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The Carboxy-Terminal Portion of the CheA Kinase Mediates Regulation of Autophosphorylation by Transducer and CheW

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The CheA kinase is a central protein in the signal transduction network that controls chemotaxis in *Escherichia coli*. CheA receives information from a transmembrane receptor (e.g., Tar) and CheW proteins and relays it to the CheB and CheY proteins. The biochemical activities of mutant CheA proteins truncated at various distances from the carboxy terminus were examined. The carboxy-terminal portion of CheA regulates autophosphorylation in response to environmental signals transmitted through Tar and CheW. The central portion of CheA is required for autophosphorylation and is also presumably involved in dimer formation. The amino-terminal portion of CheA was previously shown to contain the site of autophosphorylation and to be able to transfer the phosphoryl group to CheB and CheY. These studies further delineate three functional domains of the CheA protein.

The sensory transduction pathway controlling bacterial chemotaxis links changes in external chemoeffector concentrations to altered swimming behavior via a cascade of transient protein phosphorylation events (reviewed in references 4 and 35). A central protein in this scheme is the CheA kinase. CheA integrates information from a variety of chemosensory receptors, such as the transmembrane transducer Tar, by regulating autophosphorylation activity up or down in response to receptor occupancy. The CheW protein is necessary for CheA-transducer coupling; however, the mechanistic role of CheW remains obscure. CheA distributes information by phosphorylating the CheY protein, which produces an excitation signal. CheA also phosphorylates the CheB protein, which changes the degree of modification of the receptor, thus modulating sensory adaptation.

It is apparent that CheA interacts with numerous proteins and small molecules. We would like to understand how the CheA protein is organized to carry out these various tasks. Several biochemical properties of CheA have been described. The *Escherichia coli* CheA protein is 654 amino acids in length (17). CheA uses the γ-phosphoryl group of ATP to autophosphorylate histidine residue 48 (11, 13, 14, 39). Tar and CheW regulate CheA autophosphorylation in a ligand-dependent manner (1, 2, 27). CheA forms complexes with CheW (9, 23, 24); complexes of Tar, CheW, and CheA have also been observed (2, 10). These complexes are presumably involved in regulating CheA function. CheA transfers its phosphoryl group to either CheB or CheY (13, 39). Finally, CheA exists as a dimer in solution (9).

Additional information about the functional domains of CheA is also available from previous genetic studies. Intragenic complementation between mutations in the 5′ and 3′ portions of the *cheA* gene suggests the existence of at least two distinct functional domains (32). One such domain has been identified. An isolated tryptic peptide corresponding to the N-terminal ~18 kDa of CheA is capable of phosphotransfer to CheB and CheY but cannot autophosphorylate (11). Missense mutations which generate CheA proteins deficient in phosphorylation are also found in the middle of the gene, suggesting that the central portion of the protein may be involved in phosphorylation function (29).

In the present work, we have assessed the effect of removing the C-terminal portion of CheA on kinase, regulation, phosphotransfer, and dimerization functions. The results indicate that the C-terminal domain is involved in regulating CheA activity as a function of its interaction with CheW and the receptor.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* KO685 (14) and RBB382 (5) are Δ*cheA* Δ*recA*. HCB721 is a "gutted" strain, lacking all chemotaxis functions (38). pDV4 contains the *cheAW* genes under the control of the *Serratia marcescens trp* promoter (33). pRBB28 was made by filling in the *Hin*dIII site of pDV4 to create an *Nhe*I site and converting the *Sty*I site in *cheW* to a *Hin*dIII site with a synthetic linker; thus, only CheA is expressed from pRBB28. pRBB30 was made by filling in the *Hin*dIII site of pDV4 to create an *Nhe*I site and converting the *Xba*I site 3′ of *cheW* to a *Hin*dIII site with a synthetic linker. pRBB41 was constructed by replacing the *Hin*cII-*Hin*dIII fragment of pACYC84 (6) containing the Tet^r gene with the *Sca*I-*Hin*dIII fragment of pRBB30 carrying p_{trp} *cheAW*. Thus, pRBB30 and pRBB41 express both CheA and CheW. Nonsense mutations in *cheA* were created by the *dut ung* method of site-directed mutagenesis (18). Mutations are named with the number of the codon changed, followed by the single-letter code for the original amino acid, and then two letters in parentheses indicating the nature of the nonsense codon created (Am [amber] or Oc [ochre]). All mutations were confirmed by DNA sequencing.

