

A bifunctional kinase-phosphatase in bacterial chemotaxis

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Phosphorylation based signaling pathways employ dephosphorylation mechanisms for signal termination. Histidine to aspartate phosphosignaling in the two-component system controlling bacterial chemotaxis has been studied extensively. *Rhodobacter sphaeroides* has a complex chemosensory pathway with multiple homologues of the *Escherichia coli* chemosensory proteins, although it lacks homologues of known signal terminating CheY-P phosphatases such as CheZ, CheC, FliY or CheX. Here we demonstrate that an unusual CheA homologue, CheA₃, is not only a phosphodonor for the principal CheY protein, CheY₆, but is also a specific phosphatase for CheY₆-P. This phosphatase activity accelerates CheY₆-P dephosphorylation to a rate that is comparable with the measured stimulus response time of ~1 s. CheA₃ possesses only two of the five domains found in classical CheAs, the Hpt (P1) and regulatory (P5) domains, which are joined by a novel 794 amino acid sequence that is required for phosphatase activity. The P1 domain of CheA₃ is phosphorylated by CheA₄ and it subsequently acts as a phosphodonor for the response regulators. A CheA₃ mutant protein deleted for the 794 amino acid region lacked phosphatase activity, retained phosphotransfer function but did not support chemotaxis, suggesting that the phosphatase activity may be required for chemotaxis. Using a nested deletion approach we show that a 200 amino acid segment of CheA₃ is required for phosphatase activity. The phosphatase activity of previously identified non-hybrid histidine protein kinases depends upon the dimerization and histidine phosphorylation (DHp) domains. CheA₃, however, lacks a DHp domain, suggesting that CheA₃ is a novel phosphatase.

Keywords: chemotaxis/phosphatase/response regulator/two-component/signal termination

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Introduction

Dephosphorylation is required for signal termination in phosphorylation based signaling pathways. The most common phosphorylation based signaling pathways in bacteria are two-component signal transduction systems, which can detect and mediate responses to a wide range of different environmental stimuli, with some bacteria having over 100 distinct systems (1, 2). These systems comprise sensor histidine protein kinases (HPKs) and response regulators (RRs). HPKs detect sensory stimuli and these regulate the rate at which the HPK autophosphorylates on a conserved histidine residue. Subsequently the phosphoryl group is transferred from the histidine residue of the HPK onto an aspartate residue in the receiver domain of the RR. Phosphorylation of the RR causes a conformational change, allowing it to mediate an output appropriate to the original stimulus, often a change in transcription (3). The phosphosignal is terminated by hydrolysis of the aspartyl-phosphate residue of the RR.

Receiver domains have intrinsic autodephosphorylation activity, although in many systems a dedicated specific aspartyl-phosphate phosphatase is employed to accelerate this process. Such phosphatases can be found in separate protein molecules e.g. RapA and CheZ dephosphorylate Spo0F-P and CheY-P, respectively (4, 5); alternatively phosphatases can be integral parts of the HPKs. Hybrid HPKs, which are components of multistep phosphorelays, contain one or more receiver domains and all show phosphatase activity due to the autodephosphorylation activity of their receiver domains (6). Many non-hybrid HPKs also show phosphatase activity e.g. NtrB and EnvZ dephosphorylate their RRs, NtrC-P and OmpR-P, respectively (7, 8). The dimerization and histidine phosphorylation (DHp) domain of these HPKs

has been implicated in the phosphatase reaction (8, 9). In this study we report the discovery of an aspartyl-phosphatase activity within the chemotaxis protein, CheA₃, of *Rhodobacter sphaeroides*. Interestingly, unlike all previously identified non-hybrid HPKs with phosphatase activity, CheA₃ lacks a DHp domain and is not homologous to known phosphatases suggesting that the CheA₃ phosphatase activity is novel.

The two-component system controlling chemotaxis allows bacteria to move towards environments that are better for growth (reviewed in (10-12)). Chemoreceptors modulate the autophosphorylation rate of CheA in response to chemotactic stimuli. In *E. coli*, unliganded receptors activate CheA, whereas attractant-occupied receptors inhibit CheA autophosphorylation. The phosphoryl group is transferred from CheA-P to specific aspartate residues on its cognate RRs, CheY and CheB. CheY-P binding to the FliM component of the flagellar motor brings about a change in flagellar rotation and therefore swimming direction, whilst CheB-P demethylates the chemoreceptors, mediating adaptation. Hydrolysis of the phosphoryl-aspartate residues in CheY-P and CheB-P allows signal termination. *E. coli* CheY-P autodephosphorylates with a half-time of ~14 s (13), however, the phosphatase CheZ can increase this rate by a factor of ~100 (14). Many bacteria lack CheZ homologues and some of these instead use CheY-P phosphatases belonging to the CheC/FliY/CheX family of proteins (15-17). Other bacteria, including *Sinorhizobium meliloti* and *R. sphaeroides* do not have homologues of any of these CheY-P phosphatases but do have multiple CheYs. *S. meliloti* has two CheYs, one that can bind the motor, CheY2, and one that can not bind the motor, CheY1. CheY1 mediates dephosphorylation of CheY2-P by functioning as a phosphate sink (18). Like *S. meliloti* CheY2 but unlike *S. meliloti* CheY1, all of the *R. sphaeroides* CheYs are capable of

binding to FliM, suggesting that a phosphate sink may not be used for signal termination in the *R. sphaeroides* chemotaxis pathway (19).

