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A Fragment Liberated from the *Escherichia coli* CheA Kinase That Blocks Stimulatory, but Not Inhibitory, Chemoreceptor Signaling

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CheA, a cytoplasmic histidine autokinase, in conjunction with the CheW coupling protein, forms stable ternary complexes with the cytoplasmic signaling domains of transmembrane chemoreceptors. These signaling complexes induce chemotactic movements by stimulating or inhibiting CheA autophosphorylation activity in response to chemoeffector stimuli. To explore the mechanisms of CheA control by chemoreceptor signaling complexes, we examined the ability of various CheA fragments to interfere with receptor coupling control of CheA. CheA[250-654], a fragment carrying the catalytic domain and an adjacent C-terminal segment previously implicated in stimulatory control of CheA activity, interfered with the production of clockwise flagellar rotation and with chemotactic ability in wild-type cells. Epistasis tests indicated that CheA[250-654] blocked clockwise rotation by disrupting stimulatory coupling of CheA to receptors. In vitro coupling assays confirmed that a stoichiometric excess of CheA[250-654] fragments could exclude CheA from stimulatory receptor complexes, most likely by competing for CheW binding. However, CheA[250-654] fragments, even in vast excess, did not block receptor-mediated inhibition of CheA, suggesting that CheA[250-654] lacks an inhibitory contact site present in native CheA. This inhibitory target is most likely in the N-terminal P1 domain, which contains His-48, the site of autophosphorylation. These findings suggest a simple allosteric model of CheA control by ternary signaling complexes in which the receptor signaling domain conformationally regulates the interaction between the substrate and catalytic domains of CheA.

CheA, a cytoplasmic histidine autokinase, plays a central role in the chemotactic signaling pathway of *Escherichia coli*. CheA autophosphorylates (14) and then donates its phosphoryl groups to two aspartate autokinases, CheY and CheB, which in turn control flagellar rotation (5, 39) and sensory adaptation (21). The CheW protein couples CheA to the cytoplasmic signaling domains of membrane-bound chemoreceptors, forming a stable ternary complex (6, 13, 19). These chemoreceptor complexes govern chemotactic behavior by modulating the autophosphorylation activity of CheA in response to attractant and repellent stimuli (7, 28).

Ternary receptor complexes exhibit two signaling modes, one that augments clockwise (CW) flagellar rotation in vivo and stimulates CheA autophosphorylation in vitro, and another that augments counter-clockwise (CCW) flagellar rotation in vivo and inhibits CheA autophosphorylation in vitro. Soluble fragments of the serine chemoreceptor, Tsr, exhibit comparable signaling activities (2). Tsr fragments locked in the CW mode stimulate CheA autophosphorylation approximately 100-fold, whereas fragments locked in the CCW mode reduce CheA autophosphorylation to about 20% the rate of CheA alone. CheA stimulation by CW-signaling Tsr fragments is CheW dependent, as is formation of stimulatory signaling complexes by native Tsr molecules. In contrast, CheA inhibition by CCW Tsr fragments is CheW independent, implying that receptors in the CCW-signaling conformation make direct contact with CheA to inhibit the autophosphorylation reaction (2). Identification of the CheA contact site(s) involved in receptor-mediated inhibition could shed important light on the mechanisms of CheA control by receptor signaling complexes.

A variety of structure-function studies have shown that CheA is a modular protein (Fig. 1). The catalytic, or transmitter (T), domain is flanked on the N-terminal side by two domains (P1 and P2) involved in phosphotransfer operations. P1 contains the autophosphorylation site (His-48) (14), whereas P2 contains binding sites for CheB and CheY that facilitate the subsequent phosphotransfer reactions (17, 24, 35). The C-terminal third of CheA contains two regions (M and C) needed for chemoreceptor control (9, 18). Truncated CheA molecules lacking this region can autophosphorylate but are not subject to stimulatory control by chemoreceptors and CheW (9). Missense mutations in the MC region also alter the ability of CheA to respond to receptor control (18, 31).

It seems likely, but is not yet proven, that the MC segment of CheA contains CheW binding determinants essential for formation of ternary signaling complexes. If the MC segment is also involved in inhibitory receptor coupling, it might make direct contact with the receptor signaling domain. We investigated the signaling roles of the MC segment of CheA by examining the ability of MC-containing CheA fragments to interfere with stimulatory or inhibitory receptor coupling. We reasoned that MC polypeptides able to interact with coupling targets in CheW or the receptor should compete with native CheA for those interactions. In vivo, such competition might disrupt chemotaxis; in vitro, it might alter the receptor-coupled control of CheA activity. In this report, we demonstrate that a polypeptide corresponding to the T, M, and C domains of CheA was able to block stimulatory receptor coupling in vivo and in vitro, suggesting that it contains one or more of the contact sites needed for stimulatory complex formation. However, this fragment failed to affect inhibitory coupling, implying that other parts of the CheA molecule are involved in receptor-mediated inhibition of CheA. Based on these findings, we suggest a simple allosteric model of CheA control in receptor signaling complexes.