Protein purification. HCB721 carrying plasmid pBR322, pNT201, or pNT201-N15 was used as the source for control, Tar-containing, or smooth-mutant Tar-containing membranes, respectively, for purification, as described previously (2). Some preparations were a gift from Kathy Borkovich. CheW was purified as previously described (3) and was a gift from Lisa Alex. CheY was purified as previously described (12).

Preliminary attempts to purify the truncated CheA pro-

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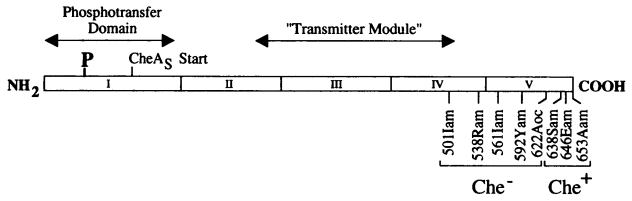

FIG. 1. Important features of mutant CheA proteins used in this study. The location, nonsense codon, and phenotype of each mutant are given. P denotes the site of autophosphorylation, histidine 48 (11). The first amino acid of the CheA$_S$ protein, methionine 98, is also indicated (17). The approximate extents of the 18-kDa proteolytic fragment that mediates phosphotransfer (11) and of the conserved transmitter module sequences believed to comprise kinase function (16, 30) are shown. Regions I to V are arbitrarily defined from restriction sites in the *cheA* gene; mutants with altered phosphorylation properties were previously identified in regions I, III, and IV (29). Diagram is drawn to scale.

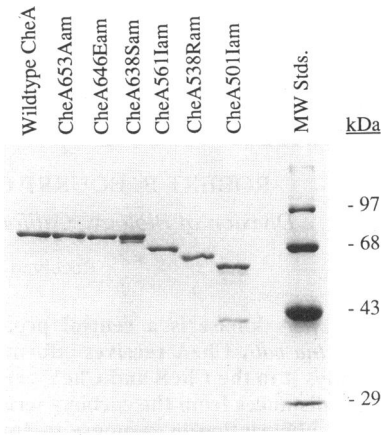

FIG. 2. Coomassie blue-stained gel of six purified truncated CheA proteins. The CheA592Y(Am) and CheA622A(Oc) proteins were also successfully purified but are not shown here. Size standards were run in the far right lane.

teins by our previously described procedure (12) failed because the mutant proteins have lower affinity for an Affi-Gel Blue column than full-length CheA. Ion-exchange chromatography was therefore used in a modified procedure, prompted by the purification protocol for *Salmonella typhimurium* CheA (34). Briefly, cultures of KO685/pRBB28 coding for the various mutant proteins were induced with β-indoleacrylic acid, harvested by centrifugation, and lysed by sonication in TEDG with 1 mM phenylmethylsulfonyl fluoride (12). After ultracentrifugation to remove membranes, ammonium sulfate precipitation, and dialysis, the crude preparation containing CheA was loaded onto a MonoQ fast protein liquid chromatography (FPLC) column. CheA was eluted at a flow rate of 0.4 ml/min, with a linear NaCl gradient from 0.188 to 0.413 M in TEDG plus 1 mM phenylmethylsulfonyl fluoride. Fractions containing essentially pure CheA were identified by gel electrophoresis and pooled.

