Chemotaxis in *Rhodobacter sphaeroides* Requires an Atypical Histidine Protein Kinase*

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The chemotaxis system of *R. sphaeroides* is encoded in three major chemosensory loci (15, 18, 19). Although *R. sphaeroides* lacks a CheZ homologue, each locus encodes homologues of the remaining proteins of the *E. coli* chemosensory pathway. The proteins of immediate relevance to this study are: CheA1, CheY1, CheY2, and CheY6 encoded by cheP1, (18, 20); CheA2, CheB1, and CheY3 encoded by cheP2 (19); CheA3, CheA4, CheB2, and CheY4 encoded by cheP3 (15); CheY4 encoded in a separate genetic locus with the chemoreceptor McpG (21). Genetic studies have shown that, although CheA2, CheA3, CheA4, CheB1, CheB2, and CheY4 are essential for chemotaxis (15, 22, 23), all of the proteins encoded by cheP3 have no detectable role in chemotaxis (18). Chemotaxis also requires either of CheY1 or CheY2.

Bacterial chemotaxis uses a two-component phosphotransfer pathway to integrate information from multiple sensory inputs reflecting changes in chemoelector concentration to produce an appropriate change in swimming behavior. Two-component phosphotransfer pathways comprise dimeric histidine protein kinases and response regulators (reviewed in Ref. 1). Histidine protein kinases form homodimers, where each subunit transphosphorylates the other subunit on a conserved histidine residue using ATP as the phosphodonor. The rate of this auto phosphorylation reaction is controlled by sensory stimuli. Phosphorylated histidine protein kinases are able to transfer the phosphoryl group from their conserved histidine residue onto a conserved aspartate residue on their cognate response regulator. Phosphorylation of the response regulators modulates their activity, producing an appropriate response to the original stimulus. Some bacteria have over 50 distinct phosphotransfer pathways, and the extent to which they interact with one another is an active area of current research (2–4).

The chemotaxis histidine protein kinase is CheA, whose rate of autophosphorylation is increased by the chemoreceptors via CheW in response to decreases in attractant concentration (5, 6). CheA-P phosphotransfers to its cognate response regulators, CheY and CheB (7, 8). In *Escherichia coli*, CheY-P interacts with the flagellar motor to promote a switching in the direction of flagellar rotation, causing the cell to change its swimming direction (9). CheB-P is a methyltransferase that demethylates methylated glutamate residues on the chemoreceptors and promotes adaptation to the current environmental conditions (10). *E. coli* CheA has five domains: P1 is a histidine phosphotransferase (Hpt) domain that contains the conserved phosphotransferable histidine residue (11); P2 binds the response regulators and, although it is not essential for phosphotransfer from the P1 domain to the response regulators, it does accelerate the process (12, 13); P3 is the dimerization domain; P4 is the kinase domain that binds ATP and phosphotranslates the P1 domain; it is characterized by the conserved N, G1, F, and G2 sequence boxes (11); and P5 is the regulatory domain that interacts with CheW and the chemoreceptors (14). In this report we will call proteins with an identical domain organization to *E. coli* classic CheAs, whereas chemotaxis proteins containing at least two of the *E. coli* CheA domains but either lacking some of the other domains or containing additional domains are called atypical CheAs. Atypical CheAs have been found in a range of organisms for example *Rhodobacter sphaeroides* (15), *Myxococcus xanthus* (16), and *Pseudomonas aeruginosa* (17).

The chemotaxis system of *R. sphaeroides* is encoded in three major chemosensory loci (15, 18, 19). Although *R. sphaeroides* lacks a CheZ homologue, each locus encodes homologues of the remaining proteins of the *E. coli* chemosensory pathway. The proteins of immediate relevance to this study are: CheA1, CheY1, CheY2, and CheY3 encoded by cheP1, (18, 20); CheA2, CheB1, and CheY3 encoded by cheP2 (19); CheA3, CheA4, CheB2, and CheY4 encoded by cheP3 (15); CheY4 encoded in a separate genetic locus with the chemoreceptor McpG (21). Genetic studies have shown that, although CheA2, CheA3, CheA4, CheB1, CheB2, and CheY4 are essential for chemotaxis (15, 22, 23), all of the proteins encoded by cheP3 have no detectable role in chemotaxis (18). Chemotaxis also requires either of CheY1 or CheY2.

