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METHYLATION OF THE CheA AUTOKINASE IN THE BACTERIAL CHEMOTAXIS SIGNAL TRANSDUCTION PATHWAY

By

FENG-CHING CHANG

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1996

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This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

METHYLATION OF THE CHEA AUTOKINASE IN THE BACTERIAL CHEMOTAXIS SIGNAL TRANSDUCTION PATHWAY

by

Feng-Ching Chang

Advisor: Professor Simon A. Simms

Responses of *Escherichia coli* to chemical stimuli are mediated by regulating the direction of flagellar rotation and are the end result of a cascading series of regulatory reactions. Two types of reversible protein modification reactions are involved, methylation and phosphorylation. The reversible methylation of a family of sensory transducer proteins has been correlated with adaptation. The phosphorylation is involved in response to repellents stimulation.

Computer analysis of the GenBank database indicated the presence of a 27 amino acid region from Leu^{169} to Lys^{195} in CheA which is highly homologous to the methylation region, termed K1 site, of the chemoreceptor, Tar. This analysis also showed that CheA contains an identical glutamic acid that is methylated in Tar by CheR methyltransferase. These findings prompted me to investigate possible methylation of CheA by CheR. My results showed that purified CheR could not methylate ovalbumin, BSA, or a peptide of isoaspartic acid but it methylated the two forms of CheA, $CheA_L$ and $CheA_S$, *in vitro*, in the presence of AdoMet. The methylated CheA protein, $CheA_L$, whose molecular weight is 73 kDa, has

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autophosphorylation activity. The methylation rate of CheA was found to be very sensitive to the ionic strength of the medium.

In an effort to investigate the nature of this reaction, the methylation behavior of CheR on Tar, phosphorylated CheA and unphosphorylated CheA were compared. The results showed that both phosphorylated CheA and unphosphorylated CheA were specifically methylated by CheR but at a slower rate than that of Tar. CheB methylesterase which demethylates membrane receptor-transducer proteins failed to remove the methyl group in methylated CheA *in vitro*.

Protease digestion and site-specific mutagenesis were used to further confirm the methylation site of CheA. Taken together, these results suggested that CheA could be methylated by CheR and that both Glu^{182} and Glu^{183} in CheA were involved in CheA methylation.

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It has taken me quite a long time to complete this degree, but I am so glad that I had the patience and endurance to reach this goal. I have been very lucky and proud to have professors, colleagues, family, and friends that have been wonderfully supportive and understanding.

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I also thank Dr. Horst Schulz and Professor Michael E. Green, Chairman of the Chemistry Department of City College, for their support and help. Thanks are extended to the technical staff at the Chemistry Department of City College for their kind assistance.

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LIST OF ABBREVIATIONS

AdoMet: S-adenosyl-methionine

BSA: bovine serum albumin

DTT: dithiothreitol

EDTA: ethylenediamine tetraacetic acid

kDa: kilodalton

MCP: methyl-accepting chemotaxis protein

PBS: phosphate buffered saline

PMSF: phenylmethylsulfonyl fluoride

SDS: sodium dodecyl sulfate

Tar receptor: aspartate receptor

TCA: trichloroacetic acid

TEDG: Tris-HCl, EDTA, dithiothreitol and glycerol buffer

IPTG: isopropyl-β-D-thio-galactopyranoside

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Introduction

Motile bacteria are able to respond to a variety of environmental stimuli. These organisms will swim toward higher concentrations of attractants and away from elevated levels of repellents. This relatively simple chemotactic behavior has been most studied in *Escherichia coli* and *Salmonella typhimurium* because these systems are amenable to both biochemical and molecular genetic techniques.

In uniform chemical environments, bacteria swim in a pattern which resembles a three-dimensional random walk (Figure 1. A). This is produced by periods of smooth swimming interrupted by tumbling that reorients the cell in a new, randomly chosen direction (Berg and Brown, 1992). Positive chemotaxis (Figure 1. B) refers to the movement toward an attractant and negative chemotaxis refers to the movement away from a repellent. Usually, an attractant is of some benefit to the cell such as a nutrient and a repellent is one that is harmful to the cell, e.g., metabolic wastes. The behavior is controlled by the duration and frequency of smooth swimming versus its tumbling behavior. The direction of flagellar rotation determines whether the cell swims smoothly or tumbles. Counter-clockwise rotation of the flagella leads to smooth swimming while clockwise rotation generates tumbles (Figure 2). Attractants induce a brief suppression of tumbling and allow the cell to swim smoothly in any direction whereas repellents cause the cell to reorient itself and swim in a random and new direction. These periods of smooth

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Figure 1. The path of bacterial movement

(A) In an isotropic medium, the cell swims in a straight line, randomizes its direction by tumbles, swims again, tumbles, yielding a three-dimensional random walk.

