JOURNAL OF BACTERIOLOGY, Feb. 1997, p. 825–830 Vol. 179, No. 3
0021-9193/97/$04.00+0
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Genetic Analysis of the Catalytic Domain of the Chemotaxis-Associated Histidine Kinase CheA

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Received 9 September 1996/Accepted 15 November 1996

Escherichia coli **cells express two forms of CheA, the histidine kinase associated with chemotaxis. The long form, $CheA_L$, plays a critical role in chemotactic signal transduction by phosphorylating two chemotaxis-associated response regulators, CheY and CheB. $CheA_L$ first autophosphorylates amino acid His-48 before its phosphoryl group is transferred to these response regulators. The short form, $CheA_S$, lacks the amino-terminal 97 amino acids of $CheA_L$ and therefore does not possess the site of phosphorylation. The centrally located transmitter domain of both forms of CheA contains four regions, called N, G1, F, and G2, highly conserved among histidine kinases of the family of two-component signal transduction systems. On the basis of sequence similarity to highly conserved regions of certain eukaryotic kinases, the G1 and G2 regions are purported to be involved in the binding and hydrolysis of ATP. We report here that alleles mutated in the G1, G2, or F region synthesize CheA variants that cannot autophosphorylate in vitro and which cannot support chemotaxis in vivo. We also show that in vitro, the nonphosphorylatable $CheA_S$ protein mediates transphosphorylation of a $CheA_L$ variant defective in both G1 and G2. In contrast, $CheA_L$ variants defective for either G1 or G2 mediate transphosphorylation of each other poorly, if at all. These results are consistent with a mechanism by which the G1 and G2 regions of one protomer of a CheA dimer form a unit that mediates transphosphorylation of the other protomer within that dimer.**

Chemotaxis by cells of enteric bacteria, such as *Escherichia coli*, depends on the ability of chemoreceptors to communicate with switch components of flagellar motors to modulate swimming behavior in response to the cells' chemical environment. This communication requires the cooperative effort of the cytoplasmic gene products of six genes, *cheA*, *cheW*, *cheR*, *cheB*, *cheY*, and *cheZ*. Of these genes, *cheA* is the most central. This gene encodes two proteins, $CheA_L$ (78 kDa) and $CheA_S$ (69 kDa), translated in frame from two different initiation sites (14, 25, 28).

Found in vitro as a dimer (6, 33), the two-component histidine kinase $CheA_L$ autophosphorylates a conserved histidine residue (His-48) located near its amino terminus (10, 11). Phosphorylated-$CheA_L$, in turn, acts as a phospho-donor for two other chemotaxis proteins: CheY, required for clockwise signal generation, and CheB, required for adaptation (reviewed in references 4, 30, and 31). $CheA_L$ homodimers form ternary complexes with CheW and chemoreceptors, e.g., Tar (7). Within these complexes, the autokinase activity of $CheA_L$ is significantly accelerated relative to that of uncomplexed $CheA_L$ homodimers alone. Binding of ligand, e.g., the Tar-specific attractant L-aspartate, to the chemoreceptor within these ternary complexes greatly diminishes $CheA_L$ autokinase activity (2, 3, 21). Thus, CheW and chemoreceptors modulate $CheA_L$ autokinase activity in a ligand-dependent manner.

Although $CheA_S$ lacks the site of autophosphorylation, it retains domains that in $CheA_L$ are required for kinase activity, ternary complex formation, and phospho-transfer to CheY and CheB (5, 10, 15, 19, 35). Evidence obtained in vivo and in vitro indicates that the $CheA_L$ domains responsible for kinase activity and ternary complex formation also function in $CheA_S$. In vitro, $CheA_S$ mediates receptor-modulated transphosphorylation of mutant $CheA_L$ proteins either deficient for kinase activity or truncated for the carboxy-terminal segments required for ternary complex formation (34, 38, 39). In vivo, $CheA_S$ restores chemotactic ability to cells that express either kinase-deficient or truncated $CheA_L$ mutant proteins (38, 39).

$CheA_L$ and $CheA_S$ are composed of several distinct functional domains (reviewed in reference 15) (Fig. 1). The amino-terminal P1 domain contains the site of autophosphorylation, His-48 (22, 25). This domain, present in $CheA_L$ but not in $CheA_S$, is required for phospho-transfer to CheY and CheB (11). The P2 domain assists in the interaction between the phospho-donor site in P1 and amino acid sequences located in CheY (19, 34). Two other domains, M and C, located at the carboxy terminus, appear to play important roles in enabling $CheA_L$ and/or $CheA_S$ to receive sensory information from the chemoreceptors (5, 25). Finally, the centrally located transmitter (T) domain contains both highly variable (T_L) and highly conserved (T_R) subdomains. The T_R subdomain contains four regions, highly conserved among histidine kinases of the family of two-component signal transduction systems, named N, G1 (also called D), F, and G2 (also called G) (25, 31). On the basis of sequence similarity and structure-averaging algorithms, Parkinson and Kofoid (25) proposed that the T_R subdomain likely consists of two β-pleated sheets, the N and F boxes, interspersed by two large glycine-rich regions, G1 and G2, that resemble the unstructured loops of eukaryotic nucleotide-binding sites (8, 26, 29). Thus, they hypothesized that this region plays a role in the binding and hydrolysis of ATP (25).