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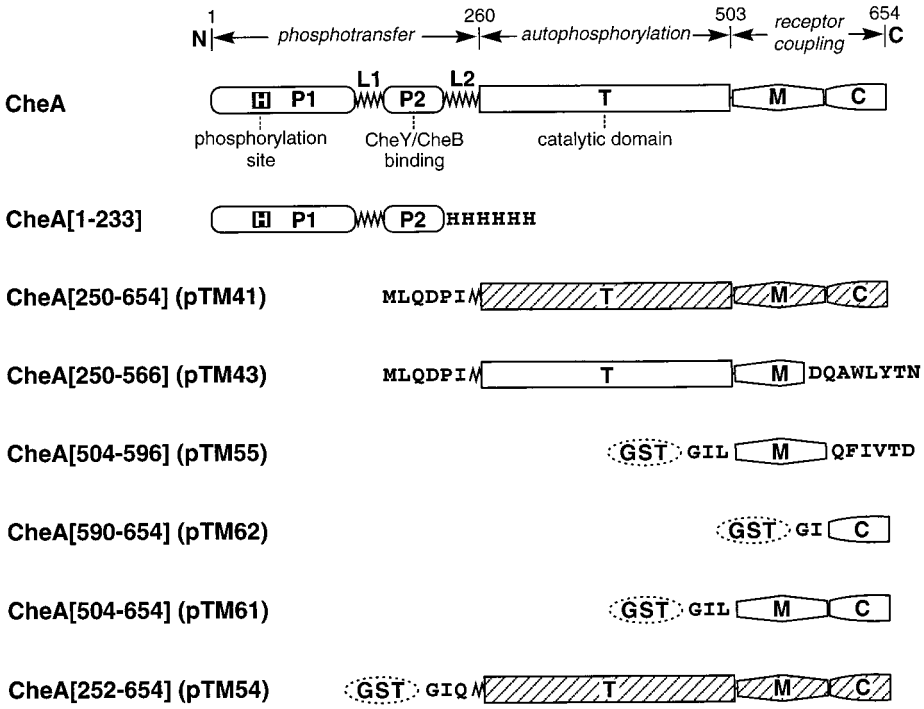

FIG. 1. CheA fragments used in this work. The domain organization of CheA is shown at the top with amino acid coordinates for the boundaries of each functional region. P1, P2, T, M, and C correspond to discrete structural or functional domains; L1 and L2 are flexible linkers (26). Amino acids at the N or C termini of various fragment constructs that are not present in native CheA are indicated by single-letter designations. GST is the GST domain present in GST-CheA fusion constructs. The two cross-hatched fragments strongly interfered with the chemotactic ability of RP437 and were the principal subjects of this study. Names of the corresponding fragment-producing plasmids are listed in parentheses. The plasmids were constructed by subcloning portions of the *cheA* coding region into an IPTG-inducible expression vector as follows: pTM41, codons 250 to 654 in an *Eag*I/S1 nuclease-*Pvu*I fragment from pJL153 (19) inserted into pTM30 cut with *Eco*RV and *Pvu*I; pTM43, codons 250 to 566 in an *Xmn*I fragment from pTM41 inserted into pTM30 cut with *Eco*RV and *Xmn*I. GST fusions were made by PCR amplification of *cheA* sequences in pJL153 and insertion into pGEX-3X cut with *Eco*RI and *Bam*HI.

MATERIALS AND METHODS

Bacterial strains. RP437, an *E. coli* K-12 strain wild type for chemotaxis (30), was used to assess the ability of CheA-fragment plasmids to interfere with chemotactic ability. Derivatives of RP437 with multiple chemotaxis defects for epistasis studies are listed in the legend to Fig. 3. RP3098 [Δ(*flhD-flhA*)*4*] (33) was used to synthesize proteins from expression plasmids for subsequent purification.

Plasmids. Fragments of the *cheA* coding region were cloned and expressed in pTM30 (24, 26), which contains a multiple cloning site flanked on one side by the ribosome-binding site and translational start of the *cheY* gene (22) and on the other side by TAA stop codons in all reading frames. In-frame inserts produce polypeptide products with different N- and C-terminal residues specified by codons in the vector, depending on the point of insertion and the exiting reading frame. Transcription of insert fragments is driven by a p_{tac} promoter, which is in turn controlled by lactose repressor expressed constitutively from a *lacI*^{q} gene also carried on the plasmid. The pGEX-3X plasmid (Pharmacia) was used as the expression vector for CheA fragments fused to the glutathionine *S*-transferase (GST) domain. Cells carrying pTM30, pGEX-3X, or one of their derivatives were identified and maintained by selection for resistance to ampicillin at 50 to 100 μg/ml. The CheA fragment plasmids used in this work are listed in Fig. 1; details of their construction are given in the legend to Fig. 1.

Behavioral assays. Chemotactic ability was assessed by rate of colony expansion on semisolid tryptone swarm agar (29). Flagellar rotation patterns were determined by cell tethering as described previously (29). Measurements of flagellar motor torque output were made by determining the rotation rates of tethering, plasmid-containing cells. To eliminate reversals, which confound measurements of rotational speed, plasmids were introduced into strain RP4160, which has a chemoreceptor alteration (*tsr-192*) that locks signal output in the CCW mode (1). Torque calculations were performed as described previously (37, 38).

Measurement of CheA[250-654] expression levels. Strain RP437 carrying plasmid pTM41 was grown to mid-log phase at 30°C in tryptone broth containing 50 μg of ampicillin per ml and various concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were lysed by boiling for 10 min in 3 volumes of sample loading buffer (15), and the cell extracts were subjected to electrophoresis in sodium dodecyl sulfate-containing 15% polyacrylamide gels (SDS-PAGE). Gels were blotted to polyvinylidine difluoride membranes to visualize the CheA and CheA[250-654] bands by immunoblotting, using a monoclonal antibody directed against the T domain of CheA (CA2.62.2; kindly supplied by Phil Matsumura, University of Illinois Medical School), followed by a rat anti-mouse immunoglobulin antibody and ^{35}S-labeled protein A (Amersham). The amount of radiolabeled CheA and CheA[250-654] in each lane was measured with a Molecular Dynamics PhosphorImager.

