The CheC Phosphatase Regulates Chemotactic Adaptation through CheD*

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The bacterial chemotaxis system is one of the most extensively studied signal transduction systems in biology. The response regulator CheY controls flagellar rotation and is phosphorylated by the CheA histidine kinase to its active form. CheC is a CheY-P phosphatase, and this activity is enhanced in a CheC-CheD heterodimer. CheC is also critical for chemotactic adaptation, the return to the prestimulus system state despite persistent attractant concentrations. Here, CheC point mutants were examined in Bacillus subtilis for in vivo complementation and in vitro activity. The mutants were identified separating the three known abilities of CheC: CheD binding, CheY-P binding, and CheY-P phosphatase activity. Remarkably, the phosphatase ability was not as critical to the in vivo function of CheC as the ability to bind both CheY-P and CheD. Additionally, it was confirmed that CheY-P increases the affinity of CheC for CheD, the later of which is known to be necessary for receptor activation of CheA. These data suggest a model of CheC as a CheY-P-induced regulator of CheD. Here, CheY-P would cause CheC to sequester CheD from the chemoreceptors, inducing adaptation of the chemotaxis system. This model represents the first plausible means for feedback from the output of the system, CheY-P, to the receptors.

In the signal transduction process, a cell detects extracellular stimuli, transmits a signal across the cell membrane, and generates a response. Notably, the bacterial chemotaxis system must constantly respond to effectors and then adapt to their continued presence. This adaptation allows the cell to detect minute changes in an attractant gradient. Chemotaxis, the ability of an organism to direct movement toward more favorable environments, is accomplished in the bacteria and archaea through a modified two-component signaling system (1, 2). The cytoplasmic histidine kinase CheA is regulated by chemoreceptor proteins known as methylated chemotaxis proteins (MCP)† and coupled to them by the CheW-like domain. In Bacillus subtilis, CheA autophosphorylation increases when the receptors bind an attractant (4). The phosphoryl group is subsequently transferred to the response regulator CheY. This active protein (CheY-P) regulates the rotational direction of the flagella of the cell by binding the flagellar switch protein FlhM (5, 6). In B. subtilis, the default rotation is clockwise and causes the cells to “tumble” in space. High CheY-P levels cause the rotation to reverse to counter-clockwise, and the bacterium swims smoothly (7). Controlled modulation of tumbles and smooth swims results in a biased random walk up a concentration gradient to optimal conditions.

Adaptation is accomplished by desensitizing the system to effector concentrations already detected and responded to. This returns CheA to basal activity and leads to normal CheY-P levels (8). Traditionally, adaptation has involved modification of the chemotaxis receptors by the addition and removal of methyl-esters from conserved glutamate residues by the CheR methyltransferase (9, 10) and CheB methylesterase (11, 12), respectively. The activity of CheR is not directly regulated, but CheB is activated by CheA phosphorylation of its N-terminal response regulator domain. Consequently CheB activity parallels that of CheA and forms a feedback loop between CheA and the receptors. A second, less common adaptation system involves CheV (13), a response regulator protein with a CheW-like domain. CheV-P is thought to decouple CheA and the receptors to bring about adaptation. CheC is also known to be involved in chemotactic adaptation in B. subtilis (14, 15), although the mechanism for CheC involvement has been unclear. However, there has been no mechanism identified by which the primary output of the chemotaxis system, CheY-P, was involved in a feedback loop.

Removal of the CheY-P signal is accomplished by accelerating the natural auto-dephosphorylation of CheY (2). In Escherichia coli, the CheY-P phosphatase CheZ is absolutely necessary for chemotaxis (16). However, CheZ has only been found in proteobacterial genomes (17, 18). The CheC, FliY, and CheX protein family members have been shown to be CheY-P phosphatases and are found throughout the Bacteria and Archaea domains (19–21). These proteins share a common tertiary structure and a consensus sequence (D/S-X3-E-X32-N-X32-P) thought to define the phosphatase active site (17).

CheC has been shown to form a heterodimer with the receptor deamidase CheD (22). This interaction increases the effectiveness of CheC phosphatase activity (20). The CheD-binding site on CheC was shown to be an α-helix, which mimics the enzymatic target of CheD, the MCP chemoreceptors (23). Because a cheC mutant has defective adaptation, it was pro-

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The abbreviations used are: MCP, methylated chemotaxis protein; GST, glutathione S-transferase.
posed that CheD is released from the receptors post stimulus to activate the CheC phosphatase activity (20). CheC has been shown in vitro to inhibit CheD deamidation activity on the MCP chemoreceptors for both Thermotoga maritima (23) and B. subtilis.4 The addition of CheY-P led to further inhibition of deamidation, suggesting that CheY-P augments CheD binding by CheC (23). CheD has been shown to have other possible MCP interactions, including demethylation (23), and an interaction with the HAMP domain found N-terminal to the methylation region (24). Indeed, mutants lacking cheD show almost no CheA kinase activity (14).