Phosphorylation assays. For phosphorylation assays, the various chemotaxis proteins were incubated at room temperature in a final volume of 20 μl containing 5 mM $MgCl_2$, 100 mM KCl, 50 mM Tris (pH 7.5), and 0.1 mM [γ-^{32}P]ATP (specific activity, 5 to 10 Ci/mmol). Specific reaction times and protein quantities are given in the individual figure legends. Reactions were terminated by the addition of sodium dodecyl sulfate sample buffer containing a final concentration of 10 mM EDTA to stabilize CheY-phosphate against Mg^{2+}-catalyzed autodephosphorylation (22), and reaction mixes were subjected to electrophoresis and autoradiography as described before (3).

The bacterial strain used to prepare membranes for transducer-coupled reactions involving mutant CheA proteins is important. Some CheA is localized to the inner membrane (31). Preliminary phosphorylation experiments with membranes from the transducer deletion strain KO607 (1) gave results consistent with contamination of the membranes with wild-type CheA, in spite of the 2 M NaCl wash. Thus, all reported experiments were done with membranes prepared from HCB721.

RESULTS

C-terminal portion of CheA essential for chemotaxis. A series of eight nonsense mutations in the 3′ portion of the *cheA* gene were constructed in order to explore the functional role of the C-terminal portion of CheA (Fig. 1). The ability of the resulting truncated CheA proteins to support chemotaxis in the Δ*cheA* host RBB382 was examined. Bacteria carrying the *cheA*638S(Am), *cheA*646E(Am), or *cheA*653A(Am) mutation on pRBB41 behaved indistinguishably from bacteria carrying the wild-type *cheA* gene in terms of swarm rate on tryptone motility plates and flagellar rotation as assayed by tethering (data not shown). In contrast, bacteria carrying the *cheA*501I(Am), *cheA*538R(Am), *cheA*561I(Am), *cheA*592Y(Am), or *cheA*622A(Oc) mutation behaved indistinguishably from the Δ*cheA* host with no plasmid (data not shown). Thus, removal of ≤17 amino acids from the C terminus of CheA has no effect on function, whereas removal of ≥33 amino acids abolishes chemotaxis, as assayed on swarm plates.

C-terminal portion of CheA not essential for stability, autophosphorylation, or phosphotransfer. All eight truncated CheA proteins, lacking from 2 to 154 amino acids, were successfully purified as the first step in assigning a functional role to the C-terminal portion of CheA (Fig. 2). Significant degradation was observed only for the shortest polypeptide, CheA501I(Am), but even in this case the prominent species was of the expected size. Thus, the failure of the truncated CheA proteins to support chemotaxis is not due to degradation of mutant polypeptides.

The simplest biochemical assay for CheA function is autophosphorylation (14, 39). The truncated CheA proteins were incubated with [γ-^{32}P]ATP, and the products of the reaction were separated by gel electrophoresis. Autoradiography showed that all the truncated CheA proteins except CheA501I(Am) autophosphorylated (Fig. 3). The central conclusion drawn from this experiment is unaffected by the observed variation in apparent autophosphorylation levels. Removal of up to 117 amino acids from the C terminus does not prevent autophosphorylation, whereas removal of 154 amino acids abolishes autophosphorylation. Truncated CheA proteins that do not support chemotaxis are nevertheless capable of autophosphorylation.

A more complex assay is to incubate the CheA proteins with [γ-^{32}P]ATP in the presence or absence of CheY (13, 39). All truncated CheA proteins that were capable of autophosphorylation were also capable of phosphotransfer to CheY (Fig. 4). This result is consistent with the previous observation that the N-terminal portion of CheA mediates phos-

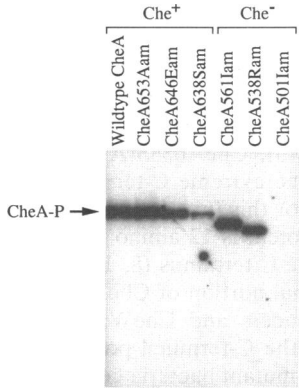

FIG. 3. Autophosphorylation of truncated CheA proteins. Each CheA protein (5 pmol) was incubated with 0.1 mM [γ-^{32}P]ATP for 10 min. An autoradiograph of the reaction products after separation by gel electrophoresis is shown. Autophosphorylation of the CheA592Y(Am) and CheA622A(Oc) proteins was demonstrated in a separate experiment (data not shown).