(B) The presence of an attractant causes the cell to swim longer by suppressing tumbles. Therefore, the cell will swim up the gradient (Berg and Brown 1972).

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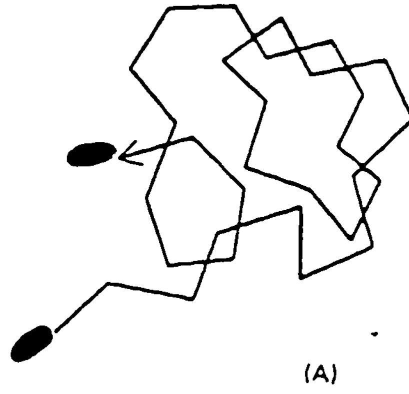

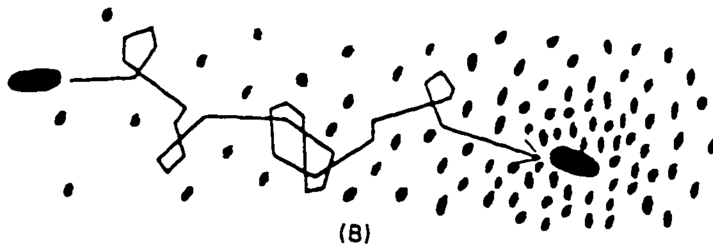

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Figure 2. Flagella behavior

Flagella behavior of *E. coli* or *S. typhimurium* during swimming (a & b) and tumbling (d). During counter-clockwise rotation, the flagellar filaments form a propulsive bundle leading the cell to smooth swim, whereas in clockwise rotation, the bundle is dispersed and the cell tumbles randomly. (c) is the polymorphic transition state between smooth swimming and tumbling. (Copied from Jones *et al.*, 1991)

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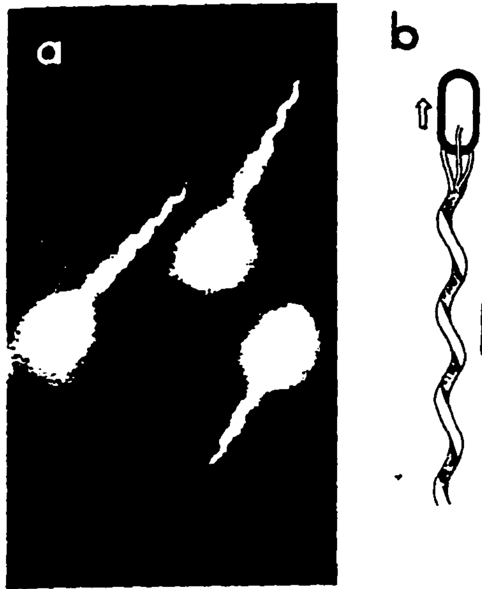

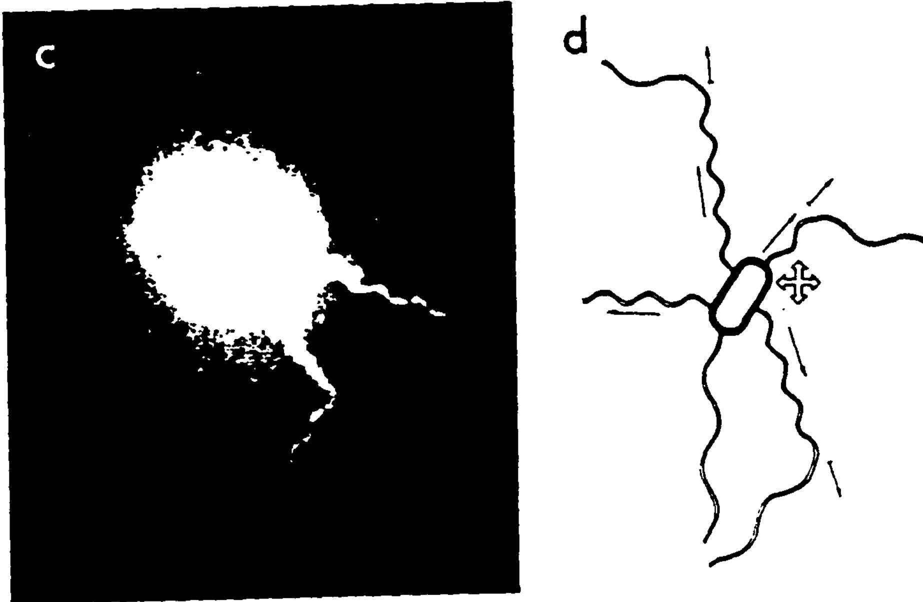

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swimming and tumbling result in a net movement of the cell toward attractants and away from repellents.

