Low pH VcCheY4 (VcCheY4low) was refined to Rsym 23.0% and Rfree 26.0% and VcCheY4high was refined to Rsym 22.5% and Rfree 26.0%. VcCheY3-BeF2− structure was solved by using the coordinates of E. coli activated CheY i.e. EcCheY-BeF2− (PDB code: 1FAV) as the search model. Strong electron density of beryllofluoride was found close to the active-site residue D60. The structure was refined up to Rsym of 23.1% and Rfree of 24.3% by several rounds of refinements and manual rebuilding by using the programs CNS [27] and COOT [28], respectively. The structure of VcCheY3-Q97A was solved using VcCheY3 as template and refined by the similar protocol to Rsym of 22.5% and Rfree of 25.2%. The structure of Mg2+ bound VcCheY3 was also solved using VcCheY3 (Ca2+ bound) as template after removing the coordinates of Ca2+ and waters and refined by the similar protocol to Rsym of 20.0% and Rfree of 22.3%. Details of the refinement parameters for all the structures along with the geometric parameters determined by PROCHECK [29] are given in Table 2.

Calculation of Normalized B Factor

Since VcCheY4free and VcCheY4sulf crystals grew in different space groups and their diffraction resolutions are different, to compare their B factors we have plotted their normalized B-factor or B‘-factor. Crystallographic B-factors of proteins determined even at high resolutions show large variations from one structure to another but the B-factors expressed in units of standard deviation about their mean value (normalized B-factor or B‘-factor) shows consistent behaviour [30–32]. The equation used by us to calculate the normalized B-factor is B‘ = B – <B> / σ(B‘); where <B> is the average B value for the whole molecule based on Cα atoms and σ(B‘) is the standard deviation of the B values.

Results

Overall Structures of VcCheY3 and VcCheY4

As expected, both VcCheY3 and VcCheY4 possess β\(\alpha_2\)β fold (Figure 1 b, c) typical of the response regulators. Structure of VcCheY3 in free state superposes on S. typhimurium CheY (β\(\alpha_2\)β; PDB code: 2CHE) with a root mean square deviation (rmsd) of 0.4 Å for 100 Ca atoms (Figure 1b). VcCheY4 was crystallized in two different states; one is in free state with no ligand attached (VcCheY4free) and another with a sulfate and a Ca2+ ion bound at the active site (VcCheY4sulf). Interestingly, when VcCheY4free is superposed on VcCheY4sulf significant differences are observed at the active site, together with helix \(\alpha_4\) and \(\beta_4\) loop and \(\beta_3\) loop (Figure 1c). Since VcCheY4free and VcCheY4sulf were crystallized in different space groups, we have checked the probable influence of crystal packing on the observed structural differences. Our packing analysis suggests that, in either case, these regions are rather loosely packed and their conformations are not influenced by crystal packing. VcCheY4, in either state, is significantly different from that of VcCheY3 (Figure 1b) and superposition of VcCheY4free and VcCheY4sulf on VcCheY3 produces rmsd values of 1.4 Å and 1.2 Å respectively. VcCheY4, in either state, differs from VcCheY3 mainly in the \(\alpha_1\), \(\alpha_5\), \(\alpha_4\) loop and \(\beta_3\) regions and in the \(\beta_3\) loop (Figure 1b). It is to be noted that \(\alpha_1\) and \(\alpha_5\) were implicated previously in CheA and FlhM binding respectively [33].

We have solved the structures of VcCheY3 in Ca2+ and Mg2+ bound states to the resolutions of 1.57 Å (Figure S1a) and 2.2 Å (Figure S1b) respectively. The location of the Ca2+ (or Mg2+) ion in VcCheY3 is similar to that of Mg2+ in SbCheY. The Ca2+ (or Mg2+) of VcCheY3 is heptacoordinated where four coordinations occur with protein atoms and three with water molecules (Figure 2a). In contrast to that, the Mg2+ of SbCheY is hexacoordinated. Although D12 of SbCheY is not coordinated to Mg2+, D15 of VcCheY3 that corresponds to D12 of SbCheY, coordinates with the metal ion (Figure 2a). Except this residue the disposition of the side chains of the other residues that coordinate with the metal ion are more or less similar in these structures (Figure 2a). The average coordination distance between Ca2+ and the protein atoms is about 2.4 Å while this is of about 2.1 Å in case of Mg2+ which is due to the size difference of the ions.
Identification of a Conformational Barrier Towards Activation of VcCheY3

In StCheY or EcCheY, upon phosphorylation at D57, a series of structural changes occur near the active site. T87 along with b4a4 loop moves toward the active site and stabilizes the bound phosphate through hydrogen bonding. Y106 of b5 executes an ‘inward’ movement (shown in line in Figure 2b) with minimal conformational adjustments of W58 and M85 and that inward movement of Y106 is essential for the binding of FliM at a4-b5-a5 face of CheY. K109 and the Mg2+ contribute to stabilize phosphorylated D57 [34]. In the free state StCheY, W58 stays more on the surface (with x1 of 174 u, x2 of -101 u) and M85 side chain adopts such a x1 value (2155 u) that together these residues leave a preformed cavity for the ‘inward’ positioning of Y106 upon activation (Figure 2b).

