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Chemotactic Signaling by the P1 Phosphorylation Domain Liberated from the CheA Histidine Kinase of *Escherichia coli*

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CheA is a histidine kinase central to the signal transduction pathway for chemotaxis in *Escherichia coli*. CheA autophosphorylates at His-48, with ATP as the phosphodonor, and then donates its phosphoryl groups to two aspartate autokinases, CheY and CheB. Phospho-CheY controls the flagellar motors, whereas phospho-CheB participates in sensory adaptation. Polypeptides encompassing the N-terminal P1 domain of CheA can be transphosphorylated in vitro by the CheA catalytic domain and yet have no deleterious effect on chemotactic ability when expressed at high levels in wild-type cells. To find out why, we examined the effects of a purified P1 fragment, CheA[1-149], on CheA-related signaling activities in vitro and devised in vivo assays for those same activities. Although readily phosphorylated by CheA[260-537], the CheA catalytic domain, CheA[1-149], was a poor substrate for transphosphorylation by full-length CheA molecules, implying that the resident P1 domain monopolizes the CheA catalytic center. CheA-H48Q, a nonphosphorylatable mutant, failed to transphosphorylate CheA[1-149], suggesting that phosphorylation of the P1 domain in *cis* may alleviate the exclusion effect. In agreement with these findings, a 40-fold excess of CheA[1-149] fragments did not impair the CheA autophosphorylation reaction. CheA[1-149] did acquire phosphoryl groups via reversible phosphotransfer reactions with CheB and CheY molecules. An H48Q mutant of CheA[1-149] could not participate in these reactions, indicating that His-48 is probably the substrate site. The low level of efficiency of these phosphotransfer reactions and the inability of CheA[1-149] to interfere with CheA autophosphorylation most likely account for the failure of liberated P1 domains to jam chemotactic signaling in wild-type cells. However, an excess of CheA[1-149] fragments was able to support chemotactic signaling by P1-deficient *cheA* mutants, demonstrating that CheA[1-149] fragments have both transphosphorylation and phosphotransfer capability in vivo.

CheA, a cytoplasmic histidine autokinase, plays a central role in the chemotactic signaling pathway of *Escherichia coli*. CheA autophosphorylates (12) and then donates its phosphoryl groups to two aspartate autokinases, CheY and CheB, which in turn control flagellar rotation (3, 35) and sensory adaptation (19). The CheW protein couples CheA to the cytoplasmic signaling domains of membrane-bound chemoreceptors, forming a stable ternary complex (4, 11, 18). These chemoreceptor complexes govern chemotactic behavior by modulating the autophosphorylation activity of CheA in response to attractant and repellent stimuli (5, 24).

A variety of structure/function studies have shown that CheA is a modular protein (see Fig. 1). The catalytic or transmitter (T) domain is flanked on the C-terminal side by two domains (M and C) needed for coupling control by chemoreceptors and CheW (6). The N terminus of the CheA molecule contains two domains (P1 and P2) involved in phosphotransfer operations. P1 contains the autophosphorylation site (His-48) (12), whereas P2 contains a binding site(s) for CheB and CheY that facilitates the subsequent phosphotransfer reactions (17, 21, 32). The *cheA* coding region also contains an internal, in-frame translational start that produces a CheA variant ($CheA_S$) lacking the first 98 amino acids, including His-48, the phosphorylation site (15, 30). However, start site mutants that cannot make $CheA_S$ are fully chemotactic; thus, its signaling role is unclear (29).

In vitro studies have demonstrated that the P1 domain interacts with at least three protein targets. P1 polypeptides can be phosphorylated in *trans* by CheA catalytic domains, indicating that P1 contains docking and substrate sites for interaction with the catalytic center (32). Phosphorylated P1 fragments are also able to donate phosphates to CheB and CheY (12, 32), suggesting that P1 specifies interaction sites for its phosphotransfer partners. Given these in vitro activities, P1 fragments might be expected to interfere with CheA autophosphorylation or phosphotransfer in vivo by competing for interaction targets. However, in a domain liberation study of CheA, Morrison and Parkinson found that P1 fragments did not inhibit chemotaxis when expressed at high levels in wild-type cells (22). In contrast, P2 fragments were potent inhibitors of chemotaxis, evidently because they bind to CheY and prevent its interaction with the flagellar switch (22).

The failure of liberated P1 domains to disrupt chemotactic signaling could mean that P1 polypeptides are functional in vitro but not in vivo—an interesting but unlikely scenario. Alternatively, P1 fragments may be active in vivo but are inefficient competitors of critical signaling interactions. To distinguish these possibilities, we examined the effects of P1 fragments on CheA-related signaling activities in vitro and devised in vivo assays for those same activities. Our findings indicate that P1 fragments can exchange phosphoryl groups with CheB and CheY molecules; however, in wild-type cells, these phosphotransfer reactions are too inefficient to divert CheA signaling phosphates from their CheB and CheY targets. Moreover, P1 fragments are poor substrates for transphosphorylation by CheA molecules because the resident P1 domain monopolizes the catalytic center. However, CheA molecules lacking a P1 domain in *cis* can phosphorylate P1 domains in *trans* at rates sufficient for chemotactic signaling.