Since it functions as a dimer, CheA could possess as many as two sites that bind ATP independently. Each independent site could reside entirely within one protomer of a dimer or could include regions contributed by both protomers. To distinguish between these two possibilities, we generated *cheA* mutants defective in the G1, G2, or F subdomain. Here, we demonstrate that such mutants do not support chemotactic ability

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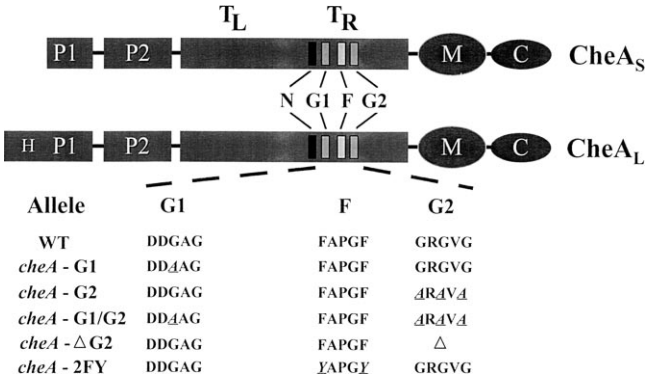

FIG. 1. Functional organization of CheA and schematic of *cheA* alleles used in this study. The shaded bars within T_R represent the N, G1, F, and G2 blocks of highly conserved amino acids shared by histidine kinases of the family of two-component signal transduction systems. Mutant amino acids are italicized and underlined. See text for details.

in vivo. Similarly, $CheA_L$ variants defective in the G1, G2, or F subdomain cannot autophosphorylate in vitro. We also show that in vitro, the nonphosphorylatable $CheA_S$ and $CheA_L$48HQ proteins mediate transphosphorylation of $CheA_L$ variants defective for G1 alone, G2 alone, or both G1 and G2. In contrast, $CheA_L$ variants defective for either G1 or G2 mediate transphosphorylation of each other poorly, if at all. These results are consistent with a mechanism by which the G1 and G2 regions of one protomer of a CheA dimer form a unit that mediates transphosphorylation of the other protomer of that dimer.

MATERIALS AND METHODS

Bacterial strains, alleles, and plasmids. All strains were derivatives of *E. coli* K-12 and are listed in Table 1. Alleles *cheA*$_S$ (39), *cheA*, and *cheA48HQ* (11, 22) encode wild-type $CheA_S$ alone, both wild-type $CheA_S$ and wild-type $CheA_L$, and both wild-type $CheA_S$ and the mutant protein $CheA_L$48HQ, respectively. This mutant protein cannot autophosphorylate because the histidine residue at position 48 has been replaced by a glutamine residue. The following alleles are shown in Fig. 1. Alleles *cheA422GA* (herein called *cheA*G1), *cheA470GA472GA474GA* (*cheA*G2), *cheA422GA470GA472GA474GA* (*cheA*G1/G2), and *cheA455FY 459FY* (*cheA*2FY) encode $CheA_S$ and $CheA_L$ proteins disrupted in G1 alone, G2 alone, both G1 and G2, or F alone, respectively. Each mutation and unique restriction sites used to track these mutations during various in vitro manipulations were generated by using standard oligonucleotide-directed mutagenesis procedures (16). The following oligonucleotides were used: (i) to generate the allele *cheA*G1, 5′-ACCGACGATGCCGCGGGGCTAA-3′, which substitutes an alanine residue for the glycine at position 422 and which introduces a unique *Sac*II restriction site into G1; (ii) to generate the allele *cheA*G2, 5′-ACGTCTC CGCGCGCGCCGTCGCCATGGACGTC-3′, which substitutes alanine residues for the glycines at positions 470, 472, and 474 and which introduces a unique *Bss*HII restriction site into G2; and (iii) to generate the allele *cheA*2FY, 5′-AT GCTGATATACGCACCCGGGTACCCACGGCAG-3′, which substitutes tyrosine residues for the phenylalanines at positions 455 and 459 and introduces a unique *Xma*I restriction site in F. Allele *cheA*Δ(*468-477*) (herein called *cheA*ΔG2) encodes $CheA_S$ and $CheA_L$ proteins deleted for nine amino acids, including those that comprise the entire G2 region.

The alleles *cheA*G1, *cheA*G2, and *cheA*G1/G2 were introduced into the chromosome by means of homologous recombination in the *polA*(Ts) strain CP366 (23). The resultant recombinants were screened for nonchemotactic behavior. Those that proved nonchemotactic then were tested for the ability to exhibit wild-type chemotactic behavior when transformed with pAR1.*cheA*, a plasmid that encodes both $CheA_S$ and $CheA_L$. The existence of each mutation was verified by restriction analysis of PCR products, using primers designed to amplify the entire G1-F-G2 region. P1 transduction (27) was used to transfer each mutant allele out of its Pol(Ts) host into the Pol^+ Rec^+ Che^+ recipient strain RP437 and to make the resultant transductants *recA* by using either strain $MH6^-$ or KO685 as the donor.

Media and growth conditions. Cells were grown with aeration in tryptone broth (TB; 1% [wt/vol] tryptone, 0.5% [wt/vol] sodium chloride) or in Luria broth (LB; TB supplemented with 0.5% [wt/vol] yeast extract). The optical density at 610 nm (OD_{610}) was monitored.

Swarm assays. Cells were grown at 32°C to mid-exponential phase (OD_{610} of ~0.3) in TB supplemented with the requisite antibiotics. Tryptone swarm plates were 0.20% agar in the same broth. Antibiotics were not added. A 5-μl aliquot of the culture (10^6 to 10^7 cells) was placed on the surface of the swarm plate near its center, and the plate was incubated at 32°C in a humid environment. Swarm plates were handled and measured as described previously (37).

