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Regulation of the Chemotaxis Histidine Kinase CheA: A Structural Perspective

Alise R. Muok[a], **Ariane Briegel**[a], **Brian R. Crane**[b,*]

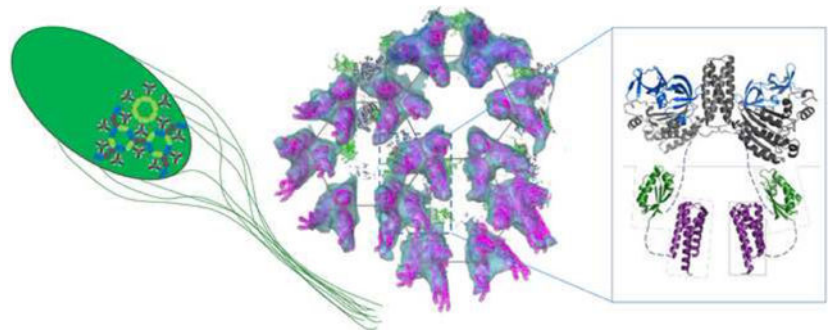
[a]Institute for Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden

[b]Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14850

Abstract

Bacteria sense and respond to their environment through a highly conserved assembly of transmembrane chemoreceptors (MCPs), the histidine kinase (CheA), and the coupling protein CheW, hereafter termed “the chemosensory array”. In recent years, great strides have been made in understanding the architecture of the underlying chemosensory arrays and how these assemblies engender sensitive and cooperative responses. Nonetheless, a central outstanding question surrounds how receptors modulate the activity of the chemotaxis kinase CheA, the enzymatic output of the sensory system. With a focus on recent advances, we summarize the current understanding of array structure and function to comment on the molecular mechanism by which CheA, receptors and CheW generate the high sensitivity, gain and dynamic range emblematic of bacterial chemotaxis. The complexity of the chemosensory arrays has motivated investigation with many different approaches. In particular, structural methods, genetics, cellular activity assays, nanodisc technology and cryo-electron tomography have provided advances that bridge length scales and connect molecular mechanism to cellular function. Given the high degree of component integration in the chemosensory arrays, we ultimately aim to understand how such networked molecular interactions generate a whole that is truly greater than the sum of its parts.

Graphical Abstract



*Corresponding Author: bc69@cornell.edu.

1. Introduction

Motile bacteria control their movement in response to changes in their environment through a behavior known as bacterial chemotaxis (reviewed in [1–6]). 50 years ago chemotaxis in the γ-proteobacterium *Escherichia coli* (*Ec*) was shown to be a receptor-mediated process [7–9]. Since that time, our understanding of the underlying sensory system that mediates chemotaxis has steadily grown. Nonetheless, unresolved questions regarding molecular mechanism still remain [1–6]. The principal molecular components of the chemotaxis system are the transmembrane chemoreceptors, also called methyl-accepting chemotaxis proteins (MCPs). MCPs sense external ligands directly or through periplasmic binding proteins. The receptors transduce binding signals across the cellular membrane to regulate the autophosphorylation activity of the histidine kinase CheA (Fig. 1, 2). CheA is composed of five domains (P1 through P5) each with a distinct function (see below). Once activated, CheA initiates an intra-cellular phosphorelay that ultimately controls flagella rotation sense (Fig. 1, 3A). For the *Ec* system, an increase in repellent or decrease in attractant increases CheA autophosphorylation [1–6]. As an example of a “two-component” regulatory system [10, 11], phosphorylated CheA donates phosphate from phosphoryl-histidine to an aspartate residue on the response regulator protein CheY. Phosphorylated CheY (CheY-P) directly binds the flagellar rotor protein FliM to favor clockwise (CW) rotation of the flagella, promoting disassembly of the flagellar bundle, and cell tumbling. When CheA is deactivated by attractant, a reduction in CheY-P favors counterclockwise (CCW) rotation of the rotor, bundling of the flagellar filaments, and smooth straight swimming (generating a so-called run). By alternating between tumbling and smooth swimming, the cell tracks gradients of attractants and repellents through what is effectively a biased random walk. The action of two additional enzymes – the methyltransferase CheR and the methylesterase CheB – produce a slower adaptation response that aids the tracking of chemoattractant gradients. CheR and CheB alter the methylation status of specific glutamate residues on the receptors and thereby tune receptor ligand affinity and modulation of CheA activity (Fig. 1).

MCPs interact with CheA and a coupling protein CheW to form a supramolecular assembly called the chemosensory array (Fig. 2). High signal sensitivity, cooperative responses and a wide dynamic range emerge from direct interactions among the components and the methylation system involving CheR and CheB [12–21]}. Chemoreceptors function as trimers-of-receptor dimers [18, 19, 22–24] that further assemble into a large hexagonally packed lattice arrangement [25–28] (Fig.1, 2). The hexagonal lattice of the receptor trimers-of-dimers is upheld by interactions among CheA P5, CheW, and the receptor tips [27, 28] (Fig. 2, 4C, 4D). Cryo-electron tomography (cryo-ET) maps generated from whole bacterial cells demonstrate that this general receptor arrangement is conserved in all classes of chemotactic bacteria studied [27, 29]. Reconstitution experiments with full-length receptors captured in defined oligomeric states [16, 17, 30–32] complement cellular studies [18, 19, 24] to show that the receptors must assume a trimer-of-dimer arrangement to fully modulate CheA activity. Protein crystal structures and solution models generated with fragments of receptors, domains of CheA, and CheW allow for interpretation of *in vivo* cryo-ET maps in terms of the general arrangement of molecular components [27, 29, 33–35] (Fig. 4). However, resolution limitations of the cellular tomograms, coupled with regions of

indiscernible electron density as well as symmetries inherent to the assemblies leave some ambiguity with respect to component identity and positioning. Furthermore, the conformational changes in the array that generate CheA on-off switching are not yet fully defined. A detailed molecular explanation for how receptors engage and modulate CheA is the next step in understanding the emergent properties of this remarkable sensory system.

The five domains of CheA each have distinct functions. The P1 domain contains the substrate histidine that becomes phosphorylated, P2 docks the response regulator proteins CheY and CheB, P3 dimerizes the protein, P4 binds Mg^{+2}-ATP and catalyzes phosphoryl transfer to the histidine substrate on P1, and P5 couples CheA to the other array components by binding both CheW and the chemoreceptors. CheA functions as a dimer in that the P4 domain of one subunit *trans* phosphorylates the P1 domain of the adjacent subunit [36, 37]. The P1 and P2 domains are separated from the P3-P5 domains by long flexible linkers that vary considerably in length and protein sequence among CheA orthologs [38] (Fig. 3A). The mobility of the P1 and P2 domains makes structural characterization of full-length CheA challenging. Although high-resolution crystal structures of each domain have been determined, a full-length structure of CheA has been elusive (Fig. 3A). However, a structure of the full-length kinase free from array incorporation is perhaps of limited relevance. More complex assemblies must be either reconstituted for study or probed *in situ*. Below we discuss aspects of chemotaxis signaling from the perspective of the CheA kinase and build to the central question of how CheA autophosphorylation activity is regulated by chemoreceptors.

2. Roles of individual CheA domains and array components

2.1 The P1 substrate domain and the P2 docking domain

The P1 domain is an HPt domain (**H**istidine **P**hospho**t**ransfer) that consists of 5 helices (A-E), with A-D comprising a tightly associated four-helix bundle (Fig. 3B) and helix E associated with and connected to the bundle by a flexible linker [39, 40] (Fig. 4A). HPt domains transfer phosphate groups to target proteins via phosphorylation of a histidine residue and can either function as an independent protein or as fused to a histidine kinase [41]. The substrate histidine of CheA P1 corresponds to residue H48 on helix B in *Ec* and H45 in the thermophile *Thermotoga maritima* (*Tm*), which has served as a useful source for well-behaved chemotaxis proteins (Fig. 3B). Site-directed mutagenesis of residues adjacent to *Ec* H48 indicates that phosphorylation depends on a local hydrogen bonding network [42]. In the *Tm* enzyme, residues K48, H64 and E67 elevate the pKa of the substrate H45 to 6.9 and stabilize the $N^{\delta 1}H$ tautomer that is necessary for CheA autophosphorylation [42]. In this tautomeric form the nucleophilic N^{e2} atom of the substrate histidine attacks the γ-phosphate of the P4-bound ATP [42]. Key aspects of P1 autophosphorylation and phosphotransfer have been elucidated, including the association of P1 with CheY bound to P2 [43]. However, the docking interaction between P1 and P4 has yet to be fully defined, despite models from both computation and experiment [37, 44–46].