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant marker(s) or feature(s)	Source or reference
Strain		
RP437	Wild type for chemotaxis	27
RP3098	Δ(*flhD-flhB*)*4*	30
RP3440	*recA1 cheA157*(Am)	30
RP3471	*recA1 cheA169*(Am)	30
RP9005	Δ(*motB-cheA*)*m1111 recD*::*mini*Tn*10*	29
UU1118	*cheA*Δ*(7-247)*	This study
Plasmid		
pTM30	IPTG-inducible P_{tac} expression vector	21, 22
pKJ9	pTM30 *cheA*[a]	14
pAG3	pKJ9 *cheA*(*1-149*)	This study
pAG10	pAG3 *cheA*(*1-149*)-*H48Q*	This study
pEK46	pUC118 *motA-cheW*	15
pAG13	pEK46 *cheA*Δ(*7-247*)	This study

[a] The *cheA* coding region in pKJ9 and its derivatives is preceded by four in-frame codons from the vector and consequently produces a CheA protein with four extra residues (MLQP) at the amino end and a fifth (V) which differs from the first residue (M) of wild-type CheA. The pKJ9 CheA protein is functionally indistinguishable from wild-type CheA both in vivo and in vitro.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this work are listed in Table 1. The bacterial strains were all close relatives of RP437, an *E. coli* K-12 strain wild type for chemotaxis (27). The *cheA*Δ[*7-247*] mutation in strain UU1118 was initially constructed by deleting codons 7-247 of the *cheA* gene carried by plasmid pEK46 (15). The deletion was made by replacing a *Bsa*BI-*Eag*I restriction fragment spanning the P1-P2 portion of the *cheA* coding region with a double-stranded oligonucleotide linker having a blunt *Bsa*BI site at one end and a single-stranded *Eag*I site at the other. The intervening sequence encoded a short polypeptide (LYPAPPA). The resulting mutation was confirmed by sequencing and transferred into the *E. coli* chromosome by linear transformation of strain RP9005, as previously described (29). The *cheA*Δ[*7-247*] allele was subsequently moved to RP437 by cotransduction with the *eda* locus, yielding UU1118.

Plasmids used to produce CheA and various CheA fragments were derivatives of pTM30, an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression vector (21, 22). pKJ9 (CheA) carries the entire *cheA* coding region preceded by four in-frame codons of pTM30. The additional residues have no discernible effect on CheA autophosphorylation rate or ability to respond to chemoreceptor control (14). pAG3 (CheA[1-149]) was constructed by deleting the *cheA* sequence of pKJ9 from an engineered *Sac*II site at codon 149 through an *Eco*RV site at the downstream end of the coding region. pAG10 (CheA[1-149]-H48Q) was constructed by replacing an *Mfe*I-*Stu*I restriction fragment in pAG3 that includes the triplet coding for His-48 with the same fragment from a derivative of plasmid pDV4 that carried the H48Q allele of *cheA* (12).

Media and culture conditions. T broth (10 g of tryptone and 5 g of NaCl per liter) was routinely used for growth of bacterial strains. The growth temperature was generally 35°C. HCG is H1 medium (1, 26) supplemented with 10 g of Casamino Acids and 4 g of glycerol per liter and was used for IPTG induction of protein synthesis. IPTG was purchased from Promega Corporation. Ampicillin was purchased from Sigma Chemical Co. and was used at a final concentration of 100 μg/ml unless otherwise indicated.

Genetic methods. Phage P1 was used for transductional crosses as described previously (26). Plasmid transformations were performed as described previously (9).

Behavioral assays. The chemotactic abilities of strains were measured on semisolid tryptone agar (swarm) plates (10 g of tryptone, 5 g of NaCl, 2.6 g of agar per liter) as described elsewhere (26). For strains harboring plasmids, ampicillin was added to the swarm plate at a final concentration of 50 μg/ml.

Protein purification. CheA[1-149] was purified from cultures of strain RP3098 carrying plasmid pAG3. Cells were grown in HCG plus 50 μg of ampicillin per ml to mid-log phase, induced by the addition of IPTG to a final concentration of 200 μM, and grown for an additional 4 h. The cells were harvested by centrifugation, resuspended in buffer A (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 2 mM β-mercaptoethanol), and passed twice through a French press (10,000 lb/in^2). The extracts were clarified by centrifugation at 100,000 × *g* for 1 h and then precipitated with ammonium sulfate at 45% saturation. The precipitate was resuspended in buffer A, dialyzed against buffer A, and loaded onto a 50-ml column packed with Q-Sepharose (Sigma). After washing with 10 volumes of TEDG10 buffer (50 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 2 mM dithiothreitol, 10% [vol/vol] glycerol), the adherent protein was eluted with a 0 to 400 mM KCl gradient in TEDG10. Fractions containing CheA[1-149] were pooled, concentrated, and dialyzed against TEDG10. To avoid proteolytic degradation, 1 mM phenanthroline and 1 mM phenylmethylsulfonyl fluoride were present throughout the purification.