Plasmids used for in vivo swarm plate analyses were derivatives of pAR1 (6) in which the *cheA* alleles were under the control of tandem *tac* promoters.

Protein purification. To purify $CheA_L$, the 2.1-kb *Nde*I-*Bam*HI fragment from pAR1.*cheA* (38) was subcloned into pET14b (Novagen Inc., Madison, Wis.) to generate plasmid pDE1. This plasmid permits expression, by means of the bacteriophage T7 ϕ10 promoter, of a six-histidine (6×His) sequence fused to the amino terminus of $CheA_L$. To purify mutant $CheA_L$ proteins, the 5.9-kb *Stu*I-*Mlu*I restriction fragment of pDE1 was ligated to 1.2-kb *Stu*I-*Mlu*I fragments from each of the respective pAR1 derivatives. To purify $CheA_S$, the 1.8-kb *Nde*I-*Bam*HI fragment from pAR1.*cheA*$_S$ (38) was subcloned into pET14b (Novagen). Each protein used in this study was purified by transforming the plasmid which expressed the appropriate 6×His-CheA variant fusion protein under control of the T7 promoter into BL21(DE3), a strain which expresses T7 RNA polymerase under the control of *lac* promoter (32). Cells of the resultant transformant were grown overnight in 5 ml of LB in the presence of ampicillin (100 μg/ml). The overnight culture was diluted 1:100 in LB containing ampicillin (200 μg/ml) and grown to an OD_{610} of ~0.4 at 30°C before addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and more ampicillin (200 μg/ml). After a 3-h incubation under inducing conditions, the cells were harvested by centrifugation at 3,000 × *g* for 20 min at 4°C. Purification of the fusion protein and cleavage of the 6×His sequence were performed as instructed by the manufacturer (Novagen) (13) except that the thrombin/protein ratio was 1:1,000. Protein concentrations were determined by the bicinchoninic acid assay (Pierce Biochemicals).

Protein labeling, SDS-PAGE, autoradiography, and immunoblot analysis. Phosphorylation reaction mixtures contained the indicated proteins in TKMD buffer (50 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol [pH 7.5]) and 50 μM [γ-^{32}P]ATP (~2,500 cpm $pmol^{-1}$). Reactions were terminated by the addition of 3× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (39). Samples were subjected to SDS-PAGE (10% polyacrylamide gels) at 10 to 15°C in a Hoefer SE600 gel apparatus under the conditions of Laemmli (17). Following electrophoresis, the gels were incubated in drying buffer (45% methanol, 7.0% glacial acetic acid, 10% glycerol, 0.5% dimethyl sulfoxide), dried for 2 h, and visualized by autoradiography. To quantify phosphorylated CheA, gels were scanned for 1 h on a Betascope 603 blot analyzer (Betagen).

Immunoblot analyses, using affinity-purified rabbit anti-CheA and anti-$CheA_L$ antisera, were performed by standard procedures (9). The anti-$CheA_L$ antiserum was generated by coupling bovine serum albumin to the oligopeptide IKGGAG TFGFS. This sequence exists exclusively in $CheA_L$, adjacent to His-48.

RESULTS

CheA variants defective for G1, F, or G2 cannot autophosphorylate in vitro, nor can they support chemotaxis in vivo. We purified wild-type $CheA_L$, wild-type $CheA_S$, and the $CheA_L$ mutant proteins $CheA_L$G1, $CheA_L$G1/G2, $CheA_L$G2, $CheA_L$ΔG2, $CheA_L$2FY, and $CheA_L$48HQ to near homoge-

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Source
AJW531	RP437 *cheA470GA472GA474GA recA*::*cml eda*$^+$	This study
AJW953	RP437 *cheA422GA470GA472GA474GA* Δ*recA*(*Sst*II-*Eco*RI) *srl*::Tn*10 eda*$^+$	This study
AJW955	RP437 *cheA422GA* Δ*recA*(*Sst*II-*Eco*RI) *srl*::Tn*10 eda*$^+$	This study
AJW1071	RP9535 *recA*::*cml*	This study
BL21(DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *lon dcm* (λ*imm21 lacI lacUV5* T7 polymerase *inf*)	32
CP366	*polA*(Ts)	23
KO685	Δ*recA*(*Sst*II-*Eco*RI) *srl*::Tn*10*	12
$MH6^-$	*recA*::*cml*	A. Binney[a]
RP437	*thr*(Am)*1 leuB6 his-4 metF*(Am)*159 eda-50 rpsL136 thi-1 lacY1 ara-14 mtl-1 xyl-5 tonA31 tsx-78*	24
RP9535	RP437 Δ*cheA1643 eda*$^+$	18

[a] A. Binney, Purdue University, West Lafayette, Ind.

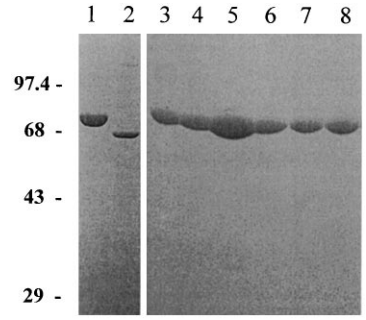

FIG. 2. Purified CheA variant proteins isolated from cells of strain BL21 (DE3) transformed with various CheA expression vectors, separated by SDS-PAGE (10% gel), and stained with Coomassie blue. Lane 1, wild-type $CheA_L$; lane 2, wild-type $CheA_S$; lane 3, $CheA_L$G1; lane 4, $CheA_L$G1/G2; lane 5, $CheA_L$G2; lane 6, $CheA_L\Delta$G2; lane 7, $CheA_L$2FY; lane 8, $CheA_L$48HQ. The image was derived by scanning a photograph of the resultant gel with a Hewlett-Packard Scanjet model IIc.

neity, as judged by SDS-PAGE (Fig. 2), and confirmed them as CheA variants by immunoblotting with the anti-CheA antibody (data not shown).