Specific sites on P1 have been identified to interact directly with CheA P4, CheY and the methyl-esterase CheB (see below) [42, 46, 47]. Separation of the P1 domain from the kinase core (P3P4P5) demonstrates that a covalent connection to P1 is not essential for

phosphorylation *in vitro* [46, 48, 49] or *in vivo* [50]. However, overexpression of P1 is needed to attain normal *in vivo* chemotaxis behavior [46, 50]. Therefore, the long P1 linker may be important for increasing the local concentration of P1 in the vicinity of the kinase core. Progressive shortening of the linker between P1 and P3 in a *Tm* CheA variant devoid of the P2 domain disfavors *trans* subunit autophosphorylation relative to *cis*, but activity does not increase to the degree predicted by a random chain model [37]. These data suggest that domain arrangements and possibly interactions within the CheA dimer influence the ability of P1 to access P4. Crystal structures of truncated CheA variants also reveal interactions between P4 and linker segments N-terminal to P3 [37].

The P2 domain docks CheY and CheB to CheA [51–53]. Although P2 is not essential for phosphotransfer to CheY or CheB, it greatly accelerates the rate of phosphotransfer [54]. Despite a conserved structural fold, P2 orthologs do not bind CheY in the same manner, suggesting that the specific mode of recognition is not critical for phosphotransfer [55]. CheA devoid of P2 still supports chemotaxis, albeit tumbling responses are impaired [56]. Interestingly, overexpression of the P1 domain largely rescues the defect caused by removal of P2, further indicating that productive interactions between CheY and phosphorylated P1 do not require P2, and that P2 likely functions to increase the CheY concentration local to P1 [56]. Overall, tethering both P1 and P2 to the kinase core (P3-P4-P5) facilitates rapid phosphate transfer from ATP to CheY.

Mutational analyses of P1 and P4 define the P1:P4 interaction site required for phosphotransfer [46]. Random mutagenesis screens in *Ec* suggest that residues on helices A and B of P1 directly interact with the P4 domain [46] (Fig. 4A). These data, along with docking simulations done with crystal structures and homology models [57], cysteine-scanning studies carried out with *Salmonella enterica* (*Se*) CheA, [58] and cross-linking and linker-length experiments with *Tm* CheA [37] all suggest a similar interaction mode between P1 and P4. Intriguingly, NMR chemical shift perturbations with liberated P1 and P3-P4 from *Tm* CheA indicate that the strongest interaction between P1 and P4 involves helix D of P1 (Fig. 3B). This region is distal from the substrate histidine (H45) and interacts with a site on P4 that is remote from the nucleotide binding region [47]. Furthermore, residue substitutions in this region do moderately increase basal activity. These findings suggest that there may be a non-productive interaction between P1 and P4 that does not facilitate autophosphorylation but could be regulatory in nature.

2.2 The P4 kinase domain

The kinase domain of CheA (P4) that binds Mg^{2+}-ATP is composed of seven α-helices and a five-stranded β-sheet [59, 60]. The central part of the structure consists of an α-β sandwich with three of the helices packed against the β-sheet. Three other helices surround the Mg^{2+}-ATP pocket, and a short loop between two helices comprises the lid to the nucleotide pocket. Mg^{2+} is essential for histidine activation, and significantly increases nucleotide affinity. Crystal structures of *Tm* P4 without nucleotide or reconstituted with nucleotides (ADP, ADPCP, TNP-ATP, ADP-MTSL) reveal that the conformation of the ATP-lid changes dramatically upon nucleotide binding [60, 61]. When P4 is reconstituted with the non-hydrolysable ATP-analog ADPCP and Mg^{2+}, the ATP-lid folds into a short helix to partially

sequester the binding pocket. In all other crystal structures, the ATP lid is conformationally variable (Fig. 4A). Spatial ordering and folding of the ATP lid likely influence interactions between P4 and P1 (Fig. 4A). Molecular dynamics studies of docked P1 and P4 complexes further suggest that motions of ATP itself induce conformational changes within the ATP-lid that "open" the binding site and facilitate P1 engagement [44].

The P4 domain is structurally homologous to a class of bacterial ATPases called the GHL family, named after three representative proteins (Gyrase B, Hsp90, and MutL), and to the kinase domains of sensor kinases [59, 62]. Although the overall structure of CheA and the GHL proteins vary, they all possess a deep ATP-Mg^{2+} binding pocket formed by a conserved topology of four α-helices, five β-strands and an ATP lid that changes conformation upon nucleotide binding (Fig. 4A). However, CheA diverges from the GHL proteins in several aspects. Whereas GHLs possess a strictly conserved glutamate residue essential for ATP hydrolysis, the glutamate residue responsible for histidine phosphorylation in CheA is instead located on the P1 domain near the histidine substrate [42] (Fig. 3B, 4A). The divergent mechanism for ATP hydrolysis in CheA results in phospho-transfer as opposed to phosphate release as catalyzed by the GHLs. Additionally, structures of GHLs and CheA bound to nucleotide analogs reveal that GHLs have a more extensive network of residues that recognize and bind ATP than does CheA. Presumably, additional interactions with the P1 domain may further stabilize ATP-binding (Fig. 4A). Furthermore, CheA P4 has a larger ATP-lid than do the histidine sensor kinases [62]. Although the function of the increased ATP-lid size is unclear, it may interact with other components of the array in a regulatory function in addition to involvement in P1 autophosphorylation.

2.3 The P5 regulatory domain and the CheW adaptor protein

CheW and the CheA P5 domain are paralogs that compose hexagonal ring structures of the chemosensory arrays (Fig. 2, 4B). They each share a duplicated SH3-domain-like topology that domain-swaps one β-hairpin, thereby producing two intertwined five-stranded β-barrels (designated subdomains 1 and 2). The P3-proximal barrel of P5 (subdomain 1) binds CheW subdomain 2 through a pseudosymmetric contact that involves conserved hydrophobic residues on each domain [34, 35, 63] (Fig. 4B, 4C). This so-called "interface 1" interaction is mirrored by a pseudosymmetric "interface 2" interaction involving P5 subdomain 2 and CheW subdomain 1 [35]. When modified, sites in P5 subdomains 1 and 2 produce chemotaxis defects or affect CheW binding [64–67]. Notably, Cys-substitutions (and their subsequent modification) in subdomain 2, several of which localize to interface 2, cause defects in CheA regulation, primarily deactivation [64]. Thus, the ring contacts that couple two CheA dimer core complexes play an important role in kinase regulation, as will be discussed further below.

