Protein Phosphorylation is Involved in Bacterial Chemotaxis

J. Fred Hess; Kenji Oosawa; Philip Matsumura; Melvin I. Simon

Proceedings of the National Academy of Sciences of the United States of America,

Stable URL:
http://links.jstor.org/sici?sici=0027-8424%2819871101%2984%3A21%3C7609%3APPIIB%3E2.0.CO%3B2-H

Your use of the JSTOR archive indicates your acceptance of JSTOR’s Terms and Conditions of Use, available at http://www.jstor.org/about/terms.html. JSTOR’s Terms and Conditions of Use provides, in part, that unless you have obtained prior permission, you may not download an entire issue of a journal or multiple copies of articles, and you may use content in the JSTOR archive only for your personal, non-commercial use.

Each copy of any part of a JSTOR transmission must contain the same copyright notice that appears on the screen or printed page of such transmission.

Proceedings of the National Academy of Sciences of the United States of America is published by National Academy of Sciences. Please contact the publisher for further permissions regarding the use of this work. Publisher contact information may be obtained at http://www.jstor.org/journals/nas.html.

Proceedings of the National Academy of Sciences of the United States of America
©1987 National Academy of Sciences

JSTOR and the JSTOR logo are trademarks of JSTOR, and are Registered in the U.S. Patent and Trademark Office. For more information on JSTOR contact jstor-info@umich.edu.

©2003 JSTOR
Protein phosphorylation is involved in bacterial chemotaxis

(signal transduction/chemotaxis proteins/ATP/mutagenesis)

J. Fred Hess*, Kenji Oosawa*, Philip Matsumura†, and Melvin I. Simon*

*Division of Biology 147-74, California Institute of Technology, Pasadena, CA 91125; and †Department of Biological Sciences, University of Illinois at Chicago, Chicago, IL 60680

Contributed by Melvin I. Simon, July 22, 1987

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 proteins that are required for the integration and transduction of information. Two other genes encoded by cheR and cheB are responsible for adaptation to wide ranges of ligand concentration. These 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 SYS402 ΔtrpE-A, recA1, ina-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 SYS402 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 NaDodSO4/polyacrylamide gels (see Fig. 1).

Protein Labeling Experiments. Proteins were labeled for analysis by NaDodSO4/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-32P]ATP (500 cpm/ pmol) and 5 mM MgCl₂ 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 CCl₃COOH 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 NaDodSO₄ sample buffer (0.25 M Tris-HCl, pH 6.8/4% NaDodSO₄/20% 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 CCl₃COOH 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% CCl₃COOH containing 1% sodium pyrophosphate. After 30 min in ice-cold 10% CCl₃COOH, the filters were washed three times for 30 min each in ice-cold 5% CCl₃COOH 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-32P]ATP and 5 mM MgCl₂ 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
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 MgCl₂ and 0.4 mM [γ-³²P]ATP for 30 min at room temperature. These reactions were terminated by CCl₄-COOH precipitation and analyzed by NaDodSO₄/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 co-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 adenyllylation, identical samples of CheA were incubated for 30 min with 0.4 mM ATP containing ³²P 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-³²P]ATP (Fig. 2). No radioactivity was associated with CheA in preparations containing [α-³²P]ATP. Thus, CheA is modified by the
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-32P]ATP and Mg²⁺; 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-32P]ATP. At various times the reaction was terminated by CCl₃COOH precipitation of an aliquot containing 10 pmol of CheA (Fig. 3A). 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-32P]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. 3B. 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 reaction were analyzed by TLC as shown in Fig. 4A. In the presence of MgCl₂, CheA donated phosphate to unlabeled ADP to produce [32P]ATP (lane 6). The absolute dependence of this reaction upon additional Mg²⁺ is shown in lane 5. A small amount of labeled ATP was also produced when unlabeled ATP and Mg²⁺ were added to phosphorylated CheA. This may be due to slow turnover of the bound phosphate or to the presence of contaminating...
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 phosphorolyzing 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. 4B. The addition of CheY and CheZ in molar excess with respect to phosphorylated CheA resulted in the release of P, (lanes 4 and 8). Both the CheY and CheZ reactions were inhibited by EDTA (lanes 3 and 7) and appeared to be 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 — had no detectable effect on phosphorylated CheA.

The chemical nature of the covalent bond between CheA and P, 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 P, 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 dyes-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 methyl esterase 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. 3B) 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.

This work was supported by Grant AI19296-05 from the National Institutes of Health. Dr. Fred Hess is supported by a Damon Runyon–Walter Winchell Cancer Fund Fellowship, DRG-915. The work in Dr. Philip Matsuzawa’s laboratory is supported by Grant AI18895 from the National Institutes of Health.

Genetics: Hess et al.