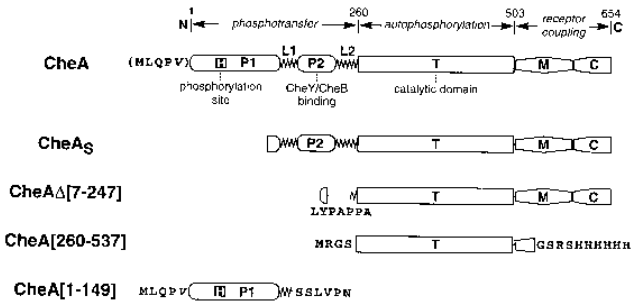

FIG. 1. Structural features and functional organization of CheA and the CheA-derived fragments used in this study. The scale at the top gives the amino acid coordinates of the three major functional regions of CheA. P1, P2, T, M, and C correspond to discrete structural or functional domains; L1 and L2 are flexible linkers (22, 28). Amino acids at the N or C termini of various constructs that are not present in native CheA are indicated by single-letter designations.

CheA protein was purified from cultures of RP3098 carrying plasmid pKJ9 as described (13). CheY protein was purified from cultures of RP3098 carrying plasmid pRL22 (20). Purified CheB was kindly supplied by Peter Ames and purified CheA-H48Q by Tom Morrison. Purified CheA[260-537] was a gift from Ron Swanson.

Phosphorylation assays. All reactions were carried out in phosphorylation buffer (50 mM Tris-HCl [pH 7.5], 50 mM KCl, 5 mM MgCl) at room temperature. CheA autophosphorylation assays were performed as previously described (2). Phospho-CheA was purified for phosphotransfer assays as described (23). All phosphotransfer assays between CheA or CheA[1-149] and CheY or CheB also followed the general methods previously described (23). Reactant concentrations for CheA and CheA[1-149] phosphotransfer experiments are listed in the legends of Fig. 3, 5, and 6. Transphosphorylation assays of CheA[1-149] by CheA[260-537] or full-length CheA were performed in 20 μl of phosphorylation buffer. After mixing of the purified proteins (reactant concentrations are given in the legend of Fig. 2), reactions were started by addition of γ-^{32}P-labeled ATP (~1,000 cpm/pmol) to a final concentration of 1 mM. At various times, 2-μl reaction samples were removed and added to 10 μl of sodium dodecyl sulfate (SDS) protein sample buffer (16) to stop the reaction. Reaction products were separated by electrophoresis on SDS-containing 16.5% polyacrylamide gels and quantified with a Molecular Dynamics PhosphorImager (23).

RESULTS

Failure of liberated P1 fragments to jam chemotactic signaling in vivo. In our initial domain liberation study of CheA, high-level expression of CheA[1-169], a P1-containing polypeptide, had no deleterious effect on the chemotactic ability of wild-type cells (22). To confirm that P1 fragments do not block chemotactic signaling, we constructed an IPTG-inducible plasmid (pAG3) that expresses a different P1-containing polypeptide, CheA[1-149] (Fig. 1). Strain RP437 containing pAG3 formed normal chemotactic swarms at all inducer concentrations tested (up to 2 mM IPTG; data not shown). A derivative of pAG3 (pAG10) that produces CheA[1-149] fragments with an H48Q replacement at the phosphorylation site in P1 also failed to inhibit the chemotactic behavior of RP437 (data not shown). At full induction, P1 expression from both plasmids accounted for at least 5% of total cell protein (data not shown); thus, their failure to inhibit chemotaxis cannot be ascribed to defects in the expression or stability of the CheA[1-149] polypeptide.

Liberated P1 domains might fail to inhibit chemotactic signaling because they do not assume native structure in vivo when expressed as polypeptides. To assess the biochemical functionality of the P1 fragment made by pAG3, CheA[1-149] peptides were purified and tested in vitro for three signaling-related activities: (i) ability to serve as phosphorylation sub-

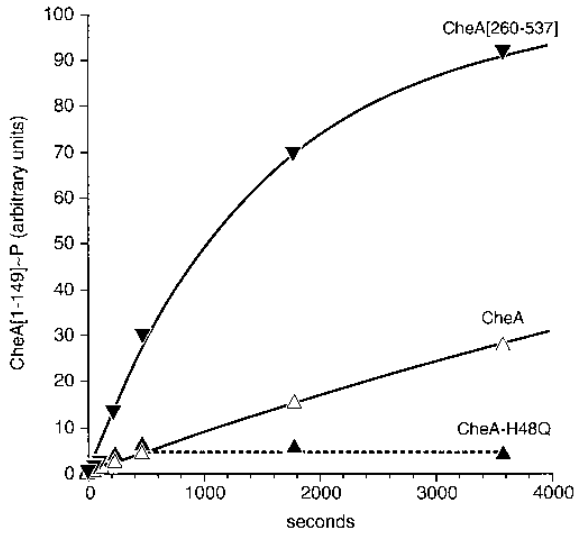

FIG. 2. Phosphorylation of CheA[1-149] by CheA[260-537] or CheA. Reaction mixtures contained 10 μM CheA[1-149], 1 mM [γ-^{32}P]ATP, and either 10 μM CheA[260-537] (▼), 10 μM CheA (△), or 10 μM CheA-H48Q (▲). Solid lines connecting the CheA[260-537] and CheA datum points represent nonlinear least-squares best fits to the following equation: fraction phosphorylated = $1 - e^{-kt}$, where t is reaction time in seconds and k is the pseudo-first-order rate constant for the reaction. Reaction half-times were approximately 760 s for CheA[260-537] and 6,100 s for CheA.