R. sphaeroides has two sets of flagellar genes and three chemotaxis operons (20). The fla1 set encodes a single unidirectional flagellum that is controlled by the chemotaxis proteins encoded by *cheOp*₂ and *cheOp*₃ (21), while the fla2 set encodes polar flagella that are controlled by *cheOp*₁ (22, 23). *R. sphaeroides* has four CheA homologues. Phosphosignaling from CheA₂, CheA₃ and CheA₄ is essential for fla1 driven chemotaxis (24). CheA₂ localizes with the transmembrane chemoreceptors to the cell poles, while CheA₃ and CheA₄ localize to a cytoplasmic chemotaxis cluster along with the cytoplasmic chemoreceptors (25). CheA₁, CheA₂ and *E. coli* CheA function as homodimers. Each protomer contains five domains (P1-P5) with the P3 domain mediating dimerization. In *E. coli*, the P5 domain has been shown to bind CheW and the chemoreceptors, and to couple the rate of CheA autophosphorylation to receptor control. During the autophosphorylation reaction, the kinase domain (P4) phosphorylates the histidine residue within the Hpt (P1) domain using ATP as the phosphodonor. The phosphorylated P1 domain donates phosphoryl groups to aspartate residues on CheY and CheB. The P2 domain binds CheY and CheB and therefore increases their local concentration, which accelerates the phosphotransfer reaction mediated by the P1 domain. CheA₃ and CheA₄ from *R. sphaeroides* are atypical CheAs in that they each lack some of the domains found in *E. coli* CheA. CheA₄ is a homodimeric protein containing only the P3, P4 and P5 domains while CheA₃ has only the P1 and P5 domains, separated by a novel 794 amino acid sequence containing no identifiable domains. CheA₃ and CheA₄ both localize to the cytoplasmic chemotaxis cluster (25). Neither CheA₃ nor CheA₄ are capable of autophosphorylation; however, CheA₄ can phosphorylate the P1

domain of CheA₃ on residue H51. Subsequently CheA₃-P acts as a phosphodonor for a specific subset of the chemotaxis RRs (24).

R. sphaeroides has eight chemotaxis RRs; six CheYs and two CheBs. While both CheBs are required for normal chemotaxis only CheY₆ plus either of CheY₃ and CheY₄ are needed for control of the fla1 flagellum (21, 26). The CheAs all show different patterns of phosphotransfer to the RRs: CheA₁-P phosphorylates CheY₁, CheY₂, CheY₃ and CheY₅; CheA₂-P phosphorylates all eight chemotaxis RRs; CheA₃-P phosphorylates CheY₁, CheY₆ and CheB₂ (24, 27). Here we demonstrate an additional activity for CheA₃, the ability to specifically catalyze the hydrolysis of the aspartyl-phosphate residue in CheY₆-P.

Results

The P1 domain of CheA₃ is a specific phosphodonor for CheY₁, CheY₆ and CheB₂. CheA₃-P is a phosphodonor for only CheY₁, CheY₆ and CheB₂ (24). To determine whether this specificity depends solely on the interactions between the RRs and the P1 domain of CheA₃, phosphotransfer experiments were performed comparing the specificity of the isolated P1 domain of CheA₃ (CheA₃P1) with that of full length CheA₃. Transfer of phosphoryl groups to the eight chemotaxis RRs was measured under multiple turnover conditions in the presence of CheA₄ and ATP, allowing CheA₃ or CheA₃P1 phosphorylation to continue throughout the course of the reactions. The progress of these phosphotransfer reactions after 30 s is shown in Fig. 1. Phosphotransfer occurred in reactions where a decrease in CheA₃-P or CheA₃P1-P levels was accompanied by an increase in CheY/B-P levels. CheA₃-P and CheA₃P1-P phosphorylated the same RRs (CheY₁, CheY₆ and CheB₂) indicating that it is the interaction between the RRs and the P1 domain of CheA₃-P that determines the phosphotransfer specificity of CheA₃-P. Interestingly, despite using equimolar concentrations of phosphodonor, CheY₆-P levels were greater when CheA₃P1-P was used as the phosphodonor rather than CheA₃-P. One possible explanation for this is that full length CheA₃ is both a phosphodonor and a phosphatase for CheY₆-P. The isolated CheA₃P1 domain would lack this phosphatase activity retaining only phosphodonor function allowing more CheY₆-P to accumulate.

