Protein phosphorylation is involved in bacterial chemotaxis

(signal transduction/chemotaxis proteins/ATP/mutagenesis)

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ABSTRACT   The nature of the biochemical signal that is involved in the excitation response in bacterial chemotaxis is not known. However, ATP is required for chemotaxis. We have purified all of the proteins involved in signal transduction and show that the product of the *cheA* gene is rapidly autophosphorylated, while some mutant CheA proteins cannot be phosphorylated. The presence of stoichiometric levels of two other purified components in the chemotaxis system, the CheY and CheZ proteins, induces dephosphorylation. We suggest that the phosphorylation of CheA by ATP plays a central role in signal transduction in chemotaxis.

Bacteria can respond to chemical changes in their environment by altering their pattern of motility, resulting in swimming toward higher concentrations of attractants and away from repellents. The chemotaxis response is mediated by a series of transmembrane receptor–transducer proteins that bind specific ligands and transmit information about changes in ligand concentration as a function of time to the bacterial flagellar apparatus (for reviews, see refs. 1, 2, and 3). The components involved in the intracellular signal transduction pathway for chemotaxis in *Escherichia coli* and *Salmonella* have been identified by genetic techniques. Four genes, *cheA*, *cheW*, *cheY*, and *cheZ* elaborate products that are required for the integration and transduction of information. Two other gene products encoded by *cheR* and *cheB* are responsible for adaptation to wide ranges of ligand concentration. They reversibly methylate specific glutamic acid residues on the cytoplasmic portion of the receptor–transducer, modulating its function.

While a great deal is known about the components of the information-processing system, little is known about the biochemical nature of the chemotactic signal. A number of laboratories have found that ATP is required for signal transduction (4–7). However, the exact nature of its involvement was not clear. Indirect experiments have led to the formulation of models for the function of the chemotaxis proteins and their interaction with ATP (2, 8). To measure these interactions directly, we purified all of the proteins known to be involved in the central pathway for information transduction. In this paper we show that the *cheA* gene product can autophosphorylate with ATP. We can isolate a phosphorylated CheA intermediate and show that the CheY and CheZ proteins can influence the course of CheA phosphorylation. Furthermore, mutations that eliminate chemotaxis and map within the *cheA* gene result in proteins that are defective in the phosphorylation reaction.

MATERIALS AND METHODS

Protein Purifications. CheA and CheW were overexpressed from a plasmid, pDV4 (P.M., unpublished data), containing

the *cheA* and *cheW* genes. The plasmid was maintained in an *E. coli* W3110 derivative SVS402 Δ*trpE-A*, *recA1*, *tna-2*, *bglR*, obtained from R. Bauerle (University of Virginia, Charlottesville, VA). CheA was purified by a protocol including the use of dye-ligand chromatography and gel filtration and will be described in greater detail elsewhere. CheW was purified essentially as described by Stock *et al.* (9). CheY and CheZ were purified from SVS402 containing the plasmid pRL22 (10). CheY was purified as described by Matsumura *et al.* (10). CheZ was purified in a procedure using dye-ligand chromatography, ion-exchange chromatography, and gel filtration that will be described in greater detail elsewhere. Each protein was purified to approximately 90% homogeneity as judged by Coomassie blue staining of NaDodSO$_4$/polyacrylamide gels (see Fig. 1).

Protein Labeling Experiments. Proteins were labeled for analysis by NaDodSO$_4$/polyacrylamide gel electrophoresis in TEG buffer [50 mM Tris, pH 7.5/0.5 mM EDTA/10% (vol/vol) glycerol] containing 0.4 mM [γ-^{32}P]ATP (5000 cpm/pmol) and 5 mM MgCl$_2$ in a volume of 15 μl. After incubation for 30 min at room temperature, reactions were terminated by the addition of 700 μl of 5% (wt/vol) ice-cold CCl$_3$COOH containing 1% sodium pyrophosphate, and then the preparation was placed on ice for 20 min prior to centrifugation for 10 min in a Microfuge. The resulting pellet was resuspended in NaDodSO$_4$ sample buffer (0.25 M Tris·HCl, pH 6.8/4% NaDodSO$_4$/20% sucrose/10% 2-mercaptoethanol/0.02% bromphenol blue) and analyzed on 12.5% polyacrylamide gels. After electrophoresis the gels were stained with Coomassie blue, destained, dried under vacuum, and subjected to autoradiography for 18 hr using Kodak XAR-5 film.