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1 The abbreviations used are: Hpt, histidine phosphotransferase; GFP, green fluorescent protein.
2 Deletion of either CheY1 or CheY2 had no effect on chemotaxis; however deletion of both genes abolished chemotaxis (S. L. Porter, E. Byles, and J. P. Armitage, unpublished observation).
The Atypical CheAs of Rhodobacter sphaeroides

The chemosensory proteins of *R. sphaeroides* are found in two distinct clusters, the polar chemotaxis cluster and the cytoplasmic chemotaxis cluster, each containing a specific subset of chemosensory proteins. The transmembrane chemoreceptors localize to the polar chemotaxis cluster along with *CheA*2, *CheW*2, and *CheW*3 (24–26). The cytoplasmic chemotaxis cluster, each containing a specific subunit, P4 (kinase), and P5 (regulatory) domains.

The absence of the histidine kinase catalytic core (P3 and P4) lacks the P1 (Hpt) and P2 (CheYB binding) domains found in classic CheAs but retains the P3 (dimerization), P4 (kinase), and P5 (regulatory) domains.

A previous biochemical study focusing on the classic CheAs, *CheA*1 and *CheA*2, showed that both proteins can autophosphorylate and phosphotransfer to the response regulators (28). *CheA*2-P can phosphorylate all eight chemotaxis response regulators, whereas *CheA*1-P is more selective and will only phosphotransfer to the response regulators encoded within its own genetic locus (*CheY*1, *CheY*2, and *CheY*5) and *CheY*3 (28). Here we describe the roles of the atypical CheA proteins of *R. sphaeroides*, *CheA*3 and *CheA*4, and present data showing that the homodimeric *CheA*4 protein phosphorylates *CheA*3. We also examined the role of *CheA* phosphorylation in *R. sphaeroides* chemotaxis and determined whether the classic CheAs could participate in phosphorylation reactions between different CheAs.

### Experimental Procedures

**Strains and Plasmids**—The strains and plasmids used in this study are shown below in Tables I and II. *E. coli* strains were grown in LB medium at 37 °C. *R. sphaeroides* strains were grown in succinate medium at 30 °C either aerobically with shaking without illumination or phototrophically in an anaerobic cabinet (Don Whitley Scientific) with illumination at 50 μmol m⁻² s⁻¹. Where appropriate antibiotics were used at concentrations of 100 μg ml⁻¹ for ampicillin and 25 μg ml⁻¹ for kanamycin, nalidixic acid, and tetracycline.

**Molecular Genetic Techniques**—All standard genetic techniques were performed as described previously (29). *Pfu* polymerase (Promega) was used for all PCR reactions. All primers were synthesized by Sigma-Genosys. The Plasmid midi-prep kit (Qiagen) was used to prepare sequencing quality DNA, which was sequenced by the DNA sequencing service (Department of Biochemistry, Oxford University). DNA se-
The Atypical CheA of Rhodobacter sphaeroides

TABLE II

<table>
<thead>
<tr>
<th>CheA mutant</th>
<th>Strain name</th>
<th>Reference</th>
<th>Chemotaxis on swarm plates*</th>
<th>Tethered cell responses to propionate*</th>
<th>Phototropism, stop on light removal</th>
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<td></td>
<td></td>
<td></td>
<td>Aerobic</td>
<td>Phototrophic</td>
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<td>cheA4 G220K</td>
<td>JPA1211</td>
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<td>None</td>
</tr>
</tbody>
</table>

*a All strains either showed wild-type levels of chemotaxis on swarm plates or showed no chemotaxis at all to all attractants tested; no intermediate phenotypes were seen.

*b Normal tethered cell responses to propionate were: 1) a slight increase in rotation rate upon propionate addition and 2) a stop followed by adaptation upon removal of propionate. Inverted responses were a stop upon addition of propionate, which persisted until the propionate was removed; no adaptation was seen. Weak inverted responses were a reduction in rotation rate, which persisted following rotation upon addition of propionate, which persisted until the propionate was removed; no adaptation was seen.

sequence was analyzed using Clone Manager Version 7 (Scientific and Educational Software).

Site-directed Mutagenesis of CheA in the Genome of R. sphaeroides—
The overlap extension method of PCR mutagenesis (30) was used to produce the phosphorylation site and kinase domain CheA mutants. For phosphorylation site mutants the codon for the phosphorylatable histidine residue was replaced with CAG, the preferred R. sphaeroides codon for glutamine. For kinase domain mutants the codon for a conserved glycine residue (corresponding to Gly-470 in E. coli CheA) was mutated to AAG, the preferred R. sphaeroides codon for lysine. The overlap extension PCR products comprised the entire coding sequence of the CheAs flanked by ~500 bp of native genomic sequence. These PCR products were cloned into the allelic exchange suicide vector pK18mobACAB to generate the mutation plasmids (Table I). The mutation plasmids were checked by sequencing and then used to introduce the point mutations into the genome of R. sphaeroides by allelic exchange as described previously (Table II) (19, 31).