3. Architecture of the chemosensory arrays

MCPs form transmembrane homodimers that are composed of mostly helical domains that stack together to form long thin molecules that can extend ~380 Å in some receptor classes [68] (and reviewed in [2–4]). Most MCPs, including the *Ec* receptors Tar (for aspartate sensing) and Tsr (for serine sensing) are shorter (~310 Å) and contain periplasmic,

transmembrane and intracellular domains that are primarily helical in topology. The extracellular domains, which are the most variable among MCPs, bind ligands whereas the intracellular domains, which conserve a mainly 4-helix bundle structure (~220 Å for Tar and Tsr), consist of regions with defined functions for transducing receptor response and regulating CheA activity (Fig. 1). The intracellular region most proximal to the cell membrane is called the HAMP domain (for proteins in which it is found: **H**istidine kinases, **A**denylyl cyclases, **M**ethylaccepting chemotaxis proteins, and **P**hosphatases). The HAMP domain undergoes structural rearrangements upon ligand binding that are transmitted to the receptor tip (Fig. 1; reviewed in [69]) (Fig. 1). C-terminal to the HAMP domain are residues that undergo reversible methylation as part of an adaptation process that allows cells to keep a temporal record of ligand concentration (Fig. 1) [70–77]. Glutamate residues in this region are methylated and demethylated by the SAM-dependent methyltransferase CheR [78, 79] and the methylesterase CheB [80–82], respectively (Fig. 1). CheB possesses an N-terminal CheY-homology domain that is phosphorylated by CheA to activate the methylesterase, thereby allowing the level of CheA activity to influence adaptation [83, 84] (Fig. 1). The methylation status of the adaptation region influences receptor dynamics near the intracellular tip, which modulates CheA kinase activity [85–88]. Methylation of glutamate residues by CheR biases the receptor to an activated (kinase-on) conformation in *Ec* (Fig. 1). Adjacent to the methylation region, a glycine hinge provides flexibility to assemble the receptor tips into trimers and also facilitates on-off switching [89–92]. Notably, different types of chemoreceptors can assemble into heterotrimeric “teams” of trimers, provided they belong to the same length class [23, 68, 93]. The region at the intracellular tips of the receptors, called the protein interaction region (PIR), directly interacts with CheA and CheW to form the chemoreceptor ternary complex [2, 3, 35, 85] [94, 95] (Fig. 1, 4C, 4D). In a case of structural quasi-equivalence, every dimeric receptor that binds CheA or CheW via the PIR has a symmetry-related PIR that associates with PIRs from two other receptors on the trimer axis [18, 19, 22–24]. Thus, the PIR region can interact with either P5, CheW or another receptor (Fig. 2).

A model of a receptor:CheA:CheW cytoplasmic ternary complex generated by application of site-specific spin labeling and pulsed-dipolar electron spin resonance (ESR) spectroscopy [33] indicated that the receptor tip binds CheW but also interacts near the P4 and P5 domains of CheA. The ESR model also suggested that the receptor stalk aligns along the CheA dimerization domain, and the P1 substrate and P4 kinase project away from the receptor tips [33], in agreement with data based on chemical modification studies of the complex [58] (Fig. 4D). Furthermore, taken with the receptor trimer-of-dimer crystal structure [22], the ESR model suggested that the P5 and CheW units may form rings [33]. Several crystal structures comprising a receptor fragment in complex with CheA P5 and CheW revealed important interactions for array formation and signal propagation [27, 35] (Fig. 4A–D). These structures served as components to interpret cryo-ET reconstructions of intact arrays [27–29, 96–98] (Fig. 2B). The crystal structures contained hexagonal rings of CheA P5 and CheW that agree well with the cryo-ET images. In the crystal structures the receptor fragments bind on the outside of the rings at a groove between the β-barrels that is common to both P5 and CheW (Figs. 2 and 4), These interactions, which are consistent with the native-cell tomograms, serve to anchor the CheA:CheW assembly to the cell membrane.

CheW-P5 rings are then held together by the receptor trimers and by the P3 dimerization domain of CheA (Figs. 2 and 4). Within each receptor dimer one subunit directly contacts CheW or P5 and the other interacts with two other receptor subunits within the trimer arrangement (Figs. 2 and 4). Cryo-ET data from *in vitro* assembled arrays also indicate that the otherwise "open" hexagons of the array may be occupied by CheW-only rings [98]. Mutational studies of the *Ec* Tsr receptor [94, 95] and molecular dynamic simulations of an isolated receptor dimer [99] demonstrate that residues within the PIR are important for trimerization and signaling [88, 100].

Notably, a high degree of pseudo-symmetry in the arrays raises the possibility of alternative configurations across species or even receptor class. Pseudosymmetry relates subdomains 1 and 2 of CheW and P5 and the two proteins are very similar in structure to each other. In addition, equivalent receptor N-terminal helices run in opposite polarity when binding the analogous recognition grooves of CheW and P5. Crystal structures of receptor fragments bound to P5 and CheW do define these orientations in the context of the known crystal lattices [27, 35], but only small thermodynamic differences may distinguish the alternative conformations. Targeted disulfide cross-linking experiments provide *in vivo* validation of the current array "handedness" (i.e. the relative locations of P5 and CheW in the rings) [101] (Fig. 4), but even in these studies the "up" and "down" configurations of the receptor interactions are difficult to distinguish, owing in part to the two-fold symmetry of the receptors themselves. As discussed below, arrays from different bacterial species display some variations in their assembly states; and thus, changes in handedness or ring composition may also be possible.

The CheA dimerization domain P3 plays a critical role in the array by associating the hexagonal rings; however, P3 itself may exhibit substantial mobility. Cryo-ET of native arrays generally reveal only sparse electron density for the P3 domain [27, 28, 92, 102] and spin-labeling experiments of reconstituted CheA complexes indicate highly variable P3 orientations [33]. However, cryo-ET of *in vitro* reconstituted arrays provide accurate placement of the P3 domains [98], likely owing to the selection of highly ordered volumes of the tomograms for classification and averaging.

4. Chemosensory arrays in bacteria other than *Escherichia coli*.

4.1 Salmonella enterica, Bacillus subtilis and Thermotoga maritima

Whereas *Ec* has the best understood chemotaxis system, research on several other model organisms provides a more comprehensive understanding of chemotaxis across Bacteria. The *Salmonella enterica* (*Se*) system, which very closely resembles the *Ec* system, has elucidated aspects of CheA modulation and also provided a useful model to study chemotaxis effects on host-pathogen enteric interactions [103]. Research with the gram-positive bacteria *Bacillus subtilis* (*Bs*) has identified a more complex chemotaxis system that includes proteins without counterparts in the *Ec* and *S*e systems [104, 105]. Furthermore, chemotaxis homologs from the thermophile *Tm* have provided thermostable proteins that are well-suited for *in vitro* biochemical and structural experiments [62]. As such, many chemotaxis protein crystal structures have been determined by utilizing *Tm* homologs [27, 34, 106]. Importantly, chemotaxis studies from diverse bacteria reveal key differences in

molecular signaling. For instance, the *Ec* and *Se* systems are very similar and they utilize four common chemoreceptors (Tsr, Tar, Aer, Trg); however, they also each employ unique chemoreceptors specific to their environmental needs [107]. Furthermore, in *Ec* and *Se* attractants downregulate CheA kinase activity and repellants upregulate activity [107], but in organisms such as *Bs* the opposite signaling logic is used, i.e. attractants activate CheA. To compensate for this switch, CheY-P causes counterclockwise, rather than clockwise, flagellar rotation in *Bs* [108]. Bioinformatics analyses reveal that the *Ec* and *Se* chemotaxis systems belong to the same evolutionary class (F7), whereas those from *Bs* and *Tm* belong to a different class (F1) [38]. Receptor sequences from these organisms also indicate that *Ec*/*Se* fall into one signaling class, whereas *Bs*/*Tm* also fall into a different signaling domain classes (36H and 44H, respectively, where "#H" stands for the number of helical heptads within the cytoplasmic signaling domain) [68]. Therefore, the signaling logic used by the chemotaxis systems appears to correlate with the evolutionary class of the chemotaxis proteins [109].

Both *Bs* and *Se* also contain the CheV protein, which was first identified as a component of the adaptation system in *Bs* [110–112]. CheV is a homolog of CheW but also contains a CheY-like response regulator domain that CheA phosphorylates [111, 113]. Bioinformatics analyses indicate that CheV co-evolved with certain classes of MCPs and interacts preferentially with them. It has been suggested that CheV either substitutes for CheW in the arrays or perhaps only interacts with itself in CheV-only rings [114]. The mechanism through which CheV acts is unclear, but it has been suggested that the response-regulator domain may serve as a sink to buffer phosphate transfer to CheY [114, 115]. Interestingly, *Helicobactor pylori* contains three CheV proteins, one of which is critical for chemotaxis [116, 117].