CheA₃ is a specific phosphatase for CheY₆-P. The ability of CheA₃ to act as a chemotaxis RR phosphatase was measured *in vitro* using a RR dephosphorylation assay that quantified the loss of ³²P-labelled phosphoryl groups from RR-P as a function of time. Parallel timecourse experiments were performed where a phosphodonor (either CheA₃P1-³²P or CheA₂-³²P) was

mixed with a vast excess of RR (400 μM) in the presence and absence of the putative phosphatase, CheA₃ (Fig. 2 and Table 1). Following phosphotransfer, no rephosphorylation of the phosphodonor could occur since ATP was not present. Under these conditions, phosphotransfer from phosphodonor to the RR occurred rapidly and was complete before the first timepoint. Therefore, the only reaction occurring after the first timepoint was the dephosphorylation of the phosphorylated RR that had been generated by the phosphotransfer reaction. By monitoring the subsequent decrease in RR-P levels over time it was possible to measure the dephosphorylation rate constant by fitting the timecourse data to a first order exponential decay model (Table 1). No significant change in CheY₁₋₅-P or CheB_{1&2}-P dephosphorylation rate constants was observed in the presence of CheA₃ (Fig. 2A and Table 1), indicating that CheA₃ is not a phosphatase for these RRs. In contrast, CheY₆-P levels fell faster in the presence of CheA₃ than in the absence of CheA₃ indicating that CheA₃ is a phosphatase for CheY₆-P (Fig. 2B and Table 1). Under these reaction conditions, where the concentration of CheA₃ used was 2.5 μM and the CheY₆ concentration was 400 μM giving a molar ratio of CheY₆ to CheA₃ of 160:1, CheA₃ increased the dephosphorylation rate of CheY₆-P by a factor of 3. No CheA₃-P was detected at any point in the assay (Fig. 2B), suggesting that the phosphatase mechanism does not involve reversed phosphotransfer to CheA₃. The effect of varying [CheA₃] on the rate of CheY₆-P dephosphorylation is shown in supporting information (SI) *Text*, and Fig. S1. In summary, CheA₃ is an aspartyl-phosphate phosphatase that is specific for CheY₆-P.

Requirements for CheA₃ phosphatase activity. To determine whether the phosphorylation site (H51) of CheA₃ has any involvement in phosphatase activity, as has been seen for other HPKs with intrinsic phosphatase activity, the phosphatase activity of CheA₃(H51Q) was measured. The

phosphatase activities of CheA₃ and CheA₃(H51Q) were indistinguishable, indicating that the phosphorylation site (H51) of CheA₃ has no role in phosphatase activity (Fig. 3).

The CheA₃(Δ 155-948) mutant protein retains only the P1 and P5 domains and lacks the intervening 794 amino acid region. Like the isolated CheA₃P1 domain, the CheA₃(Δ 155-948) mutant protein was phosphorylatable by CheA₄ and functioned as a phosphodonor for the cognate RRs of CheA₃ (data not shown). This mutant protein did not, however, show any CheY₆-P phosphatase activity (Fig. 3), indicating that either the deleted 794 amino acid region is required for phosphatase activity or that this large deletion causes misfolding of the remainder of the protein such that phosphatase activity but not phosphodonor ability was abolished. The 794 amino acid region of CheA₃ was then arbitrarily sub-divided into four subregions (Fig. 3). Mutant CheA₃ proteins with various nested deletions of these regions were purified and assayed for CheY₆-P phosphatase activity. The P5 domain and subregion 4 were not required for phosphatase activity. Subregion 3 (residues 549-749) was essential for phosphatase activity, while deletion of subregion 2 or subregions 1 and 2 together caused a partial reduction in phosphatase activity (Fig. 3). The sequence in subregions 1 and 2 may therefore either be required for correct folding of the phosphatase activity or may have a regulatory effect on the phosphatase activity. Subregion 3 (residues 549-749) was the only segment of CheA₃ that was been shown to be essential for phosphatase activity and is therefore presumed to contain the phosphatase activity. Within subregion 3, there is a partial match to the consensus sequence of CheC-type phosphatases (*SI Text*). However, changing the predicted catalytic residues (E585 and N588) did not alter phosphatase activity (Fig. 3), indicating that CheA₃ is not a homologue of CheC.