To assess the ability of these CheA variants to autophosphorylate, we added [γ-^{32}P]ATP to purified preparations of each mutant protein. At the conclusion of a 5-min labeling period at room temperature, we terminated each reaction by adding 3× SDS-PAGE sample-loading buffer and analyzed the results by SDS-PAGE and autoradiography (Fig. 3). As expected, the reaction mixture containing only wild-type $CheA_L$ (lane 1) resulted in phosphorylation of a protein with a molecular mass of about 78 kDa. In contrast, the reaction mixture containing $CheA_S$ (lane 2), $CheA_L$G1 (lane 3), $CheA_L$G2 (lane 4), $CheA_L$G1/G2 (lane 5), $CheA_L$2FY (lane 6), $CheA_L$48HQ (lane 7), or $CheA_L\Delta$G2 (lane 8) did not.

To determine whether these in vitro results correspond to physiologically relevant phenomena, we inoculated cells carrying either the wild-type allele *cheA* (strain RP437) or the mutant allele *cheA*G1 (strain AJW955), *cheA*G2 (strain AJW531), or *cheA*G1/G2 (strain AJW953) at the center of tryptone swarm plates and, after 6 h of incubation, compared the displacements of the outermost edges of their resultant swarms (Fig. 4). Wild-type cells (Fig. 4A) formed two rapidly migrating concentric bands (0.72 cm h^{-1} for the outermost band), indicating that such cells are chemotactic to both L-serine and L-aspartate (1). In contrast, cells of each mutant strain formed dense irregular swarms that migrated at a rate (0.02 cm h^{-1}) (data not shown) characteristic of nonchemotactic cells that

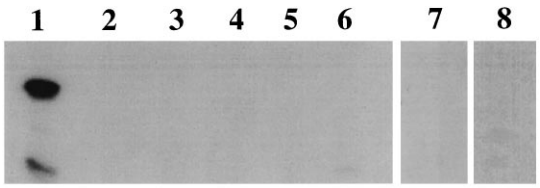

FIG. 3. Autophosphorylation of $CheA_L$ variants. CheA samples (each protein at 5 μM [final concentration]) were incubated with 50 μM [γ-^{32}P]ATP (~2,500 cpm $pmol^{-1}$) in TKMD buffer (see Materials and Methods), the reactions were terminated by the addition of 3× SDS-PAGE sample buffer, and the samples were analyzed by SDS-PAGE (10% gel) followed by autoradiography for 60 min. Lane 1, wild-type $CheA_L$; lane 2, wild-type $CheA_S$; lane 3, $CheA_L$G1; lane 4, $CheA_L$G2; lane 5, $CheA_L$G1/G2; lane 6, $CheA_L$2FY; lane 7; $CheA_L$48HQ; lane 8, $CheA_L\Delta$G2. The image was derived by scanning the autoradiograph with a Hewlett-Packard Scanjet model IIc.

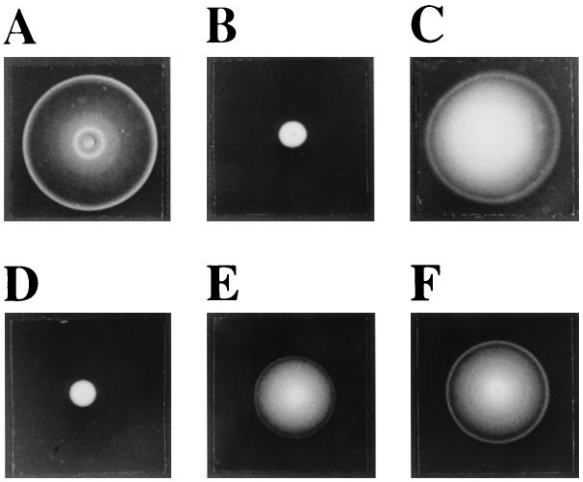

FIG. 4. Photographs of swarms produced by wild-type cells (A); those deleted for *cheA* (B); those deleted for *cheA* and transformed with either pAR1.*cheA*, a plasmid that expresses both wild-type $CheA_L$ and wild-type $CheA_S$ (C), or its vector parent, pAR1 (D); those carrying the allele *cheA*G1 and transformed with pAR1.$cheA_S$, a plasmid that expresses $CheA_S$ (E); and those carrying the allele *cheA*G2 and transformed with pAR1.$cheA_S$ (F). Cells were inoculated near the center of tryptone swarm plates containing 0.20% agar, and the plates were incubated at 32°C for 6 h. Expression of CheA variants from each plasmid was induced with 25 μM IPTG.

cannot tumble, e.g., the Δ*cheA* strain AJW1071 (Fig. 4B). To determine whether increased expression of the mutant proteins could confer chemotactic ability, we transformed the mutant allele *cheA*G1, *cheA*G2, *cheA*G1/G2, *cheA*ΔG2, or *cheA*2FY (each carried by the vector pAR1) into the Δ*cheA* strain AJW1071. To construct positive and negative controls, we also transformed this Δ*cheA* strain with the alleles *cheA* and $cheA_S$ (also carried by the vector pAR1). We inoculated cells of the resultant transformants at the center of tryptone swarm plates containing 25 μM IPTG and, after 6 h of incubation, compared their swarms. Only cells that expressed both $CheA_L$ and $CheA_S$ formed two rapidly migrating concentric bands (Fig. 4C). Those that expressed only $CheA_S$ or mutant variants of CheA formed dense nonchemotactic swarms indistinguishable from those produced by the cells of AJW1071, the Δ*cheA* strain, transformed with the vector pAR1 (Fig. 4D).