4.2 Nonstandard arrays in Vibrio cholerae

Although the general arrangement of the chemotaxis machinery in the arrays is conserved across several bacterial phyla [29, 96, 97, 118], the proteins involved in these systems and their general mechanisms are variable [119]. Bioinformatics analyses of bacterial genomes reveals that there are nineteen different chemotaxis classes but only two of these classes have been studied extensively—class F1 (*Bs* and *Tm*) and F7 (*Ec* and *Se*) [119]. Research with less commonly used bacterial models has revealed variations in array composition and structure. For example, cryo-ET of arrays from *Vibrio cholerae* show that the abundance of CheA is much lower compared to *Ec* in the supramolecular assembly and cooperativity may therefore play an even greater role in signal transduction in this organism [120]. Furthermore, one class of array in *V. cholerae* is purely cytoplasmic and is stabilized by an unusual chemoreceptor with a doubled signaling domain [121].

4.3 Spirochetes

Many spirochetes possess variants of chemotaxis proteins that have yet to be fully characterized. For example, the pathogens *Borrelia burgdorferi* (*Bb*) and *Treponema denticola* (*Td*) both contain atypical CheA and CheW proteins [122–124]. CheA from these organisms have longer linkers connecting the P1–P2 and P2–P3 domains. While the function of the increased linkers is unknown, the connection length between P1 and the kinase core

influences CheA activity and the effective concentration of P1 in the vicinity of the P4 domain [37] (Fig. 3). Therefore, it is possible the linker lengths modulate CheA in a manner relevant to function. Furthermore, *Td* and *Bb* CheA also have additional residues between the two conserved helices of the P3 domain. Secondary structure prediction suggests that these residues form additional helices, and thus may allow an extension of the P3 domain toward the top of the array. An increased length in the P3 domain could provide additional stability to the CheA dimer or supply a direct interaction with adjacent receptors. *Bb* contains three CheW paralogs. Of these an atypical CheW contains a C-terminal domain with homology to some CheR proteins [122, 123]. Deletion of the CheR-like domain in *Bb* does not impair chemotaxis under standard laboratory conditions, and thus the function of the domain remains unclear [125].This unusual CheW/CheR-like fusion protein is also present as the sole CheW in *Td,* but the importance of the CheR-like domain on chemotaxis has yet to be explored [123].

4.4 Rhodobacter sphaeroides

Rhodobacter sphaeroides (*Rs*) contains transmembrane chemosensory arrays for the detection of external attractant and repellant concentrations and cytoplasmic chemosensory arrays for monitoring the metabolic state of the cell [126]. *Rs* possesses three homologs of CheA that vary in length, cellular localization, and interacting response regulators [127]. The *Rs* $CheA_2$ protein contains the typical five domains of the kinase and associates with transmembrane arrays. However, this organism also encodes the two proteins, $CheA_3$ and $CheA_4$ that localize to cytoplasmic arrays and only contain the P1P5 and P3P4P5 domains, respectively [126]. Neither protein can autophosphorylate on its own. Instead, $CheA_4$ phosphorylates the P1 of $CheA_3$ [128]. As a photosynthetic organism *Rs* is metabolically diverse. The complexity of its chemotaxis system likely reflects integration from both external and internal signals that together allow the cell to assess the energy status of its environment [127].

5. How then is CheA regulated by chemoreceptors?

5.1 CheA activation

Central to the chemotaxis response is the large change in activity that the CheA kinase undergoes in response to receptor stimulation. Early work demonstrated that *Ec* CheA operates at three general levels of autophosphorylation: a “closed” state in its free form, an “open” or “on” state when complexed with chemoreceptors and a “sequestered” or “off” state when the receptors were bound to chemoattractants [129, 130]. The receptor-inhibited or off state was designated as “sequestered” because, unlike the open state, the sequestered state cannot exchange phosphate with ATP or ADP [129]. Moreover, although sequestered CheA can be dephosphorylated by CheY, further autophosphorylation is blocked [129]. The progression of studies on CheA autophosphorylation illustrate the challenges of reconstituted systems when key features of assembly state are yet to be understood. Initial work with isolated *Ec* membranes showed a roughly 300-fold increase of activation between the closed (free) and open (receptor-bound state) for CheY-P production, but only a minimal change in CheA autophosphorylation rates [130]. Subsequent studies on purified and reconstituted systems with receptors in isolated membranes or liposomes revealed a roughly

10-fold increase in CheA autophosphorylation in the open form [129, 131, 132]; albeit these were likely underestimates owing to the challenges associated with reforming the then unknown chemosensory arrays. Experiments with recombinant receptor fragments produced much larger increases in CheA autophosphorylation, with activation levels seen in excess of 300-fold [133–136]. Considerable biochemical data and cryo-ET imaging have indicated that changes in CheA activity do not result from association or disassociation of components within the complexes and that the integrity of the arrays do not change with signaling state [25, 102, 137–140]. Indeed, engineered disulfide cross-linking and targeted protein modification suggest that relatively subtle changes in CheA conformation are involved in its activation and regulation by receptors [58, 64, 65, 101, 141, 142]. These conformational changes may involve domain motions within CheA. Specific residues in linkers that connect P4 to both P3 and P5 are critical for signal propagation through the kinase [143–145]. Residue substitutions in the P3–P4 linker decrease basal kinase activity and also receptor-mediated activation to some extent [143, 145], whereas those in the P4–P5 linker can both decrease and increase kinase activity but consistently hamper receptor activation [144, 145]. Interestingly, residue substitutions in the P4–P5 linker that impair receptor coupling also affect CheA activity even in the absence of the P5 domain [145]. Thus, the receptors may modulate kinase activity by controlling the positioning or catalytic efficiency of the P4 domain via the P3–P3 and P4–P5 linker conformations. Indeed, alterations to the P4–P5 linker directly influence the structure and dynamics of the ATP binding site [143].

5.2 In vivo FRET assays to measure chemotaxis gain

Two advances in assaying kinase activity have been critical for delineating key aspects of CheA regulation. The first was the development of an *in vivo* FRET assay by Sourjik and Berg that allows kinase activity to be monitored directly in *Ec* cells upon stimulation with attractant [146–148]. This assay, designed to monitor the interaction between the CheA product CheY-P and its phosphatase CheZ, has been employed to investigate cooperativity in the chemotaxis response and provide a quantitative measure of the so-called chemotaxis gain [74], which relates the change in fractional kinase activity to the change in receptor:ligand occupancy [12, 14] (Fig. 1). Sourjik and Berg revealed that ~35-fold gain derives from the receptor response [12] and modeled this gain in terms of a Monod-Wyman-Changeux (MWC) model of allostery [14]. The MWC model has subsequently undergone modification and refinement by several groups to account for receptor modification state, stimulation by different ligands, and physical details of the receptor-kinase array architecture [149–151] (and reviewed in [152]). Parkinson and coworkers have further exploited the *in vivo* FRET assay to study the effects of extensive residue substitutions on the core components of the receptor arrays [67, 95, 153]. Array cooperativity depends on receptor composition and to some degree modification state (i.e. which and how many glutamate residues are methylated) [12, 14, 20, 154], although strains with only one type of homogeneous receptor that have either uniform high or low methylation states can both exhibit cooperative responses [14, 154, 155]. Importantly, higher order interactions within the P5-CheW layer underlie cooperativty [66, 67]. In particular, the so-called interface 2 contacts between CheW and P5 subdomain 2 link core particles both structurally and functionally (Fig. 4B) [64–67]. Genetic experiments confirm that disruption of interface 2 produces arrays with aberrant organization and diminished cooperativity [65, 66]. These specific lesions were

further investigated to demonstrate that the coupling of core complexes through CheW:P5 interactions increases the sensitivity of the array to chemoattractants and maintains appropriate adaptational responses [67]. These results, particularly the lower ligand response thresholds for the interface 2 mutants, indicate that interface 2 may be weakened in the kinase-off state; interestingly, targeted disulflide cross-linking experiments suggest that the same may be true for interface 1 [141] (Fig. 6A). These studies show that minor conformational changes can have pronounced effects on activity and response without substantial disruption of the array structure or destabilization of the core components. Nonetheless, the strength of interactions within the CheW/P5 rings likely depends on signaling state. Early mutational work and genetic suppressor studies pointed to functionally key contacts between the receptor and CheW [156–158]. In fact, contacts between the receptor and CheW appear to dominate signal conveyance to the kinase over contacts between receptor and the P5 domain, despite these interactions being pseudosymmetric [27, 153]. In addition, *in vivo* crosslinking experiments identified two contacts between CheW and the Tsr receptor that are increased in the presence of a chemoattractant, thereby demonstrating that receptor conformational changes alter interactions with CheW to perhaps distribute signals across the molecular lattice [142].