D60 is the site of phosphorylation in VcCheY3 as it corresponds to D57 of StCheY (Figure 1a). Both in the Mg2+ and Ca2+ bound free state structures of VcCheY3, the side chain of W61 (that corresponds to W58 of StCheY) is observed in a conformation, substantially different from that of StCheY (Figure 2c, 2d). In the free state structure of VcCheY3, the side chain of W61 buries unusually deeply with a x1 of 2135 u and x2 of 2133 u (Figure 2c). Y109 stays in its ‘out’ position and the side chain of M88 (with x1 of 64 u, x2 of 175 u) stays between W61 and Y109, packing snugly with W61, Y109 and V106 through hydrophobic interactions (Figure 2c). This

Table 1. Data collection and processing statistics.

|                      | VcCheY3 Mg2+ bound | VcCheY3-Q97A | VcCheY3-BeF3 |
|----------------------|--------------------|--------------|--------------|
| Space group          | R3                 | R3           | R3           |
| Unit-cell parameters (Å) | a = b = 67.48, c = 74.46 | a = b = 65.858, c = 65.039 | a = b = 67.320, c = 72.660 |
| Oscillation range (°) | 0.5                | 0.5          | 0.5          |
| Number of images     | 92                 | 138          | 88           |
| Maximum resolution (Å) | 30.0–2.2          | 30.0–2.8     | 30.0–2.1     |
| No. of molecules per ASU | 1                | 1            | 1            |
| Mathews coefficient (VMD Å3 DA-1) | 2.23              | 1.86         | 2.19         |
| Solvent content (%)  | 44.9               | 33.77        | 43.86        |
| No. of observations  | 16597              | 9361         | 10428        |
| No. of unique reflections | 6341            | 4141         | 7391         |
| Mosaicity (°)        | 1.59               | 0.5          | 0.35         |
| Completeness (%)     | 98.9(100)          | 97.9(98.8)   | 94.3(92.2)   |
| Rmerge(%)            | 8.45(44.3)         | 7.39 (27.80) | 3.12(22.47) |
| Average I/s(I)       | 7.5(2.7)           | 5.3(2.0)     | 6.5(2.0)     |

Table 2. Refinement statistics.

|                      | VcCheY3 Ca2+ bound | VcCheY3 Mg2+ bound | VcCheY3-Q97A | VcCheY3-BeF3 | VcCheY4off | VcCheY4off*** |
|----------------------|--------------------|--------------------|--------------|--------------|------------|--------------|
| Rcryst (%)a          | 20.2               | 20.0               | 22.5         | 23.1         | 21.8       | 22.5         |
| Rfree (%)b           | 22.9               | 22.5               | 25.2         | 24.3         | 24.6       | 26.0         |
| r.m.s.d bond (Å)     | 0.005              | 0.016              | 0.019        | 0.012        | 0.006      | 0.009        |
| r.m.s.d angle (°)    | 1.3                | 1.6                | 2.17         | 1.6          | 1.3        | 1.59         |
| No. of waters        | 176                | 94                 | 93           | 107          | 114        | 144          |
| β-factors (Å2)       | 19.05              | 25.69              | 48.194       | 27.350       | 19.215     | 46.52        |

Ramachandran plot (%)

|                | VcCheY3 Mg2+ bound | VcCheY3-Q97A | VcCheY3-BeF3 | VcCheY4off | VcCheY4off*** |
|----------------|--------------------|--------------|--------------|------------|--------------|
| Most favored (%) | 97.5               | 95.1         | 97.5         | 92.7       | 98.3         | 94.9         |
| Allowed(%)      | 2.5                | 4.8          | 2.5          | 5.7        | 1.7         | 34.2         |
| Disallowed(%)   | 0.0                | 0.0          | 0.0          | 1.6        | 0.0         | 0.9          |
| PDB code        | 3T05               | 4LX8         | 4HNQ         | 4HNS       | 4H60        | 4HNRI        |

1 Rmerge = Šhkl|Ihkl|−<Ihkl>|/Šhkl|<Ihkl>, where Ihkl is the intensity of an individual reflection and <Ihkl> is the average intensity over symmetry equivalents.
2 Rfree = ŠFobs|Fobs|−<Fobs>|/ŠFobs|<Fobs>, where Fobs and Fcalc are the observed and calculated structure factor amplitudes, respectively.
3 VMD is the equivalent of VMD, calculated for a randomly chosen set of the reflections (9%) that were omitted throughout the refinement process. VM is the partial specific volume.
4 As defined by PROCHECK.

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packing essentially fills up the pocket, required for the 'inward' positioning of Y109 upon activation (Figure 2c, 2d).