Purification of CheA[252-654]. Strain RP3098 carrying plasmid pTM54 (GST-CheA[252-654]) was grown at 37°C to ~5×10^8 cells/ml in 1 liter of Luria broth containing 100 μg of ampicillin per ml. IPTG was added to a final concentration of 40 μM to induce expression of the GST-CheA fusion protein, and the cells were incubated at 37°C for 6 h. Cells were harvested by centrifugation, and the cell pellets were resuspended in 20 ml of TEDG-10 (50 mM Tris [pH 7.5], 0.5 mM EDTA, 2 mM dithiothreitol [DTT], 10% glycerol) plus 1 mM phenylmethylsulfonyl fluoride and 1 mM phenanthroline. Cells were disrupted in a French press, and 4 ml of deoxycholate mixture (0.2 M NaCl, 1% [wt/vol] deoxycholate, 1% [vol/vol] Nonidet P-40, 20 mM Tris [pH 7.5], 2 mM EDTA) was added. Inclusion bodies containing the GST-CheA fusion protein were pelleted at 5,000 × *g* for 10 min, washed with 9 volumes Triton-X mixture (0.5% Triton X-100, 10 mM EDTA), and then pelleted at 5,000 × *g* for 10 min (27). Inclusion body material was solubilized in 40 ml of urea mixture (8 M ultrapure urea, 10 mM EDTA, 50 mM Tris [pH 7.5], 10 mM DTT). Proteins were renatured by dialysis twice against 1 liter of phosphate-buffered saline (10 mM Na_2HPO_4, 10 mM NaH_2PO_4 [pH 7.2])–2 mM DTT, and insoluble matter was removed by centrifugation at 100,000 × *g*. Factor Xa (Promega) was added to the solubilized sample in a 50:1 weight ratio in 25 mM Tris (pH 8.0)–1 mM $CaCl_2$ and incubated for 2 h at room temperature. Digested proteins were allowed to bind to glutathione-Sepharose matrix (Pharmacia) for 2 h at 4°C and then poured onto a 15-ml disposable column (Bio-Rad). The flowthrough volume plus 1 additional column volume of a phosphate-buffered saline–DTT wash was collected. The CheA[252-654] polypeptide was denatured by addition of 4 M ultrapure urea and renatured by dialysis twice against 1 liter of TEDG-10. Small amounts of contaminants were removed by applying the renatured CheA[252-654] sample to an Affi-Gel Blue column, washing with 500 mM NaCl, then eluting with a 0.5 to 2 M NaCl linear gradient. Pooled fractions were concentrated by Centra-Prep 10s and dialyzed against TEDG-10. Protein concentration was determined by the Bradford assay.

Phosphorylation assays. Purified CheA and CheW were kindly provided by Peter Ames (University of Utah) (2). Purified CheA[1-233] was kindly provided by Ron Swanson (Recombinant BioCatalysis). Tsr-containing membranes for coupling assays were prepared from cells of strain RP3098 carrying plasmid pJC3 (*tsr*$^+$) (10), pJC3 *tsr-1006* (AV413) (1, 2), or pTM30 (vector control) essentially as described previously (8). [γ-^{32}P]ATP was purchased from New England Nuclear. Conditions and reactant concentrations for particular experiments are given in the legends to Fig. 4 to 7. In all cases, reaction products were separated by SDS-PAGE, and the distribution of radiolabel was quantified with a Molecular Dynamics PhosphorImager.

RESULTS

Fragments of the CheA coupling region that interfere with chemotaxis. Plasmid pTM41 was generated in the course of a domain liberation study of CheA (26). It carries codons 250 to 654 of the *cheA* coding region, encompassing the T, M, and C domains (Fig. 1), inserted at the expression site of pTM30, a cloning vector that supplies an IPTG-inducible promoter and efficient translation initiation signals (24). Wild-type strains containing pTM41 failed to form chemotactic swarms on semisolid agar plates containing IPTG, suggesting that a TMC polypeptide from CheA interferes with chemotaxis. To identify the portion of the TMC region responsible for this interference, we constructed inducible plasmids expressing various segments of the TMC region (Fig. 1) and tested them for the ability to block chemotactic swarming of a wild-type host. Plasmid pTM43, which encodes CheA[250-566] (corresponding to T and part of M), interfered only slightly, even though upon induction it made high levels of the CheA fragment that it encodes (data not shown). Purified CheA[250-566] fragments catalyzed transphosphorylation of the CheA[1-233] fragment (data not shown), indicating that CheA[250-566] retains native structure and function. Thus, some portion of the MC region of CheA[250-654] is needed for its interference effect.

Attempts to express coding segments from the MC portion of CheA in pTM30 were unsuccessful (data not shown), suggesting that M- or C-containing polypeptides are unstable in the absence of an adjoining T domain. However, we were able to express polypeptides corresponding to the M (CheA[504-596]), C (CheA[590-654]), and MC (CheA[504-654]) segments in the form of GST fusion proteins (Fig. 1). The fusion proteins could be expressed at high levels in wild-type cells, migrated with the expected mobilities through SDS-PAGE, and reacted with CheA antisera (data not shown). But unlike CheA[250-654], the fusion proteins did not interfere with chemotaxis (data not shown), suggesting that their M, C, or MC component, even though protected from proteolysis, might not retain its native structure or function in the presence of the adjoining GST domain. Conceivably, a structural or functional interaction between the T and MC components in the CheA[250-654] fragment is responsible for its unique interference properties. Accordingly, the in vivo interference effects of CheA[250-654] and the corresponding in vitro effects of a closely related TMC fragment (CheA[252-654]) (Fig. 1) were characterized in detail to probe the intermolecular contacts involved in receptor-mediated control of CheA.