5.3 Kinetic considerations of autophosphorylation

In the second advance, nanodisc technology has quantified kinetic parameters for CheA in controlled and defined assembly states [16, 17, 30–32]. Nanodisc complexes with CheA truncated to its core components (domains P3P4P5) have been used to phosphorylate a free P1 domain and thereby allow for P1 steady-state turnover to be studied directly, instead of through the accumulation of CheY-P [48]. Basal kinase activity k_{cat} values for free P1 are similar to those characterized previously for full-length CheA coupled to CheY [159]. However, CheA engagement by nanodisc particles greatly increases k_{cat} for P1 phosphorylation while only producing a modest change to the Michaelis-Menten constants (K_M) for ATP and P1 [48]. Thus, receptors may modulate the catalytic efficiency of the CheA P4 domain by communication through the P5-CheW layer, and/or the P4 linkers to the catalytic center. These changes then effectively alter the active fraction of the kinase. Because the steady-state parameters reflect only active kinase, K_M values for P1 and ATP are relatively unchanged. In both isolated membranes and in reconstituted systems with full-length CheA, P1-P accumulates with the expected first order kinetics; however, the amplitude of the response drops dramatically upon receptor inhibition, which suggests that a large percentage of CheA cannot autophosphorylate to an appreciable degree in the off state [49, 129]. The ability of receptors to produce a large range of kinase activity with free P1 implies that kinase regulation does not depend on imposing constraints on P1 through its attachment to the kinase core [48]. Rather, occlusion of P1 or possibly the ATP binding site on P4 may better embody the inactive form. Nevertheless, the off state could also involve an inhibitory binding site for P1 that interferes with the productive site and does not depend on covalent attachment of P1. Kinetic modeling of extensive rate data from nanodiscs and membranes further indicates that receptors activate CheA by regulating both phosphoryl transfer from ATP (which could include both P1 binding and the catalysis step) as well as ATP binding [160]. Remarkably, receptors appear to alter the kinetic barriers between key

steps in the reaction without changing the free energy differences between states [160]. A P1/P4 occlusion mechanism would be consistent with such behavior.

5.4 Structural implications for CheA down-regulation

From identifying the unusual assemblies of chemoreceptors [26, 97, 161, 162], through providing details on component arrangements [27, 28, 98], to suggesting mechanisms of regulation [25, 102, 163], the impact of cryo-ET on our understanding of chemosensory array structure and function cannot be overstated. For example, cryo-ET of native receptor arrays indicates that the kinase becomes more ordered in the inhibited state [92, 102] (Fig. 5). Reconstructions of the core complex from *Ec* cell membranes with a set of variant receptors that shift the system through a range of activity levels show more ordered density at the tip of the receptors as CheA inhibition increases [102]. The additional electron density has the shape of a “sailboat keel” joining two trimers-of-receptor dimers and is of a volume consistent with the binding of the P1 and P2 domains to the core P3–P4–P5 unit (Fig. 5). Although limitations in resolution prevent unambiguous placement of the P1, P2 and P4 domains, keel density was lost when arrays were produced with truncated CheA proteins lacking P1 and P2 (Fig. 5). In support of an ordered kinase in the inhibited state, CheA is also more resistant to proteolytic digestion when inhibited [102].

CheA heterodimers that contain only one P1 domain have higher autophosphorylation activity than full-length symmetric dimers, suggesting that the P1 domains may interfere with each other [37, 164]. Furthermore, P1 domains can be cross-linked to each other [37] and P1 contributes to CheA dimer stability (by presumably providing inter-subunit contacts, perhaps in the form of P1–P1 interactions) [165]. Thus, despite assuming a broad distribution of states [33], the P1 domains may not always be widely separated, even in the free kinase. Indeed, recent spin-labeling studies provide evidence for organization of the P1 and P2 domains by their linkers [166]. The P4 domains may also participate in functionally important contacts with other array components. Several different modeling studies with crystals structures, tomogram density and MD simulations position the P4 ATP-lid of P4 next to the P5-CheW layer [37, 98]. In addition, α10 of P5 was found to partially block the ATP pocket in one subunit of the original CheA P3–P4–P5 dimer structure [59, 62]. Such interactions may provide means for the P5-CheW rings to affect the P4 catalytic center [37] (Fig. 4D, 6). Movement of P4 away from the P5:CheW layer would then allow P1 to access ATP in the P4 pocket (Fig. 6). Such a P4 dipping motion was observed in large-scale molecular dynamics simulations in combination with high-resolution cryo-ET reconstructions of *in vitro* assembled arrays [98]. If the inhibited state of CheA involves a constrained conformation that blocks access of P1 and perhaps ATP to the kinase active center, specific mutations would be expected to destabilize key domain interfaces and increase the active fraction of kinase. However, few residue substitutions have been found to activate CheA, and those that do generally have modest effects. Of these, several mutations have intriguing mechanistic implications given that they alleviate the non-productive binding site for P1 on P4 [47], affect positions far removed from the P4 active center [167] or reside on the P4–P5 linker [145]. Interestingly, a monomeric P4 domain phosphorylates P1 very poorly, but this activity substantially increases when P3 dimerizes P4 [37, 133]. Thus, either high kinase activity requires two P4 domains or P3 is needed to provide a structural interface

for one P4 domain and its flanking linkers. Furthermore, high negative cooperativity of ATP binding in *T. maritima* CheA [168] implies that the two kinase domains influence each other in some manner, despite the two ATP binding sites being far apart in most models [27, 28, 37, 98]. Finally, experiments with isolated core complexes reconstituted into nanodiscs demonstrate allosteric coupling between CheA subunits in the active dimer [17]. Thus, changes in P4 juxtaposition may be a consequence of receptor regulation.

6. Conclusions

The CheA kinase should be thought of as an integrated system of protein modules that assumes a fully functional state when networked with receptors and CheW. In the absence of the other core components, *Ec* CheA does not attain high levels of autophosphorylation activity. Array incorporation imparts the kinase-on state and enables regulation of that state by chemoattractant. However, the conformational changes that tune CheA activity within the arrays are relatively subtle (Fig. 6). Structural alterations within the P5-CheW layer induced by signals primarily through the receptor:CheW interface may be transmitted to P4 through its flanking P5 linker. P3 may also repack in response to a conformational change in the receptors and convey signals to P4 through the P3 linker. Indeed, the entire lattice may reorganize in a highly cooperative manner. It is likely that the P1, P2 and P4 domains are less mobile and more closely associated at the base of the P5-CheW layer when the kinase is less active (Fig. 6). Occlusion of both ATP and P1 from the P4 catalytic center, through either interaction of P4 with P5-CheW, or its symmetric subunit, may embody the essential feature of the aptly named sequestered state (Fig. 6). That said, the low activity of free *Ec* CheA must be reconciled with the ability of ATP and ADP to exchange in this state and the ability of P1 to access the P4 active center. Thus, array incorporation is likely doing more to the catalytic machinery than simply controlling binding of substrates to the ATP binding pocket. In the end, the next advance in understanding CheA regulation may come from determining structures of the kinase in array assemblies of defined activity at relatively high resolution – a formidable task, but not one out of reach.