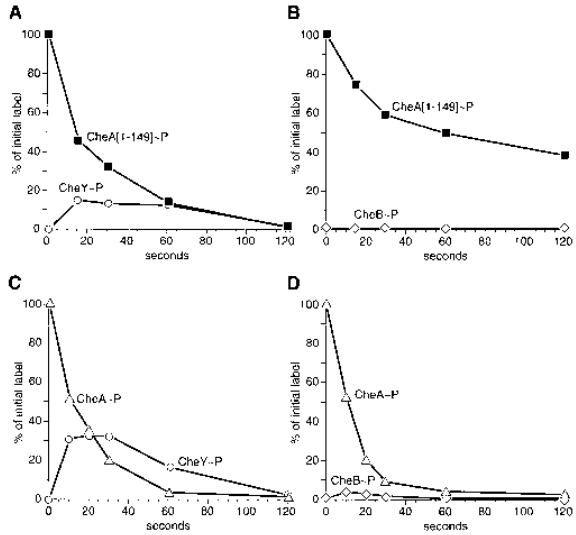

FIG. 3. Phosphotransfer from CheA[1-149] and CheA to CheB and CheY. Transfer reaction mixtures contained 1 μM phosphorylated CheA[1-149] (A and B) or CheA (C and D) and 0.2 μM CheY (A and C) or CheB (B and D). CheA[1-149] fragments were phosphorylated with CheA[260-537], as described in the legend to Fig. 2. After 1 h, the reaction was halted by a 10-fold dilution in phosphorylation buffer as CheB or CheY was added. Datum points are the mean values from two experiments. Error bars were omitted for clarity but are given in Fig. 4. ■, CheA[1-149]~P; △, CheA~P; ○, CheY~P; ◇, CheB~P.

strates; (ii) ability, following phosphorylation, to serve as phosphodonors to CheB and CheY; and (iii) ability to interfere with the phosphorylation reactions of intact CheA molecules.

Transphosphorylation of CheA[1-149]. Swanson et al. have shown that CheA[1-134] polypeptides could be phosphorylated in *trans* by CheA[260-537], a CheA fragment corresponding to the catalytic domain (32) (Fig. 1). We used this transphosphorylation test to determine whether purified CheA[1-149] fragments were also functional as phosphorylation substrates. CheA[1-149] and CheA[260-537] fragments were mixed in the presence of γ-^{32}P-labeled ATP, and the appearance of radiolabel in the P1 fragments was monitored over time (Fig. 2). The reaction proceeded with a half-time of approximately 13 min. The initial rate slowed substantially at lower concentrations of either fragment, in agreement with a bimolecular reaction (data not shown), but was not impeded by a 20-fold excess of CheA[1-149]-H48Q (data not shown). By contrast, the rate of CheA autophosphorylation under the same reaction conditions was much faster (half-time of approximately 17 s) and essentially insensitive to dilution effects (at concentrations higher than 0.5 μM), in agreement with an intramolecular reaction (data not shown). We conclude that CheA[1-149] is competent as a phosphorylation substrate in vitro and presumably in vivo as well. Thus, the failure of these P1 fragments to block chemotactic signaling in vivo is probably not due to a defect in their ability to interact properly with the CheA catalytic domain.

CheA[1-149] also became phosphorylated in reactions containing ATP and full-length CheA (Fig. 2). However, the rate of phosphorylation by intact CheA was approximately eightfold lower than that by CheA[260-537], suggesting that the resident P1 domain in CheA molecules may limit access of free P1 fragments to the catalytic center. CheA-H48Q, whose P1 domain lacks a functional phosphorylation site, was not able to phosphorylate CheA[1-149] fragments in *trans* (Fig. 2), even though complementation tests indicate that its catalytic domain is functional (31a, 36). This implies that transphosphorylation of P1 fragments by CheA depends on prior phosphorylation of the P1 domain in *cis*. Perhaps phosphorylation of the P1 in *cis* reduces its affinity for the catalytic center, thereby enhancing access of free P1 domains. Alternatively, the P1 in *cis* might relay phosphates to P1 molecules in *trans* through an exchange reaction.

To distinguish these possibilities, we labeled CheA molecules in an autophosphorylation reaction, purified them to remove free ATP, and tested the phosphorylated CheA for ability to donate phosphate to CheA[1-149] fragments. After mixing phospho-CheA with CheA[1-149], there was no migration of phosphate label from CheA to the P1 fragment (data not shown). We conclude that the kinase activity of CheA is directly responsible for transphosphorylation of P1 fragments by wild-type CheA in vitro. Evidently, the resident P1 domain in CheA molecules blocks access of free P1 fragments to the catalytic center. This may be one reason why liberated P1 domains fail to interfere with chemotactic signaling by CheA. The transphosphorylation reaction could conceivably divert some CheA phosphates to P1 fragments in vivo but is probably too slow to have a significant physiological effect. In agreement with this view, a 40-fold molar excess of CheA[1-149] fragments had no discernible effect on either the rate or the final level of CheA autophosphorylation in vitro (data not shown).