photransfer (11) and rules out the possibility that C-terminal truncations of CheA result in an aberrant structure that inhibits phosphotransfer. Once again, the ability of truncated CheA proteins to support chemotaxis did not correlate with the biochemical function assayed, i.e., a number of these mutant proteins showed no change in autophosphorylation or phosphoryl group transfer yet were not able to complement *cheA*-deleted strains.

C-terminal portion of CheA essential for regulated autophosphorylation. The in vitro assay that most completely mimics the in vivo signaling pathway includes Tar-containing membranes and CheW in addition to CheA, CheY, and [γ-^{32}P]ATP (1). Tar and CheW have previously been shown to directly regulate autophosphorylation of CheA (2, 27). The CheA autophosphorylation reaction may be sensitively but indirectly assayed by coupling it to the CheA-CheY phosphotransfer reaction. A large molar excess of CheY is used so that the phosphotransfer step is not rate limiting. Tar

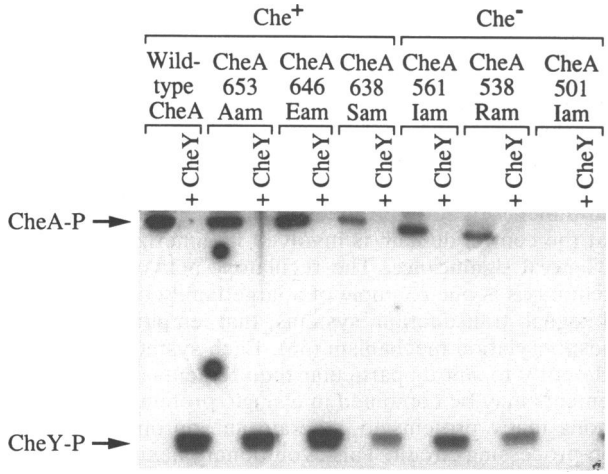

FIG. 4. Phosphorylation of CheY by truncated CheA proteins. Each CheA protein (5 pmol) was incubated with 0.1 mM [γ-^{32}P]ATP for 10 s in the absence or presence of 200 pmol of CheY. An autoradiograph of the reaction products after separation by gel electrophoresis is shown.

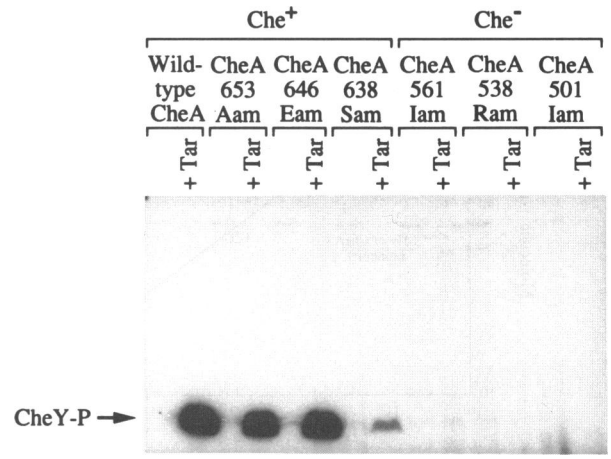

FIG. 5. Phosphorylation of CheY by truncated CheA proteins in the presence of transducer-containing membranes and CheW. Membranes lacking or containing Tar (11 μg of membrane protein in either case), 40 pmol of CheW, 5 pmol of each CheA protein, and 200 pmol of CheY were incubated with 0.1 mM [γ-^{32}P]ATP for 10 s. An autoradiograph of the reaction products after separation by gel electrophoresis is shown. The presence of membranes inhibits CheA autophosphorylation (see references 2 and 13). Thus, in this experiment, phosphorylated CheY (CheY-P) is observed only under conditions that stimulate CheA autophosphorylation, in contrast to Fig. 4.

and CheW were found to enhance formation of CheY-phosphate by the same truncated CheA proteins that supported chemotaxis (Fig. 5). The truncated CheA proteins that did not support chemotaxis did not show receptor-mediated stimulation of CheY-phosphate formation.