Purification of Chemotaxis Proteins—His-tagged CheA1, CheA2, CheA3, CheY1, CheY2, CheY3, CheY4, CheY5, CheY6, CheB1, and CheB2 were overexpressed and purified as described (22, 28). CheA3 and CheA4 were overexpressed from His-tagging expression vectors and purified in the same way as CheA1 and CheA2, except that for CheA3 the His tag was C-terminal instead of N-terminal. The His-tagged CheA proteins containing point mutations were purified in the same way as the wild type His-tagged CheA proteins. Protein purity and protein concentrations were measured as described (28). Purified proteins were stored at −20 °C; activity remained constant for at least 1 year.

Cloning CheAs into Expression Vectors—The construction of the pQE91, pQE92, and pQE4A expression plasmids has been described previously (15, 22); the kinase domain and phosphorylation site mutants of cheA1, cheA4, and cheA6 were cloned into the expression plasmid pQE91 in the same way. The coding sequences of cheA2 and cheA5 H51Q (excluding the stop codones) were amplified by PCR from genomic DNA of R. sphaeroides strains WSSN and JPA1210, respectively. The primers used incorporated NcoI restriction sites, which were located at both ends of the PCR products, allowing in-frame cloning of the PCR products into the NcoI site of the C-terminal His-tagging expression vector pQE91 (NcoI and NcoI generate compatible sticky ends).

Phenotypic Analysis of R. sphaeroides Strains—The swarm plating responses, the behavior of tethered cells in a flow chamber and the photoresponses of R. sphaeroides strains were characterized as described previously (15). Nine data sets were obtained for each tethered cell analysis. Three data sets that together contained at least ten cells were obtained for each tethered cell analysis. The localization of MCP-G-GFP was analyzed using the method described previously (25). A minimum of 50 cells for each strain was examined.

CheA Phosphorylation Reactions—All reactions were performed at 20 °C in TGMNK buffer (50 mM Tris-HCl, 10% (v/v) glycerol, 5 mM MgCl2, 150 mM NaCl, 50 mM KCl, 1 mM diithiothreitol, pH 8.0). Reaction mixtures contained CheA proteins as described under “Results” (unless otherwise stated, the concentration of each CheA present in the reaction mixtures was 5 μM) prior to the addition of ATP. These reaction mixtures were allowed to equilibrate for 1 h at 20 °C. All reactions were initiated by the addition of [γ-32P]ATP (specific activity, 14.8 GBq mmol−1; this was used at a concentration of 0.5 mM unless otherwise stated, Amersham Biosciences). Reaction aliquots of 10 μl were taken at the specified time points and quenched immediately in 5 μl of 3× 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 gel electrophoresis and phosphorimaging as described previously (28).

Phosphotransfer from CheA4 to the Response Regulators—Phosphotransfer assays were performed at 20 °C in TGMNK buffer. Reaction mixtures contained 2 μM CheA4 and 10 μM CheA5. The reactions were initiated by the addition of 10 μM response regulator. These phosphotransfer reaction mixtures were sampled, quenched, and analyzed at the times indicated, using the same method as the phosphorylation reactions (see above).

Determination of Native Molecular Weight by Gel Filtration Analysis—This was performed essentially as described (32) by the Protein Production Division of the University of York Technology Facility. A Superdex 200 gel filtration column (Amersham Biosciences) was used in conjunction with an AKTA Purifier 10 high performance liquid chromatography system (Amersham Biosciences) with TGMNK buffer as the column running buffer. The column was calibrated using the following molecular mass standards (Amersham Biosciences): ribonuclease A (13.7 kDa), chymotrypsinogen A (25.0 kDa), ovalbumin (43.0 kDa), albumin (67.0 kDa), aldolase (158 kDa), catalase (232 kDa), and ferritin (440 kDa). 500 μl of an 8 μM CheA4 solution was applied to the column, and its molecular mass was estimated by comparison with the elution profile of the calibration standards.