Phosphotransfer between CheA[1-149] and CheB and CheY. CheA[1-149] fragments, labeled by transphosphorylation with CheA[260-537], were compared with autophosphorylated CheA molecules for their abilities to donate phosphate to CheB or CheY. The phosphotransfer reaction was assessed by following the loss of label from phosphorylated CheA or CheA[1-149] upon addition of substoichiometric amounts of CheB or CheY (Fig. 3). With no CheB or CheY, both phosphodonors were stable (data not shown); thus, the overall rate of substrate dephosphorylation in this assay reflects the slower of two reaction steps: (i) phosphotransfer from phospho-CheA or phospho-CheA[1-149] to CheB or CheY; and (ii) sponta-

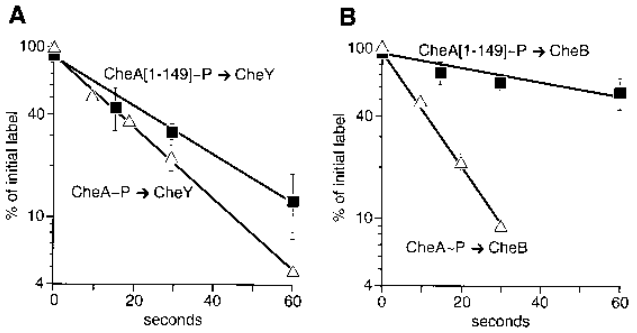

FIG. 4. Rates of dephosphorylation of CheA and CheA[1-149] by CheY and CheB. Data from the early time points of the phosphotransfer reactions of Fig. 3 were plotted on semi-log coordinates and best fit to a single exponential to facilitate rate comparisons. (A) Dephosphorylation by CheY; (B) dephosphorylation by CheB. △, CheA~P; ■, CheA[1-149]~P.

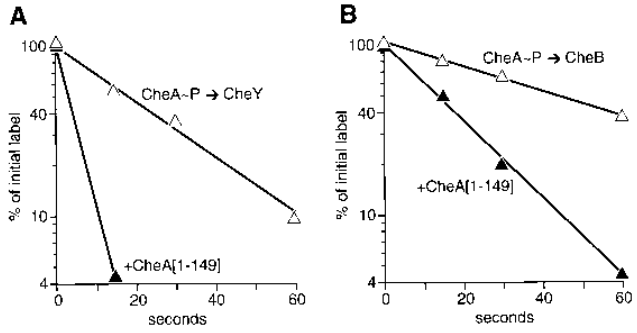

FIG. 5. Effect of CheA[1-149] on dephosphorylation of CheA by CheY and CheB. The reaction mixtures contained 1 μM [^{32}P]phospho-CheA, 0.2 μM CheY (A) or CheB (B), and, when indicated, 20 μM CheA[1-149]. Data from early time points were plotted as in Fig. 4. (A) Dephosphorylation by CheY; (B) dephosphorylation by CheB. △, CheA~P alone; ▲, CheA~P plus CheA[1-149]. Note that the CheB used in this experiment had a lower specific activity than that used in the experiment described in Fig. 3.

neous hydrolysis of phospho-CheB or phospho-CheY to regenerate the phosphoacceptor species.

Both CheY (Fig. 3A) and CheB (Fig. 3B) dephosphorylated CheA[1-149], indicating that the failure of P1 fragments to block chemotactic signaling in vivo is probably not caused by an inability to function as a donor in phosphotransfer reactions. However, CheA[1-149] appeared to be a less efficient phosphodonor than full-length CheA, particularly with CheB as the phosphoacceptor. There was little rate difference with CheY as phosphoacceptor (Fig. 3A versus 3C) because turnover of phospho-CheY is apparently the rate-limiting step in the reactions, as evidenced by the substantial buildup of label in CheY at early time points. With CheB as the phosphoacceptor, which turns over faster than CheY (7), there was little accumulation of phospho-CheB in the reactions (Fig. 3B versus D), indicating that phosphotransfer could be the rate-limiting step. CheB dephosphorylated CheA[1-149] approximately ninefold more slowly than it did CheA (Fig. 4B), whereas the corresponding dephosphorylation rates by CheY differed by less than twofold (Fig. 4A). Thus, phosphotransfer from CheA[1-149] to CheB (and perhaps to CheY as well) is less efficient than that from CheA. This rate difference most likely reflects the presence of the P2 domain in CheA, which facilitates the phosphotransfer reaction by binding CheB and CheY.