Moreover, in this inactivated structure of VcCheY3, the crucial T90 of b4a4 loop (that corresponds to T87 of StCheY), which stabilizes the bound phosphate on D60 upon activation, is hydrogen bonded with Q97 (Figure 2c). To the best of our knowledge, this kind of interaction involving the Thr of b4a4 loop was not observed so far in any other response regulator. In VcCheY3, T90 and Q97 are oriented in such a fashion that together they form a capping on the aforesaid hydrophobic packing and at the same time block the 'out to in' trajectory of Y109 (Figure 2d). Additionally, the side chain carboxylate group of E100 (which is Ala in EcCheY or StCheY) forms a water mediated hydrogen bond with NE1 of W61 (Figure 2c; Figure S3a, S3b). Therefore, the hydrophobic packing of W61, M88, V106, hydrogen bond between T90 and Q97, and water mediated hydrogen bond between E100 and W61 that make a conformational barrier in VcCheY3, (d) superposition of 'b' and 'c' showing the buried conformation of W61 and its packing with M88 in VcCheY3 (violet) compared to StCheY (grey), 'in' position of Y109 (thin line) makes clashes with VcCheY3 residues, doi:10.1371/journal.pone.0073923.g002

Figure 2. Metal binding and conformational barrier in VcCheY3. (a) Stereo representation to compare the Ca$^{2+}$ and Mg$^{2+}$ binding at the active site of VcCheY3 (violet) with the Mg$^{2+}$ binding of StCheY3 (grey). Ca$^{2+}$ bound to VcCheY3 is shown as pink sphere, Mg$^{2+}$ bound to VcCheY3 is shown as dark green sphere and Mg$^{2+}$ bound to StCheY3 is shown as light green sphere. Waters bound to of Ca$^{2+}$ and Mg$^{2+}$ are shown as light and dark red spheres respectively. Only the hydrogen bonds, observed in Ca$^{2+}$ bound VcCheY3 are shown for clarity; (b) preformed pocket for the 'in' position for Y106 in EcCheY (thin line), coordinates for the 'in' position of Y106 is taken from the activated EcCheY structure (PDB code:1F4V), (c) The hydrophobic packing of W61, M88, V106, hydrogen bond between T90 and Q97, and water mediated hydrogen bond between E100 and W61 that make a conformational barrier in VcCheY3, (d) superposition of 'b' and 'c' showing the buried conformation of W61 and its packing with M88 in VcCheY3 (violet) compared to StCheY (grey), 'in' position of Y109 (thin line) makes clashes with VcCheY3 residues.

Comparison of Phosphorylation Events Through Fluorescence Spectroscopy

To investigate the contribution of the proposed 'conformational barrier' of VcCheY3 towards its activation, we prepared three mutants VcCheY3-Q97A, VcCheY3-Q97A/E100A and VcCheY3-D60A. Since W61 is within the Forster distance of D60, tryptophan quenching study was performed with VcCheY3 and its mutants to monitor the phosphorylation event using acetyl phosphate (acP) as substrate. Interestingly, VcCheY3 showed very low quenching (Figure 3a) indicating that phosphorylation at D60 does not induce any conformational change in W61 and W61 remains buried even after the treatment with acP. VcCheY3-Q97A showed low quenching (Figure 3a) indicating that phosphorylation at D60 does not induce any conformational change in W61 and W61 remains buried even after the treatment with acP. VcCheY3-Q97A and VcCheY3-Q97A/E100A, on the other hand, showed considerable quenching in the presence of acP (Figure 3a), suggesting that in the absence of the hydrogen bond between T90 and Q97 (and also in absence of E100), conformational alteration of W61 may take place more easily and it can move toward the surface of the molecule. As expected, quenching is almost negligible for the nonphosphorylatable analog VcCheY3-D60A (Figure 3a). Based on these experiments we have calculated the $K_m$ ($K_m = K_s \cdot k_3/k_2$)
values where a higher $K_m$ value implies a decrease in the binding affinity between CheY and the phosphodonor (i.e. larger $K_s$), a slower rate of phosphorylation of bound CheY (i.e. smaller $k_2$) or a faster rate of autodephosphorylation (i.e. larger $k_3$) \[35\]. $K_m$ value, obtained by us, was the highest for $Vc$CheY3 (6.4±0.45 mM) followed by $Vc$CheY3-Q97A (2.3±0.4 mM) and $Vc$CheY3-Q97A/E100A (2.0±0.2 mM) (Figure 3b) which are in accordance with our structural observations.