Incorporation of label into protein was also analyzed by CCl$_3$COOH precipitation onto Whatman GF/C glass fiber filter disks. Reactions were terminated by spotting the preparation onto the disks, which were immediately plunged into ice-cold 10% CCl$_3$COOH containing 1% sodium pyrophosphate. After 30 min in ice-cold 10% CCl$_3$COOH, the filters were washed three times for 30 min each in ice-cold 5% CCl$_3$COOH containing 1% sodium pyrophosphate. The disks were then washed briefly in ethanol, dried, and placed into Amersham ACS scintillation fluid for assay of radioactivity.

Anti-CheA antibody was raised in rabbits to purified CheA protein. CheA protein was detected by immunoblotting according to the instructions of the manufacturer (Bio-Rad).

Purification of Phosphorylated CheA. Purified CheA (150 μg) was incubated with 0.4 mM [γ-^{32}P]ATP and 5 mM MgCl$_2$ for 30 min. The reaction mixture was placed on ice, and ammonium sulfate solution was added to 45% saturation to precipitate CheA. Precipitated CheA was redissolved in TEG buffer, applied to a Pharmacia Superose-12 fast protein liquid chromatography column, and eluted with a flow rate of 0.4 ml/min. Fractions of 0.5 ml were collected and assayed for radioactivity.

Thin-Layer Chromatography. Polygram CEL 300 PEI (polyethyleneimine-cellulose) plates (Brinkmann) were washed overnight in water and air-dried. Samples containing 1000
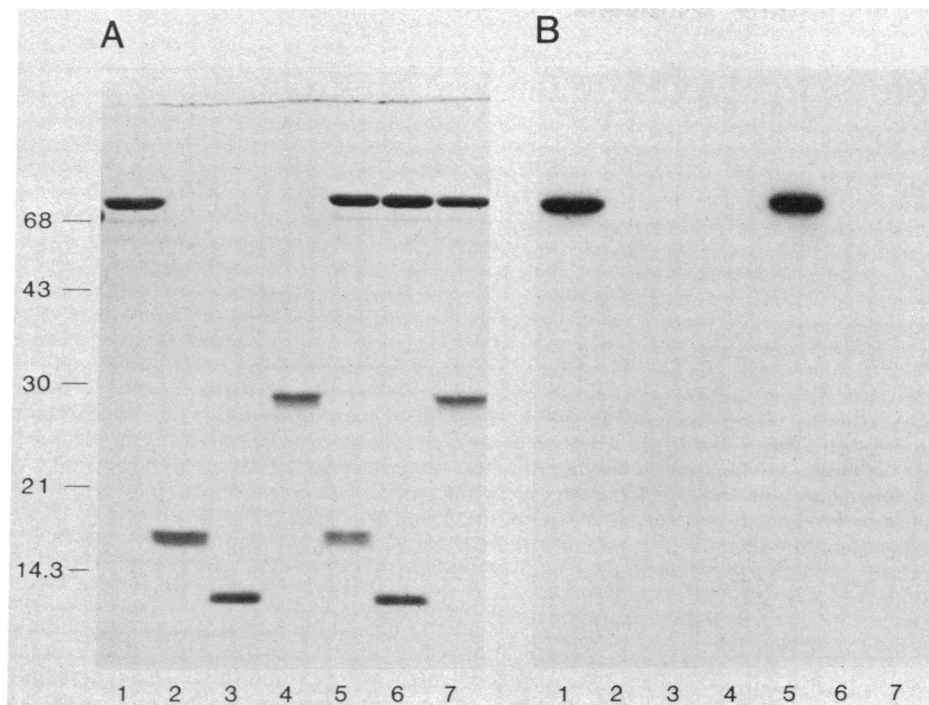
FIG. 1. Phosphorylation of chemotaxis proteins. Purified chemotaxis proteins were incubated with [γ-^{32}P]ATP. Protein was precipitated from the reaction by CCl$_3$COOH and dissolved in NaDodSO$_4$ sample buffer. (*A*) Coomassie blue stain of NaDodSO$_4$/12.5% polyacrylamide electrophoresis gel. (*B*) Autoradiogram of the gel in *A*. Lanes: 1, CheA; 2, CheW; 3, CheY; 4, CheZ; 5, CheA and CheW; 6, CheA and CheY; 7, CheA and CheZ.