In vitro transphosphorylation of kinase-deficient $CheA_L$ mutants by $CheA_S$ or $CheA_L$48HQ. To assess the abilities of the nonphosphorylatable CheA variants $CheA_S$ and $CheA_L$48HQ to promote phosphorylation of the glycine-rich region mutants $CheA_L$G1, $CheA_L$G2, $CheA_L$G1/G2, and $CheA_L\Delta$G2, we added [γ-^{32}P]ATP to purified preparations of each mutant protein preincubated for 30 min in the presence of either $CheA_S$ or $CheA_L$48HQ. At the conclusion of a 30-min labeling period at room temperature, we terminated each reaction by adding 3× SDS-PAGE sample-loading buffer and analyzed the results by SDS-PAGE and autoradiography (Fig. 5). Reactions performed with either $CheA_S$ (Fig. 5A) or $CheA_L$48HQ (Fig. 5B) and mutant $CheA_L$G1 (lane 1), $CheA_L$G2 (lane 2), $CheA_L$G1/G2 (lane 3), or $CheA_L\Delta$G2 (lane 4) resulted in phosphorylation of a protein of about 78 kDa. The level of phosphorylation was not uniform, however. Reactions performed with $CheA_S$ repeatedly transphosphorylated more $CheA_L$G1 or $CheA_L$G2 (about 6.0% of wild-type $CheA_L$ levels of autophosphorylation) than $CheA_L$G1/G2 or $CheA_L\Delta$G2 (about 1.5% of wild-type levels).

To assess the abilities of the mutant proteins $CheA_L$G1 and $CheA_L$G2 to promote phosphorylation of each other, we

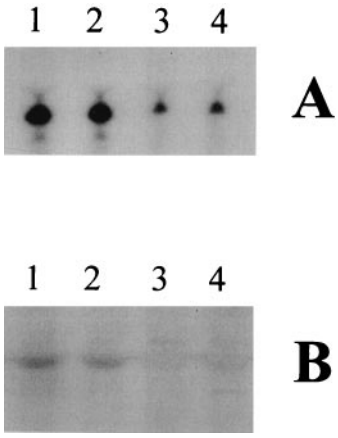

FIG. 5. Phosphorylation of $CheA_L$ variants in the presence of $CheA_S$ (A) or $CheA_L$48HQ (B). CheA samples (each protein at 5 μM [final concentration]) were preincubated for 30 min in TKMD buffer (see Materials and Methods) before the addition of 50 μM [γ-^{32}P]ATP (~2,500 cpm pmol^{-1}). The reactions were terminated by the addition of 3× SDS-PAGE sample buffer, and the samples were analyzed by SDS-PAGE (10% gel) followed by autoradiography for 60 min. Lanes 1, $CheA_L$G1; lanes 2, $CheA_L$G2; lanes 3, $CheA_L$G1/G2; lanes 4, $CheA_L$ΔG2. The image was derived by scanning the autoradiograph with a Hewlett-Packard Scanjet model IIc. Intensities of the bands in panel A were quantified with a Betascope 603 blot analyzer. The gel was scanned for 1 h within the linear range of the instrument.

added [γ-^{32}P]ATP to purified preparations of wild-type $CheA_L$ or each of the two mutant proteins preincubated separately or together for 30 min (Fig. 6A). In contrast to the reaction mixture containing wild-type $CheA_L$ alone (lane 1), those containing $CheA_L$G1 alone (lane 2) and $CheA_L$G2 alone (lane 3) did not result in a phosphorylated 78-kDa protein. Reactions performed with both $CheA_L$G1 and $CheA_L$G2 together (lane 4) resulted in very poor phosphorylation of a protein that migrated slightly more slowly than wild-type $CheA_L$. For several reasons, it seems unlikely that this band corresponds to a phosphorylated form of either $CheA_L$G1 or $CheA_L$G2 or of their 6×His-tagged variants. First, the phosphorylated forms of $CheA_L$G1 and $CheA_L$G2 migrate with the same mobility as phosphorylated wild-type $CheA_L$. Second, after cleavage with thrombin, we detected no 6×His-CheA variant by immunoblot analysis. Third, we detected no phosphorylation of the 6×His tag itself when fused to either of the nonphosphorylatable proteins, $CheA_S$ and $CheA_L$48HQ (data not shown).

Interestingly, reactions involving $CheA_L$G1 (lanes 2 and 4) yielded a phosphorylated protein fragment of about 30 kDa. We observed a phosphorylated fragment of similar molecular mass in reactions involving wild-type $CheA_L$ alone (lane 1) or the F mutant, $CheA_L$2FY (lane 5). To determine whether the 30-kDa fragment included the site of phosphorylation, His-48, we performed immunoblot analyses using anti-CheA (Fig. 6B) and anti-$CheA_L$ (Fig. 6C) antisera. Whereas $CheA_L$ and the 30-kDa fragment reacted with both antisera (lanes 1 and 3, respectively), $CheA_S$ reacted only with the anti-CheA antiserum (lanes 2). On the basis of its size, its immunoreactivity with an antiserum specific for an epitope unique to the P1 domain, and its ability to accept a phosphoryl group, we conclude that this degradation product corresponds to the N-terminal P1 and P2 domains of CheA (22).