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Highlights

- The histidine kinase CheA is five-domain dimeric enzyme that is central to the signal transduction pathway that underlies bacterial chemotaxis.
- Transmembrane chemoreceptors assemble with CheA and the coupling protein CheW to form extended molecular arrays that allow for integration of signals and cooperative responses.
- In recent years, cellular activity assays, nanodisc reconstitution and *in vivo* cryo-electron tomography have revealed much new information regarding how receptors regulate CheA activity.
- Plausible models for CheA regulation involve domain sequestration and release mechanisms as well as direct modulation of the kinase active site by conformational switching of structural elements.
- Whereas studies of the *E. coli* chemotaxis system have defined the fundamental principles of array architecture and CheA regulation, other bacterial species display interesting variations of array composition and function.

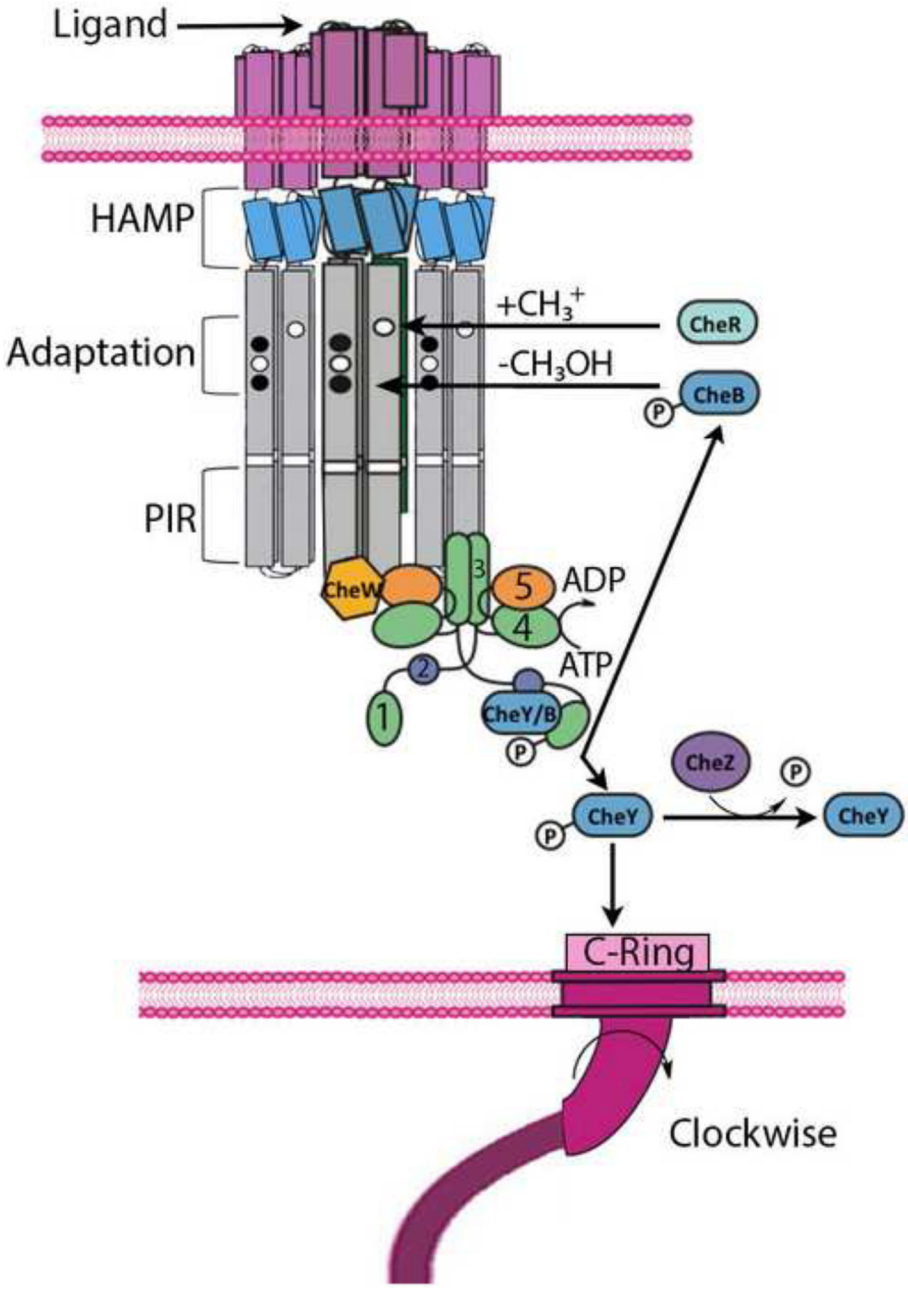

Fig. 1.

The bacterial chemotaxis system of *E. coli*. Attractants and repellents stimulate transmembrane chemoreceptors that function in a trimer-of-dimers. Binding of ligands to the extracellular domain elicits a conformational change in the receptor that is propagated through the HAMP to the intracellular protein interaction region (PIR) where the receptor directly interacts with the adaptor protein CheW (yellow) and the dimeric histidine kinase CheA (green, purple, orange). CheA has five domains (P1–P5) and once activated by receptors, the P4 kinase domain (orange) will *trans* phosphorylate the P1 substrate domain. CheA binds the response regulator CheY through the P2 domain (purple) and activates CheY by phospho-transfer. Phospho-CheY interacts directly with the flagellar motor at the

C-ring to induce clockwise rotation of the flagella and cell tumbling. The concentration of phospho-CheY is modulated by the phosphatase CheZ. CheA also phosphorylates the methyl-esterase CheB, which removes methyl groups on glutamate residues located at the receptor adaptation region. The SAM-dependent methyltransferase CheR adds methyl groups to glutamate residues to reactivate receptors inhibited by attractant.

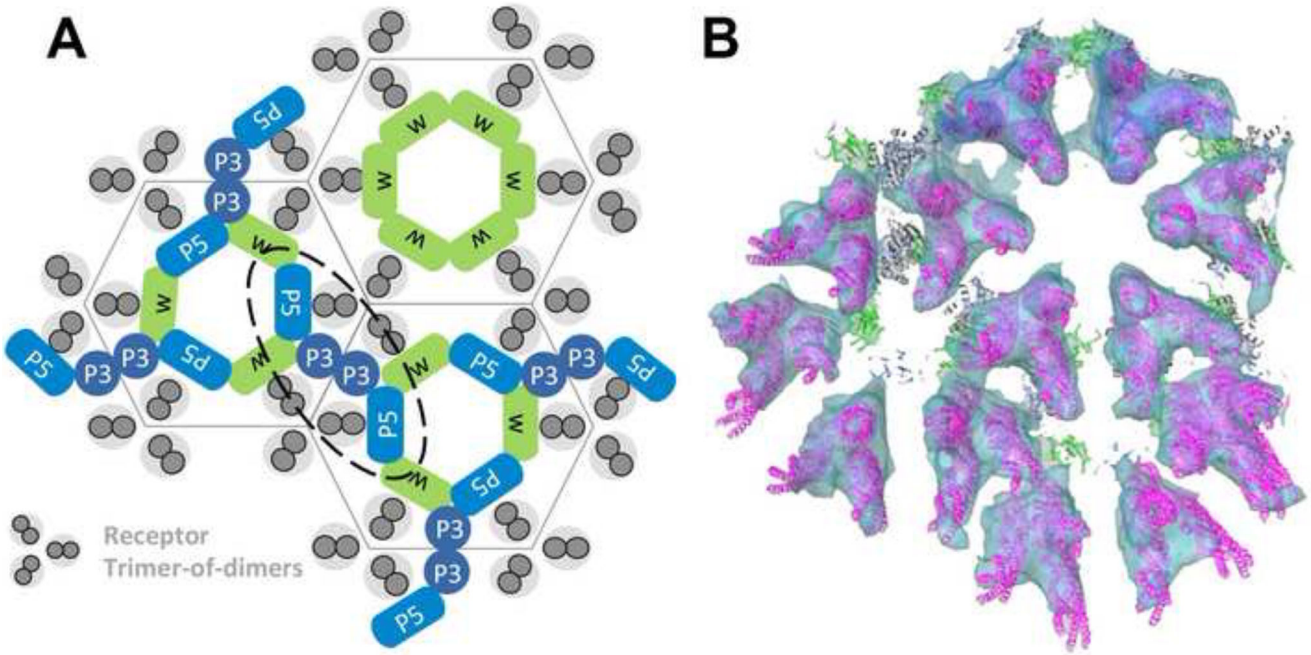

Fig 2.