RESULTS

CheA4 Phosphorylates CheA3—CheA3 and CheA4 both lack some of the domains required for autophosphorylation. Both proteins were purified and used for in vitro studies. Unsurprisingly, CheA3 and CheA4 were unable to autophosphorylate using [γ-32P]ATP as phosphodonor (Fig. 1); however, CheA3 was phosphorylated in a reaction mixture containing CheA3, CheA4, and [γ-32P]ATP. The simplest explanation for this is the P4 (kinase) domain of CheA3 phosphorylated the P1 (Hpt) domain of CheA3. To test this hypothesis we generated two mutants: first, the predicted phosphorylation site of CheA3 (H51) contained within the H-box of the P1 domain was mutated to the non-phosphorylatable glutamine residue, yielding CheA3 H51Q, and second, a highly conserved glycine residue within the G2-box of the P4 (kinase) domain of CheA4 was...
mutated to a lysine, yielding CheA4 G220K. The corresponding mutations in E. coli CheA (H48Q and G470K) abolish kinase activity (33, 34). The CheA4 H51Q mutant was not phosphorylated by CheA4 suggesting that His-51 is the phosphorylation site of CheA4. The CheA4 G220K mutant did not phosphorylate CheA3; taken together these results are consistent with our hypothesis that the kinase domain of CheA4 phosphorolyses the P1 domain of CheA3. These data show that CheA3 and CheA4 interact to form a chemotaxis histidine protein kinase, subsequently referred to as CheA3A4.

CheA4 Is a Dimeric Protein—The kinase core of E. coli CheA comprising the P3 and P4 domains forms homodimers; indeed, kinase activity requires dimerization of the kinase catalytic core (35). To test whether CheA4 would also form homodimers, gel filtration chromatography of purified CheA4 was carried out. More than 90% of the protein eluted in fractions with a predicted molecular mass of 90 kDa. The predicted molecular mass of CheA3 (from its sequence) is 44 kDa; the gel filtration results are therefore consistent with the hypothesis that CheA4 forms a homodimer. In the subsequent kinetic analysis of CheA4 we have therefore made the simplifying assumption that all of the CheA4 present in the reaction mixture is homodimerized.

Rate of Phosphorylation of CheA3 as a Function of CheA4 Concentration—The rate of CheA3 phosphorylation was measured in reaction mixtures containing 2 μM CheA3, 0.5 mM [γ-32P]ATP, and varying concentrations of CheA4 monomers (1–90 μM of CheA4 monomers). The pseudo-first order rate constant (kobs) for each phosphorylation reaction was determined from a semi-logarithmic plot of the time course (Fig. 2A). The reaction rate and therefore kobs were observed to increase as the CheA4 concentration was increased (Fig. 2B). These data fit a reaction model (Equation 1) in which a CheA4 dimer has two independent binding sites for CheA3,

\[ k_{\text{obs}} = \frac{k_{\text{max}}(\text{[CheA3]}_T)}{[\text{CheA3}]_T} = \frac{k_{\text{max}}([\text{CheA3]}_T + [\text{CheA3}]_T + K_0 - \sqrt{([\text{CheA3]}_T + [\text{CheA3}]_T + K_0)^2 - 4[\text{CheA3]}_T[\text{CheA3}]_T)}}{2[\text{CheA3}]_T} \]  

(1)

where kobs is the pseudo-first order rate constant, kmax is the maximal value of the pseudo-first order rate constant (occurs when all of the CheA3 is complexed with CheA4), [CheA3]T is the total concentration of CheA3 (i.e. free CheA3 plus CheA3 in complex with CheA4), [CheA3]T is the total concentration of CheA3 monomers (it is assumed that all of the CheA3 is dimerized), [CheA3A4] is the concentration of CheA3 that is bound to CheA4, and K0 is the dissociation constant for the complex between CheA3 and CheA4. The value of K0 obtained from this analysis was 48 ± 8 μM.

To test this reaction model, we measured the rate of phosphorylation of CheA3 as a function of [CheA4]. The results of this analysis were consistent with the above reaction model (data not shown), although these experiments were constrained by the limited solubility of CheA3; the maximum [CheA4] that could be used in the phosphorylation reactions was 5 μM.

Rate of Phosphorylation of CheA3 as a Function of ATP Concentration—The rate of CheA3 phosphorylation was measured in reaction mixtures containing 2 μM CheA3, 10 μM CheA4, and varying concentrations of [γ-32P]ATP (0.01–2 mM). As before the pseudo-first order rate constant for these reactions (kobs) was determined from a semilogarithmic plot of the reaction time course. The reaction rate and therefore kobs were found to increase in accordance with the following equation as the ATP concentration increased (Fig. 2C),

\[ k_{\text{obs}} = \frac{k_{\text{cat app}}[\text{ATP}]}{K_{\text{D}_{\text{ATP}}}[\text{ATP}]} \]  