To further understand the function of CheC, we have mutationally analyzed the two putative phosphate active sites and the CheD-binding site. The mutant genes were analyzed for their ability to complement the ΔcheC mutant, and the corresponding proteins were assayed for their ability to dephosphorylate and bind CheY-P. We were able to identify mutants defective in the known functions of CheC: CheY-P binding, phosphatase activity, and CheD binding. Based on this data we have created a model for the physical association of CheY with CheC. Further, the ability to bind CheY-P and/or CheD was shown to be critical for chemotaxis, whereas a mutant defective only in phosphatase activity retained some function. Based on this data, we present a model of the chemotaxis system where CheC is a mediator of CheY-P feedback to the receptors via regulation of CheD.

4 W. Yuan and G. W. Ordel, unpublished data.

### EXPERIMENTAL PROCEDURES

**Chemicals, Enzymes, and Growth Media**—All of the chemicals were of reagent grade from Sigma-Aldrich. All of the restriction enzymes were purchased from Invitrogen. The media components were from Difco.

**Plasmid and Strain Construction**—All of the strains used in this study are listed in Table 1. *B. subtilis* strains are derived from strain OI1085 (che−). All of the cheC mutations were created in pMR108 by QuikChange mutagenesis (Stratagene, La Jolla, CA). The mutant genes were then subcloned into pDR67 to create pMR130 variants. The cheC mutants in pMR130 were introduced to strain OI3135 by transformation, selected for CmR, and screened for loss of amyE function. The cheX gene from *Bacillus halodurans* (BH1088) was cloned into pDR67 to create pTM50 with the Shine-Delgarno sequence from *B. subtilis* cheC. pTM50 was transformed into OI3135 to create OI4226 (ΔcheC cheX−).

To create GST fusion proteins for expression and purification, cheC mutants were subcloned into pGEX-6P-2 to create pTM18 variants. The fliY gene was also cloned into pGEX-6P-2 to create pTM0. These pGEX-6P-2 derived plasmids were expressed in strain BL21.

**Protein Overexpression and Purification**—All of the proteins used for *in vitro* experiments were purified as GST fusions from BL21 lysates expressing the proteins from pGEX-6P-2 variant plasmids. 5-ml GSTrap columns (GE Healthcare, Piscataway, NJ) were used with an AKTA Prime system (GE Healthcare, Piscataway, NJ) for purification as the manufacturer described.