The chemosensory arrays. (A) Schematic of the bacterial chemotaxis transmembrane array viewed from the membrane. CheA P5 (blue) and CheW (green) interact to form hexagonal rings that are linked together by dimerization of the CheA P3 domain (dark blue). One CheA dimer is shown in the dashed circle. The rings are anchored to the membrane and further stabilized by direct interaction with chemoreceptors (grey), which form a trimer-of-dimer oligomeric state. Within the trimer-of-dimer module, the subunits of the receptor dimer either interact with an adjacent receptor subunit within the trimer or with P5/CheW. In areas of the lattice where there is no CheA P5 domain, CheW assembles into all-CheW rings. (B) ECT of *Ec in vivo* arrays show trimer-of-receptor dimers; crystal structures of chemotaxis protein fragments are fit into the electron density [27].

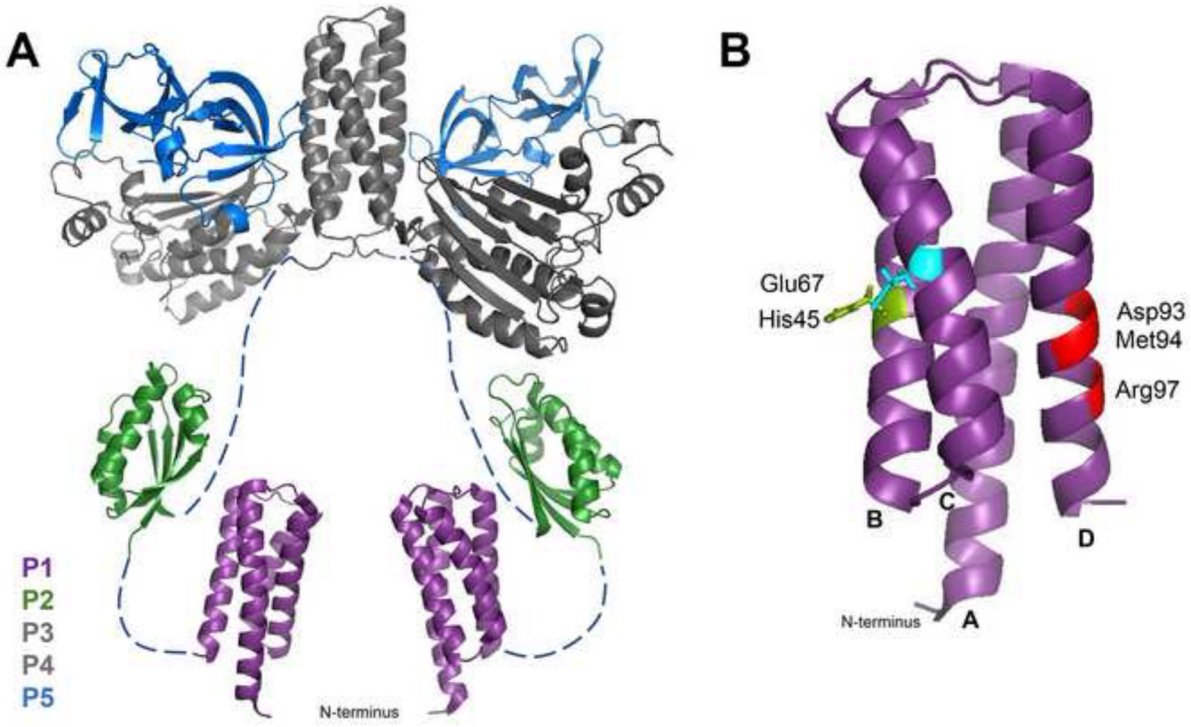

Fig 3.

Crystal structures of *Tm* CheA domains. (A) Crystal structures of CheA fragments P1 (PDB ID: 1TQG), P2 (PDB ID: 1U0S), and P3P4P5 (PDB ID: 1B3Q) arranged to represent a full-length kinase. Dashed lines denote flexible linkers between the P1/P2 and P2/P3 domains with undetermined structure. (B) The P1 structure (PDB ID:1TQG) consists of helices A-D. Residues D93, M94 and R97 (red) participate in non-product interactions with P4. Residues near the H45 substrate residue (green) mediate productive interactions with P4. E67 (cyan) activates H45 for phosphorylation.

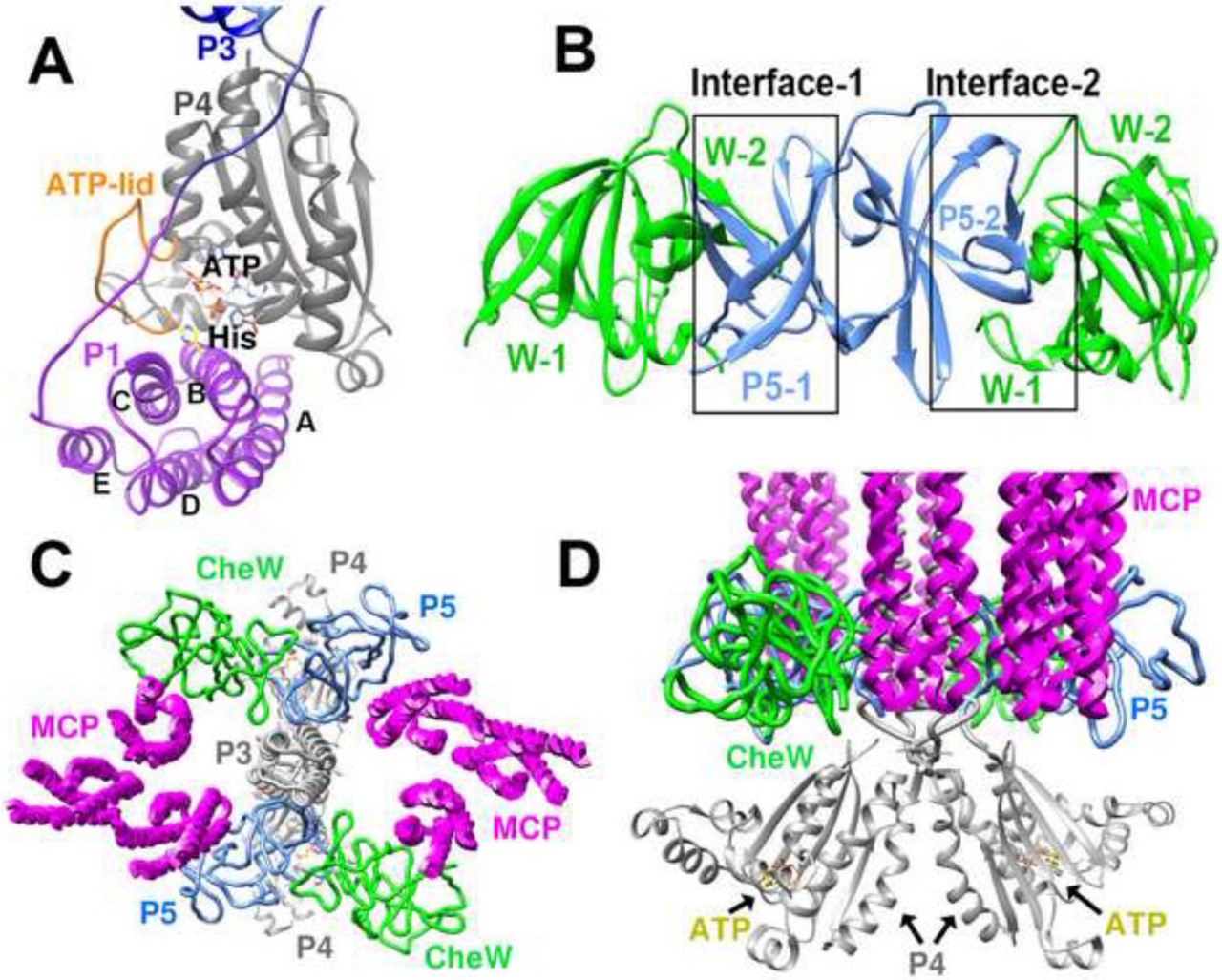

Fig. 4.