CheA₃(Δ155-948) localizes correctly but does not support chemotaxis. CheA₃ has previously been shown to localize to the cytoplasmic chemotaxis cluster along with CheW₄, CheA₄ and the putative cytoplasmic chemoreceptors (25). Localization of CheA₃ to this cluster may be required for it to function in the chemotaxis signal transduction system, therefore, the localization of CheA₃(Δ155-948) was examined by replacing the wild-type *cheA₃* gene in the *R. sphaeroides* genome with *yfp-cheA₃(Δ155-948)* generating strain JPA1741. YFP-CheA₃(Δ155-948) localized to the cytoplasmic chemotaxis cluster in a pattern indistinguishable from that of YFP-CheA₃ (Fig. 4A-C), indicating that the localization determinants for CheA₃ were still present and correctly folded in CheA₃(Δ155-948). Western blotting using an antibody that recognizes YFP showed that expression levels of YFP-CheA₃ and YFP-CheA₃(Δ155-948) were similar (data not shown).

CheA₃(Δ155-948) retains all of the known activities of full-length CheA₃ except for the phosphatase activity i.e. it can be phosphorylated by CheA₄, it is a specific phosphodonor for the cognate RRs of full-length CheA₃, it is expressed at wild-type levels in *R. sphaeroides* and it localizes to the cytoplasmic chemotaxis cluster. To assess the importance of the CheY₆-P phosphatase activity *in vivo*, the *cheA₃* gene in the *R. sphaeroides* genome was replaced with *cheA₃(Δ155-948)*. As expected, and unlike the Δ*cheA₃* strain, the *cheA₃(Δ155-948)* strain was capable of responding to step changes in chemoeffector concentration (1 mM to 0 mM sodium propionate) in tethered cell assays (data not shown), suggesting that CheA₃(Δ155-948) is capable of transducing signals *in vivo*. However, the *cheA₃(Δ155-948)* strain was non-chemotactic in swarm plate assays (Fig. 4D), suggesting that although cells lacking the phosphatase activity of CheA₃ are capable of responding to changes in chemoeffector levels they do so in a time-frame

that is too slow to allow migration up a chemoeffector gradient. The lack of chemotaxis exhibited by the *cheA₃(Δ155-948)* strain suggests that the phosphatase activity of CheA₃ may be required for chemotaxis.

Discussion

In this study we have shown that CheA₃ is a novel aspartyl-phosphate phosphatase that is specific for CheY₆-P. CheA₃ localizes with its partner protein CheA₄ to the cytoplasmic chemotaxis cluster (25), where CheA₄ phosphorylates CheA₃ (24). CheA₃-P is a phosphodonor for CheY₁, CheY₆ and CheB₂ (Fig. 5). Chemotactic stimuli, possibly reflecting the metabolic state of the cell, are presumed to control the rate at which CheA₄ phosphorylates CheA₃ (12). Both CheA₃ and CheA₄ contain a P5 (regulatory) domain, and the detection of phosphatase activity within CheA₃ raises the intriguing possibility that the phosphatase activity of CheA₃ and the kinase activity of CheA₄ could be reciprocally regulated by chemotactic stimuli. These results may also explain why the activities of CheA₃ and CheA₄ are encoded within separate proteins rather than within a single polypeptide chain. The chemotactic response of *R. sphaeroides* has been shown to depend upon growth condition (12). Variation of the expression level of CheA₃ relative to that of CheA₄ would alter the phosphatase to kinase ratio and could allow tuning of the CheY₆-P output of the signaling pathway according to growth conditions. Consistent with this hypothesis, an internal promoter has recently been discovered within *cheOp*₃ that could allow CheA₃ and CheA₄ expression levels to be independently regulated (M. Gould, M.A.J.R. & J.P.A., unpublished).

The phosphatase activity of CheA₃ is novel. Outside of chemotaxis, many non-hybrid histidine protein kinases have built in aspartyl-phosphatase activity (28, 29); some of the most extensively studied examples are the HPKs, NtrB, EnvZ and PhoR. The phosphatase activity of these proteins has been shown to depend upon their dimerization and histidine phosphorylation (DHp) domains (8, 9, 29). Interestingly, the DHp domain is not present in CheA₃ suggesting that the

phosphatase activity of CheA₃ is novel and differs substantially from those found in previously characterized HPKs.

The ability of the CheA₃ phosphorylation site mutant protein, CheA₃(H51Q) to act as a phosphatase indicates that the CheY₆-P dephosphorylation mechanism employed by CheA₃ does not require reversed phosphotransfer from CheY₆-P to CheA₃. Deletion of the 794 amino acid region between the P1 and P5 domains of CheA₃ abolished the CheY₆-P phosphatase activity (Fig. 3) but not the ability of the protein to be phosphorylated by CheA₄ and subsequently act as a phosphodonor for its RRs. Nested deletion analysis showed that residues 549-749 (subregion 3) are essential for phosphatase activity, and since no other region of CheA₃ was shown to be essential for phosphatase activity, the phosphatase activity presumably resides within this sequence. The entire 794 amino acid region contains no known conserved domains although homologues have been identified in *Roseovarius* sp. TM1035 (RefSeq accession: ZP_01878577) and in a marine metagenomic sample obtained from a hypersaline lagoon in the Galapagos Islands (CAMERA accession: JCVI_PEP_1105096654245) (30), suggesting that a range of different bacteria may employ this novel phosphatase activity in their signal transduction pathways.