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**TABLE 1**

Strains and plasmid used in this study

| Strain or plasmid | Relevant genotype or description | Reference |
|------------------|---------------------------------|-----------|
| OI1085           | che+, trpE7 hisH2 metC133        | Ref. 35   |
| OI2934           | CheA::cat                        | Ref. 14   |
| OI3135           | ΔcheC1                           | Ref. 14   |
| OI3165           | ΔcheC1 amyE5720::cheC3           | Ref. 14   |
| OI4172           | ΔcheC1 amyE5720::cheC4 (cheC-D149K) | Ref. 23   |
| OI4193           | ΔcheC1 amyE5720::cheC5 (cheC-D13A) | This work |
| OI4194           | ΔcheC1 amyE5720::cheC6 (cheC-E17A) | This work |
| OI4195           | ΔcheC1 amyE5720::cheC7 (cheC-N20A) | This work |
| OI4196           | ΔcheC1 amyE5720::cheC8 (cheC-P43A) | This work |
| OI4197           | ΔcheC1 amyE5720::cheC9 (cheC-E17A/ N20A) | This work |
| OI4198           | ΔcheC1 amyE5720::cheC10 (cheC-S14A) | This work |
| OI4199           | ΔcheC1 amyE5720::cheC11 (cheC-E118A) | This work |
| OI4200           | ΔcheC1 amyE5720::cheC12 (cheC-N121A) | This work |
| OI4201           | ΔcheC1 amyE5720::cheC13 (cheC-P144A) | This work |
| OI4202           | ΔcheC1 amyE5720::cheC14 (cheC-E118A/N121A) | This work |
| OI4203           | ΔcheC1 amyE5720::cheC15 (cheC-D13A/S144A) | This work |
| OI4204           | ΔcheC1 amyE5720::cheC16 (cheC-E17A/E118A) | This work |
| OI4205           | ΔcheC1 amyE5720::cheC17 (cheC-N20A/N121A) | This work |
| OI4206           | ΔcheC1 amyE5720::cheC18 (cheC-P43A/P144A) | This work |
| OI4207           | ΔcheC1 amyE5720::cheC19 (cheC-E117A/N20A/E118A/N121A) | This work |
| OI4208           | ΔcheC1 amyE5720::cheC20 (cheC-D149A) | This work |
| OI4209           | ΔcheC1 amyE5720::cheC21 (cheC-D149W) | This work |
| OI4210           | ΔcheC1 amyE5720::cheC22 (cheC-A153L) | This work |
| OI4211           | ΔcheC1 amyE5720::cheC23 (cheC-A153W) | This work |
| OI4233           | ΔcheC1 amyE5720::B. halodurans cheX | This work |
| BL21             | E. coli protease deficient expression host | Amersham |
| TG1              | E. coli protease deficient expression host | Amersham |
| TG1             | pBluescriptSK−::ΔcheC1            | Stratagene |
|                 | E. coli GST tag expression vector, AmpR | Amersham |
| pDR67           | amyE::integration plasmid, pSpac, AmpR, CmR | Ref. 36 |
| pMR108          | pGEX-6P-2::ΔcheC1                 | Ref. 14   |
| pMR130          | pGEX-6P-2::ΔcheC1                 | Ref. 14   |
| pHS102          | pGEX-6P-2::cheY1                  | Ref. 19   |
| pTM0            | pGEX-6P-2::fliY5                  | This work |
| pTM18           | pGEX-6P-2::cheC2                  | Ref. 20   |
| pTM25           | pGEX-6P-2::cheD2                  | Ref. 20   |
| pTM50           | pDR67::B. halodurans cheX         | This work |
Phosphate Release Assay—The assay was performed essentially as described (16, 20, 25). Briefly, the evolution of inorganic phosphate from CheY was measured using the EnzCheck phosphate assay kit (Invitrogen) and a Shimadzu Biospec-1601 spectrophotometer. The data were averaged from two separate experiments. The P_i release rates at various concentrations of phosphate were plotted versus concentration. Rates of μM P_i, min^{-1} μM^{-1} phosphatase were determined for CheC, CheC-CheD, and FliY using the slope of the initial, linear portion of the plot. Concentration of CheD was varied to see the activation effect of CheD on the phosphatase ability of CheC and mutants.

Swarm Plate Assay for Chemotaxis—Semi-solid swarm plates were inoculated by sterile toothpick from single colonies grown overnight at 30 °C. Tryptone swarm medium was 1% tryptone, 0.5% NaCl, 0.28% agar, 1 mM isopropyl β-D-1-thiogalactopyranoside (to induce expression from Pspac promoters). Tryptone plates were grown at 37 °C for 4 h, and swarm diameters were measured. Swarm sizes were averaged over four swarms for each strain and expressed relative to the wild type (WT) strain. The error bars represent S.E.

Capillary Assay for Chemotaxis—The standard capillary assay for chemotactic ability was performed essentially as described (26, 27). The strains were grown in 1 mM isopropyl β-D-1-thiogalactopyranoside to induce the expression of genes controlled by the Pspac promoter. The experiments were performed in triplicate and on two different days.

GST Pulldown Assays—Pulldown assays were performed essentially as described (25) except 20 mM acetyl phosphate was selectively added to incubations and washes to continuously generate GST-CheY-P or CheY-P. Briefly, a bait GST fusion protein (10 nmol GST-CheY, GST-CheD, or GST) was bound to glutathione-Sepharose (GE Healthcare, Piscataway, NJ) and incubated with the secondary protein (12 μM CheC) and co-factors (12 μM CheD or 70 μM CheY). After subsequent washes, the proteins were eluted from the beads, separated by SDS-PAGE, and visualized by Coomassie stain. Presence of the secondary protein indicates a protein-protein interaction.

RESULTS

Complementation Ability of cheC Mutants—Alanine mutations were made for each of the four conserved residues (D/S, E, N, and P) in each of the two CheC active sites (Fig. 1A), as well as selected double mutations. The cheC mutant genes were expressed ectopically in strain OI3135 containing an in frame deletion of cheC. Each mutant strain was inoculated onto low agar “swarm” plates. As the cells grow and metabolize the nutrients, they create a gradient detectable by the chemotaxis system and form dense rings as they swarm out from the inoculation point. The diameters of each swarm were measured and expressed normalized to the wild type swarm diameter. Mutants in the first active site were generally less deleterious than those in the second active site (Fig. 1B), suggesting that the second active site is more important for function. For both active sites, glutamate mutants were the most impaired of the four conserved residues. Double mutants cheC-E17A/N20A and -E118/N121A had swarm sizes close to those of the respective single glutamate mutants (Fig. 1B).