Similarly, the presence of a mutant Tar protein locked in the "smooth" signaling mode (25) inhibited autophosphorylation of the "Che$^+$" proteins CheA646E(Am) and CheA653A(Am) but had no effect on autophosphorylation of the Che$^-$ proteins CheA538R(Am), CheA561I(Am), or CheA622A(Oc) (data not shown). Note that CheA-phosphate rather than the surrogate CheY-phosphate was monitored directly in the inhibition experiment. The activity of the shortest Che$^+$ protein, CheA638S(Am), which was stimulated modestly by wild-type Tar in vitro (Fig. 5), was not detectably affected in the less-sensitive inhibition assay (data not shown). Thus, the C-terminal portion of CheA is necessary for Tar/CheW-mediated regulation of autophosphorylation. Furthermore, the dependence of regulation on the length of the truncated CheA proteins appears to be the same for both stimulation and inhibition of autophosphorylation.

C-terminal portion of CheA not essential for dimerization. CheA migrates on gel filtration columns with the apparent molecular weight predicted for a tetramer (14, 34), although it has been shown to behave as a dimer by equilibrium analytical ultracentrifugation (9). CheA538R(Am) migrates slightly more slowly than CheA on an FPLC gel filtration column, at a position consistent with its shorter length and quite distinct from that expected of a monomer (Fig. 6). CheA538R(Am) thus apparently forms homodimers. This result implies that the lack of regulation of phosphorylation in CheA538R(Am) and the other Che$^-$ truncation proteins is not attributable to failure to form dimers.

DISCUSSION

Model of CheA functional organization. The experiments described in this article, in conjunction with previous re-

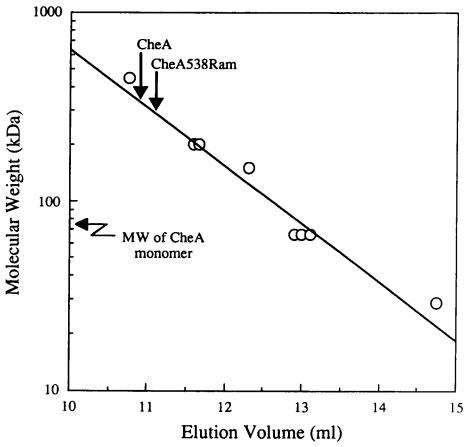

FIG. 6. Migration of CheA and CheA538R(Am) on a Superose 12 FPLC gel filtration column. Molecular size standards were apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

sults, clearly suggest that CheA is composed of at least three distinct functional domains (Fig. 7). The central catalytic domain is a kinase whose function is subject to control by a C-terminal regulatory domain. The phosphoryl group is transferred from CheA to CheB or CheY by the N-terminal phosphotransfer domain. The central portion of CheA is also involved in dimerization. We comment in more detail on each function below.

Kinase function. CheA is a member of a large family of bacterial regulatory proteins with kinase activity (34, 36). Amino acid sequence comparison reveals a common region, termed a transmitter module, believed to encode a kinase function (16, 30). Transmitter sequences extend approximately from amino acids 261 to 507 in CheA (Fig. 1). A CheA-CheZ fusion protein lacking the C-terminal portion of CheA was previously observed to autophosphorylate (19). The precise extent of *cheA* sequences retained in the fusion has not been determined, except that an *Eco*RI restriction site corresponding to amino acid 431 is present (7, 17). The observation of kinase activity in CheA538R(Am) but not in CheA501I(Am) (Fig. 2) defines the C-terminal end of the catalytic domain as occurring sharply between amino acids 500 and 537, in accord with the C-terminal end of the predicted transmitter module. The N-terminal end of the catalytic domain has not been experimentally defined. However $CheA_S$, a naturally occurring form of CheA lacking the N-terminal 97 amino acids of the full-length molecule (17, 32), possesses kinase activity (37).