Reversible covalent modification of cellular proteins, such as phosphorylation and methylation, is very important for cellular coordination and control. Phosphorylation of proteins during transmembrane signaling of an outside stimulus into a biochemical message inside the cell has been well studied (reviewed by Alex *et al.*, 1994). Protein phosphorylation controls the resulting cellular response. One can conclude that phosphorylation and methylation controls most of cellular function and growth. The effects of these covalent modifications and their regulation in bacterial sensing and adaptation systems have been studied intensively, and may provide guidelines toward similar events in eukaryotic cells.

In a bacterium, chemotaxis is the end result of a cascading series of regulatory steps. Approximately 50 genes are involved in chemotaxis (Jones and Aizawa, 1991; Macnab, 1992). Some of the most important proteins responsible for the chemotactic response and transfer of the signal to the flagella are localized in two operons, *mocha* and *meche*. These are coordinately regulated (Silverman and Simon, 1976 & 1977). *Mocha* (for motility and chemotaxis) contains *mot*A, *mot*B, *che*A and *che*W whereas *meche* (for methylation and chemotaxis) contains *tar*, *che*R, *che*B, *che*Y and *che*Z (Figure 3).

The *mot* genes encode components of the flagellar motor. These are not the focus of this study. The *che* genes, on the other hand, are responsible for sensory processing and transferring of the signals from receptor to flagella during chemotaxis. The locations of the various gene products in the bacterium and their functions are outlined in Figures 4 & 5 (reviewed by Parkinson, 1993; Alex *et al.*, 1994).

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Figure 3. The *mocha* and *meche* operons

The six *che* genes are located in two adjacent operons called *mocha* and *meche* (Silverman and Simon, 1976 & 1977).

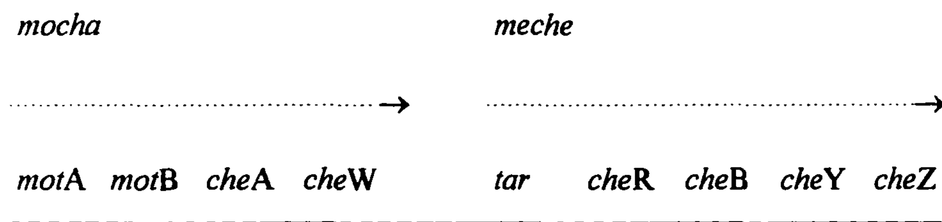

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Figure 4. Stages in intracellular signal processing

Stimuli are detected by the receptors. The activation of chemotaxis receptors by a chemoattractant has two separable consequences, one fast and one slow. (Modified from Alex and Simon, 1994)

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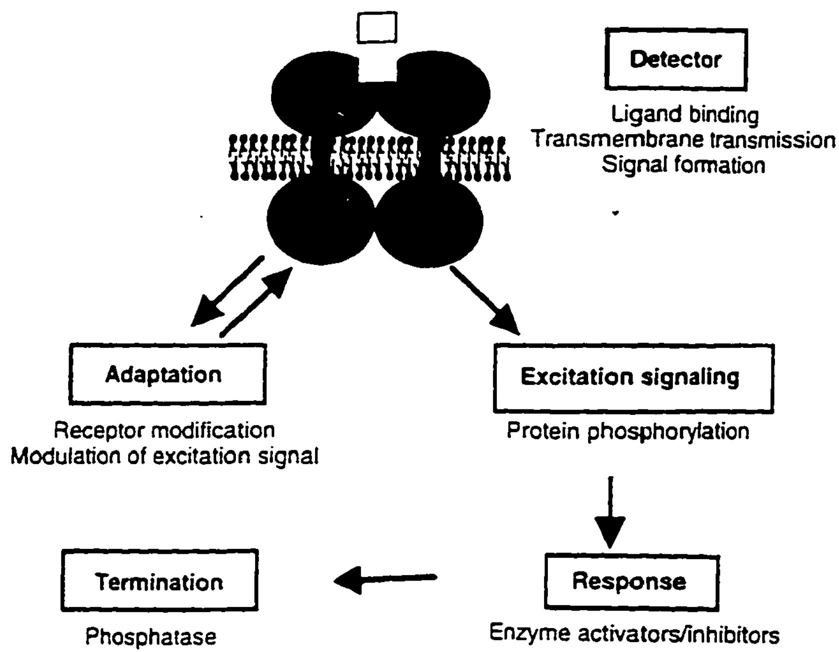

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Figure 5. A schematic outline of the signal transduction pathway involved in chemotaxis in *E. coli:* proteins in the cell membrane communicate with the flagellar motor via the Che system (Taken from Poole, 1990).