Paired mutations of the homologous conserved residues from each active site were also created. The double glutamate mutation (cheC-E18A/E118A) is the least able to complement in vivo; its swarm size is no greater than the ΔcheC mutant alone (Fig. 1B). However, the other paired mutants have less effect. The cheC-N20A/N121A and cheC-D13A/S114A mutants are 42 and 64% of the wild type swarm, respectively. The cheC-E18A/N20A/E118/N121A strain predictably looks identical to the ΔcheC mutant (Fig. 1B). Based on this assay, only the glutamate residue of the active sites were necessary for in vivo CheC function.

Mutations were generated in cheC residues likely to disrupt the interaction with CheD based on the CheC-CheD crystal structure (23). The Asp^{149} and Ala^{53} residues are conserved in
CheC throughout Gram-positive bacteria and are positioned in the CheC-CheD interface (Fig. 1A). The α2'-helix of CheC serves as the MCP mimic for CheD association. Ala153 is on this α2'-helix, and Asp149 is found between the central β-sheet and α2'-helix. Both residues proved critical for CheD association (Fig. 1B). Previously, a cheC-D149K mutant was shown to have impaired chemotaxis, and the protein was unable to bind to CheD (23). Additionally, mutation of Asp149 to tryptophan and even alanine also led to loss of the ability of the gene to complement the cheC strain on swarm plate (Fig. 1B). The Ala153 residue was shown to be intolerant of substitution to tryptophan or leucine (Fig. 1B). These mutants demonstrate that the ability to bind CheD is critical for the function of CheC in vivo.

The ΔcheC strain was previously shown to have a "sharp" ring morphology compared with the wild type strain on minimal medium asparagine swarm plates, whereas a cheD::cat mutant did not form a ring (15). Selected mutants were tested on asparagine swarm plates to see whether they retained the cheC morphology. Both the cheC-E17A/E118A and -D149K strains displayed the small, sharp swarm rings associated with the ΔcheC strain (Fig. 2). The cheC-N20A/N121A mutant, however, had a swarm ring similar in morphology to that of the wild type in addition to having a larger swarm size than the ΔcheC strain. A strain deleted for cheC but expressing cheX had a similar swarm size to the cheC-N20A/N121A strain, although it retained the cheC morphology (Fig. 2). Although the swarm sizes of the cheC-N20A/N121A and ΔcheC cheX+ were similar, the differences in swarm morphology further demonstrate that the CheX phosphatase is unable to truly complement the ΔcheC strain as was recently reported (28).

Phosphatase Activity of CheC and the CheC-CheD Complex—Previous studies have shown that CheC is a much weaker CheY-P phosphatase than FliY (20). However, when CheD was added, the phosphatase activity of CheC was greatly increased. A kinetic assay was employed to quantitate the rates of dephosphorylation of CheY-P (16) in the presence of the phosphatases CheC, CheC-CheD, and FliY. The concentration of phosphatase was varied and plotted against the resulting Pi release/min (Fig. 3A). A rate of μM P1 min−1 μM−1 phosphatase was obtained from the initial linear portion of the curve. The specific activities for CheC, CheC-CheD, and FliY were 1.7 ± 0.1, 7.8 ± 0.2, and 19 ± 1 μM P1 min−1 μM−1 phosphatase, respectively. The CheC-CheD complex showed an increase of 4.6 times the activity of CheC alone. Although CheC alone seems

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**FIGURE 2. Asparagine swarm plate.** Strains on an asparagine swarm plate demonstrating the morphological swarm differences. Note the ring of the cheC-N20/N121 swarm is diffuse and similar to the wild type, whereas the ΔcheC cheX+ swarm is more defined and similar to the ΔcheC strain.

**FIGURE 3. Phosphate release assays.** A, phosphatase effects on CheY of CheC (white squares), CheC-CheD (black squares), and FliY (white diamonds) were measured through quantization of the inorganic phosphate released from CheY under steady state conditions. B and C, CheD activation of CheC mutant phosphatase ability. B, shown are wild type CheC (black squares), -E17A (white circles), -N20A (white triangles), -E17A/N20A (white diamonds), -E118A (black circles), -N121A (black triangles), and -E118A/N121A (black diamonds). C, shown are wild type CheC (black squares), -D149A (white diamonds), -D149K (black diamonds), -E17A/E118A (black circles), and -N20A/N121A (white triangles). The error bars represent S.E. in each graph.
**CheC Regulation of CheD**

Weak at only 9% of FliY activity, the addition of CheD enhanced CheC activity up to almost half that of FliY. Furthermore, FliY and CheC-CheD have the same maximum activity (Fig. 3A).