**Structure of VcCheY3-Q97A**

To investigate whether the hydrogen bond between T90 and Q97 affects the hydrophobic packing of W61, M88 and V106, we have solved the structure of VcCheY3-Q97A. As expected, the overall structure of VcCheY3-Q97A is almost identical to that of VcCheY3 and the Mg$^{2+}$ ion bound at the active site occupies the equivalent position to that of Mg$^{2+}$ (or Ca$^{2+}$) of VcCheY3 (Figure 4a). Interestingly, even in the absence of the hydrogen bond between T90 and Q97, the conformation and packing of W61, M88 and V106 are found to be unaltered with respect to the wild type VcCheY3 (Figure 4b). However, the water mediated hydrogen bond between W61 and E100 is not seen in this mutant. E100 is slightly reoriented here and has moved toward the CD1 atom of the adjacent I69 (Figure 4b). These observations, coupled with the quenching results, point to the fact that although the hydrophobic packing of W61, M88 and V106 is independent of the hydrogen bond between T90 and Q97 in free state, in the

![Figure 3. Activation of VcCheY3 and its mutants, measured through fluorescence quenching.](file:///C:/Users/.../en.jpg)
absence of the later interaction, reorientation of W61 and M88 occurs more smoothly upon phosphorylation.

Structure of VcCheY3-BeF$_3^-$

Quenching data using acP (Figure 3a) clearly indicate that obtaining of stable VcCheY3-P for crystallographic study is not possible. Since BeF$_3^-$ readily forms persistent activated complexes with many response regulators, regardless of the half-lives of their phosphorylated states, this is regularly used to structurally mimic the phosphorylated state of the response regulators [36]. Fluorescence quenching experiment for VcCheY3 and its mutants, performed in the presence of BeF$_3^-$, showed approximately 30 fold lowering of the $K_m$ values (219.0±0.6 μM, 110.0±2.1 μM, and 96.4±1.4 μM for VcCheY3, VcCheY3-Q97A and VcCheY3-Q97A/E100A respectively) compared to that of acP (Figure 3c). Thus, to visualize the structural changes in VcCheY3 upon phosphorylation, we have activated VcCheY3 using BeF$_3^-$ and solved the structure of VcCheY3-BeF$_3^-$ to 2.1 Å.

The active site of VcCheY3-BeF$_3^-$ largely resembles to that of EcCheY-BeF$_3^-$ (PDB code: 1F4V) (Figure 4c). In VcCheY3-BeF$_3^-$, BeF$_3^-$ is covalently linked with D60 and Mg$^{2+}$ is properly poised to interact with BeF$_3^-$ (Figure 4c, 4d). To stabilize the
bound BeF$_3^-$, the side chain of K112 reorients and T90 along with the β4α4 loop moves toward the active site with a conformational change, hallmark for the activation of this type of CheY (Figure 4d). The hydrogen bond between T90 and Q97 is abolished and Q97 side chain moves away from T90 (Figure 4e). Breaking the hydrophobic packing with M88, the side chain of W61 moves toward the surface (with χ1 of −166°, χ2 of −34°) acquiring a conformation similar to that observed in EcCheY-BeF$_3^-$ (Figure 4e). Under that situation, M88 occupies the space left by W61 and creates a pocket, sufficient to accommodate the ‘in’ position of Y109 which is essential for FliM binding (Figure 4e).

**Free and Sulfated Structures of VcCheY4**

Although the overall structures of VcCheY$_{4\text{free}}$ and VcCheY$_{4\text{sulf}}$ are similar, substantial conformational differences are observed between these two, especially around the active site, in helix α4 and β4α4 loop. A Ca$^{2+}$ ion is located at the active site of VcCheY$_{4\text{free}}$ which coordinates with D9, D52 and main chain carbonyl oxygen of N54 with an average coordination distance of 2.4 Å (Figure 5a; Figure S2a). A tetrahedral positive electron density was observed in the active-site pocket of VcCheY$_{4\text{sulf}}$ during refinement which was interpreted as a sulfate ion because VcCheY4 was crystallized using ammonium sulfate as precipitant (Figure S2a). In contrast to that, neither a metal ion nor a sulfate ion was observed at the active site of VcCheY$_{4\text{free}}$ although both of these components were added during crystallization (Figure 5a; Figure S2b). Absence of the divalent metal ion does not cause any change in the side chain conformation of D9 and D52 compared to VcCheY$_{4\text{free}}$, but the carbonyl oxygen of N54 points away from the metal binding side (Figure 5a). As a result, the β3δ3 loop of VcCheY$_{4\text{free}}$ takes a different conformation and moves about 3 Å away from the active site (Figure 5a). In VcCheY$_{4\text{free}}$, helix α4 is shorter and β4α4 loop is unusually longer compared to that of VcCheY$_{4\text{sulf}}$ (Figure 1c). Electron density around the β4α4 loop of VcCheY$_{4\text{free}}$ is shown in the Figure S2c. The plot of B-values indicated that the crystallographic B-factor of the β4α4 loop is much lower in VcCheY$_{4\text{free}}$ compared to that of VcCheY$_{4\text{sulf}}$ (Figure 3b). In VcCheY$_{4\text{free}}$ part of the β4α4 loop is stabilized and adopts a helical structure effectively extending the length of α4 (Figure 5c) and overall, the VcCheY$_{4\text{free}}$ structure seems to be more compact compared to VcCheY$_{4\text{sulf}}$.