cpm of purified phosphorylated CheA were spotted onto the plate. Plates were developed with 1 M acetic acid/4 M LiCl, 8:2 (vol/vol) (11). With this solvent, phosphorylated CheA remains at the origin, ATP migrates with an R_f of approximately 0.16, and P$_i$ migrates with an R_f of approximately 0.68. After development the plates were air-dried and subjected to autoradiography for 24 hr.

Mutagenesis. Hydroxylamine mutagenesis of plasmid pDV4 was performed as described by Mutoh *et al.* (12). Mutants in *cheA* were screened on swarm plates after transformation of the mutagenized plasmid DNA into KO685, which is a Δ*recA* derivative of RP1788 (Δ*cheAm102-11*) obtained from J. S. Parkinson (University of Utah, Salt Lake City, UT). Extensive analysis of *cheA* mutants will be described in detail elsewhere.

RESULTS

We examined covalent modifications of purified preparations of the cytoplasmic chemotaxis proteins CheA, CheW, CheY, and CheZ. The protein preparations were incubated with 5 mM MgCl$_2$ and 0.4 mM [γ-^{32}P]ATP for 30 min at room temperature. These reactions were terminated by CCl$_3$-COOH precipitation and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis followed by autoradiography (Fig. 1). In reactions containing only CheA, the protein was labeled (lane 1); therefore, CheA is presumably autophosphorylated. None of the other cytoplasmic chemotaxis proteins examined were radiolabeled. The addition of either CheY or CheZ in molar excess with respect to CheA decreased the level of CheA labeling (lanes 6 and 7), whereas the addition of similar amounts of CheW (lane 4) had no detectable effect. The *cheA* gene encodes two polypeptides—a large and a small form that are identical except that the large form contains additional amino acids on the N terminus (13). The small form is thought to arise because of a second translational start site in *cheA* (13). In our preparations the large form of 72 kDa predominated (Fig. 1). We detected two minor bands at 65 kDa and 60 kDa, both of which cross-reacted with anti-CheA antibody (see Fig. 5). Only the large form of CheA appeared to be phosphorylated, suggesting that

the N terminus of CheA is essential for CheA phosphorylation.

To establish whether the observed labeling of CheA was due to phosphorylation or adenylylation, identical samples of CheA were incubated for 30 min with 0.4 mM ATP containing ^{32}P label in either the α or γ phosphate. The reaction mixtures were then placed on ice, and CheA was precipitated by ammonium sulfate. The precipitated CheA was redissolved in buffer and immediately applied to a fast protein liquid chromatography gel filtration column. CheA was eluted from this column as a complex of approximately 300 kDa. Radioactive material was found to cochromatograph with CheA only in preparations containing [γ-^{32}P]ATP (Fig. 2). No radioactivity was associated with CheA in preparations containing [α-^{32}P]ATP. Thus, CheA is modified by the
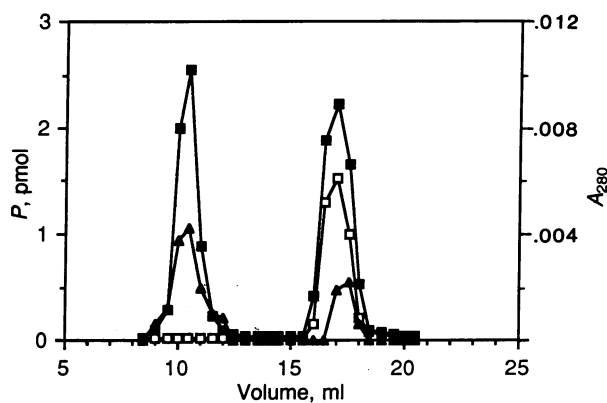