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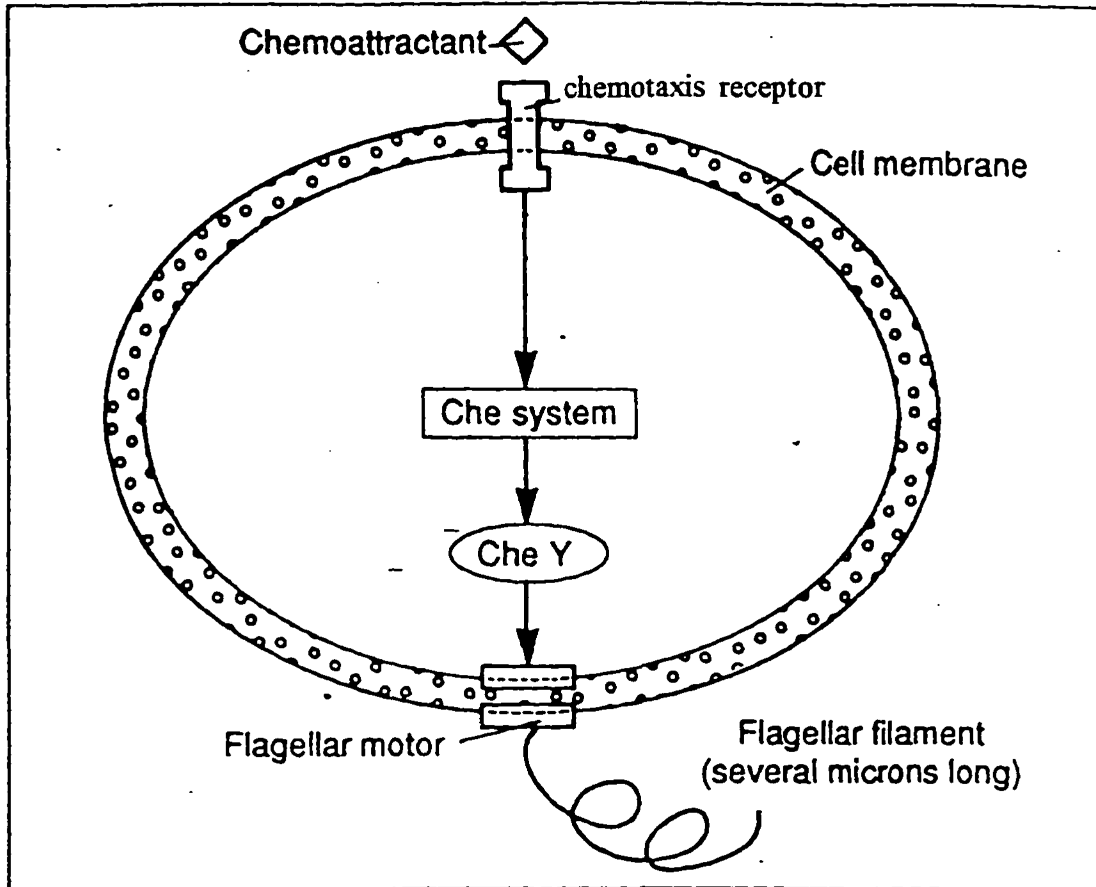

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Stimuli are detected by receptors at the inner membrane. The activation of chemotaxis receptors by a chemoattractant has two separable consequences, one fast and one slow (Figure 4). First, a rapid excitation occurs because the activated receptor generates an intracellular signal (by affecting a cascade of phosphorylation reactions of the Che proteins) to the effector outputs (by changing the direction of the flagellar rotation). Thus, the information passes from a receptor through a cascade of signal transduction components, to a target activity. In addition, a slower adaptation occurs. While activated, the receptor is methylated by an enzyme (CheR) in the cytoplasm. This methylation reaction reverses the activation reaction over a period of a few minutes. That is, the fast reaction acts as an excitation signal to the switch, which is then sequentially canceled as the adaptation process catches up. These two sequential reactions reset the cell to the original state. Without the second reaction, any small stimulus would cause complete and irreversible behavioral transitions.

Bacteria sense changes in attractant or repellent concentrations and elicit flagellar responses (Segall *et al.*, 1982). This mechanism (Figure 5) involves a family of receptors at the inner membrane and six cytoplasmic components, the Che proteins. After binding or releasing of the ligands, the receptor goes through a conformational change which initiates the signal transduction. Phosphorylation reactions are involved in this process which eventually affect the "gear shift" of the flagella (Richarme 1982).

Four homologous transmembrane receptors in *E. coli* and *S. typhimurium* (Tsr, Tar, Trg, and Tap) have been identified as chemotaxis transducer proteins (Figure 6)

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Figure 6. A family of four homologous receptors has been identified as chemotaxis transducer proteins: Tsr, Tar, Trg, and Tap, each of these detects a different set of chemical stimuli.