The phosphotransfer ability of CheA[1-149] indicates that this P1 fragment interacts with CheB and CheY, perhaps through specific binding contacts. If so, CheA[1-149] fragments might interfere with the phosphotransfer activity of CheA by sequestering phosphoacceptors, but surprisingly, a 20-fold molar excess of CheA[1-149] actually produced a substantial increase in the rate of CheA dephosphorylation by CheY (Fig. 5A) and by CheB (Fig. 5B). CheA[1-149]-H48Q fragments neither augmented nor inhibited the overall reaction rate (data not shown), suggesting that phosphorylation of CheA[1-149] might be involved in the acceleration effect. Examination of the reaction kinetics revealed that at very early time points, a substantial proportion of the CheA label appeared in CheA[1-149] (Fig. 6). Because CheA[1-149] cannot dephosphorylate CheA in the absence of CheB or CheY (see above), it seems likely that CheA first transfers its phosphate to CheB or CheY, which in turn donate it to CheA[1-149] through a reverse phosphotransfer reaction. Thus, CheA[1-149] probably accelerates the overall loss of phosphate from CheA by acting as a phosphate sink to facilitate the turnover of CheB and CheY phosphoacceptors. This effect was especially dramatic in the CheY reaction (Fig. 6A), in which the buildup of phospho-CheY indicates that phospho-CheY hydrolysis is the rate-limiting step (see also Fig. 3). The effect is less dramatic in the CheB reaction (Fig. 6B), in which the rates of CheB phosphorylation and dephosphorylation are probably similar, as evidenced by the negligible level of phospho-CheB intermediate.

Chemotactic signaling by P1 fragments in vivo. The in vitro assays described above indicate that CheA[1-149] polypeptides retain the principal signaling activities ascribed to the P1 domain of CheA, namely, phosphorylation by the catalytic domain and phosphotransfer to CheB and CheY. However, CheA[1-149] overexpression in wild-type cells had no effect on chemotactic behavior; thus, we cannot be certain that these P1 fragments are functional in vivo. To answer this question, we looked for *cheA* mutants in which expression of P1 fragments could restore chemotactic ability, presumably by relaying phosphates between CheA and CheB/CheY. Two types of mutants lacking a functional P1 domain in *cis* responded to free P1 fragments in *trans*: those which make only $CheA_S$ (Fig. 1), owing to a nonsense mutation (*cheA157* or *cheA169*) between the *cheA* translational start sites (15); and a strain that produces CheAΔ[7-247], from which both the P1 and P2 domains are deleted (Fig. 1). $CheA_S$ and CheAΔ[7-247] possess wild-type catalytic domains but lack P1 domains and cannot autophosphorylate. Importantly, both carry the C-terminal domains needed to couple CheA catalytic activity to chemoreceptor control. Upon induction, plasmid pAG3, which produces CheA[1-149], enhanced the swarm size of both types of mutants. The cooperation between CheA[1-149] and CheAΔ[7-247] was particularly dramatic and is shown in Fig. 7. Plasmid pAG10, which produces the H48Q mutant of CheA[1-149], failed to enhance chemotaxis in either type of strain (Fig. 7), indicating that the signaling pathway probably involves stimulus-modulated transphosphorylation of CheA[1-149] by the catalytic domains of the mutant CheA molecules.

DISCUSSION

The CheA[1-149] polypeptide studied in this report failed to disrupt chemotactic signaling in vivo but nevertheless exhibited several signaling-related functions in vitro (Fig. 8) that shed new light on the phosphorelay transactions involved in chemotactic signaling.

Transphosphorylation. CheA[1-149] was a substrate for transphosphorylation by CheA[260-537], the CheA catalytic domain. Because phospho-CheA[1-149] can donate its phosphate

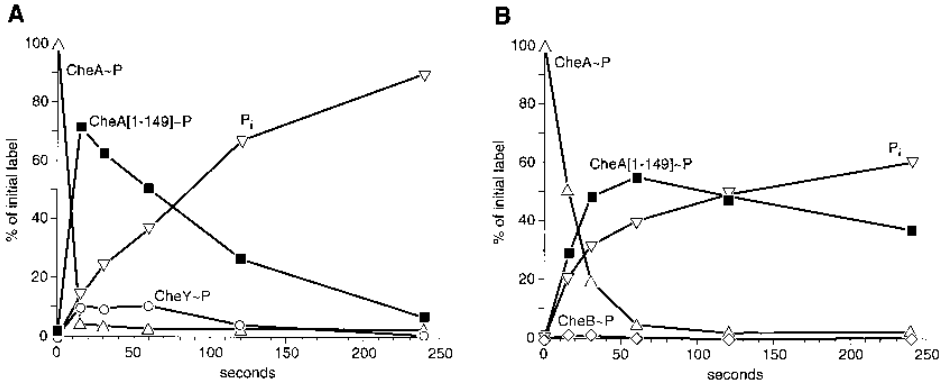

FIG. 6. Phosphate flux through the components of CheA dephosphorylation reactions in the presence of CheA[1-149]. The reaction mixtures contained 1 μM [^{32}P]phospho-CheA, 0.2 μM CheY (A) or CheB (B), and, when indicated, 20 μM CheA[1-149]. △, CheA~P; ■, CheA[1-149]~P; ○, CheY~P; ◇, CheB~P; ▽, P_i.

to CheB and CheY (see below) and because CheA[1-149]-H48Q cannot be phosphorylated, the site of phosphorylation is most likely histidine-48. Thus, the transphosphorylation reaction seems to be an intermolecular version of the CheA autophosphorylation reaction, implying that CheA[1-149] contains recognition determinants that promote functional interaction with the catalytic domain. The structural basis for that interaction has not been explored, but the failure of competing CheA[1-149]-H48Q fragments to impede the transphosphorylation reaction could mean that the partners bind with low affinity.