FIG. 2. Gel filtration of CheA incubated with [α-^{32}P]ATP or [γ-^{32}P]ATP. CheA samples were chromatographed on a Pharmacia Superose-12 fast protein liquid chromatography column. Fractions of 0.5 ml were collected, and a 10-μl aliquot of each fraction was placed in scintillation fluid and assayed for radioactivity; cpm were converted to pmol of phosphate and plotted. The absorbance at 280 nm of the column effluent was monitored by a Pharmacia UV-M flow cell. □, CheA with [α-^{32}P]ATP; ■, CheA with [γ-^{32}P]ATP; ▲, absorbance at 280 nm. CheA was eluted as a single peak at approximately 10 ml, whereas ATP was eluted with the void volume at approximately 18 ml.
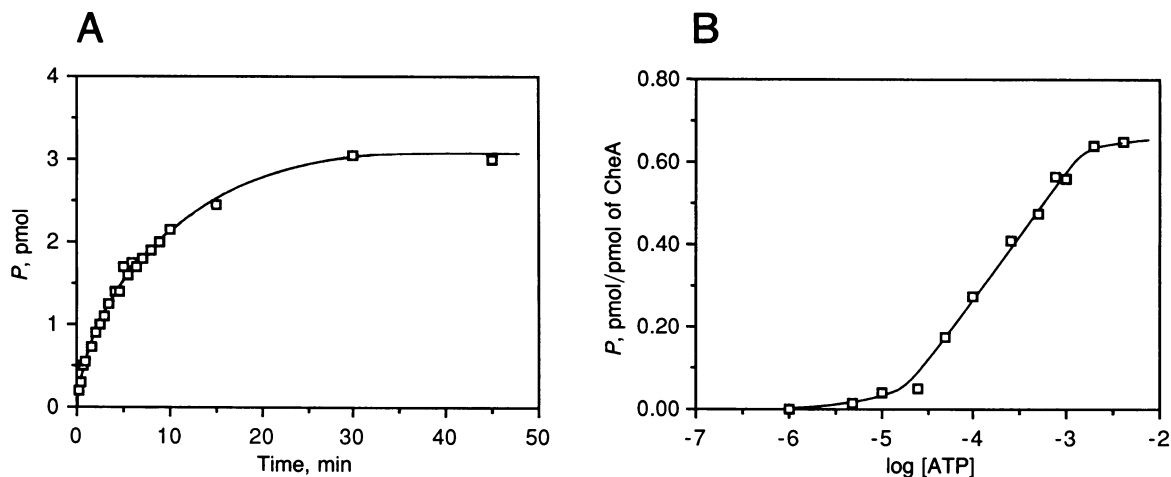
FIG. 3. Time course and dependency of CheA phosphorylation upon ATP concentration. (*A*) Time course of CheA phosphorylation. A reaction mixture containing CheA with 0.4 mM [γ-^{32}P]ATP was incubated at room temperature. At the indicated times, an aliquot containing 10 pmol of CheA was removed and precipitated with CCl₃COOH on a glass fiber disk. The CCl₃COOH-precipitable radioactivity is expressed as pmol of phosphate and is the average of two experiments. (*B*) Dependency of CheA phosphorylation on ATP concentration. CheA was incubated with the indicated concentrations of ATP for 30 min, and the reactions were terminated by precipitation with CCl₃COOH on glass fiber disks. The level of CCl₃COOH-precipitable phosphate is expressed relative to the pmol of CheA placed on each disk and is the average of two experiments with duplicate determinations in each experiment.

incorporation of the γ phosphate of ATP. In this experiment the molar ratio of phosphate bound to CheA was found to be approximately 1:1. CheA was not phosphorylated in the presence of [γ-^{32}P]GTP and Mg^{2+}; therefore, GTP does not substitute for ATP in this reaction. The presence of 5 mM EDTA blocked CheA phosphorylation, indicating that the reaction requires a divalent cation; consequently, 5 mM MgCl₂ was included in all phosphorylation experiments.