**Phosphatase Activity of CheC Mutants**—The mutant CheCs were purified and tested for CheY-P phosphatase ability via the phosphate release assay. Mutants in the first active site (CheC-D13A, -E17A, -N20A, and -P43A) had a slightly reduced phosphatase activity compared with wild type CheC; however, all second site mutant proteins (CheC-S114A, -E118A, -N121A, and -P144A) had almost no phosphatase ability (data not shown). Remarkably, when CheD was added to a concentration of 5 μM (such that most CheC should be bound to CheD) the CheC-E118A and CheC-N121A mutant proteins each were nearly indistinguishable from the wild type CheC-CheD (data not shown).

To see the effect of CheD activation on these mutant proteins, CheC concentrations were kept constant on 2 μM, and the concentration of CheD was varied from 0 to 5 μM. 2 μM CheC was chosen because it was the concentration at which maximum Pᵢ release was attained for the wild type CheC with CheD (Fig. 3A). CheC-E17A and CheC-N20A had moderately lower Pᵢ release at 0 μM CheD compared with wild type CheC (Fig. 3B), but the Pᵢ release/min increased with CheD concentration for both mutants, although less than wild type. Both mutant proteins caused maximum Pᵢ release per minute at 2 μM CheD, but at a lower maximum rate than wild type CheC. CheC-E17A/N20A had activity similar to the single mutant proteins.

Second active site mutants had no phosphatase activity alone. As CheD concentration was increased CheC-E118A and -N121A each gained phosphatase activity (Fig. 3B), but more slowly per μM CheD compared with wild type CheC. Neither reached maximum activity until a CheD concentration of 3 μM, and the CheC-N121A mutant protein exceeded the maximum activity of the wild type CheC (Fig. 3B). CheC-E118A/N121A was activated by CheD very poorly, even in the presence of 5 μM CheD.

Paired double CheC mutant proteins were also tested. CheC-N20A/N121A predictably showed no activity at 0 μM CheD but was unable to be activated by CheD at any concentration tested (Fig. 3C). Thus, the conserved asparagin residues are necessary for the phosphatase ability of CheC. CheC-E17A/E118A phosphatase activity was severely impaired as well but showed slight enhancement at high CheD concentration (Fig. 3C). Its maximum activity was still well below wild type CheC alone. The conserved glutamate residues must be important for CheC function but not absolutely critical for the dephosphorylation of CheY-P. CheC-D13A/S114A reacted similarly to the single CheC-S114A mutant; phosphatase activity was impaired but quickly restored by CheD activation (data not shown).

Because the cheC-D149K and -D149A mutant strains swarmed the least and the most, respectively, of the CheD-binding mutants, the corresponding proteins were purified and tested for CheD activation of phosphatase ability. Alone, both proteins showed wild type or higher activity, demonstrating that the mutation had little or no direct effect on the active sites (Fig. 3C). When CheD was added, no increase in phosphatase activity was seen until high concentrations (2–3 μM). Thus, CheD was unable to effectively activate either mutant.

**Binding of CheC to CheY-P**—A modified pulldown assay was used to examine binding of CheC and mutants to CheY-P. First, conditions were tested for CheC ± acetyl phosphate (to generate GST-CheY-P) and/or CheD. In the pulldown experiment (Fig. 4A), CheC was only retained in the presence of both acetyl phosphate and CheD and thus only bound GST-CheY-P with CheD. Also tested under these conditions were CheC-E17A/E118A and -N20A/N121A. The later bound GST-CheY with acetyl phosphate and CheD as wild type CheC had, but E17A/E118A would not pulldown with GST-CheY under the conditions tested (Fig. 4A). This shows that CheC only exhibits significant binding (to an extent detectable by pulldowns) toward CheY-P (versus CheY) and only when bound to CheD.

Select mutant CheC proteins were tested by the same method to observe the mutational effect on binding to CheY (Fig. 4B). In this assay, acetyl phosphate was added to each sample because it was shown to be necessary for binding, but CheD was varied as control. Again, the wild type CheC and -N20A/N121A mutant proteins both bound GST-CheY in the presence of CheD, whereas CheC-E17A/E118A did not. Less CheC-D13A/S114A bound than the wild type CheC, but considerably more than CheC-E17A/E118A (Fig. 4B). Previous data suggested that the second active site of CheC was more functional than the first site. This was confirmed as CheC-E118A/N121A had little ability to bind CheY-P, whereas the CheC-E17A/N20A mutant retained CheY-P binding (Fig. 4B). The CheC-E17A/N20A/E118A/N121A mutant also did not bind, although it appears that a small amount of the CheC-D149K mutant was retained in the presence of CheD (Fig. 4B). The concentration of CheD was 10 μM, quite high compared with the Kᵦ of 1 μM (21) and should have bound all available CheC.