The catalytic domain in full-length CheA molecules can transphosphorylate CheA[1-149] but preferentially phosphorylates the resident P1 domain. It may be that native CheA molecules are simply folded in a way that sterically hinders access of free P1 fragments to the catalytic center. Alternatively, the P1 domain in *cis*, by virtue of its covalent connection, would also be expected to monopolize reversible binding interactions with the catalytic domain, effectively excluding P1 fragments in *trans*. Whatever the exclusion mechanism, the phosphorylation state of the P1 in *cis* may play a pivotal role, because CheA-H48Q failed to transphosphorylate CheA[1-149].

His to Asp phosphotransfer. Phosphorylated CheA[1-149] transferred its phosphate to CheB and CheY, presumably to the target aspartate residues in these phospho-acceptors. We refer to this reaction as a phospho-His→Asp transfer (HD phosphotransfer). Previous studies described the phosphodonor ability of P1 fragments but did not examine the efficiency of the phosphotransfer reaction (12, 32). We found that the rate of the CheA[1-149]→CheB transfer reaction was nearly 10-fold lower than that of the corresponding CheA reaction. Our assay conditions did not allow us to measure a definitive rate for phosphotransfer from CheA[1-149] to CheY; however, we predict that this reaction is also less efficient because P1 fragments lack the CheB/CheY-binding P2 domain that facilitates CheA phosphotransfer reactions. It seems unlikely that P1 fragments bind tightly to their phosphotransfer targets, because CheA[1-149]-H48Q did not competitively interfere with the CheA transfer reaction. The nature of the recognition determinants that promote interaction of a P2-less donor fragment with target CheB or CheY molecules is not known, but several *cheA* mutations that affect relative CheB/CheY phosphotransfer efficiency lie close to His-48 (12, 25). It may be that P1 phosphodonor ability is primarily a function of surface contour around the phosphohistidine, which would dictate its

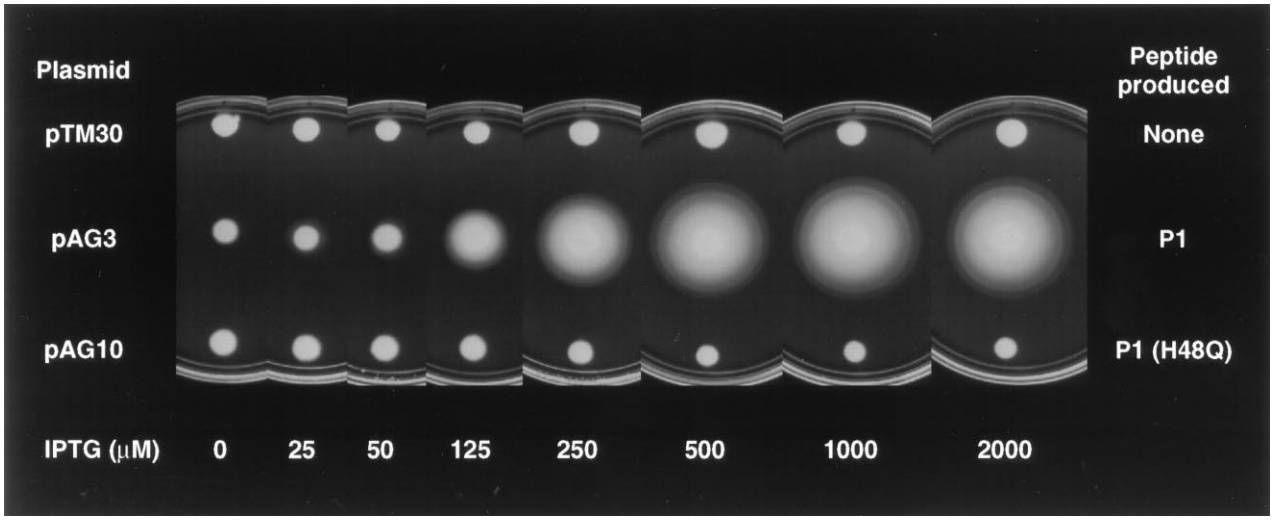

FIG. 7. Complementation of the *cheAΔ[7-247]* mutation by CheA[1-149] fragments. Tryptone swarm plates containing the indicated concentrations of IPTG were inoculated with single colonies of strain UU1118 carrying plasmids pTM30 (vector control), pAG3 (CheA[1-149]), or pAG10 (CheA[1-149]-H48Q) and were incubated at 35°C for approximately 16 h.