The relevance of the CheA₃ phosphatase to chemotactic signaling. *E. coli* CheY-P autodephosphorylates with a half-time of ~14 s (13) and its phosphatase, CheZ, can accelerate this by a factor of 100 (14) to ~0.14 s. This dramatic stimulation of CheY-P dephosphorylation is required because the stimulus response time of *E. coli* is ~0.2 s (31) and signal termination needs to occur on a comparable timescale (32). When comparing the phosphatase activity of CheA₃ towards CheY₆-P with *E. coli* CheZ towards CheY-P, the phosphatase activity of CheA₃ appears

modest giving a 3-fold versus a 100-fold stimulation, respectively. However, *R. sphaeroides* may not need such a potent phosphatase since CheY₆-P already has one of the fastest known dephosphorylation rates with a half time of ~4.1 s versus the ~14 s half-time of *E. coli* CheY. In addition, the measured stimulus response time is slower for *R. sphaeroides* (~1 s) than *E. coli* (~0.2 s) (31, 33), possibly reflecting the requirement for transport and partial metabolism in *R. sphaeroides* chemotaxis (12). The enhancement of CheY₆-P dephosphorylation by CheA₃ reduces the half-time to ~1.4 s which is comparable with the stimulus response time of ~1 s (33). The modest phosphatase activity of CheA₃ may therefore be critical for chemotaxis since this would bring the rate of CheY₆-P dephosphorylation within the physiological range required for efficient gradient sensing and chemotactic signaling. Consistent with this, *R. sphaeroides* strains deleted for the 794 amino acid region of CheA₃ and therefore lacking phosphatase activity, were non-chemotactic even though the remainder of the CheA₃ protein, CheA₃(Δ155-948), was expressed at wild-type levels and localized correctly to the cytoplasmic chemotaxis cluster (Fig. 4). These observations suggest that the phosphatase activity of CheA₃ is required to achieve a rapid rate of signal termination that is compatible with the chemotactic migration of cells in chemoeffector gradients.

Chemotaxis phosphatase localization. To the best of our knowledge this is the first time that a non-hybrid CheA protein has experimentally been shown to possess phosphatase activity.

However, this colocalization of phosphotransfer and phosphatase functions is not restricted to CheA₃ and its close homologues. *Methanospirillum hungatei* JF-1 has a CheA-CheC fusion protein (RefSeq accession: YP_501607) that sequence analysis suggests would show CheY-P phosphatase activity (34). The CheY-P phosphatases, CheC from *B. subtilis* and CheX from *Treponema denticola*, both interact with CheA in two-hybrid assays, suggesting that the principle

of colocalizing phosphotransfer and phosphatase activities is also utilized by these bacteria (35, 36). In *E. coli*, the chemotaxis phosphatase, CheZ, localizes to the polar chemoreceptor cluster via its interaction with CheA_{short}. This co-localization of the *E. coli* chemotaxis kinase and phosphatase at the cell poles prevents the formation of steep spatial gradients of CheY-P concentration that would otherwise form if the phosphatase was not localized at the cell poles and was instead distributed throughout the cytoplasm (37). This is important because steep gradients of CheY-P would expose each flagellar motor to different concentrations of CheY-P depending on their proximity to the chemoreceptor cluster and therefore motor switching would become a function of the distance between the motor and the chemoreceptor cluster (38, 39). In CheA₃, *R. sphaeroides* appears to have extended this network design principle by colocalizing phosphotransfer and phosphatase activities within the same protein molecule.

Methods

Plasmids and strains. The plasmids and strains used are shown in Table S1. *E. coli* strains were grown in LB medium at 37 °C. *R. sphaeroides* strains were grown in succinate medium at 30 °C under aerobic conditions with shaking. Where required antibiotics were used at concentrations of 100 µg ml⁻¹ for ampicillin and 25 µg ml⁻¹ for kanamycin and nalidixic acid.

Molecular genetic techniques. All standard genetic techniques were performed as described (40). *Pfu* polymerase (Promega) was used for all PCR reactions. All primers were synthesized by Sigma-Genosys. DNA sequencing was performed by Geneservice (Department of Biochemistry, Oxford University).