Behavioral effects of the CheA[250-654] fragment. The chemotactic behaviors of derivatives of strain RP437 carrying pTM41 (TMC fragment) and pTM30 (vector control) were compared at different concentrations of the inducer IPTG (Fig. 2). Unlike the control, pTM41 produced an IPTG-dependent reduction in chemotactic swarming on soft-agar medium (Fig. 2A). This progressive interference with chemotactic ability was accompanied by a corresponding increase in the intracellular level of the TMC fragment (Fig. 2A). At full induction (1 mM IPTG), swarming was reduced to about 25% of the control rate and TMC fragments were made in nearly 30-fold

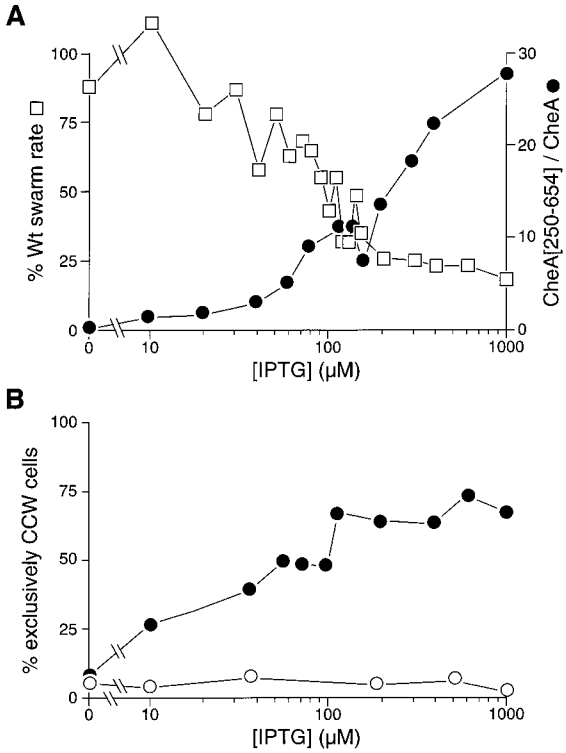

FIG. 2. Behavioral effects and expression levels of CheA[250-654]. All experiments were performed with strain RP437 carrying pTM41 (CheA[250-654] plasmid) or pTM30 (vector only; wild-type [wt] control). (A) Normalized rate of swarm expansion on semisolid tryptone agar at 30°C and expression level of the pTM41-encoded CheA[250-654] fragment relative to that of native CheA produced from the chromosome. (B) Flagellar rotation patterns of RP437/pTM41 (filled circles) and RP437/pTM30 (open circles). Cells were grown in tryptone broth at 30°C and tethered with flagellar antibody. For each strain, 100 rotating cells were observed for 15 s each, and the proportion of cells that rotated exclusively CCW during the observation period was determined.

excess of the chromosomally encoded CheA molecules. The level of expression of TMC fragments also correlated with a change in the flagellar rotation pattern of the cells (Fig. 2B). Upon induction with IPTG, cells containing pTM41 became increasingly CCW biased, showing fewer and fewer episodes of CW rotation (Fig. 2B). Because frequent CW reversals are essential for chemotaxis, the CCW shift caused by pTM41 is probably responsible for its interference effect on chemotaxis.

To identify the in vivo target for TMC interference, we first determined whether pTM41 caused a general decline in cell physiology that might lead to reduced chemotactic ability. Cells were grown in the presence of 200 μM IPTG, which produces a nearly maximal interference effect (Fig. 2A), and examined for changes in growth rate or motility that could account for slower swarming. We found that the experimental (RP437/pTM41) and control strains (RP437/pTM30) had similar doubling times and flagellar torque outputs (data not shown), indicating that the TMC fragment has no appreciable effect on growth rate or motor function. We conclude that the TMC fragment produced by pTM41 most likely interferes with swarming by competitively inhibiting protein-protein contacts required for chemotactic signaling and/or CW flagellar rotation.

Signaling targets of CheA[250-654] interference. The CCW rotational bias associated with pTM41 was used to identify the signaling step(s) at which the TMC fragment acts. In principle, the CCW bias could arise in at least four different ways: (i) by preventing the stimulation of CheA by ternary receptor com-

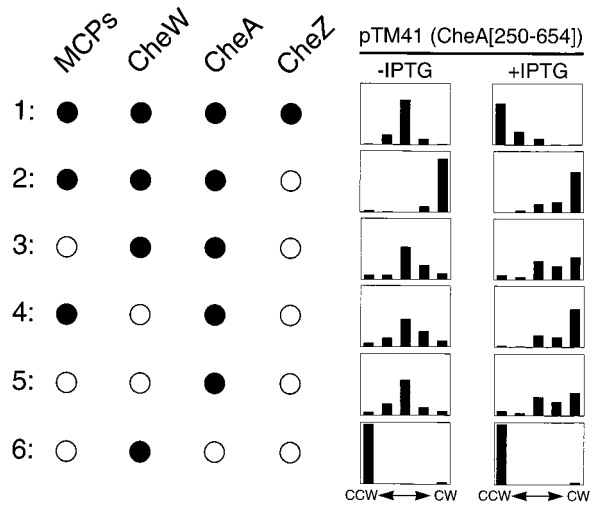

FIG. 3. Epistatic analysis of CheA[250-654]-mediated interference with CW flagellar rotation. Strains containing pTM41 were grown in tryptone broth at 30°C and tethered with flagellar antibody for rotational analysis. At least 100 rotating cells were observed for 15 s each and classified into one of five categories, from exclusively CCW to exclusively CW (19). The rotational profiles are presented as histograms showing the relative proportion of cells in each rotational category when grown with no IPTG (−IPTG) or with 1 mM IPTG (+IPTG). Each line represents the behavior of a strain with a different combination of the signaling components discussed in the text. Open circles denote missing components; filled circles denote components that were present. Strains: (1) RP437 (30); (2) RP9349 [Δ(*cheZ*)*6725*] (26); (3) RP9352 [Δ(*tsr*)*7028* Δ(*tar-tap*)*5201* Δ(*trg*)*100* Δ(*cheZ*)*6725*] (19); (4) RP9351 [Δ(*cheW-tap*)*2217* Δ(*cheZ*)*6725*] (19); (5) RP9411 [Δ(*tsr*)*7028* Δ(*trg*)*100* Δ(*cheW-tap*)*2217* Δ(*cheZ*)*6725*] (19); (6) RP9543 [Δ(*cheA*)*1643* Δ(*tsr*)*7028* Δ(*trg*)*100* Δ(*tar-tap*)*5201* Δ(*cheZ*)*6725*] (24).