**CheY-P-mediated Binding of CheC to CheD**—CheC can inhibit CheD deamidation of MCP receptors in vitro, and CheY-P increases this inhibition (23). These data suggested that CheY-P binding to CheC increases the affinity of CheC for CheD. A pulldown experiment was previously used to test CheC binding to GST-CheD (23, 25) and was employed to directly see the effect of CheY-P. As expected, CheC was retained by GST-CheD in the absence of CheY-P as compared with a GST-only control. The addition of CheY-P noticeably increased the amount of CheC bound to GST-CheD (Fig. 4C); image analysis (ImageQuant, Molecular Dynamics) showed an increase of 50–70% CheC binding in the lane with CheY-P. Therefore, CheY-P increases the binding affinity of CheC for CheD.

**Capillary Assay Confirms Swarming Assay Results**—To confirm the swarm plate data and assure that the effects seen were not the result of pseudotaxis, selected strains were tested via the standard capillary assay for chemotaxis. Strains OI4172 (cheC-D149K), OI4204 (cheC-E17A/E118A), and OI4205 (cheC-N20A/N121A) were compared with the ΔcheC strain (O13135) and the cheC⁺ strain (O13165) at three concentrations of the attractant proline. The cheC-D149K and -E17A/E118A mutants both had cell accumulations equal to the ΔcheC, demonstrating that the ability to bind both CheD and CheY-P are
necessary for CheC function in vivo (Fig. 5). However, the cheC-N20A/N121A mutant, with no phosphatase ability, had intermediate accumulations between the ΔcheC and cheC + strains (Fig. 5). Hence, CheY-P dephosphorylation is only part of CheC function.

DISCUSSION

In this study, the individual active sites and conserved active site residues of CheC were analyzed for their contribution to protein function. Swarm plate data suggested that the first active site on CheC is much weaker or less critical to the function of CheC (Fig. 1B). The results of the P_1 release assay confirmed this, because the CheC-E17A/N20A is comparable with wild type, whereas CheC-E118A/N121A has severely impaired phosphatase ability even in the presence of CheD (Fig. 3B). CheC-E118A/N121A also showed greatly reduced binding to GST-CheY-P in a pull-down assay, whereas CheC-E118A/N20A is retained nearly as well as the wild type CheC (Fig. 4B). Therefore, the second active site binds CheY-P better and is a more potent phosphatase active site, as well as showing more activity in vivo.

The double mutants of similar CheC active site residues proved most telling for uncovering the function of each residue. The CheC-N20A/N121A mutant was completely unable to dephosphorylate CheY-P even with excess CheD (Fig. 3B) but was able to bind CheY-P in the presence of CheD (Fig. 4B). Conversely, the CheC-E17A/E118A was able to (poorly) dephosphorylate CheY-P with CheD (Fig. 3B) but was unable to bind GST-CheY-P even with CheD present (Fig. 4B). Therefore, the asparagine residue is absolutely necessary for the phosphatase activity of CheC, and this defect is not due to a disruption of the association of CheC and CheY-P. However, the active site glutamate is not necessary for phosphatase activity but is critical for proper binding of CheC to CheY-P.

Alanine mutants of the conserved aspartate/serine or proline residues showed little loss of function when expressed in vivo (Fig. 1B). The CheC-D13A/S114A mutant did have a reduced ability to bind CheY-P (Fig. 4B), but it was still greater than CheC-E17A/E118A. The aspartate/serine residue likely helps to stabilize the interaction with CheY-P but is not absolutely necessary for function. The proline residue is likely neither catalytic nor important for CheY association. In the crystal structure of CheC, the second active site proline residue points toward the other active site residues but points away in the first active site. The proline is likely a conserved structural element. It is found in the middle of the β1 and β1' and forms a “kink” in the β-strand that flips the β-strand orientation.

Comparison of CheC and CheZ Active Sites—Because CheC and CheZ both dephosphorylate CheY-P, this is likely accomplished through an evolutionarily convergent common mechanism. This phosphate transfer mechanism has other analogs in two component systems. CheZ has been shown in the crystal structure (29) to associate with CheY-P in a manner similar to that of the sporulation protein Spo0B (30) with response regulator Spo0F-P. Spo0B is an Hpt domain protein that
CheC Regulation of CheD
dephosphorylates Spo0F-P by transferring the phosphate to its own histidine residue for later transfer to Spo0A. CheZ Gln147 has an analogous function to the Spo0B histidine, but instead Gln118 is thought to position a water to which the phosphate is transferred.