Mutagenesis of *cheA₃* in the *R. sphaeroides* genome. Overlap extension PCR was used to generate constructs for i) overexpressing mutant CheA₃ proteins and ii) for replacing either *cheA₃* in strain WS8N or *yfp-cheA₃* in strain JPA1425 in the *R. sphaeroides* genome with mutant versions of *cheA₃* (41).

Behavioral analysis. The swarm plate and tethered cell responses to propionate of the *R. sphaeroides* strains were characterized as described previously (21). Swarm plates were used to assess motility by reference to known non-motile, non-chemotactic and chemotactic strains; non-motile cells form smaller colonies on swarm plates than motile but non-chemotactic cells which in turn form smaller colonies than chemotactic cells. Nine data sets were obtained.

Fluorescence microscopy. DIC and fluorescence images of YFP fusion expressing *R. sphaeroides* strains were acquired as described previously (25). At least seven fields of view each containing at least 30 cells from independent cultures were analyzed for each strain.

Protein purification. His-tagged and GST-tagged *R. sphaeroides* CheA, CheY and CheB proteins were purified as described previously (24, 27). All CheA₃ derivatives were purified in the same way as wild-type CheA₃ (24). Protein purity and protein concentrations were measured as described (27). Purified proteins were stored at -20 °C.

Phosphotransfer from CheA₃-P and CheA₃P1-P to the response regulators. Phosphotransfer assays were performed at 20 °C in TGMNKD buffer (50 mM Tris HCl, 10% (v/v) glycerol, 5 mM MgCl₂, 150 mM NaCl, 50 mM KCl, 1 mM DTT, pH 8.0). Reaction mixtures contained 10 μM CheA₄ and 4 μM of either CheA₃ or CheA₃P1. The reactions mixtures were incubated at 20 °C for 1 hour prior to addition of 0.5 mM [γ -³²P] ATP (specific activity 14.8 GBq mmol⁻¹; PerkinElmer). The ATP dependent phosphorylation of CheA₃/CheA₃P1 was allowed to proceed for 30 minutes and then the phosphotransfer reactions were initiated by the addition of 5 μM RR. Reaction aliquots of 10 μl were taken after 30 s and quenched immediately in 5 μl of 3 X SDS-PAGE loading dye (7.5% (w/v) SDS, 90 mM EDTA, 37.5 mM Tris HCl, 37.5% glycerol, 3% (v/v) β-mercaptoethanol, pH 6.8). Quenched samples were analyzed using SDS-PAGE and phosphorimaging as described previously (27).

Preparation of CheA₃P1-³²P and CheA₂-³²P. Proteins were phosphorylated in reactions performed at 20 °C in TGMNKD buffer. The final reaction volumes were 4.5 ml. For production of CheA₃P1-³²P, reaction mixtures contained 300 μM CheA₃P1 (His-tagged) and 20 μM CheA₄ (GST-tagged), while for production of CheA₂-³²P, reaction mixtures contained 60 μM CheA₂ (His-tagged). Reactions were initiated by addition of 0.5 mM [γ -³²P] ATP (specific activity 14.8 GBq mmol⁻¹). Following a 1 hour incubation, samples were purified using Ni-NTA columns (Qiagen) as described previously for unphosphorylated His-tagged CheA₂ and CheA₃ (24, 27).

This purification step removed the unincorporated ATP from the CheA₂-³²P and CheA₃P1-³²P preparations and also removed the GST-tagged CheA₄ protein from the CheA₃P1-P preparation as judged by Coomassie stained SDS-PAGE gels of the eluted proteins. Purified proteins were stored at -20 °C.

Dephosphorylation assays. CheY/B-P dephosphorylation rates were measured using a modification of the method previously described (42, 43). All assays were performed at 20 °C in TGMNKD buffer. The final reaction volume was 150 µl. An excess of CheY/B (400 µM final concentration) was added to either CheA₃P1-³²P (for CheY₁, CheY₆ and CheB₂) or CheA₂-³²P (for CheY₂, CheY₃, CheY₄, CheY₅ and CheB₁); the phosphodonor used for each RR was chosen on the basis of fastest phosphotransfer rate. For all RRs except CheY₆, the concentration of phosphodonor used in these assays was 2 µM, although due to the rapid autodephosphorylation of CheY₆-P it was necessary to increase the concentration of CheA₃P1-³²P to 30 µM in order to obtain detectable levels of CheY₆-P throughout the 30 s timecourse. For assessing CheA₃ phosphatase activity, parallel reaction mixtures were set up with and without 2.5 µM CheA₃, allowing RR dephosphorylation rates to be compared in the presence and absence of CheA₃. Following addition of the RR to the reaction mixture, 10 µl aliquots were removed at regular time intervals and quenched immediately in 20 µl of 1.5 X SDS-PAGE loading dye. Six samples were taken for each timecourse. The duration of the timecourse was optimized according to the rate of dephosphorylation of each RR-P; for CheY₆ the timecourse covered 30 s, for CheB₁ the timecourse covered 3600 s, and for all other RRs the timecourse covered 120 s. Quenched samples were analyzed using SDS-PAGE and phosphorimaging as described previously (41). Owing to the vast molar excess of CheY/B used in these assays, the phosphotransfer reactions were completed within 10 s of mixing. Since ATP was not present in the reaction mixtures, no