The location of the sulfate ion at the active site of VcCheY$_{4\text{sulf}}$ is somewhat similar to BeF$_3^-$ of VcCheY3-BeF$_3^-$ (Figure 5a). T82 and K104, which are well known to stabilize the phosphoryl group in the other reported CheY structures, stabilize the sulfate ion in VcCheY$_{4\text{sulf}}$ through hydrogen bonding. A movement of about 2 Å towards the active site occurs for T82 along with the β4α4 loop (Figure 5a). Interestingly, in VcCheY$_{4\text{sulf}}$, an additional hydrogen is generated between T82 and K99 (K99 corresponds to Q97 of VcCheY3) which might further contribute to the compactness of α4 in VcCheY$_{4\text{sulf}}$ (Figure 5a, 5c).

Molecular Mechanism of FliM Binding in V. Cholerae

To investigate the binding ability of VcCheY3 and VcCheY4 with VfFIM, we performed an in-vitro pull down assay. VfFIM$_{5\text{NM}}$ (a construct having the N-terminal and the middle domain of VfFIM with a C-terminal 6×His-tag) was immobilized on Ni-NTA resin, which was then incubated with VcCheY3, VcCheY3-Q97A, VcCheY3-Q97A/E100A, VcCheY3-D60A and VcCheY4, individually, in presence of Mg$^{2+}$ but with or without BeF$_3^-$ . Our results showed that while the activated VcCheY3, VcCheY3-Q97A and VcCheY3-Q97A/E100A can interact with VfFIM$_{5\text{NM}}$, VcCheY3-D60A and VcCheY4 do not show any significant interaction with VfFIM$_{5\text{NM}}$ even in presence of BeF$_3^-$ and Mg$^{2+}$ (Figure 6a). VcCheY3-D60A was used as the negative control, and the experiment performed with BeF$_3^-$ and without VfFIM$_{5\text{NM}}$ quantified the basal level of adherence of VcCheY3s in Ni-NTA agarose during experiment.

To identify the structural features of VcCheY3 and VcCheY4, responsible for the difference in affinity towards VfFIM, it was necessary to critically analyse their FIM binding surface. To start with, we prepared a model of the N-terminal 16 peptide of VfFIM$_{5\text{NM}}$ by 3D-JIGSAW and VfFIM$_{5\text{NM}}$, thus prepared, was docked at the FIM binding face of VcCheY3-BeF$_3^-$ and VcCheY$_{4\text{sulf}}$. The FIM$_{5\text{NM}}$ part of the coordinates of EcCheY-BeF$_3^-$-complex structure (PDB code: 1FIV) were used as a template to prepare the model of VfFIM$_{5\text{NM}}$ and VfFIM$_{5\text{NM}}$-EcCheY structure as a whole was used as template for the docking. The resulting models of VfFIM$_{5\text{NM}}$-VcCheY3-BeF$_3^-$ or VfFIM$_{5\text{NM}}$-VcCheY4$_{4\text{sulf}}$ were then analysed to identify the structural determinants responsible for the differential FIM binding of VcCheY3 and VcCheY4. VfFIM$_{5\text{NM}}$ is observed to fit properly at the α4-β5-β5' cleft of VcCheY3-BeF$_3^-$ with considerable number of hydrogen bonds and hydrophobic interactions (Figure 6b, 6c) which are comparable with those of EcCheY-BeF$_3^-$ (Table 3). In contrast to that, the probable interactions of VcCheY4$_{4\text{sulf}}$ with VfFIM$_{5\text{NM}}$ are inadequate (Figure 6d, 6e; Table 3). The FIM binding face of VcCheY4$_{4\text{sulf}}$ is not compatible enough for VfFIM. In VcCheY4$_{4\text{sulf}}$, the space between α4 and α5 is ~2 Å wider compared to that of VcCheY3-BeF$_3^-$ which might cause a loose fit of VfFIM$_{5\text{NM}}$ at α4-β5-β5' face of VcCheY4$_{4\text{sulf}}$. Residues T2 and D3 of VfFIM$_{5\text{NM}}$ are found to interact with VcCheY3-BeF$_3^-$, but no such interaction is possible with VcCheY4$_{4\text{sulf}}$ (Figure 6e, 6c). Furthermore, in VcCheY3-BeF$_3^-$, K122 of α5 is poised to form a salt bridge with D12 of VfFIM$_{5\text{NM}}$, corresponding residue of VcCheY4$_{4\text{sulf}}$ is T114 which is spatially away from D12 of VfFIM$_{5\text{NM}}$ and naturally no interaction is expected between this pair (Figure 6e). As a result, the overall interactions between VfFIM$_{5\text{NM}}$ and VcCheY4$_{4\text{sulf}}$ are reduced significantly (Figure 6e, Table 3) supporting the observation of the pull down assay (Figure 6a).