(2)

where kcat app is the apparent catalytic rate constant, and KD_{ATP} is the dissociation constant for ATP from CheA3A4 (see Ref. 36 for application of this method to E. coli CheA). This analysis gives a K_{D_{ATP}} of 39 ± 3 μM and a k_{cat app} of 0.826 ± 0.015 min⁻¹. kcat app but not K_{D_{ATP}} was found to increase as the CheA4 concentration in the reactions was increased (data not shown); this occurred because sub-saturating levels of CheA4 were used for the initial determination of k_{cat app}. Consequently, an increase in the total CheA4 concentration increased the concentration of the CheA3A4 complex, which in turn increased the reaction rate. Using the value of K_{D_{ATP}} determined above for the CheA3A4 complex, we estimate that in a mixture of 2 μM CheA3 and 10 μM CheA4 (total concentrations) the concentration of CheA4 that was bound to CheA4 was 0.336 μM. Equation 3 allows the true kcat (i.e. the maximal rate of CheA3 phosphorylation at saturating ATP and CheA4 concentrations) to be calculated.

\[ k_{\text{cat app}} = \frac{k_{\text{cat}}[\text{CheA3A4}]}{[\text{CheA3}]} \]  

(3)

This analysis produces a kcat of 4.9 ± 0.1 min⁻¹. The CheA3A4 kcat is ~3-fold higher than E. coli CheA kcat and at least 6-fold higher than the kcat for the other R. sphaeroides CheAs (Table III).

CheA4-P Phosphotransfers to CheY1, CheY6, and CheB—Phosphotransfer rates from CheA4-P to the six CheYs and two CheBs were measured under multiple turnover conditions in the presence of CheA3 and excess ATP, allowing CheA4-P phosphorylation to continue throughout the course of the reactions. A mixture of CheA3 (2 μM) and CheA4 (10 μM) was preincubated with 0.5 mM [γ-32P]ATP for 15 min, allowing phosphorylation of CheA4. The response regulators (10 μM) were added (t = 0), and the reaction was followed by taking samples for SDS-PAGE analysis at intervals. The progress of these phosphotransfer reactions after 30 s is shown in Fig. 3; phosphotransfer occurred in reactions where a decrease in CheA4-P levels was accompanied by an increase in CheY/P-B-P levels. Phosphotransfer was observed from CheA4-P to CheY1 (cheOp1), CheY6 (cheOp3), and CheB2 (cheOp5); the remaining response regulators were not phosphorylated by CheA4-P even after prolonged incubations (60, 90, 240, and 1920 s; data not shown). Time courses showing the progress of the reactions where phospho-
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The relationship between CheA4 phosphorylation time course. Reactant concentrations were: CheA3 and ATP. The concentrations of the other reactants were not varied; CheA3 phosphorylation of CheA4 has a homodimer complex between CheA3 and CheA4 (CheA3A4) in which CheA3 is phosphorylated by CheA4. The data are fitted to a model (Equation 1) where a CheA4 homodimer has two independent binding sites for CheA3; the enzyme substrate catalyzes the reaction (Equation 2) was used to determine the dissociation constant of CheA4 for ATP (Kd, ATP) = 39 ± 3 μM and the apparent kcat (kcat_app) = 0.826 ± 0.015 min⁻¹ for phosphorylation of CheA3A4. The error bars in B and C show the S.E. obtained by measuring kobs in triplicate.

**FIG. 2. The effect of varying [CheA3] and [ATP] on the pseudo-first order rate constant (kobs) for the ATP-dependent phosphorylation of CheA3 by CheA4. A. Semilogarithmic plot of CheA3 phosphorylation time course. Reactant concentrations were: [CheA3] = 2 μM, [CheA4] = 10 μM, and [ATP] = 2 mM. The gradient of the fit line is kobs.B, the relationship between kobs and [CheA3]. The concentrations of the other reactants were not varied; [CheA4] = 2 μM and [ATP] = 0.5 mM. The data are fitted to a model (Equation 1) where a CheA4 homodimer has two independent binding sites for CheA3; the enzyme substrate catalyzes the reaction (Equation 2). A fit to Equation 2 was used to determine the dissociation constant of CheA4 for ATP (Kd, ATP) = 39 ± 3 μM and the apparent kcat (kcat_app) = 0.826 ± 0.015 min⁻¹ for phosphorylation of CheA3A4. The error bars in B and C show the S.E. obtained by measuring kobs in triplicate.**