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Chemoeffectors		Receptors
Serine, Leucine	→	
Aspartate, Maltose, Co, Ni	→	
Ribose, Galactose	→	
Dipeptides	→	

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(reviewed by Hazelbauer, 1992; Hazelbauer *et al.*, 1990). They have similar molecular weights of approximately 60 kDa (about 550 amino acids in length). Each detects different sets of chemical stimuli. Each of these transducers consists of an N-terminal periplasmic ligand binding domain, a transmembrane region, and a cytoplasmic domain (Figure 7). The N-terminal periplasmic domains tend to be variable, while the C-terminal cytoplasmic domains are nearly identical (Bollinger *et al.*, 1984; Krikos *et al.*, 1983). This implies that they recognize different ligands but they all use the same intracellular signaling mechanism.

It appears that CheW, an auxiliary protein, couples CheA to the chemoreceptor which results in the formation of a stimulus-regulated autophosphorylation kinase (Gegner *et al.*, 1992; Chervitz *et al.*, 1995). Attractant-free receptors stimulate CheA's autophosphorylation, whereas attractant-bound receptors inhibit CheA's autophosphorylation (Borkovich *et al.*, 1989; Sanatinia *et al.*, 1995). After either binding or dissociation of the ligand (Figure 8), the membrane chemoreceptor-transducer proteins stimulate the transfer of a phosphoryl group from CheA to either CheY or CheB. Thus, the information is passed from receptor through CheW to CheA as a protein kinase.

After CheA is phosphorylated, it quickly transfers its covalently-bound high-energy phosphate directly to the response regulators, CheY or CheB (Wylie *et al* ., 1988). The phosphorylated form of the CheY protein (phospho-CheY) functions as the response regulator of the chemotaxis system that will interact with the flagellar motor switching apparatus (FliG, FliM, and FliN) to control motility. Phospho-CheY will cause the

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Figure 7. Structure of chemotaxis receptor (Taken from Parkinson, 1994)