**Discussion**

Unlike _E. coli_ two-component chemosensory pathway that relies on a single copy of response regulator CheY, _V. cholerae_ possesses four CheY homologues. Occurrence of multiple CheYs is not unusual in bacteria as these are also found in _R. sphaeroides_ and _B. burgdorferi_ [5]. Recent studies have demonstrated that multiple copies of CheY play specific roles in the chemotactic signal transduction mechanisms. As for example, among the three CheYs of _B. burgdorferi_ only CheY3 directly regulates motor action while the other two cannot bind to the motor and act as signal terminating phosphate sink [37]. Similarly, in _R. sphaeroides_ only CheY6 can change the direction of the flagellar motor, although the others bind FliM probably to regulate the level of the phosphodonor [38,39]. An intriguing question, therefore, arises...
about the role of multiple copies of CheY in *V. Cholerae*, especially of *Vc* CheY3 and *Vc* CheY4.

Together, phosphorylation at the active site Asp, hallmark movement of the Thr and the β4α4 loop toward the active site to stabilize the bound phosphate, ‘in’ positioning of the crucial hydrophobic residue of β5 and FliM binding at the α4-β5-α5 face to reverse the flagellar motion constitute the general mode of action of the chemotactic response regulators. In *Ec* CheY or *St* CheY, a preformed pocket was seen to accommodate the ‘in’ position of the crucial β5 residue Y106 upon activation (Figure 2b). In contrast to that, in *Vc* CheY3, this pocket is preoccupied by the hydrophobic packing of W61, M88 and V106 (Figure 2c). A unique hydrogen bond between T90 and Q97 additionally restricts the outward movement of W61, which is necessary to make a pocket for the ‘in’ positioning of Y109. This hydrogen bond also obstructs the movement of T90 toward the active site essentially hindering the stabilization of the phosphoryl group by T90. *Vc* CheY3 shows minimum quenching in the presence of acP which further support the hindered movement of W61 upon phosphorylation at D60 (Figure 3a), *Vc* CheY3-Q97A and *Vc* CheY3-Q97A/E100A, on the other hand, show considerable quenching in the presence of acP indicating that in the absence of the hydrogen bond between T90 and Q97, W61 can easily be reoriented toward solvent and T90 can move toward the active site to stabilize the phosphoryl group.

Higher *Km* value of *Vc* CheY3 compared to its mutants *Vc* CheY3-Q97A and *Vc* CheY3-Q97A/E100A further establishes the hindrance caused by the hydrogen bond between T90 and Q97 in stabilizing the acyl phosphate on D60. The lower *Km* values of *Vc* CheY3-Q97A and *Vc* CheY3-Q97A/E100A are due to the loss of the coupling between T90 and Q97 which facilitates the movement of T90 toward the active site and stabilize the acyl phosphate. A comparison of the *Km* value of *Vc* CheY3 with the CheYs from *Helicobacter pylori* or *E. coli* shows that the *Km* of *Vc* CheY3 is also higher than that of *Hp* CheY1 (1.07 ± 0.31 mM) and *Ec* CheY (3.2 ± 0.4 mM). As mentioned by Lam et al. (2010), *Km* increases with the increase in the ionic strength of the buffer used in the experiment [24]. While 200 mM salt was used in the experiment of *Ec* CheY, only 50 mM salt was used for *Hp* CheY1 and *Vc* CheY3 (and its mutants). Since our experimental condition is same as that of *Hp* CheY1, we can clearly infer that the *Km* value of *Vc* CheY3 is about six fold higher than that of *Hp* CheY1.

![Figure 5. Structure of VcCheY4 in free and sulphated states.](https://doi.org/10.1371/journal.pone.0073923.g005)

(a) stereo view of the superposition of *Vc* CheY4<sub>free</sub> (yellow) and *Vc* CheY4<sub>sulf</sub> (orange) on activated *Vc* CheY3-BeF<sub>3</sub> (green) showing the location and the interactions of the sulfate ion in *Vc* CheY4<sub>sulf</sub>, relative movement of T82, hydrogen bond between K89 and T82 in *Vc* CheY4<sub>sulf</sub> and the interactions of the metal ion with the neighbouring residues; (b) *B*’ plot of *Vc* CheY4<sub>free</sub> (black) and *Vc* CheY4<sub>sulf</sub> (orange) showing reduction of flexibility of the β4α4 loop (*) in *Vc* CheY4<sub>sulf</sub>; (c) superposition of *Vc* CheY4<sub>free</sub> (yellow) on *Vc* CheY4<sub>sulf</sub> (orange) showing the conformational difference at the β4α4 loop and packing of W101 in its exclusive ‘in’ position.