rephosphorylation of the phosphodonor occurred, so once the phosphotransfer reaction had completed (before the first timepoint) the only reaction occurring was CheY/B-P dephosphorylation. Consequently, by measuring the decrease in CheY/B-P levels over time it was possible to directly calculate the dephosphorylation rate. All dephosphorylation reactions displayed kinetics that gave a good fit to single exponential decay ($R^2 > 0.998$) allowing the rate constants to be determined using Microcal Origin software.

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Figure legends

Fig. 1. Phosphorimages of SDS-PAGE gels showing phosphotransfer from (A) CheA₃ and (B) CheA₃P1 (the isolated P1 domain of CheA₃) to the *R. sphaeroides* chemotaxis response regulators. CheA₃ (4 μM) and CheA₄ (10 μM) were preincubated together with 0.5 mM [γ -³²P] ATP for 30 min. RRs (5 μM) were then added. 10 μl reaction samples were removed after 30 s and quenched in 5 μl of 3X SDS/EDTA loading dye. The samples were analyzed by SDS-PAGE and detected by phosphorimaging. Lane C shows a control reaction in which an equal volume of buffer was added instead of the RRs. The remaining lanes are labeled according to which RR was used; for example, CheY₁ was used in the lane labeled Y₁. Phosphotransfer is indicated by the appearance of phosphorylated RR and a reduction in the amount of (A) CheA₃-P or (B) CheA₃P1-P.

Fig. 2. Phosphorimages of SDS-PAGE gels showing the response regulator dephosphorylation timecourses. (A) 400 μM CheY₁ was added to 2 μM CheA₃P1-P in the absence (left half of gel) and presence of 2.5 μM CheA₃ (right half of gel). (B) 400 μM CheY₆ was added to 30 μM CheA₃P1-P in the absence (left half of gel) and presence of 2.5 μM CheA₃ (right half of gel). 10 μl reaction samples were taken at the time points indicated and quenched in 20 μl of 1.5 X SDS/EDTA loading dye. The quenched samples were analyzed by SDS-PAGE and detected by phosphorimaging. ATP was not present in any of the reactions, so following the phosphotransfer reactions which were completed before the first-time point, the only reaction occurring was RR-P dephosphorylation. As has been observed for *E. coli* CheA, a small fraction of CheA₃P1-P (< 4 %) failed to transfer phosphoryl groups to the RRs (42). Phosphatase activity is indicated by a

reduction in CheY-P levels in the presence of 2.5 μM CheA₃ when compared with those in the absence of CheA₃ (seen in (B) but not in (A)).

Fig. 3. The effect of CheA₃ mutant proteins on the dephosphorylation rate of CheY₆-P. [†]2.5 μM of each CheA₃ mutant protein was used in the phosphatase assays. The molar ratio of CheY₆ to CheA₃ mutant protein was 160:1. [‡]Each experiment was performed six times and mean values \pm standard error are shown (values are rounded to two significant figures). NP = could not be overexpressed and purified.

Fig. 4. The 794 amino acid region between the P1 and P5 domains of CheA₃ is not required for CheA₃ localization but is required for chemotaxis. (A) YFP fluorescence image of wild-type cells (strain WS8N). (B) YFP fluorescence image of JPA1425 (*yfp-cheA₃*). (C) YFP fluorescence image of JPA1741 (*yfp-cheA₃(Δ 155-948)*). (D) Swarm plate chemotaxis assay comparing the chemotactic ability of JPA1739 (*cheA₃(Δ 155-948)*) with wild-type (WS8N), non-chemotactic (JPA1314 & JPA1210) and non-motile (JPA1213) strains. The swarm plates contained 100 μM propionate and were incubated for 48 hours under aerobic conditions. Error bars show the standard error of the mean obtained from nine independent experiments.

Fig. 5. The phosphorylation reactions involving CheA₃. The domain structures of CheA₃ and CheA₄ are shown. The P1 domain of CheA₃ is phosphorylated by a CheA₄ dimer. CheA₃-P then acts as a phosphodonor for either CheY₁, CheY₆ or CheB₂. These RRs all autodephosphorylate. However, CheA₃ acts as a phosphatase on CheY₆-P (red arrow) and can accelerate the rate of dephosphorylation by at least a factor of 3 over the rate of autodephosphorylation.