Besides Gln147, the CheZ Asp143 residue has been shown to be critical for function (16). This residue is one turn up the helix from Gln147 and possibly interacts with many conserved CheY residues, notably forming a salt bridge with Lys109, which is conserved in all response regulators. In CheC, the important active site residues are conserved glutamates and asparagines (for simplicity, we will discuss the Glu118 and Asn121 residues of the more active second site). It is likely that because CheC Asn121 has proven to be absolutely necessary for phosphatase activity, it is analogous to CheZ Gln147. Both residues have been shown to be necessary for phosphatase activity yet have little effect on the binding affinity for CheY-P (29). Likewise, CheC Gln118 and CheZ Asp143 are analogous given that both are critical for CheY-P binding.

CheC/CheY-P Interaction Model—The analogous relationship between the CheZ Asp143/Gln147 and CheC Glu118/Asn121 pairs is not absolute. The position of the nucleophile is critical for the hydrolysis of the CheY aspartyl phosphate. In CheC, the amide-containing amino acid side chain is one carbon shorter than in CheZ, whereas the carboxylate side chain is one carbon longer. “Tilting” CheY toward the C terminus of the CheC a3-helix relative to its position on CheZ would allow for proper positioning of the longer glutamate residue and shorter asparagine in CheC and CheX.

Additionally, the CheZ interaction with CheY-P is aided by a necessary C-terminal CheY-P-binding domain analogous to those found in FliY and FliM. CheC and CheX do not contain similar binding domains, so there may be a different association between the active sites of these proteins and CheY-P. CheC and CheX have large planar surfaces at their active sites (21), whereas the CheY contact with CheZ (besides the binding domain) is fairly small and limited to the active site a3-helix (29). This planar surface of CheC has the potential for more protein-protein contacts with CheY than seen with CheZ. The proposed “tilting” of CheY could allow for these additional contacts to be made.

The positions of CheY on CheC and CheX are likely similar because they have homologous active sites (21). The CheY-P binding area on CheX is limited by the other monomer in the CheX dimer (CheX’), which is situated very close to the CheX active site. The CheX’ likely makes contact with CheY-P, further increasing the protein-protein interaction between the CheX dimer and CheY-P. However, comparing the active sites of the CheX dimer and CheC-CheD, it is clear that no direct contact between CheY and CheD is possible as was previously proposed (23) due to the CheX’ position in the CheX dimer. Also, CheD binding to CheC does not appear to cause a large conformational change in the second active site in that formulation (23). However, the CheD interaction with CheC does cause a great increase in the binding affinity for CheY-P. It is likely that CheD stabilizes the conformation of CheC for optimal CheY-P association.

CheC Functional Model—Previous studies have shown that CheC was the weakest phosphatase of the FliY, CheC, and CheX family of CheY-P phosphatases (28). Here we have shown through an enzyme-coupled assay that the activity of CheC with CheD is enhanced nearly 5-fold from that of CheC alone (Fig. 3A), confirming previous estimates (20). However, this is still less than half of the activity of FliY, the main chemotaxis phosphatase in *B. subtilis*, and one-third that of CheX (28). Further, the defect of a cheC mutant is that it is unable to adapt to the presence of a chemoattractant after the initial excitation (14, 15). This is different from cheZ or fliY mutants, the main chemotactic phosphatases, which both show a dramatic overall increase in CheY-P levels (16, 19). To account for differences in phenotype and activity between CheC and FliY, it was postulated that CheC is activated dynamically by CheD (20). In this scenario, the MCPs would change conformation upon binding a chemoattractant, and CheD would be released from them. CheD would then be free to activate CheC as a phosphatase leading to a reduction of CheY-P and adaptation.

The data presented in this study bring into question the role of CheC as a phosphatase in the chemotaxis system. First, a CheC-N20A/N121A mutant was found to have no phosphatase ability, even in the presence of CheD (Fig. 3). When this mutant protein was expressed in the ΔcheC strain, partial complementation was observed in both the swarm plate and capillary assays. This complementation is inconsistent with the presumed role of CheC as a phosphatase; no phosphatase ability in vitro should lead to a loss of function in vivo. Second, when CheX, a stronger CheY-P phosphatase, was expressed in the ΔcheC mutant, only partial complementation was observed (28) and the strain retained a “ΔcheC” swarm morphology (Fig. 2). Further, when CheX was overexpressed to achieve 80% complementation of the ΔcheC mutant, the cells were found to have a tumbly (clockwise) flagellar rotational bias compared with wild type (28). This effect on the rotational bias suggested that CheY-P levels had an overall reduction, and pseudo-complementation was being observed with CheX expression. These results suggest that CheC is more than a phosphatase and has another, more important function (namely, in adaptation).