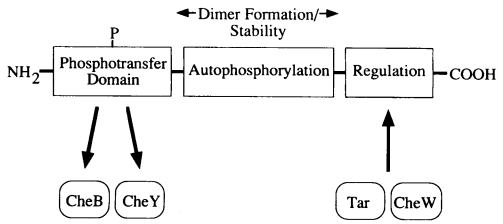

FIG. 7. Model of CheA functional organization. Information about chemoeffector concentrations is relayed to the C-terminal regulatory region of CheA via the transmembrane transducers (e.g., Tar) and CheW. The regulatory region controls the rate at which the central region catalyzes the autophosphorylation of histidine 48. The N-terminal phosphotransfer domain transfers the phosphoryl group to CheB or CheY. Dimer formation and protein stability are mediated by the central portion of the molecule.

Regulation of autophosphorylation. The C-terminal portion of CheA mediates regulation of autophosphorylation by Tar and CheW (Fig. 5). CheA638S(Am) supports chemotaxis, suggesting that the extreme C-terminal sequences of CheA are dispensable for this function. Similarly, the wild-type *B. subtilis* CheA protein is 12 amino acids shorter than the *E. coli* protein at the C terminus (8, 17).

If the C-terminal portion of CheA is required for interaction with transducers and CheW, then the phenotype of bacteria lacking the C-terminal portion of CheA should be similar to that of mutant bacteria containing intact CheA but lacking transducers or CheW. This is in fact the case, as each exhibits nonchemotactic smooth-swimming behavior. Direct binding of CheW and/or the transducers to the C-terminal portion of CheA would provide a simple explanation for the regulatory role of this region. Two previous observations are consistent with this proposal. First, allele-specific suppression between *tsr* and *cheA* mutations suggests a physical interaction in the C-terminal region of CheA (20). Second, $CheA_S$ forms complexes with CheW (24), indicating that the N-terminal portion of CheA is not required for binding to CheW. Experiments are currently in progress to test the ability of the various truncated CheA proteins to form Tar-CheW-CheA complexes.

Phosphotransfer. An isolated N-terminal CheA fragment of ~160 amino acids can mediate phosphotransfer to CheB and CheY (11). The recent discovery that phosphoramidate and other small molecules can phosphorylate CheB and CheY indicates that the amino acid residues catalyzing the phosphotransfer reaction must reside in the recipient proteins rather than CheA (21). CheA does, however, contain information that directs the specificity of phosphotransfer to CheB and CheY rather than the other potential phosphoacceptor proteins present in the cell. NtrC (26), OmpR (15), and Spo0A (28) can each transfer phosphate from CheA to themselves in vitro, but much less efficiently than CheB or CheY does. Substrate recognition specificity for phosphotransfer presumably resides in the N-terminal portion of CheA, but this has not yet been established experimentally.

Dimerization and stability. The C-terminal portion of CheA is dispensable for both dimerization and formation of a stable polypeptide (Fig. 2 and 6). The N-terminal portion is similarly dispensable, as evidenced by the ability of $CheA_S$ to form dimers (35, 37). This is consistent with the finding that mutations in the central portion of CheA affect protein stability (29, 32). A simple explanation for this correlation is that dimer formation provides resistance to proteolysis and that the central domain is involved in dimerization.

General significance. The regulatory network controlling chemotaxis is one example of a large family of environmental signal transduction systems that employ a common phosphorylation mechanism (36). Each system is organized differently to suit its particular requirements (4). Functional elements may be combined in a single protein or distributed among many proteins to generate an appropriate information-processing circuit. This evolutionary flexibility is apparently reflected in the organization of CheA into separable functional domains. Thus, we can study the mechanisms that drive the activity of each of these elements and assemble a picture of how CheA acts to transduce signals from the chemotaxis receptors to the flagellar apparatus.

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