$CheA_S$, but not $CheA_L$48HQ, restores chemotactic ability to kinase-deficient $CheA_L$ mutants. To test whether the nonphosphorylatable variant $CheA_S$ can restore chemotactic ability to mutant cells that express CheA variants defective in the glycine-rich regions, we transformed the allele *cheA*$_S$ (carried by the vector pAR1) into cells carrying the mutant allele *cheA*G1, *cheA*G2, or *cheA*G1/G2 (strain AJW955, AJW531, or AJW953, respectively). We inoculated cells of the resultant transformants at the center of tryptone swarm plates containing 25 μM IPTG and, after 6 h of incubation, compared their swarms (Fig. 4). Transformants of strains AJW955 and AJW531 (alleles *cheA*G1 and *cheA*G2, respectively) formed two concentric bands (Fig. 4E and F). The outermost bands formed by these transformants in response to L-serine migrated about 0.28 and 0.33 cm h^{-1}, respectively (cf. the migration rate of 0.49 cm h^{-1} for pAR1.*cheA* transformants of the Δ*cheA* strain AJW1071). In contrast, transformants of strain AJW953 (allele *cheA*G1/G2) formed dense nonchemotactic swarms that migrated slowly (0.02 cm h^{-1}) even when the concentration of IPTG was increased to as much as 100 μM (data not shown).

Since, in vitro, $CheA48_L$HQ did not generate the levels of transphosphorylation observed for $CheA_S$, we wondered whether we would observe similar results in vivo. Therefore, we transformed plasmid pAR1.*cheA48HQ* into cells carrying the mutant allele *cheA*G1 or *cheA*G2 (strain AJW955 or AJW531,

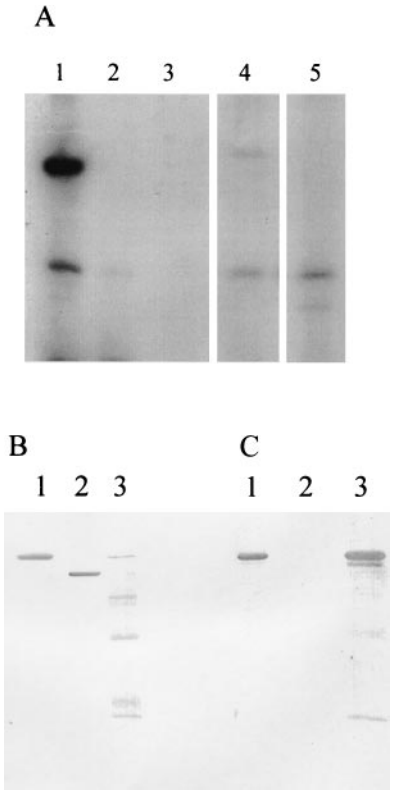

FIG. 6. (A) Phosphorylation of $CheA_L$ variants. CheA samples (each protein at 5 μM [final concentration]) were preincubated for 30 min in TKMD buffer (see Materials and Methods) before the addition of 50 μM [γ-^{32}P]ATP (~2,500 cpm pmol^{-1}). The reactions were terminated by the addition of 3× SDS-PAGE sample buffer, and the samples were analyzed by SDS-PAGE (10% gel) followed by autoradiography for 60 min. Lane 1, wild-type $CheA_L$; lane 2, $CheA_L$G1; lane 3, $CheA_L$G2; lane 4, $CheA_L$G1 and $CheA_L$G2; lane 5, $CheA_L$2FY. The image was derived by scanning the autoradiograph with a Hewlett-Packard Scanjet model IIc. (B and C) Immunoblot analyses using antisera specific for $CheA_L$ and $CheA_S$ (B) or $CheA_L$ alone (C) as primary antibodies. (B) Lane 1, 6×His-$CheA_L$; lane 2, 6×His-$CheA_S$; lane 3, 6×His-$CheA_L$ incubated at room temperature for 5 days to permit degradation. (C) Lane 1, 6×His-$CheA_L$; lane 2, 6×His-$CheA_S$; lane 3, 6×His-$CheA_L$ incubated at room temperature for 5 days to permit degradation and reabsorbed over His-bind resin (Novagen) to enrich for P1-containing fragments. The image was derived by scanning the immunoblots with a Hewlett-Packard Scanjet model IIc.

respectively), inoculated cells of the resultant transformants at the center of tryptone swarm plates containing 25 μM IPTG, and, after 6 h of incubation, compared their swarms. Transformants of both strains formed dense nonchemotactic swarms (data not shown) indistinguishable from those produced by the parent cells or those of the Δ*cheA* strain (AJW1071) transformed with the vector pAR1 (Fig. 4D).

Similarly, cells that carried the mutant allele *cheA*G1 (strain AJW955) transformed with the pAR1 derivative carrying the allele *cheA*G2 and cells that carried the mutant allele *cheA*G2 (strain AJW531) transformed with the pAR1 derivative carrying the allele *cheA*G1 formed only nonchemotactic swarms that migrated slowly regardless of IPTG concentration (data not shown).

DISCUSSION

CheA variants mutated in each of the highly conserved transmitter subdomains G1, F, and G2 did not autophosphorylate in vitro and did not support chemotactic ability in vivo. In vitro, the nonphosphorylatable $CheA_S$ protein mediated transphosphorylation of $CheA_L$ variants defective for either G1 or G2 or both. Similarly, $CheA_S$ restored chemotactic ability to cells that expressed $CheA_L$ variants defective for either G1 or G2. Such was not the case with cells that expressed the $CheA_L$ variant defective for both G1 and G2. This lack of complementation likely occurred due to the poor ability of this mutant protein to become phosphorylated. In contrast, $CheA_L$ variants defective for either G1 or G2 mediated transphosphorylation of each other poorly, if at all. Likewise, cells which expressed both mutant proteins did not perform chemotaxis.