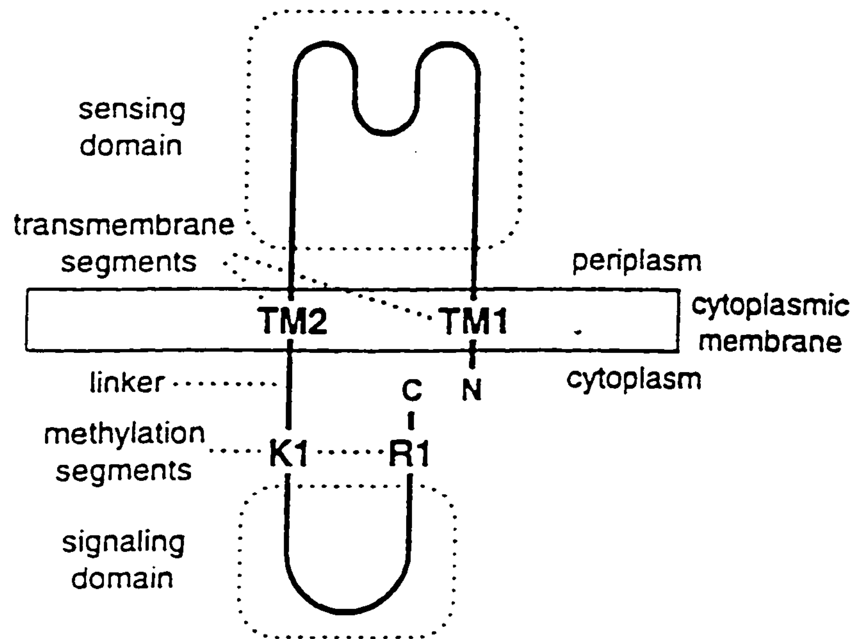

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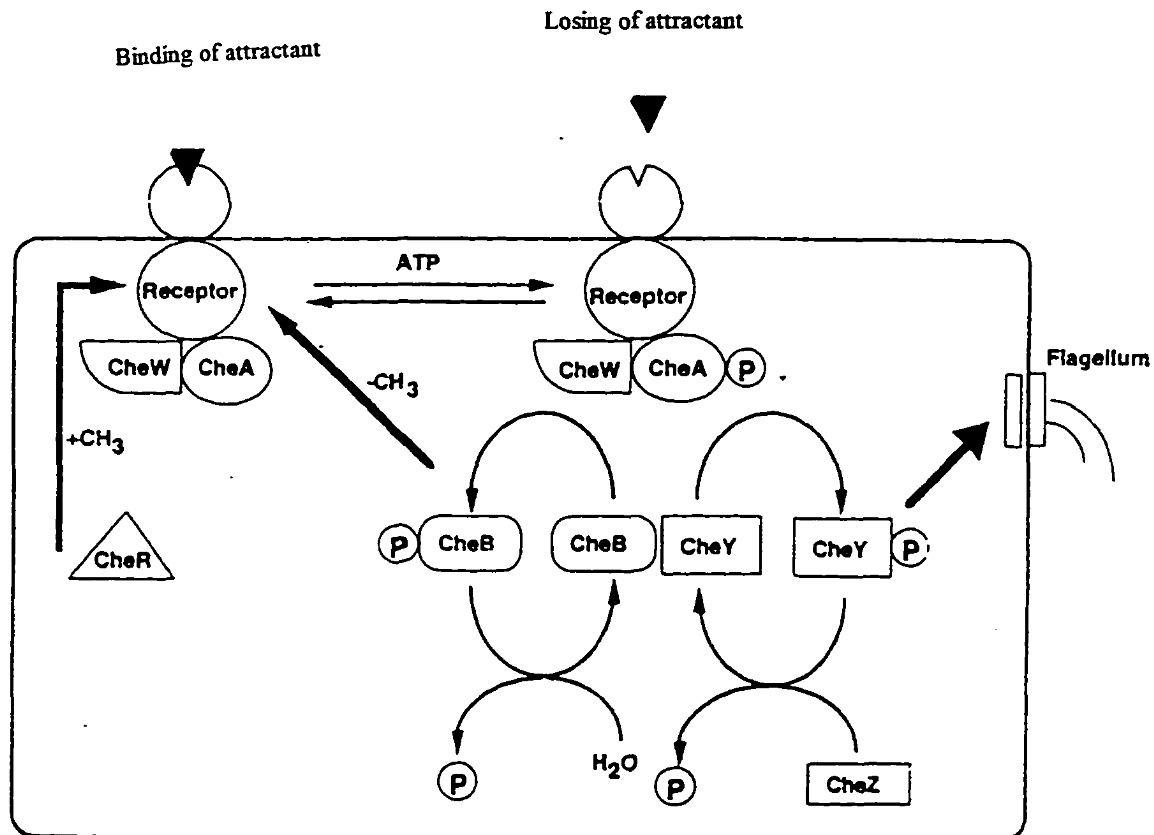
Figure 8. A schematic outline of the signal transduction pathway involved in chemotaxis in E. coli (Taken from Alex and Simon, 1994)



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flagella to rotate clockwise and thereby induce tumbling behavior (Parkinson *et al.*, 1983; Smith *et al.*, 1988).

CheZ antagonizes the action of CheY by facilitating the dephosphorylation of phospho-CheY (Hess *et al.*, 1988). Therefore, CheZ could cause the motor to rotate counterclockwise indirectly, which would result in a smooth swimming response. CheB is part of a feedback circuit. It terminates motor responses by adjusting the methylation state of the chemoreceptors, which in turn, modulates their signaling properties.

In addition to protein phosphotransfer reactions, changing the sensitivity of the membrane receptors (adaptation) is a further control mechanism in the chemotaxis system (Figure 4). Given an attractant stimulus, a bacterial cell will begin to smooth swim exclusively, but after several minutes, swimming behavior returns to the pre-stimulus level (MacNab and Koshland, 1972). This process is known as adaptation. The ability of *E. coli* and *Salmonella typhimurium* to sense temporal changes in chemoeffector levels is due to this adaptation mechanism. Adaptation continuously resets the sensitivity of the transaction machinery, so that there will be no sensory signal generated in a homogeneous environments.

Sensory adaptation is a continual process that enables the cells to make temporal comparisons. It has been hypothesized that the methylation of chemoreceptors plays a crucial role in the adaptation (Bourret *et al.*, 1991; Stock *et al.*, 1991 and Stewart *et al.*, 1989). This is based on the observation that adaptation is associated with a net change in the number of methyl groups attached to the receptors (Kort *et al.*, 1975). Chemoreceptors are also called methyl-accepting chemotaxis proteins (MCPs).

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As the MCP binds an attractant, it is methylated by CheR. As methylation proceeds, the sensitivity of the MCP to the attractant fades; that is, methylation dampens the signal from the MCP to the Che proteins. Only an increase in the concentration of the attractant will produce a continued signal from the MCP to the Che proteins and promote movement of the bacterium up a concentration gradient.

The MCP receptors (Figure 6) are methylated at specific glutamic acid residues using AdoMet by the *cheR* gene product, a methyltransferase (Springer and Koshland, 1977) and the methyl groups are removed by the *cheB* gene product, a methylesterase (Stock and Koshland, 1978) (Figure 9). In the absence of environmental stimuli, methyl groups on MCPs turn over in a balance between the activities of methyltransferase and methylesterase (Toews *et al.*, 1979).