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Figure 6. Interactions of FliMNM with VcCheY3, VcCheY4 and VcCheY3 mutants. (a) Pull-down assays of VcCheY3, VcCheY3-Q97A, VcCheY3-Q97A/E100A, VcCheY3-D60A and VcCheY4 with VcFliMNM. Purified VcFliMNM in 0.2 mg/ml was immobilized on pre-washed resin. VcCheY3, VcCheY3-D60A and VcCheY4 in a 1:1 molar ratio to VcFliMNM was incubated with immobilized VcFliMNM with or without BeF$_3^-$ at 25°C for 10 mins; (b) Docking of VcFliM$_{NM}$ (16 residues) at the FliM binding face of VcCheY3-BeF$_3^-$; (c) Zoomed view of (b) showing the probable interactions in detail; (d) Docking of VcFliM$_{NM}$ (16 residue) at the probable FliM binding face of VcCheY4$^{sulf}$; (e) Zoomed view of (d) showing the probable interactions in detail.

doi:10.1371/journal.pone.0073923.g006
the conventional conformational changes, an unusual orientation

Vc

assay shows that

the

swimming behaviour Hyakutake et al, (2005) reported that only

autodephosphorylation. Based on the swarming assay and

phosphorylation occurs slowly or it has a higher rate of

Vc

Km

higher

tively causing lower rate of activation which is reflected in its

enhanced autodephosphorylation (larger

Lesser stabilization of the bound phosphate might be implicated in

sequence comparison of

structures,

to the phosphorylated state for FliM binding.

Vc

pool might break the barrier of the free-state

Vc

therefore, acts as a molecular switch to control the level of

CheY-BeF3

CheY3 with significant number of hydrogen bonding and

interactions with

Table 3.

doi:10.1371/journal.pone.0073923.t003

As mentioned earlier, a higher

Km

(\(K_m = K_s \cdot k_3/k_2\)) implies a
decrease in the binding affinity between CheY and

CheY3 implies that either its

phosphorylation occurs slowly or it has a higher rate of

autodephosphorylation. Based on the swimming assay and

swimming behaviour Hyakutake et al, (2005) reported that only

the VcCheY3 directly switches the flagellar rotation [14]. Our pull

down assay shows that VcCheY3 and its mutants VcCheY3-Q97A and

VcCheY3-Q97A/E100A bind VfFlIM efficiently in the presence of

BeF

and Mg

2+

. Docking results suggest that

VfFlIM

can fit properly at the

\(\alpha1-\beta5-\alpha5\) face of the activated

VcCheY3 with significant number of hydrogen bonding and

hydrophobic interactions (Figure 6a, 6b; Table 3). Moreover,

sequence comparison of VcCheY3 with EcCheY or SdCheY

denotes that the crucial residues implicated in binding the kinase

CheA are conserved in VcCheY3 (Figure 1a). These observations

indicate that although VcCheY3 has all the requisites for the

phosphorylation, stabilization of the acphosphate is hindered due
to the obstructed movement of T90 towards the active site.

Lesser stabilization of the bound phosphate might be implicated in

enhanced autodephosphorylation (larger \(k_3\)) for VcCheY3, effect-
vously causing lower rate of activation which is reflected in its

higher

Km

value. The conformational barrier of VcCheY3, therefore, acts as a molecular switch to control the level of

VcCheY3-P. Elevated temperature and/or adequacy of phosphate

pool might break the barrier of the free-state VcCheY3 and flip it
to the phosphorylated state for FlIM binding.

Two distinct conformations, differing at helix

\(\alpha4\)

and the crucial

\(\beta4-\alpha4\) loop, are observed for VcCheY4. Among these two
structures, VcCheY4

possesses a bound sulfite ion near the active site which occupies a position similar to the BeF

\(\alpha4\) of SdCheY-BeF

and VcCheY3-BeF

(Figure 5a). A bound sulfite ion was also observed in

H\(b\)CheY1 structure (PDB code: 3GWG) where that sulfite ion caused conformational changes similar to

the activated structure [24]. However, in H\(b\)CheY1, along with the conventional conformational changes, an unusual orientation

of D53 was observed [24]. In VcCheY4

, the sulfate ion did not alter the side chain conformation of catalytic D52 but stayed very close (\(\sim 2.5 \AA\)) to it (Figure 5a). Since VcCheY4

was crystallized at pH 4.0, at this pH D52 might be protonated allowing the sulfate ion to come to its close vicinity. In VcCheY4

, the sulfate ion is properly coordinated with the Ca\n
\(^{2+}\) ion and is stabilized through the interactions with T82 and K104 (Figure 5a). Considering the compactness of the VcCheY4

structure having a shorter

\(\beta4-\alpha4\) loop with low B-factors, long \(\alpha4\) helix, movement of T82 and \(\beta4-\alpha4\)
loop to stabilize the sulfite ion and additional hydrogen bond between T82 and K89, it can be said that VcCheY4 has a strong tendency to be phosphorylated in the presence of a divalent metal ion and the phosphorylated state is more stable compared to its free state.