plexes; (ii) by blocking phosphotransfer between CheA and CheY; (iii) by disrupting the interaction of phospho-CheY with the flagellar switch; or (iv) by augmenting the dephosphorylation of phospho-CheY, for example, by stimulating CheZ activity. To determine which of these signaling steps was the target of TMC interference, we examined the ability of pTM41 to cause a CCW bias in strains lacking various combinations of signaling components but still able to produce detectable levels of CW rotation (Fig. 3). For example, strains that lack CheZ have an extremely high CW bias due to excessive steady-state levels of phospho-CheY. Expression of the TMC fragment in a $CheZ^-$ strain caused a modest CCW shift (Fig. 3, line 2), indicating that CheZ was not essential for the TMC interference effect.

To determine whether TMC interfered with stimulatory receptor coupling, hosts lacking chemoreceptors and/or the CheW coupling factor were tested. Ordinarily, such strains have a high CCW bias because they cannot form stimulatory ternary complexes. However, when such strains also have a CheZ defect, the autophosphorylation activity of uncoupled CheA molecules is sufficient to produce a substantial level of CW rotation (Fig. 3, lines 3 to 5). Induction of pTM41 in these strains failed to produce a CCW shift (Fig. 3, lines 3 to 5), indicating that the CCW TMC effect might disrupt the stimulation of CheA by ternary signaling complexes. These results suggest that the TMC fragment can titrate one or more of the binding interactions that form ternary signaling complexes. However, these experiments also revealed another TMC behavioral effect. Upon induction, pTM41 not only failed to cause a CCW bias but actually produced a substantial CW shift (Fig. 3, lines 3 to 5). Thus, TMC fragments enhanced CW flagellar rotation in cells lacking the ability to stimulate CheA through receptor coupling. This CW effect of TMC evidently depends on CheA function, because it did not occur in a strain lacking CheA in addition to chemoreceptors and CheZ (Fig. 3, line 6).

Catalytic activity and receptor control of CheA[252-654]. The in vivo results indicated that CheA[250-654] has two effects on chemotactic signaling. First, it appears to disrupt stimulatory coupling of chemoreceptors to CheA, perhaps by competing with CheA for a component involved in ternary complex formation. Second, in cells containing uncoupled CheA molecules, CheA[250-654] causes a CheA-dependent increase in CW flagellar signaling. This CW effect could be due either to stabilization of phospho-CheY or, more likely, to enhancement of CheA autokinase activity, perhaps by providing additional catalytic centers for the autophosphorylation reaction. To elucidate the effects of TMC fragments on CheA and its receptor-mediated control, we assayed the biochemical properties of a TMC fragment in vitro. The fragment studied, CheA[252-654], was expressed and purified in the form of a GST-TMC fusion protein but, after removal of the GST domain, differed only slightly from the CheA[250-654] fragment used in the in vivo experiments.

The purification of the CheA[252-654] fragment involved denaturation and renaturation steps that could have resulted in nonnative final structure (see Materials and Methods). To assess the functionality of the purified TMC fragment, we measured its catalytic activity, using the CheA[1-233] fragment as a phosphorylation substrate. At saturating levels of ATP, the wild-type CheA autophosphorylation reaction follows pseudo-first-order kinetics. The transphosphorylation of CheA[1-233] by CheA[252-654] also followed pseudo-first-order kinetics, with a rate of 6.6×10^{-4} s^{-1} (Fig. 4A). The bimolecular transphosphorylation reaction was, not surprisingly, several orders of magnitude slower than the intramolecular CheA reaction (Fig. 5A). Nevertheless, this result shows that the CheA[252-654] fragment survived the purification regimen with its catalytic activity intact.

Coupling assays with membrane-embedded receptor molecules showed that the catalytic activity of CheA[252-654], as assayed by transphosphorylation of CheA[1-233], was also subject to both stimulatory and inhibitory control by receptors (Fig. 4). To test for inhibitory control, we used Tsr molecules with the AV413 replacement, which locks the receptor in a CCW-signaling mode (1, 2). Under assay conditions that produced a 10-fold inhibition of CheA autophosphorylation rate (see below), these mutant receptors reduced the transphosphorylation activity of CheA[252-654] about 11-fold (Fig. 4A). Thus, CheA[252-654] and CheA[1-233] together have the contact site(s) needed for inhibitory control by Tsr-AV413. We used assays with wild-type Tsr molecules and CheW to test for stimulatory control. The reactant stoichiometries were adjusted so that the unstimulated CheA[252-654] × CheA[1-233] transphosphorylation rate was virtually undetectable (data not shown). Under these conditions, CheW and Tsr stimulated the transphosphorylation reaction over 500-fold (Fig. 4B). Addition of serine, which attenuates CheA stimulation by Tsr signaling complexes, reduced the transphosphorylation rate about sixfold (Fig. 4B), demonstrating that the receptor-stimulated CheA[252-654] fragments were also subject to chemoeffector control.