The sensitivity of each receptor is controlled by methyl esterification at glutamic acid side chains. Thus, CheR and CheB are not needed to initiate flagellar responses but rather to bring about sensory adaptation by altering MCP methylation state. The methylation level of the receptors increases when the cell is adapting to positive stimuli, and adaptation to negative stimuli is linked to demethylation of the receptors (Springer *et al.*, 1979). Approximately 100-fold decrease in sensitivity to the attractant is observed when the chemoreceptors become methylated. This action results in clockwise flagellar movement and the cell tumbles. Demethylation of the MCP brings the cell back to a state in which it can excite the flagellar motor. Levels of receptor methylation control receptor

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Figure 9. The signal transduction system

CheR catalyses the transfer of a methyl group from S-adenosylmethionine to receptor glutamyl residues. CheB demethylates γ-carboxylmethyl glutamyl residues in the receptor resulting in the production of methanol. (Taken from Stock *et al.*, 1992)

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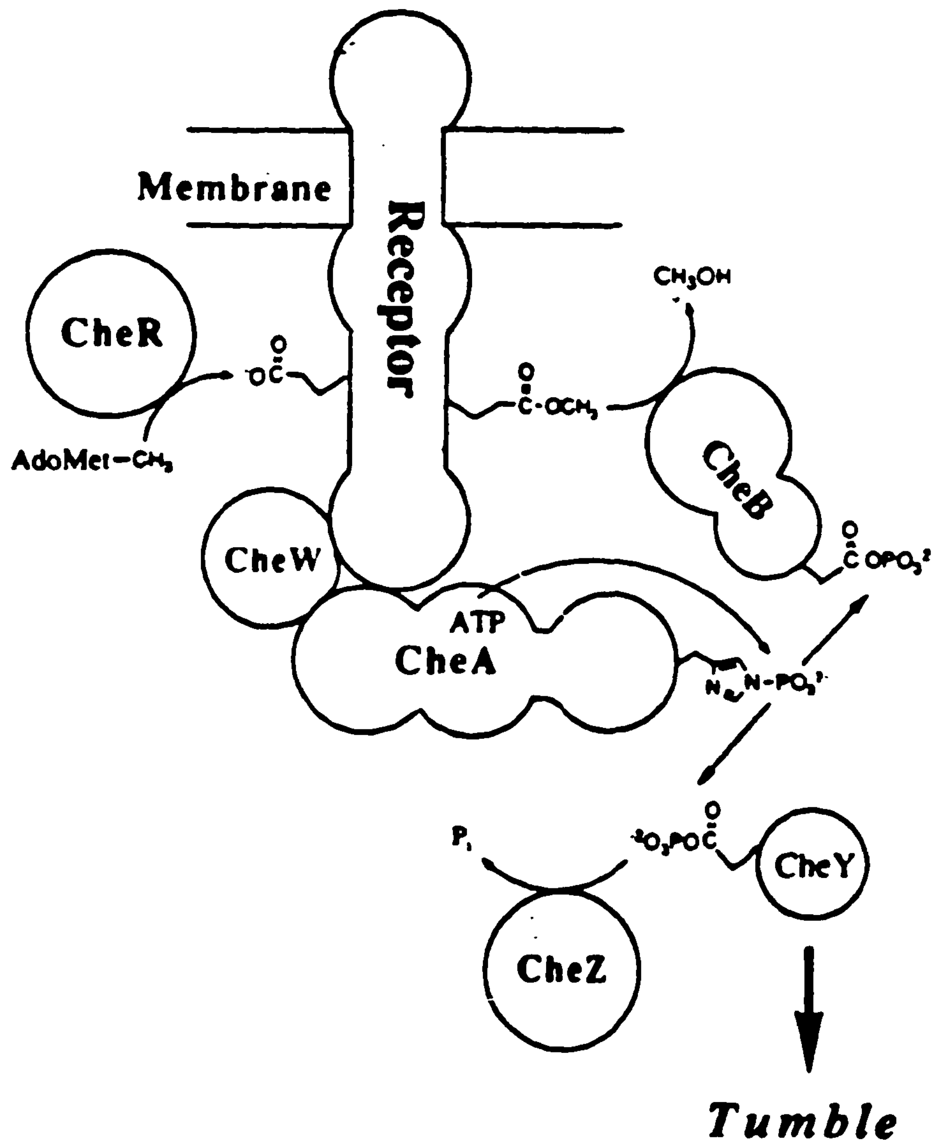

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sensitivity to stimulatory ligands. The extent of methylation increases at higher concentrations of attractant. When the attractant is removed, the receptor is demethylated.