Despite the fact that the crucial \(\beta5\) residue W101 of VcCheY4 consistently acquires ‘in’ position, VcCheY4 fails to interact with

VfFlIM

(Figure 6a). Through mutagenesis and structure-function studies Matsumura and collaborators showed that substitution of Y106 of EcCheY with tryptophan (Y106W) produces a phosphorylation-dependent, hyperactive mutant that

generates mainly clockwise rotational bias upon interacting with

FlIM [40]. In contrast to that, despite the consistent ‘in’ position of W101, VcCheY4 does not interact with VfFlIM, as the N terminal part of VfFlIM does not fit at the \(\alpha4-\beta5-\alpha5\) face of VcCheY4 because of their spatial and electrostatic incompatibility (Table 3, Figure 6c). This apparent contradiction suggest that FlIM binding by CheY is not just influenced by the ‘in’ positioning of the \(\beta5\) hydrophobic residue but the spatial and electrostatic compatibility of the \(\alpha4-\beta5-\alpha5\) face of CheY with the N-terminal part of FlIM plays a vital role in this process. Since, CheZ and FlIM share a common face of CheY for binding with similar mode of interactions [41], VcCheY4 is expected not to interact efficiently with CheZ as well. This observation corroborates with the fact that no cheZ is found in the cluster III where cheY is located. Since VcCheY4 can be phosphorylated but cannot bind FlIM and probably not CheZ as well, VcCheY4 might act as phosphate sink or it might induce the expression of some other genes upon phosphorylation which can indirectly modulate flagellar action and/or virulence.

VcCheY4 was seen to slightly enhance the spreading of an E. coli

cheZ

mutant in semisolid agar and based on that Hyakutake et al proposed that VcCheY4 can affect chemotaxis by removing a phosphoryl group from VcCheY3 [14]. Our observations intend us to hypothesise that if a phosphate pull is shared by VcCheY3 and

VcCheY4 then VcCheY4 can cause a phosphate depleted situation for VcCheY3, as phosphorylated state of VcCheY4 is more stable compared to its unphosphorylated state, which is other way round for VcCheY3. Alternatively, in a phosphate depleted situation, additional energy might help phosphorylated VcCheY4 to release the phosphoryl group through conformation dependent auto-

dephosphorylation, as proposed by Pazy et al., 2009 [42] based on their observations of the mutant EcCheY.

### Supporting Information

**Figure S1** Metal binding in VcCheY3. (a) Electron density maps (2Fo-Fc) around the active site of VcCheY3 contoured at 1.2 \(\sigma\) level, Ca\n
\(^{2+}\) is shown in pink sphere and water molecules as red dots. Ca\n
\(^{2+}\) binding residues are labelled; (b) Electron density maps

(2Fo-Fc) contoured at 1.0 \(\sigma\) level (a) around the active

Figure S2 Electron density map of VcCheY4. Electron density map (2Fo-Fc) contoured at 1.0 \(\sigma\) level (a) around the active

| E
c

CheY3-BeF

| EcFlIM

| VcCheY3-BeF

| VcFlIM

| VcCheY4

| Polar interactions |
|-------------------|
| K91               |
| D3                |
| K94 NZ            |
| T3 OG1            |
| –                 |
| K92 N             |
| S4 O              |
| R95 N             |
| D4 O              |
| –                 |
| R95 NH1, NH2      |
| D4 OD1, OD2       |
| –                 |
| A90 O             |
| L6, N             |
| A93 O             |
| L6 N              |
| T85 O             |
| V108 N            |
| Q8 OE1            |
| V111 N            |
| Q8 OE1            |
| V104 N            |
| K119              |
| D12               |
| K122 NZ           |
| D12 OD1           |
| –                 |
| Y106 N            |
| D16 OD1           |
| Y109 O            |
| H16 NE2           |
| –                 |
| Y106 O            |
| D16 N             |
| K122 NZ           |
| D16 O             |
| K125 NZ           |
| H16 O             |
| R117 NH1          |
| Hydrophobic interactions |
| I95               |
| L6                |
| R95, I98          |
| L6                |
| P87               |
| I95, A99, Y106    |
| I11, L14          |
| I98, I99, Y109,   |
| V106, A102        |
| I11, L14, L15     |
| K90, W101         |

| Role of CheY3 and CheY4 in V. cholerae Chemotaxis |

- **Table 3.** Residues of VcFlIM\(_N\) model, involved in the probable interactions with VcCheY3 and VcCheY4 structures, are compared with that of EcFlIM-EcCheY structure.
site of VcCheY\textsubscript{3} in stereo, (b) around the active site of VcCheY\textsubscript{4}\textsuperscript{free}, (c) around the βα4 loop of VcCheY\textsubscript{4}\textsuperscript{free}. (DOCX)

**Figure S3 Interaction of W61 with E100.** (a) Electron density map \((2F_o-F_c)\) contoured at 1.0 \(σ\) level around the water molecule that connects W61, M88, E100 along with the water molecule in Ca\textsuperscript{2+} bound VcCheY3; (b) Water mediated interaction of W61 with E100 in Mg\textsuperscript{2+} bound VcCheY3. (DOCX)

**Supporting Information S1.** (PDF)

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