Others have made similar but not identical observations with a number of two-component histidine kinases (20, 22, 39, 41). In particular, Yang and Inouye (40) performed studies of a design similar to ours and which serve as an important comparison for the observations and conclusions presented here. In an attempt to understand the structural relationship between the kinase and phosphatase activities exhibited by the osmoregulatory histidine kinase EnvZ, Yang and Inouye constructed a hybrid protein termed Taz1 consisting of the periplasmic domain of the aspartate chemoreceptor, Tar, fused to the cytoplasmic domain of EnvZ. This construct enabled these researchers to study signal transduction of EnvZ via an easily controllable ligand, aspartate. Using an *ompC*::*lacZ* reporter to quantify OmpR phosphorylation, Yang and Inouye determined that full catalytic function required intact N, G1, and G2 motifs with kinase activity requiring all three motifs and phosphatase activity requiring only G2. They also demonstrated that, as with CheA, a nonphosphorylatable Taz1 variant could mediate transphosphorylation of a phosphorylatable G1/G2 mutant Taz1 protein.

These results are consistent with a mechanism by which the G1 and G2 subdomains within the T_R of one protomer of a CheA (or Taz1) dimer form a unit that mediates transphosphorylation of the other protomer within that dimer (Fig. 7). This model easily reconciles with that of Tawa and Stewart (36), who demonstrated that at least one residue (i.e., 337P) in the T_L of the protomer that becomes phosphorylated helps to determine the binding affinity of CheA for ATP. We envision that the T_L of that protomer interacts with the T_R of the second protomer within the dimer to modulate ATP binding and hydrolysis.

Intriguingly, an amino-terminal 30-kDa fragment of the mutant proteins $CheA_L$2FY and $CheA_L$G1 became phosphorylated even though the full-length proteins apparently did not. Thus, it seems that these mutant proteins can mediate transphosphorylation only when the site of phosphorylation is detached (i.e., when part of the 30-kDa fragment). Perhaps the transmitter domain normally presents ATP to His-48 in a structurally constrained context. A mutation in either G1 or F might cause an alteration in the orientation of that ATP, severely limiting the ability of the P1 domain to dock with the mutated T domain and accept transfer of the γ-phosphoryl group from ATP to His-48. The removal of such constraints by decoupling the ATP-binding site from the phosphoryl acceptor site would thus enable phospho-transfer. Clearly, this observation warrants further investigation.

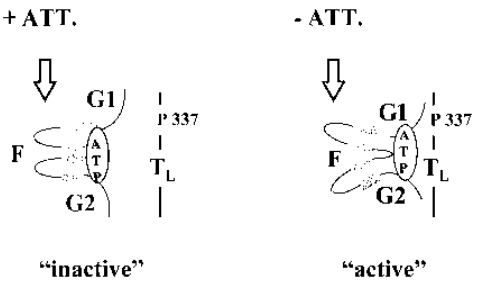

FIG. 7. Schematic demonstrating a working model representing the structural organization of the G1, F, and G2 subdomains of one CheA protomer relative to the T_L of the other protomer within the dimer in response to the addition or removal of a chemoattractant (ATT.). Shaded rectangles and arrows, putative α-helix and β-sheet, respectively, as predicted by structure-averaging algorithms (5a, 25).

ACKNOWLEDGMENTS

We thank A. Binney, C. Park, and J. S. Parkinson for strains, A. Roth and F. W. Dahlquist for plasmids, Jennifer Nelms for technical support, and Rick Stewart and Bob Bourret for their thoughts, suggestions, and critical reading of the manuscript.

This work was supported by Public Health Service grant GM46221 from the National Institute of General Medical Sciences.

REFERENCES

1. **Adler, J.** 1966. Chemotaxis in bacteria. Science **153:**708–716.
2. **Borkovich, K. A., N. Kaplan, J. F. Hess, and M. I. Simon.** 1989. Transmembrane signal transduction in bacterial chemotaxis involves ligand-dependent activation of phosphate group transfer. Proc. Natl. Acad. Sci. USA **86:**1208–1212.
3. **Borkovich, K. A., and M. I. Simon.** 1990. The dynamics of protein phosphorylation in bacterial chemotaxis. Cell **63:**1339–1348.
4. **Bourret, R. B., K. A. Borkovich, and M. I. Simon.** 1991. Signal transduction pathways involving protein phosphorylation in prokaryotes. Annu. Rev. Biochem. **60:**401–441.
5. **Bourret, R. B., J. Davagnino, and M. I. Simon.** 1993. The carboxy-terminal portion of the CheA kinase mediates regulation of autophosphorylation by transducer and CheW. J. Bacteriol. **175:**2097–2101.

5a. **Ellefson, D. D., and A. J. Wolfe.** Unpublished data.