Effect of CheA[252-654] on uncoupled CheA. CheA functions as a dimer, with the critical dimerization determinants located in the catalytic (T) domain (12, 16, 34). In cells containing wild-type CheA and a molar excess of TMC fragment, most of the full-length CheA subunits should reside in heterodimers with TMC. To evaluate the catalytic activity of such

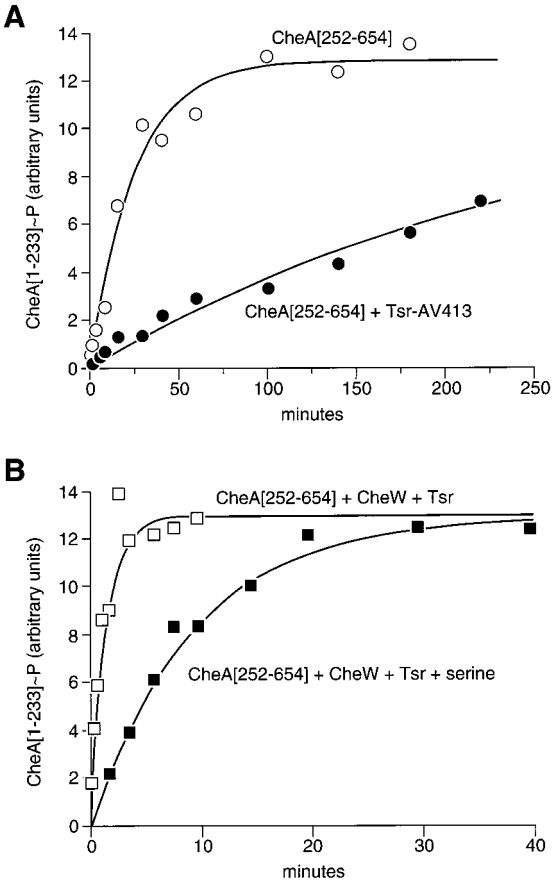

FIG. 4. Receptor-mediated control of CheA[252-654] transphosphorylation activity. Note that the time scales in panels A and B are different. (A) Inhibitory coupling reactions contained 10 μM CheA[1-233], 10 μM CheA[252-654], and either membranes containing 10 μM Tsr-AV413 (filled circles) or an equivalent amount of control membranes containing no methyl-accepting chemotaxis proteins (open circles). Components were incubated at room temperature for 60 min in TKMD buffer (2) prior to initiation of the reaction by the addition of [γ-^{32}P]ATP to a final concentration of 5 mM. The total reaction volume was 35 μl; 2-μl samples were removed at various times and mixed with 6 μl of SDS-PAGE loading buffer to stop the reaction. Lines connecting the data points represent nonlinear least-squares best fits to the equation: fraction phosphorylated = $1 - e^{-kt}$, where t is reaction time in minutes and k is the pseudo-first-order rate constant for the reaction. (B) Stimulatory coupling assays were performed and analyzed as for panel A except that reactions contained 5 μM CheA [1-233], 0.5 μM CheA[252-654], 2 μM CheW, and membranes containing 2 μM Tsr. Reactions were performed with (filled squares) and without (open squares) 1 mM serine to demonstrate that the stimulatory complexes respond to attractant ligand by down-regulating transphosphorylation activity.

heterodimers, we measured the pseudo-first-order rate of CheA autophosphorylation in the presence of increasing amounts of CheA[252-654] fragments. High levels of TMC fragments reduced the rate of CheA autophosphorylation about 3-fold at the highest concentration tested (350-fold molar excess over CheA) (Fig. 5A) and caused a concomitant increase (about 3.5-fold) in the steady-state levels of CheA phosphate attained in the reactions (Fig. 5B). The increase in steady-state level could account for the enhanced CW rotation of cells expressing the CheA[250-654] fragment. The asymmetric reactivity of phosphorylation sites in CheA heterodimers (16) may account for the increased levels of phospho-CheA in the presence of the CheA[252-654] fragment.

Effect of CheA[252-654] on stimulatory control of CheA. Because transphosphorylation of CheA[1-233] by CheA[252-654] was stimulated by Tsr and CheW (see above), it seemed likely that TMC fragments would interfere with stimulatory coupling of wild-type CheA. To monitor the receptor-stimulated CheA reaction, we used two variants of the standard coupling assay (0.5 μM CheA, 4 μM CheW, and 4 μM Tsr in membranes). A pilot experiment used saturating levels of radiolabeled ATP (5 mM) and a stoichiometric excess of CheY (15 μM) to serve as a sink for CheA-generated phosphoryl groups (8). Under these assay conditions, 30 μM CheA[252-654] reduced the level of phospho-CheY at the 5-s time point to 20% of that in the control reaction with no TMC fragment (data not shown). To measure the stimulated rate of CheA autophosphorylation more directly, we omitted CheY and used a limiting concentration of ATP (40 nM) to retard the coupled reaction. Under these conditions, CheA[252-654] slowed the initial rate of phospho-CheA production up to 10-fold (Fig. 6). Although the ATP concentration in this second experiment was much less than the concentration of CheA[252-654], it is unlikely that the TMC fragment could sequester enough ATP to account for the effect seen. The affinity of CheA for ATP is about 300 μM (36). If CheA[252-654] has a similar affinity for ATP, less than 10% of the ATP in the reaction would be bound to the fragment. Thus, the results from both coupling experiments are consistent with the principal in vivo effect of CheA [250-654], namely, an augmentation of CCW flagellar rotation by interfering with the receptor-mediated stimulation of CheA.