In order to follow the time course of the reaction, CheA was incubated with 0.4 mM [γ-^{32}P]ATP. At various times the reaction was terminated by CCl₃COOH precipitation of an aliquot containing 10 pmol of CheA (Fig. 3*A*). CheA phosphorylation occurred with an initial rate of 0.5 pmol/min, and phosphorylation reached a maximum level in about 15 min. The dependence of the level of CheA phosphorylation upon ATP concentration was examined by incubating CheA at various concentrations of ATP (1 μM to 4 mM) with constant amounts of [γ-^{32}P]ATP for 30 min, followed by CCl₃COOH precipitation onto glass filter disks. The results of several experiments using 12 pmol of CheA per reaction mixture are

shown in Fig. 3*B*. CheA phosphorylation was detected at the lowest ATP concentration examined, 1 μM. The level of phosphorylation increased rapidly between ATP concentrations of 50 μM and 0.5 mM. ATP concentrations above 1 mM caused only a slight increase in the level of CheA phosphorylation. In this experiment we found that the maximum molar ratio of CCl₃COOH-precipitable phosphate to CheA was approximately 0.7:1. The ATP concentration at which the molar ratio of phosphate to CheA was half of the maximal amount of labeling observed was 0.2 mM.

Purified phosphorylated CheA was tested for its ability to donate the covalently bound phosphate to various nucleotides. Products of the reactions were analyzed by TLC as shown in Fig. 4*A*. In the presence of MgCl₂, CheA donated phosphate to unlabeled ADP to produce [^{32}P]ATP (lane 6). The absolute dependence of this reaction upon additional Mg^{2+} is shown in lane 5. A small amount of labeled ATP was also produced when unlabeled ATP and Mg^{2+} were added to phosphorylated CheA. This may be due to slow turnover of the bound phosphate or to the presence of contaminating
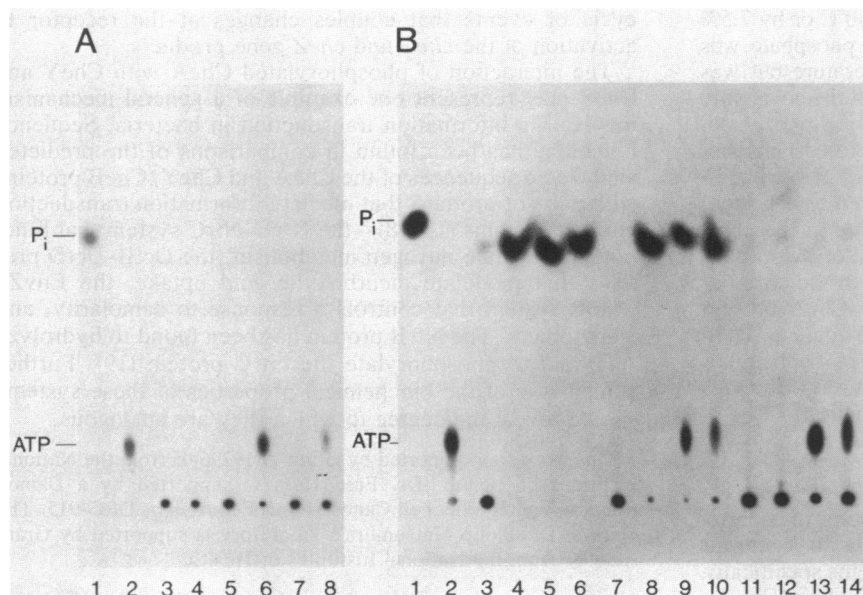