The core complex and key interactions of the chemosensory array. (A) Model of the P4 kinase domain (grey) bound to the P1 substrate domain (purple) [37]. The ATP lid (orange) shown in an open configuration may mediate interactions among nucleotide and substrate. (B) Crystal structures of P5 bound to CheW (PDB ID:3UR1) reveals conserved hydrophobic interfaces (1 and 2) that form hexagonal rings that are apparent in vivo [35]. (C) View from the membrane of a core complex composed of one CheA dimer, 2 CheW molecules and two receptor trimmers of dimers. Chemoreceptors interact with both CheA P5 and CheW at the junction between two β-barrels. (D) Side view of (C). The P4 domain resides below the P5:CheW ring and is relatively mobile [27]. P4 may move in the different activation states of CheA and perhaps interact with the P5-CheW layer to modulate access of substrates to the nucleotide-binding site. P1 and P2 are not shown.

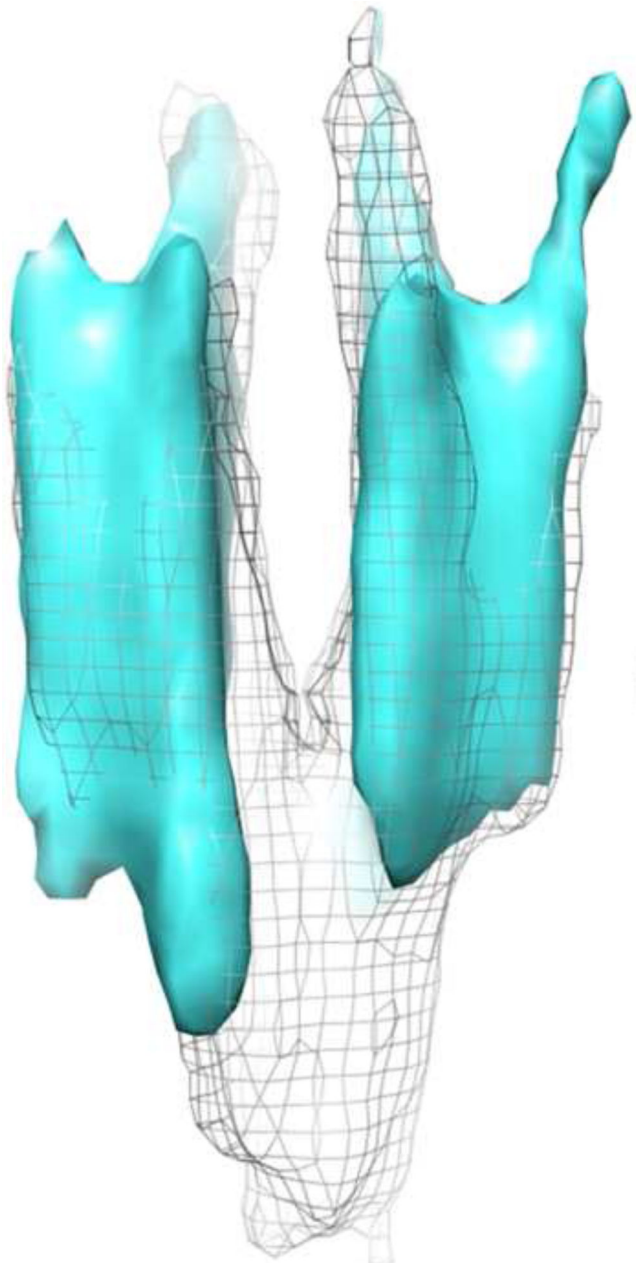

Fig. 5.

Electron density maps of the *Ec* chemotaxis core complex in a kinase-off state generated by ECT [102]. Increased density that resembles a 'keel' resides below the receptor tips in a full-length kinase (grey) but is no longer present when the arrays are generated from a CheA variant that does not contain the P1 and P2 domains (teal).

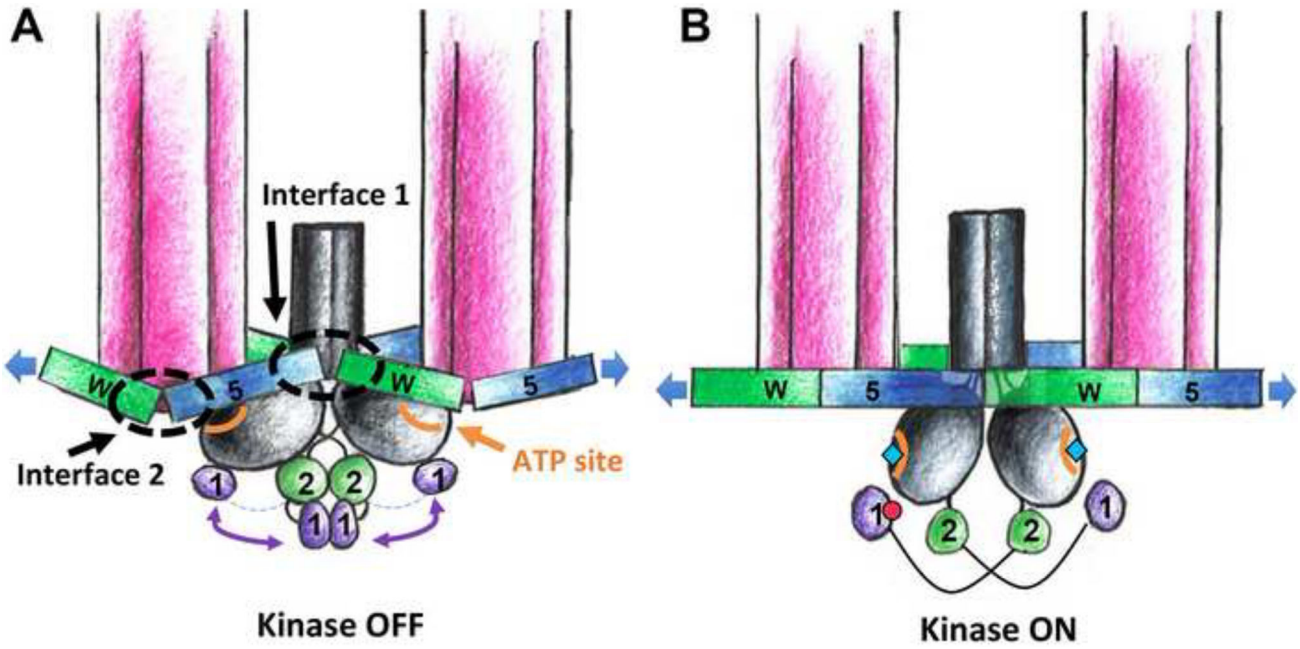

Fig. 6.

A hypothetical cartoon model for regulation of the CheA kinase. Two trimers-of-receptor dimers (magenta) are shown bound to an extended core complex of one CheA, 2 CheWs proteins, plus additional CheW and P5 domains that extend the array through interface 2. CheA P3 resides between the receptors. For detailed molecular interactions, see Fig. 4. (A) In the kinase-off state, interactions within the P5-CheW layer are relatively weakened and encourage greater interactions of P4 within the core complexes, causing either occlusion of the ATP pocket (orange) or interference with ATP binding determinants, such as the ATP-lid. P1 and P2 also associate with P3 and P4 in the sequestered conformation with P1 forming self-interactions or docking to an inhibitory site on P4 (arrows and dashed linker indicate alternative P1 docking sites). (B) In the kinase-on state, conformational signals from the receptor are transmitted through the receptor:CheW interface and perhaps the P3:receptor interface to alter the P5-CheW layer and increase interactions within both interface 1 and interface 2. Effects on the P3–P4 and P4–P5 linkers release the P4 domain, increase mobility of P1 and P2, and expose the ATP binding pocket and/or release of the lid (orange) to facilitate ATP binding (blue diamond) and P1 *trans* autophosphorylation (red circle).