Effect of CheA[252-654] on inhibitory control of CheA. Wild-type CheA was mixed with membranes containing Tsr-AV413 at stoichiometries that produced a 10-fold inhibition of CheA

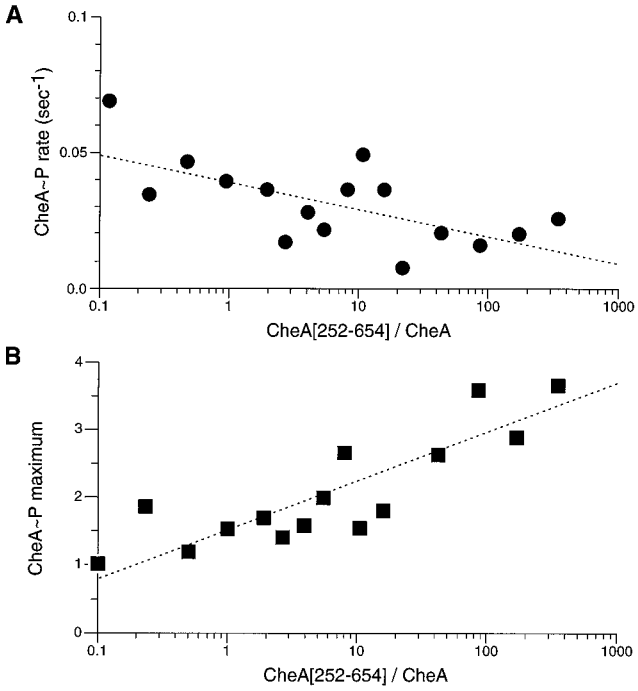

FIG. 5. Effect of CheA[252-654] on uncoupled CheA autophosphorylation activity. The assays followed the general scheme of those described in the legend to Fig. 4, with the following exceptions. Mixtures containing 0.5 μM CheA and variable amounts of CheA[252-654] were preequilibrated at room temperature for 15 min prior to the addition of [γ-^{32}P]ATP to initiate the reaction. For each CheA[252-654] concentration, a pseudo-first-order rate constant for CheA autophosphorylation was determined as described for Fig. 4. (A) Effect of CheA [252-654] on CheA autophosphorylation rate. (B) Effect of CheA[252-654] on the steady-state level of CheA phosphate attained in the reactions. Dashed lines show linear regression fits to the data points.

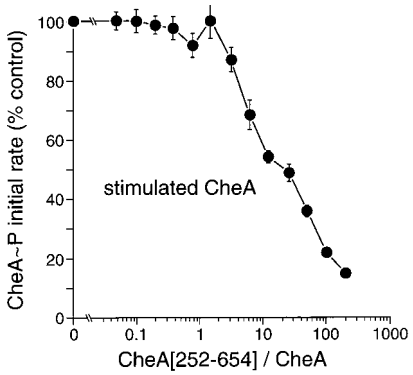

FIG. 6. Effect of CheA[252-654] on receptor-mediated stimulation of CheA autophosphorylation activity. The assay followed the general scheme of that described for Fig. 4B, with the following exceptions. Reaction mixtures contained 0.5 μM CheA, 4 μM CheW, 4 μM Tsr in membranes, and variable amounts of CheA[252-654] in a total volume of 20 μl. After 60 min of incubation at room temperature, reactions were initiated by the addition of [γ-^{32}P]ATP to a final concentration of 40 nM. The reaction was sampled at 5-s intervals for the first 30 s, and at each of the six time points, the ratio of phospho-CheA (CheA~P) formed in the presence and absence of CheA[252-654] was determined. The average (filled circles) and standard error (vertical bars) of these values are shown for each CheA[252-654] level tested in the experiment.

autophosphorylation activity (Fig. 7). Increasing amounts of CheA[252-654], up to 200-fold molar excess relative to CheA, were added to the reactions to look for changes in the extent of CheA inhibition. We reasoned that if the TMC fragment could contact the inhibitory site(s) in the receptor molecules, which seemed likely because its transphosphorylation activity was subject to inhibitory control, then CheA[252-654] should compete with CheA for those inhibitory sites. This competition would manifest itself as an increased rate of CheA autophosphorylation as more and more CheA molecules were excluded from binding to the inhibitory receptor molecules. Surprisingly, this did not happen. CheA[252-654] failed to alleviate CheA inhibition at any concentration tested (Fig. 7). We conclude from this result that the TMC segment of CheA lacks an interaction site needed for receptor-mediated inhibition of CheA. In Discussion, we consider the implications of this finding for the mechanism(s) of receptor-mediated control.

DISCUSSION

Protein-protein contacts in receptor signaling complexes. Chemoreceptors coupled to CheA exhibit two discrete signaling states, a CW mode that stimulates CheA autophosphorylation and a CCW mode that inhibits CheA autophosphorylation. The signaling domains of the receptors form stable ternary complexes with CheA and the CheW coupling factor to achieve this control. The long lifetime of these complexes (13) relative to the time scale of signaling events (32) implies that the mechanism of CheA control is an allosteric one in which the receptor, possibly assisted by CheW, manipulates CheA conformation to regulate its autophosphorylation rate. Thus, the contacts that CheA makes with chemoreceptors and the CheW coupling factor should play an important role in the control of CheA activity. The results of this study, in conjunction with recent work by others, provide tentative locations for several control sites in the CheA molecule.

CheA interacts directly with CheW (12, 23), which in turn interacts with the receptor signaling domain (13, 20). Thus, CheW physically couples CheA to the receptors to form the stable ternary complex, and CheW is essential for stimulatory control of CheA activity. However, receptors or receptor fragments in the CCW signaling mode do not require CheW to inhibit CheA, indicating that CheA also makes direct contacts with receptor molecules, at least in the inhibited conformation (2). The CheA determinants involved in interaction with CheW have not been identified, but important ones could reside in the MC region, which is essential for stimulatory control by receptors (9). In contrast, CheA molecules lacking the MC region are still subject to receptor-mediated inhibition, demonstrating that one or more sites of direct receptor contact must reside elsewhere in CheA (3).

Expression of TMC fragments of CheA in a wild-type cell caused a decrease in CW rotation and a corresponding loss of chemotactic ability, apparently resulting from interference of TMC with receptor-mediated stimulation of CheA. An excess of TMC fragments also blocked stimulation of CheA by CheW and CW-signaling receptors in vitro. TMC most likely blocks receptor-mediated stimulation of CheA by competing for CheW binding, thereby excluding CheA from ternary signaling complexes. This effect seems to depend on both the T and MC portions of the fragment, because neither alone was able to disrupt chemotactic ability. It may be that proper folding of the MC region requires a T domain in the same polypeptide or that binding of CheW requires structural determinants from both domains.