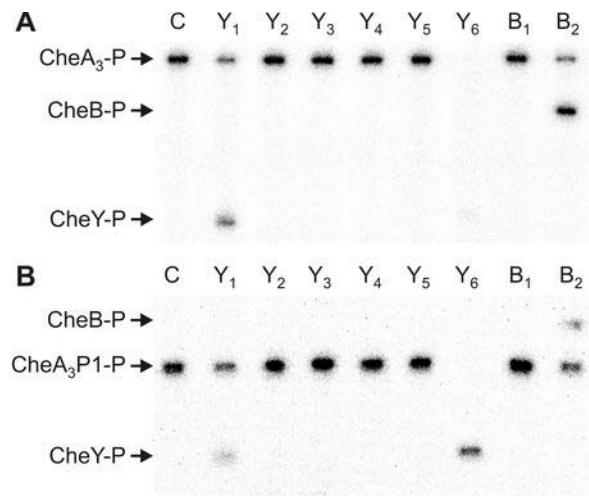
Table 1. The effect of CheA₃ on the dephosphorylation rates of the *R. sphaeroides* CheY/Bs

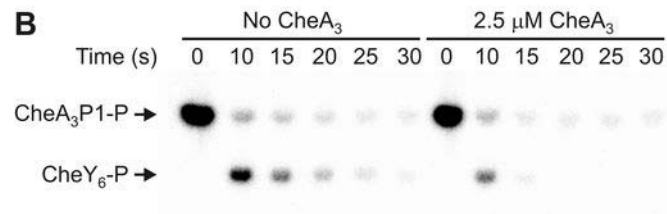
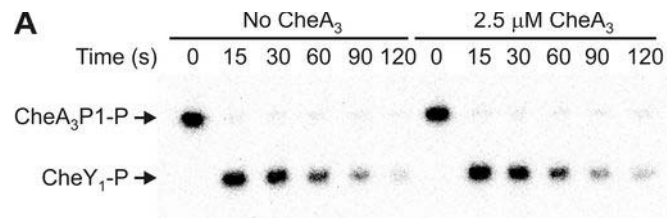
Protein	Dephosphorylation half-time (s) [*]	
	0 μM CheA ₃ [†]	2.5 μM CheA ₃ [‡]
CheY ₁ -P	27 ± 1	27 ± 1
CheY ₂ -P	63 ± 3	67 ± 4
CheY ₃ -P	36 ± 3	40 ± 4
CheY ₄ -P	38 ± 3	39 ± 2
CheY ₅ -P	27 ± 1	30 ± 1
CheY ₆ -P	4.1 ± 0.3	1.4 ± 0.1
CheB ₁ -P	4000 ± 200	4100 ± 100
CheB ₂ -P	52 ± 4	52 ± 7

* Mean ± standard error (values rounded to two significant figures). Each experiment was performed six times.

[†] Whilst most of these values are in good agreement with our previous estimates of dephosphorylation rate, some of these values differ considerably from our earlier estimates (27). The values in this table were derived from a direct assay of RR-P dephosphorylation and are more accurate. The previous estimates were based on an indirect assay that examined the steady state concentration of phosphorylated response regulator in a reaction mixture containing CheA₂ and ATP, and consequently were very sensitive to small errors in measuring the CheA₂ autophosphorylation rate and the steady state [CheY/B-P] and [CheA₂-P].

[‡] The molar ratio of RR to CheA₃ was 160:1.





Mutant protein [†]	Domain configuration of mutant protein	Dephosphorylation half-time of CheY ₆ -P (s) [‡]
No CheA ₃		4.1 ± 0.3
CheA ₃	H 1 2 3 4 P5	1.4 ± 0.1
CheA ₃ (H51Q)	Q 1 2 3 4 P5	1.5 ± 0.1
CheA ₃ (E585S,N588S)	H 1 2 **3 4 P5	1.4 ± 0.1
CheA ₃ (Δ2-154)	1 2 3 4 P5	NP
CheA ₃ (Δ155-349)	H 2 3 4 P5	NP
CheA ₃ (Δ349-549)	H 1 3 4 P5	2.4 ± 0.2
CheA ₃ (Δ549-749)	H 1 2 4 P5	4.2 ± 0.1
CheA ₃ (Δ749-948)	H 1 2 3 P5	1.4 ± 0.1
CheA ₃ (Δ950-1095)	H 1 2 3 4	1.5 ± 0.1
CheA ₃ (Δ155-549)	H 3 4 P5	2.5 ± 0.2
CheA ₃ (Δ155-749)	H 4 P5	4.1 ± 0.2
CheA ₃ (Δ155-948)	H P5	4.2 ± 0.1
CheA ₃ (Δ549-948)	H 1 2 P5	4.1 ± 0.2
CheA ₃ (Δ349-948)	H 1 P5	4.1 ± 0.2