**TABLE III**
Summary of CheA kinetic and thermodynamic constants

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<tr>
<th>Protein</th>
<th>Kd, ATP (μM)</th>
<th>kcat for phosphorylation (min⁻¹)</th>
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<td>1.56 ± 0.24</td>
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<td>0.48</td>
<td>(43)</td>
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<td>0.25 ± 0.03</td>
<td>0.38 ± 0.02</td>
<td>(38)</td>
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<tr>
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<tr>
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<td>0.039 ± 0.003</td>
<td>4.9 ± 0.1</td>
<td>This study</td>
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**FIG. 3. Phosphorimaging results of SDS-PAGE gel showing phosphotransfer from CheA3-P to the response regulators CheY, CheYr, and CheB. CheA3 (2 μM) and CheA4 (10 μM) were preincubated together with 0.5 mM [γ-32P]ATP for 30 min. Response regulators (10 μM) were then added to the reaction mix (the final volume was 100 μl). 10-μl samples were removed after 30s and quenched immediately by addition of 5 μl of 3× SDS/EDTA loading dye. The quenched samples were analyzed by SDS-PAGE and detected by phosphorimaging. The lane labeled C shows a control reaction in which an equal volume of buffer was added instead of the response regulators. The remaining lanes are labeled according to which response regulator and a reduction in the amount of CheA3-P.**

Phosphotransfer is indicated by the appearance of phosphorylated response regulator and a reduction in the amount of CheA3-P. In contrast, phosphotransfer from CheA3-P to CheY6 proceeded much more slowly with detectable levels of both CheA3-P and CheY6-P remaining at steady state. The almost undetectable levels of CheY-P at steady state are consistent with our previous observation that CheY-P dephosphorylates at least 10-fold faster than CheY1-P and CheB2-P (28).

**The Importance of CheA Phosphorylation for Chemotaxis—** Although previous deletion studies have demonstrated that CheA2, CheA3, and CheA4 are all essential for chemotaxis in R. sphaeroides (15, 19), the precise reason for their requirement has not been determined. The CheAs may be required for either their role in phosphosignaling and/or their role in localizing other components of the chemotaxis signaling pathway to either the cell poles or the cytoplasmic chemotaxis cluster. To begin to address this question, we used site-directed mutagenesis to determine whether phosphorylation of these CheAs is essential for chemotaxis. The histidine phosphorylation sites of CheA1 (His-49), CheA2 (His-46), and CheA3 (His-51) were mutated to glutamine and the conserved glycine residues within the G2-box of the P4 domains of CheA1 (Gly-501), CheA2 (Gly-480), and CheA3-P (28).

As described above, the CheA3 phosphorylation site mutant could not be phosphorylated by CheA4, whereas the CheA4 kinase domain mutant was unable to phosphorylate CheA3.
These results demonstrate that CheA autophosphorylation in vitro can be abolished by mutating either the phosphorylation site or the kinase domain.

When CheA1 H49Q was mixed with CheA1 G501K, CheA1 phosphorylation was observed, suggesting that CheA1 H49Q and CheA1 G501K formed a heterodimer in which the kinase domain of CheA1 H49Q was able to phosphorylate CheA1 G501K; similar results were obtained for CheA2 H46Q and CheA2 G472K (Table IV). These results show that, although the point mutations prevent autophosphorylation within a homodimer, the proteins can still function as part of a heterodimer and therefore are likely to have folded correctly.

These point mutations were then introduced into the native cheA genes in the R. sphaeroides genome by double homologous recombination. Previous studies using GFP labeling have shown that CheA2 is required for the polar localization of the chemoreceptor McpG (23); the CheA2 point mutations described in this study retain polar McpG localization (data not shown), demonstrating that it is possible to separate the autophosphorylation activities of CheAs from their role in the localization of other chemotaxis proteins. This result concurs with an E. coli study where it was shown that chemoreceptor localization does not require CheA autophosphorylation activity (37). The phenotypes of the R. sphaeroides point mutant cheA strains and their corresponding ΔcheA strains were characterized by swarm plates, tethered cell analysis, and phototaxis assays (Table II). The phenotypes of the cheA point mutant strains exactly matched the phenotypes of their corresponding ΔcheA strains, which for CheA2, CheA3, and CheA4 was a severely impaired chemotaxis response indicating that phosphorylation from CheA2, CheA3, and CheA4 is essential for chemotaxis.