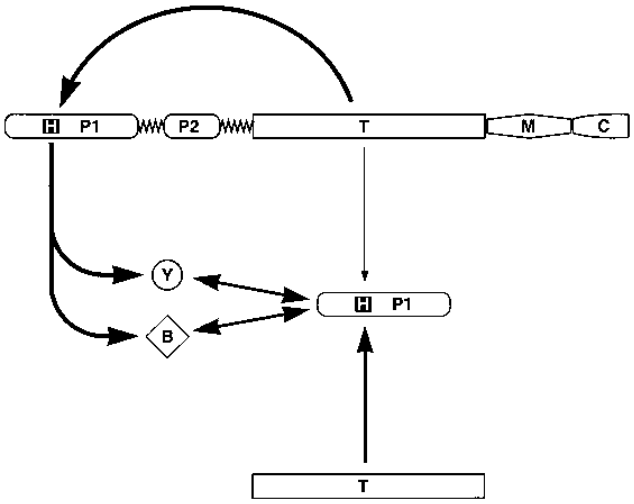

FIG. 8. In vitro phosphorylation reactions of CheA and CheA[1-149]. The thickness of the lines is a crude indicator of the relative efficiencies of the reactions. Double-headed arrows denote reversible transfers of high-energy phosphoryl groups.

accessibility to productive collisions with phosphoacceptor proteins.

Asp-to-His phosphotransfer. CheA[1-149] also accepted phosphate from phospho-CheB and phospho-CheY. We refer to this reaction as a phospho-Asp→His transfer (DH phosphotransfer). The phosphotransfer equilibria for histidine kinases, including CheA, greatly favor the forward reaction under physiological conditions (31); thus, the reverse reaction detected in our assays may reflect the considerable molar excess of CheA[1-149] fragments used in the reactions. Moreover, the catalytic domain of histidine kinases may play an active role in inhibiting the docking interactions or catalytic events needed for a reverse phosphotransfer reaction. Dutta and Inouye showed, for example, that a particular catalytic defect in the EnvZ kinase enabled the mutant enzyme to accept phosphate from phospho-OmpR, normally its phosphotransfer target (10). Thus, the lack of an adjoining catalytic domain in P1 fragments of CheA could conceivably augment DH phosphotransfer. A kinase-independent DH phosphotransfer probably occurs between Spo0F and Spo0B of the sporulation signaling pathway in *Bacillus subtilis* (8). DH phosphotransfers have also been reported in the ArcB-ArcA (33) and BvgS-BvgA (34) signaling systems.

Why don't liberated P1 fragments jam chemotactic signaling? The transphosphorylation and phosphotransfer capabilities of CheA[1-149] indicate that this P1 fragment interacts with CheB, CheY, and the catalytic domain of CheA. The inability of CheA[1-149]-H48Q fragments to interfere with the in vitro activities of CheA[1-149] implies that the binding contacts that promote these signaling transactions are relatively weak ones. Thus, CheA[1-149] fragments cannot compete effectively with CheA for common binding targets. In addition, local concentration effects probably allow the resident P1 domain in CheA molecules to monopolize the catalytic center, thereby preventing free P1 fragments from impairing the autophosphorylation reaction. Moreover, without the P2 domain dedicated to CheB/CheY binding, P1 fragments cannot sequester the phosphoacceptor targets of CheA and thus cannot reduce CheA phosphotransfer rates. The inability of liberated P1 domains to promote tight binding interactions with CheA, CheB, and CheY is probably the principal reason for their failure to disrupt chemotactic signaling.

CheA[1-149] fragments clearly have the potential to divert in vivo phosphate fluxes from their normal signaling routes, but the relatively low rates of the detour reactions probably mitigate their behavioral consequences. For example, the transphosphorylation of P1 fragments by CheA has negligible impact on CheA autophosphorylation rate. Moreover, the P1 phosphates should then transfer to their intended targets, CheB and CheY, in any event. The DH phosphotransfer activity of P1 fragments could conceivably perturb signaling in two ways: (i) by reducing the steady-state phosphorylation level of CheY and CheB; and (ii) by creating a reservoir of phosphoryl groups available for subsequent transfer to CheB and CheY. Thus, DH phosphotransfers might dampen stimulus-induced fluctuations in phospho-CheB and -CheY levels by slowing irreversible (hydrolytic) dephosphorylation of CheB and CheY. The feedback circuitry of the sensory adaptation system would be expected to counter these P1 effects. For example, whenever steady-state phospho-CheB levels drop below a hard-wired optimum set-point, receptor methylation would rise, increasing the autophosphorylation rate of CheA and consequently the flow of phosphate to CheB (and CheY).

The possibility that CheA[1-149] does not have these signaling activities in vivo can be ruled out because liberated fragments support chemotaxis in strains with mutant CheA molecules lacking P1 domains of their own. In such strains, CheA[1-149] probably serves as a substrate for transphosphorylation by the CheA catalytic domain and then transfers those phosphates to CheB and CheY. Although these P1 signaling reactions are intrinsically inefficient, the overall flux of phosphate is presumably augmented to levels sufficient for chemotaxis by mass action of the many P1 fragments in the cell. Indeed, high-level expression of CheA[1-149] was essential for chemotactic ability. This functional complementation system offers a means for obtaining and characterizing mutations that affect the substrate or transfer properties of P1 fragments and will enable us to identify the structural determinants needed for these CheA signaling activities.

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