FIG. 4. Effects of nucleotides and chemotaxis proteins on phosphorylated CheA. Nucleotides were added to purified phosphorylated CheA at concentrations of 1 mM. Proteins CheY, CheZ, and CheW were added in molar excess with respect to phosphorylated CheA. Reaction mixtures were incubated for 30 min at room temperature and then spotted onto a TLC plate. The TLC plate was developed and then subjected to autoradiography. (*A*) The effect of nucleotides on phosphorylated CheA. Lanes: 1, [^{32}P]P$_i$; 2, [α-^{32}P]ATP; 3–8, phosphorylated CheA (lane 3) with 5 mM MgCl₂ (lane 4), ADP (lane 5), MgCl₂ and ADP (lane 6), MgCl₂ and AMP (lane 7), and MgCl₂ and ATP (lane 8). (*B*) The effect of chemotaxis proteins on phosphorylated CheA. Lanes: 1, [^{32}P]P$_i$; 2, [α-^{32}P]ATP; 3–14, chemotaxis proteins CheY (lanes 3–6), CheZ (lanes 7–10), and CheW (lanes 11–14) with 5 mM EDTA (lanes 3, 7, and 11), MgCl₂ (lanes 4, 8, and 12), MgCl₂ and ADP (lanes 5, 9, and 13), and MgCl₂ and ATP (lanes 6, 10, and 14).
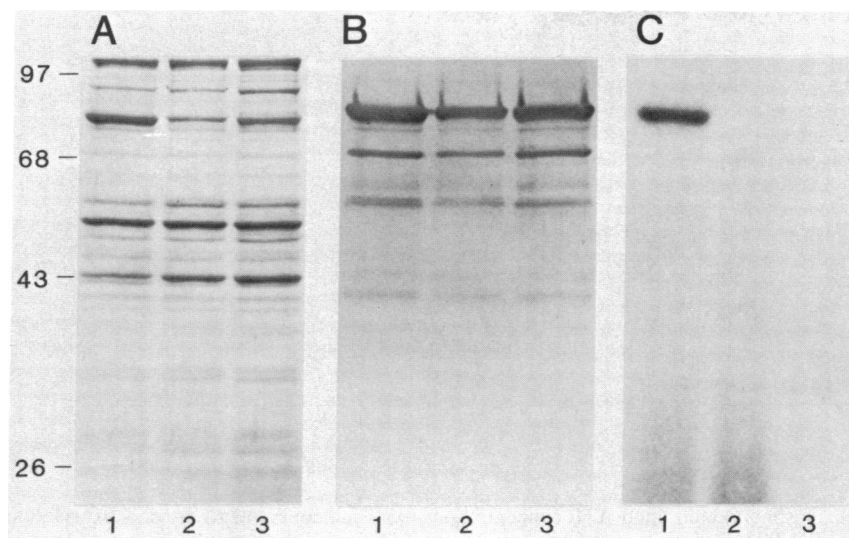
FIG. 5. Phosphorylation of CheA proteins from wild-type and mutant *cheA* plasmids. CheA protein partially purified by dye-ligand chromatography was incubated in a phosphorylation reaction mixture. The reaction was terminated by precipitation of protein with CCl₃COOH, and the precipitate was dissolved in NaDodSO₄ sample buffer for NaDodSO₄/10% polyacrylamide gel electrophoresis. (*A*) Coomassie blue stain. (*B*) Immunoblot against anti-CheA antibody. (*C*) Autoradiogram. Lanes: 1, CheA from pDV4; 2, CheA from pDV4 carrying *cheA501*; 3, CheA from pDV4 carrying *cheA510*.

ADP in the ATP. CheA is unable to donate the phosphate to other related nucleotides tested, including AMP, GMP, GDP, and GTP. Thus, phosphorylated CheA is an intermediate, capable of participating in the reverse reaction and specifically phosphorylating ADP.