Do Classic CheAs Participate in the Transphosphorylation of Other CheAs?—Having shown that CheA4 can transphosphorylate CheA3, we hypothesized that the classic CheAs of R. sphaeroides may also be capable of transphosphorylating other CheAs. This was tested in vitro by incubating all possible pairwise combinations of different CheAs (each at concentrations of 5 μM) with 0.5 mM [γ-32P]ATP for 1 h; phosphorylated proteins were then detected by SDS-PAGE followed by phosphorimaging. Because both classic CheAs can form autophosphorylating homodimers (28) and would presumably do so in mixtures with other CheAs, it was necessary to use the non-autophosphorylating kinase domain and phosphorylation site CheA mutants (described in the previous section) for these experiments. Each reaction mix contained one CheA that was potentially able to act as a kinase but could not be phosphorylated (the phosphodonor) and another CheA that could potentially be phosphorylated but lacked kinase activity (the phosphoacceptor); the results are summarized in Table IV.

Interestingly, neither of the classic CheAs could phosphorylate CheA3, indicating that only CheA4 can phosphorylate CheA3. The classic CheAs did however participate in some transphosphorylation reactions; CheA1 G501K was phosphorylated by CheA1 H49Q, CheA2 H46Q, and CheA3, whereas CheA2 G472K was phosphorylated by CheA2 H46Q and CheA4. The CheA1 H49Q G501K and CheA1 H46Q G472K mutants were unable to act as phosphoacceptors in these reactions (data not shown) suggesting that the same phosphorylation site was used for autophosphorylation and transphosphorylation. In summary, these in vitro data indicate that only CheA4 can phosphorylate CheA3, however, CheA1 can be phosphorylated by CheA1, CheA2, and CheA3, whereas CheA2 can be phosphorylated by CheA3 and CheA4.

The Relative Rates of the CheA Transphosphorylation Reactions—We reasoned that the relative physiological importance of the various transphosphorylation reactions observed in the previous section would, at least in part, depend upon the relative rates of these reactions. Therefore, time courses for these phosphorylation reactions were measured (Fig. 5). The fastest
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FIG. 5. CheA transphosphorylation time courses. Time courses for the six transphosphorylation reactions that occurred after 1 h in the experiment described in Table IV, were measured. The experimental procedure was identical to that used in Table IV except that the reactions were sampled at multiple time points as indicated on the graphs. The error bars show the S.E. obtained by performing each experiment in triplicate (error bars have been omitted from the slowest three reaction time courses to improve clarity). ■, phosphorylation of CheA3 G501K by CheA1 H49Q; □, phosphorylation of CheA3 G472K by CheA1 H46Q; ○, phosphorylation of CheA3 G501K by CheA2 H46Q; ●, phosphorylation of CheA3 by CheA4; △, phosphorylation of CheA1 G501K by CheA2 H46Q; ●, phosphorylation of CheA3 by CheA4 G472K by CheA1.

reaction was CheA3 phosphorylation by CheA4 followed by CheA2 G472K phosphorylation by CheA2 H46Q, suggesting that these reactions may have the most physiological importance. The third fastest reaction was CheA3 G501K phosphorylation by CheA4. The remaining three reactions, CheA1 G501K phosphorylation by CheA2 H46Q, CheA1 G501K phosphorylation by CheA2 H46Q, and CheA2 G472K phosphorylation by CheA1, were much slower than the other reactions and consequently could be predicted to be least important for chemotaxis.

Deletion of cheA1 (23) and inactivation of CheA1 by site-directed mutagenesis (Table II) had no effect on chemotaxis under laboratory conditions, implying that the phosphorylation reactions involving CheA1 are not required for chemotaxis. There are three phosphorylation reactions involving only the CheAs that are essential for chemotaxis: 1) CheA3 phosphorylation by CheA4, 2) CheA2 autophosphorylation, and 3) phosphorylation of CheA2 by CheA4. The slowest reaction of these three is the phosphorylation of CheA3 by CheA4, which probably does not occur in vivo because the two proteins are separated spatially; CheA2 localizes to the polar chemotaxis cluster while CheA3 colocalizes with CheA3 to the cytoplasmic chemotaxis cluster (25). In addition, strain JPA1206 (cheA1 G472K) shows no chemotaxis suggesting that CheA3 may not be phosphorylated in vivo. The remaining two reactions, 1) phosphorylation of CheA3 by CheA4, and 2) CheA2 autophosphorylation, must therefore be the most important CheA phosphorylation reactions in R. sphaeroides chemotaxis.

DISCUSSION

This study shows that R. sphaeroides has two essential chemotaxis histidine protein kinases: the CheA homodimer and a complex between CheA1 and CheA3 designated CheA3A4. CheA2 is localized to the polar chemotaxis cluster, whereas CheA3A4 is localized to the cytoplasmic chemotaxis cluster. Phosphosignaling from both of these kinases, and therefore from both polar and cytoplasmic signaling clusters, is essential for normal chemosensory and photosensory behavior. Cells must then integrate the signals produced by these clusters to produce an appropriate balanced response to the sensory stimuli.