6. **Gegner, J. A., and F. W. Dahlquist.** 1991. Signal transduction in bacteria: CheW forms a reversible complex with the protein kinase CheA. Proc. Natl. Acad. Sci. USA **88:**750–754.
7. **Gegner, J. A., D. R. Graham, A. F. Roth, and F. W. Dahlquist.** 1992. Assembly of an MCP receptor, CheW, and kinase CheA complex in the bacterial chemotaxis signal transduction pathway. Cell **70:**975–982.
8. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241:**42–52.
9. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
10. **Hess, J. F., R. B. Bourret, K. Oosawa, P. Matsumura, and M. I. Simon.** 1988. Protein phosphorylation and bacterial chemotaxis. Cold Spring Harbor Symp. Quant. Biol. **53:**41–48.
11. **Hess, J. F., R. B. Bourret, and M. I. Simon.** 1988. Histidine phosphorylation and phosphoryl group transfer in bacterial chemotaxis. Nature (London) **336:**139–143.
12. **Hess, J. F., K. Oosawa, P. Matsumura, and M. I. Simon.** 1987. Protein phosphorylation is involved in bacterial chemotaxis. Proc. Natl. Acad. Sci. USA **84:**7609–7613.

13. **Hoffman, A., and R. G. Roeder.** 1991. Purification of his-tagged proteins in non-denaturing conditions suggests a convenient method for protein interaction studies. Nucleic Acids Res. **19:**6337–6338.
14. **Kofoid, E. C., and J. S. Parkinson.** 1991. Tandem translation starts in the *cheA* locus of *Escherichia coli*. J. Bacteriol. **173:**2116–2119.
15. **Kofoid, E. C., and J. S. Parkinson.** 1988. Transmitter and receiver modules in bacterial signaling proteins. Proc. Natl. Acad. Sci. USA **85:**4981–4985.
16. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
18. **Lui, J.** 1990. Ph.D. thesis. University of Utah, Salt Lake City.
19. **Morrison, T. B., and J. S. Parkinson.** 1994. Liberation of an interaction domain from the phosphotransfer region of CheA, a signaling kinase of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **91:**5485–5489.
20. **Ninfa, E. G., M. R. Atkinson, E. S. Kamberov, and A. J. Ninfa.** 1993. Mechanism of autophosphorylation of *Escherichia coli* nitrogen regulator II (NRII or NtrB): *trans*-phosphorylation between subunits. J. Bacteriol. **175:** 7024–7032.
21. **Ninfa, E. G., A. Stock, S. Mowbray, and J. Stock.** 1991. Reconstitution of the bacterial chemotaxis signal transduction system from purified components. J. Biol. Chem. **266:**9764–9770.
22. **Oosawa, K., J. F. Hess, and M. I. Simon.** 1988. Mutants defective in bacterial chemotaxis show modified protein phosphorylation. Cell **53:**89–96.
23. **Park, C. P., and G. L. Hazelbauer.** 1986. Mutations specifically affecting ligand interaction of the Trg chemosensory transducer. J. Bacteriol. **167:**101–109.
24. **Parkinson, J. S., and S. E. Houts.** 1982. Isolation and behavior of *Escherichia coli* deletion mutants lacking chemotaxis functions. J. Bacteriol. **151:**106–113.
25. **Parkinson, J. S., and E. C. Kofoid.** 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26:**71–112.
26. **Rossman, M. G., and A. Liljas.** 1974. Recognition of structural domains in globular proteins. J. Mol. Biol. **85:**177–181.
27. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Smith, R. A., and J. S. Parkinson.** 1980. Overlapping genes at the *cheA* locus of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77:**5370–5374.
29. **Soderling, T. R.** 1990. Protein kinases: regulation by autoinhibitory domains. J. Biol. Chem. **265:**1823–1826.
30. **Stock, J. B., G. S. Lukat, and A. M. Stock.** 1991. Bacterial chemotaxis and the molecular logic of intracellular signal transduction networks. Annu. Rev. Biophys. Biophys. Chem. **20:**109–136.
31. **Stock, J. B., and M. G. Surette.** 1996. Chemotaxis, p. 1103–1129. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, 2nd ed., vol. 1. American Society for Microbiology, Washington, D.C.
32. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
33. **Surette, M. G., M. Levit, Y. Liu, E. G. Ninfa, and J. B. Stock.** 1996. Dimerization is required for the activity of the protein histidine kinase CheA that mediates signal transduction in bacterial chemotaxis. J. Biol. Chem. **271:**939–945.
34. **Swanson, R. V., R. B. Bourret, and M. I. Simon.** 1993. Intermolecular complementation of the kinase activity of CheA. Mol. Microbiol. **8:**435–441.
35. **Swanson, R. V., S. C. Schuster, and M. I. Simon.** 1993. Expression of CheA fragments which define domains encoding kinase, phosphotransfer, and CheY binding activities. Biochemistry **32:**7623–7629.
36. **Tawa, P., and R. C. Stewart.** 1994. Mutational activation of CheA, the protein kinase in the chemotaxis system of *Escherichia coli*. J. Bacteriol. **176:**4210–4218.
37. **Wolfe, A. J., and H. C. Berg.** 1989. Migration of bacteria in semisolid agar. Proc. Natl. Acad. Sci. USA **86:**6973–6977.
38. **Wolfe, A. J., B. P. McNamara, and R. C. Stewart.** 1994. The short form of CheA couples chemoreception to CheA phosphorylation. J. Bacteriol. **176:** 4483–4491.
39. **Wolfe, A. J., and R. C. Stewart.** 1993. The short form of the CheA protein restores kinase activity and chemotactic ability to kinase-deficient mutants. Proc. Natl. Acad. Sci. USA **90:**1518–1522.
40. **Yang, Y., and M. Inouye.** 1993. Requirement of both kinase and phosphatase activities of an *Escherichia coli* receptor (Taz1) for ligand-dependent signal transduction. J. Mol. Biol. **231:**335–342.
41. **Yang, Y., H. Park, and M. Inouye.** 1993. Ligand binding induces an asymmetrical transmembrane signal through a receptor dimer. J. Mol. Biol. **232:** 493–498.