The effect of CheY and CheZ on the phosphorylation of CheA was examined by using purified phosphorylated CheA and TLC. The results are shown in Fig. 4*B*. The addition of CheY and CheZ in molar excess with respect to phosphorylated CheA resulted in the release of Pᵢ (lanes 4 and 8). Both the CheY and CheZ reactions were inhibited by EDTA (lanes 3 and 7) and appeared to be relatively unaffected by the addition of ATP (lanes 6 and 10). CheY was more efficient than CheZ in competing with ADP to remove the phosphate from CheA under the conditions examined. Titrations of CheY and CheZ against CheA suggested that they interact with CheA stoichiometrically rather than catalytically (data not shown). All of the other proteins examined—CheW, bovine serum albumin, lysozyme, and cytochrome *c*—had no detectable effect on phosphorylated CheA.

The chemical nature of the covalent bond between CheA and Pᵢ was examined initially by acid and base treatment of phosphorylated CheA and analysis of the products on TLC (data not shown). The protein-bound phosphate was released by hot acid treatment (5% CCl₃COOH at 90°C for 20 min) as Pᵢ but was not significantly hydrolyzed by 5% CCl₃COOH at 0°C or by 7.5% acetic acid at room temperature. The bound phosphate was stable to alkali (0.5 M NaOH) at room temperature but was labile at 55°C. These characteristics suggest that the covalently bound phosphate may be a phosphoramidate, acylphosphate, or phosphotyrosine residue; it seems unlikely to be phosphoserine or phosphothreonine (14).

In order to examine the relationship between CheA phosphorylation and chemotaxis, mutants defective in the *cheA* gene were isolated by swarm selection. Some of the mutant CheA proteins were partially purified by dye-ligand chromatography. While partially purified wild-type CheA protein was phosphorylated, the mutant proteins were not (Fig. 5). In more extensive mutagenesis studies, examples of phosphorylated mutant CheA proteins were found (data not shown). These may be defective in another CheA function.

DISCUSSION

Our results are consistent with the conclusions that phosphorylation of CheA by ATP plays a role in bacterial chemotaxis and that the CheY and CheZ proteins specifically interact with CheA to accelerate dephosphorylation (Fig. 1).

In other experiments (J.F.H. and M.I.S., unpublished data), we have shown that the chemotaxis-specific methylesterase encoded by *cheB* also interacts with phosphorylated CheA. A role for phosphorylation is supported by the finding that the concentration of ATP required for efficient phosphorylation (see Fig. 3*B*) is similar to the intracellular concentration of ATP required to maintain chemotaxis (7). Furthermore, mutations that eliminate chemotaxis are defective in CheA phosphorylation. In addition, the *cheA* gene was found to encode a truncated product that does not mediate chemotaxis by itself (13), and this small form of CheA is also not phosphorylated (see Fig. 1).

How does the CheA protein act in signal transduction? The rate of autophosphorylation is slow compared to the *in vivo* kinetics of excitation signaling; therefore, CheA phosphorylation *per se* may not be the excitation signal. We can design experiments to test two possible functions for CheA phosphorylation; one is that it acts as an intermediate in phosphate transfer to another protein or small molecule. Alternatively, ATP binding and hydrolysis might serve to stabilize the oligomeric CheA protein in different forms. Each form of CheA could interact and activate other components of the chemotaxis system. Thus, the free protein, the ATP-bound protein, and the phosphorylated form of CheA could each facilitate different phases of information transfer and drive a cycle of events that couples changes at the receptor to activation of the *cheY* and *cheZ* gene products.

The interaction of phosphorylated CheA with CheY and CheZ may represent one example of a general mechanism involved in information transduction in bacteria. Sequence homology has been found in comparisons of the predicted amino acid sequences of the CheA and CheY/CheB proteins with pairs of proteins that mediate information transduction in other systems (15–18)—the NtrB–NtrC system that functions to regulate nitrogen metabolism, the DctB–DctD proteins that modulate dicarboxylic acid uptake, the EnvZ–OmpR system that controls a response to osmolarity, and many others. The NtrB protein has been found to hydrolyze ATP and to phosphorylate the NtrC protein (19). Further comparison of the biochemical properties of these systems should reveal the degree to which they are analogous.

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