We have identified mutants defective in each of the known functions of CheC: CheD binding (CheC-D149K), CheY-P binding (CheC-E17A/E118A), and phosphatase ability (CheC-N20A/N121A). Although the cheC-N20A/N121A mutant strain showed partial complementation, both the cheC-E17A/E118A and -D149K mutant strains had swarm plate and capillary assay phenotypes similar to the ΔcheC mutant (Figs. 1B and 5). These mutants demonstrate that the ability to bind both CheY-P and CheD is critical to the function of CheC in vivo, exclusive of their effect on phosphatase ability. The additional function of CheC then likely occurs through both CheY-P and CheD binding.

Although CheD is known to interact with and modify the MCP receptors (31, 32), a specific function in adaptation was unknown. CheD has been shown to be necessary for normal function of the receptors in that a cheD strain is very tumbly and responds to attractants poorly (14, 15). Inhibition of CheD receptor deamidation has been demonstrated by CheC for both
CheC Regulation of CheD

![Diagram of CheC-CheD/CheY-P functional model.](Image)

**Pre-stimulus**
- MCP
- A
- P

**Excited**
- MCP
- A
- P

**Adapted**
- MCP
- A
- C
- D

**FIGURE 6.** The chemotaxis system is in a balanced state. CheY-P is kept at a concentration resulting in a pre-stimulus bias of a nearly even split between clockwise and counter-clockwise flagellar rotation. This balance is achieved by the CheC-CheD/CheY-P complex, which completes the feedback loop to maintain CheA activity at a basal level and FliY dephosphorylating CheY-P. Attractant binding to an MCP causes it to become excited and activate CheA, which then phosphorylates CheY. The increased ratio of CheY-P to CheY causes a more counter-clockwise rotational bias. CheC then binds CheY-P, which increases CheC affinity for CheD. This removes CheD from the receptors, deactivating them and causing the system to adapt. Less CheY-P is generated, and CheC-CheD and FliY return CheY-P to the prestimulus levels. For illustration purposes the CheD is shown away from the receptors and bound to CheC. Likely, only some of the CheD leaves the receptors.

**T. maritima** (23) and **B. subtilis** MCP receptors. Additionally, CheY-P was shown to increase CheD inhibition by CheC (23). Here, it was confirmed that the interaction between CheC and CheD is enhanced by CheY-P (Fig. 4C). We propose that this CheY-P enhanced CheD inhibition is the critical ability of CheC (Fig. 6). In this scenario, the increase in CheY-P levels of the receptors binding attractant would induce CheC to become a better binding site for CheD. This would pull some of the CheD from the active receptors, rendering the receptor less active and inducing or aiding receptor adaptation. Thus, CheC is not only a CheD-activated CheY-P phosphatase but also a CheY-P-enhanced CheD regulator.

The chemoreceptors are rapidly demethylated in response to both the addition and removal of an attractant (33). However, the subsequent remethylation event occurs much more slowly over a matter of minutes. Based on the model presented here, the removal of CheD from the receptors by CheC in response to elevated CheY-P levels would cause a short term de-excitation of the receptors. This is followed by the remethylation event, which would keep the receptors in the unexcited state over a longer time period. The lower CheY-P levels would reduce the affinity of CheC for CheD and cause CheD to return to the receptors.

There are two possible reasons for the retention of phosphatase ability as CheC evolved from the T. maritima and B. subtilis MCP receptors. The active CheC-CheD phosphatase complex may aid FliY in reducing CheY-P to prestimulus levels. For illustration purposes the CheD is shown away from the receptors and bound to CheC. Likely, only some of the CheD leaves the receptors.

CheC-CheD/CheY-P complex represents in chemotaxis a third adaptational feedback loop in addition to receptor methylation (CheB) and receptor decoupling (CheV). It should be noted that all three of these feedback loops are generated by response regulators. This new adaptation system may represent the mechanism for the previously proposed CheY-P feedback to the receptors in **B. subtilis** chemotaxis (33). In that case, a cheY mutant was unable to remethylate the MCP receptors after the addition of attractant as wild type cells do. A connection to the methylation system is further supported in that a cheC mutant was shown to have hyper-methylated MCPs, whereas a cheD mutant had hypo-methylated MCPs (14). With the effect on methylation, it is tempting to suggest that this CheC-mediated system could be involved in controlling the selective methylation phenomena observed in **B. subtilis** chemotaxis (34). Future experiments will be necessary to determine the exact nature of CheD involvement in adaptation and how regulation by CheC affects it.

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