In coupling assays with CCW-signaling receptors, TMC fragments failed to block receptor-mediated inhibition of CheA, implying that they lack a site involved in inhibitory control. Nevertheless, the transphosphorylation activity of TMC fragments, assayed with a P1-containing substrate domain, was subject to inhibition by CCW receptors, indicating that the combination of P1 and TMC domains contains all sites needed for inhibition. Taken together, these findings suggest that the receptors contact the P1 domain of CheA during inhibitory control. However, P1 fragments alone do not interfere with chemotactic ability (11), nor do they disrupt inhibitory receptor control in vitro (25). These results suggest that CCW-signaling receptors also make inhibitory contacts with another

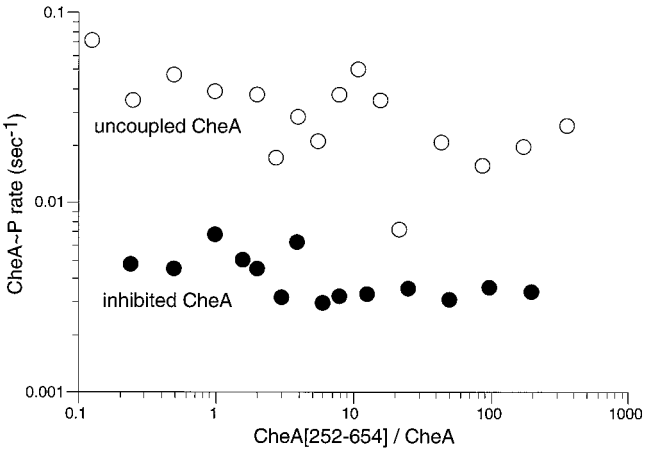

FIG. 7. Effect of CheA[252-654] on receptor-mediated inhibition of CheA autophosphorylation. The assay followed the same scheme as for Fig. 4A, with the following exceptions. Reactions contained 0.5 μM CheA, 10 μM Tsr-AV413 in membranes, and various amounts of CheA[252-654] in a total volume of 30 μl. The mixtures were preequilibrated at room temperature for 60 min prior to addition of [γ-^{32}P]ATP to a final concentration of 5 mM. For each CheA [252-654] concentration used in the experiment, a pseudo-first-order rate constant for CheA autophosphorylation was determined as described for Fig. 4. The filled circles present the rate constants for receptor-inhibited CheA. For comparison, the open circles show the corresponding rate constants for uncoupled CheA taken from Fig. 5A.

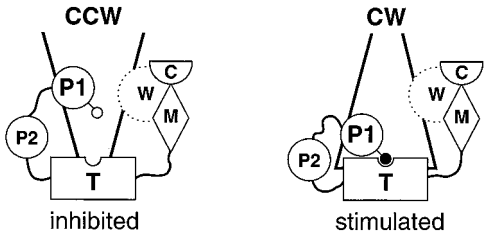

FIG. 8. Model for allosteric control of CheA in receptor signaling complexes. CheA interacts indirectly with the signaling domain of the receptor through CheW and directly through binding contacts to the P1 (substrate) and T (catalytic) domains of CheA. The model proposes that these direct contacts manipulate the P1-T geometry in response to conformational changes in the receptor signaling domain to hinder or facilitate the autophosphorylation reaction of CheA. Although receptor and CheA molecules are dimeric, these and possibly higher-order assemblies are not depicted because they are not central to the model.

part of the CheA molecule. Because the MC segment plays no role in CheA inhibition (3), these additional inhibitory control sites most likely reside in the T domain.

CheA control in receptor signaling complexes. The possibility that the receptor signaling domain makes direct contact with the P1 segment of CheA suggests a simple allosteric mechanism for CheA control in receptor signaling complexes. The CheA autophosphorylation reaction requires proper interaction of the His-48 substrate site in the P1 domain with the catalytic center in the T domain. These domains are connected through flexible linkers that undoubtedly permit collisional encounters in many orientations, only a fraction of which are productive. If proper P1-T interaction is the rate-limiting step in the CheA autophosphorylation reaction, the receptor signaling complex could regulate CheA autophosphorylation simply by controlling P1-T encounters through direct contacts to both domains (Fig. 8).

We propose that in the CCW-signaling conformation, the receptor signaling domain prevents productive interactions between the CheA substrate and catalytic sites. The fact that both sets of binding contacts seem to be needed for inhibition implies that the receptor does not block autophosphorylation by simply occluding one of the reaction partners. Rather, it probably constrains their relative orientation, for example, by holding the P1 and T domains apart. Although CheW also makes binding contacts to CheA in ternary signaling complexes, those interactions play no role in CheA inhibition (2).

The receptor-CheA contacts responsible for inhibition could also participate in stimulating CheA activity. We propose that in the CW conformation, the receptor signaling domain brings P1 close to T in the proper orientation for the autophosphorylation reaction (Fig. 8). Simply establishing a favorable P1-T geometry could account for the CheA stimulation of ternary signaling complexes. However, unlike inhibitory control, CheA stimulation requires CheW as well. CheW might serve to stabilize the CheA-receptor association through contacts to both of them, but it might play a more important role in CW signaling. For example, CheW might help to stabilize the CW conformation of the receptor signaling domain through additional direct contacts (not shown in the model in Fig. 8).

This allosteric control model makes some obvious experimental predictions. For example, several different types of receptor mutations should exist that prevent CheA inhibition, including ones that lock the receptor in a CW-signaling conformation and others that specifically block binding of the receptor to the P1 or T domains of CheA. If the same contact sites are used for both modes of CheA control, the latter class of receptor mutants should also be defective in CheA stimulation, whereas the former should not. We are currently using fragments of the Tsr signaling domain (2, 4) to test this model of receptor signaling.

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