Unlike CheA3A4-P, but like CheA1-P (28), CheA3A4-P shows no chemotaxis suggesting that CheA4 may not be phosphorylated spatially; CheA2 localizes to the polar chemotaxis cluster whereas CheA3A4 is localized to the cytoplasmic chemotaxis cluster, is essential for normal chemosensory and photosensory behavior. Cells must then integrate the signals produced by these clusters to produce an appropriate balanced response to the sensory stimuli.

Unlike CheA2-P, but like CheA1-P (28), CheA3A4-P shows selective phosphotransfer to the response regulators. A summary of the chemotaxis phosphotransfer reactions observed in this study and a previous study is shown in Fig. 6 (28). Aside from the non-essential CheY1 protein encoded by cheOp1, CheA3A4 phosphotransfers only to its response regulators (CheY6 and CheB2) encoded within its own locus. R. sphaeroides requires CheY6 and either of CheY3 and CheY4 for normal chemotaxis; the remaining CheYs are dispensable. Because CheA3A4 can only phosphorylate CheY6 and not CheY3 nor CheY4, this could explain why chemotaxis requires a further histidine protein kinase, CheA3. However, CheA3 can phosphorylate all eight chemotaxis response regulators; therefore, why is CheA3A4 also required for chemotaxis? A possible explanation is that chemotaxis requires the combined kinase output of CheA2 and CheA3A4 to generate enough CheY-P for a response; either CheA2 or CheA3A4 acting alone may not generate enough CheY-P for a response.

The histidine kinase core of CheA3A4 is a CheA homodimer; homodimer formation is presumably mediated by interaction between the P3 (dimerization) domains of the two CheA pro- 

omers. The CheA3A4 complex is formed when the CheA3 homodimer binds its substrate CheA1; in our kinetic analysis we assumed that the CheA4 homodimer has two equivalent binding sites for CheA3. Interestingly, the KD for CheA3A4 (48 ± 8 μM) is similar to the affinity of an N-terminally truncated E. coli CheA (containing only the P3, P4, and P5 domains) for separately expressed P1 domain (26 ± 1 μM) (38), raising the possibility that the interaction between CheA3 and CheA4 is mediated entirely by the affinity of the kinase domain of CheA4 for the P1 domain of CheA3. CheA3 and CheA4 localize to the cytoplasmic chemotaxis cluster independently of one another; this close association of CheA3 and CheA4 would promote the formation of the enzyme-substrate complex (CheA3A4) that is required for CheA3 phosphorylation.

The existence of two distinct chemotaxis signaling clusters allows R. sphaeroides to prevent unwanted cross-talk between the CheAs. GFP localization experiments have shown that CheA2 and CheA3 are physically separated; CheA2 is localized to the polar chemotaxis cluster while CheA3 is localized to the cytoplasmic chemotaxis cluster (25). Therefore, although CheA3 can phosphorylate CheA2 in vitro, in vivo this can not occur because the two proteins are physically separated.

The apparent lack of a P2 (CheY/B binding) domain within CheA3A4-P can be explained in two possible ways. It is possible that the region between the P1 and P5 domains of CheA3 is unique to CheAs, whereas Hpt domains (of which the CheA P2 domain is an example) are found in a range of two-component signaling pathways where they participate in selective phosphotransfer reactions without need for a P2 domain. The phosphotransfer specificity of CheA3A4 could therefore arise from selective contacts made between the P1 domain of CheA3 and its cognate response regulators.

Functionally CheA3A4 appears to perform a similar role to classic CheAs; CheA3A4 is capable of ATP-dependent phospho-

rylation and phosphotransfer to the chemotaxis response reg-

ulators. What then is the reason for deviating away from the classic homodimeric CheA architecture? One possible explana-
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FIG. 6. Summary of the in vitro chemotaxis phosphotransfer reactions. Solid lines indicate phosphorylation reactions involving both CheAs and response regulators that are essential for chemotaxis. Dashed lines indicate phosphotransfer reactions involving non-essential chemotaxis proteins. The CheAs and their phosphotransfer reactions are color coded: CheA1 (gray), CheA2 (pink), and CheA3 (green). The response regulators are color coded: unimportant CheYs are gray, important CheYs are red, and CheBs are blue.

REFERENCES

Chemotaxis in *Rhodobacter sphaeroides* Requires an Atypical Histidine Protein Kinase

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