Although the level of methylation changes during chemotactic responses, it remains constant once a bacterium is adapted because an exact balance is reached between the rates of methylation and demethylation. The relative activities of CheR and CheB determine the methylation level of each MCP species. In the absence of chemical stimuli, about half of the sites are methylated. At high attractant or low repellent levels, most sites are methylated, whereas in low attractant or high repellent levels, few sites are methylated (Russell *et al.*, 1989). Since CheR activity remains constant, changes in CheB activity produce transient fluctuations in the methylation level of all MCPs. CheB activity increases when it becomes phosphorylated (Lupas and Stock, 1989). Thus, by controlling the flux of ester phosphate from CheA to CheY or CheB (Figures 8), the receptors not only trigger behavioral responses, but also set in motion the sensory adaptation process. The same network of proteins regulates adaptation. CheA phosphorylation of CheB increases the latter activity and thereby provides a feedback regulation to the chemotaxis receptors.

Bacterial chemotaxis system is surprising because attractants produce a negative signal. The binding of an attractant leads to a decrease in the phosphorylation of CheA, CheY and CheB. Thus, when the flagella rotates counterclockwise, the cell swims smoothly, the methylation level of receptors is increased, and the sensitivity of the receptors is decreased . The phosphorylation cascade is activated when the attractant is

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released from the chemoreceptor, and is inhibited by the binding of attractant ligands to the receptors (Borkovich *et al.*, 1989).

The cytoplasmic domain of the MCP receptor (Figure 7) includes four to six methylation sites located in two separated regions termed K1 and R1. Specific glutamate residues (Kehry *et al.*, 1983; Kehry *et al.*, 1982; Terwilliger *et al.*, 1984 and Boyd *et al.*, 1983) are converted to γ-glutamyl methyl esters by CheR which may be removed by CheB (Figure 9). Changes in the MCP methylation state leads to sensory adaptation. This reaction is not considered to play a role in triggering motor responses. The actual number of methylation sites identified is five for Tsr, four for Tar, five for Trg and five to six for Tap (Stewart *et al.*, 1987). These MCP methylation sites are highly homologous (Figure 10).

The three methyl-accepting sites identified on the 23-amino acid K1 peptide of the Tsr and Tar proteins are spaced 7 amino acids apart (Figure 10). The methyl-accepting glutamyl is always the second of two adjacent glutamates (Boyd *et al.*, 1983). Except for a glutamate following the first Glu-Glu pair and the final lysine, all the other amino acids of the K1 tryptic peptide are hydrophobic. The arginine-containing R1 peptide, which has one methyl-accepting site in Tar and two in Tsr, has a similar structure to the K1 peptide (Figure 10).

The Tar K1-peptide (Figure 10) contains three methylation sites within a twenty-three amino acid region. It was used to search for homologous sites in the GenBank database. The analysis revealed the presence of several proteins from different organisms, having a similar region with extensive homology. Surprisingly, we found a twenty-three

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Figure 10. The methylation sites of the K1 and R1 peptides

Boxes enclose loci of identical residues and asterisks indicate methylation sites (From Stewart and Dahilquist 1987). A CheB-dependent modification deamidates specific glutamine residues to methyl-accepting glutamic acid (Kehry *et al.*, 1983). This CheB-dependent deamidation occurs shortly after synthesis of MCPs. CheB deamidation is irreversible and is stable under base-catalyzed demethylation conditions (Kehry et al., 1982b).

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K1 Peptide

Tar (293) TEQQASALEETAASMEQLTATVK (315)
Tsr (295) TEQQAASLEETAASMEQLTATVK (317)
Tap (291) TEQQAASLAQTAASMEQLTATVG (313)
Trg (302) TEEQAAAIEQTAASMEQLTATVK (324)

R1 Peptide

Tar (481) VTQQNASLVQESAAAAAALEEQASR (507)
Tsr (483) VTQQNAALVEESAAAAAALEEQASR (509)
Tap (479) VTQQNASLVEEAAMATEQLANQADR (505)
Trg (489) VTQQNASLVEEASAAAVSLEEQAAR (515)

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amino acid region from Leu^{169} to Lys^{195} in CheA that showed 33.3% identity and more than 85% evolutionary similarity to that of the K1-peptide (Figure 11).

It has been reported that changes in receptor methylation are correlated with the ability of bacteria to adapt to different ambient levels of attractants. CheA is the central protein in this chemotaxis signal transduction pathway. It was earlier reported that CheA plays an important role both in the initial excitation response to stimuli as well as subsequent events associated with the adaptation process. This is because CheA is an autokinase and it can transfer its phosphoryl group to CheY and CheB and regulate their activities. Increasing levels of MCP methylation favor the formation of an active receptor-CheW-CheA ternary complex. However, there is no evidence to date of any direct role for CheR in the excitation process.

The observation of a highly homologous region between Leu^{169} to Lys^{195} of the CheA protein and the K1-peptide prompted us to investigate if CheA could be methylated by CheR since CheA contains an identical glutamic acid that is methylated in Tar. Therefore, in this thesis research, attempts were made to provide evidence for the methylation of CheA *in vitro*. Over-expression vectors were used for expression and subsequent purification of CheA and CheR (Hess *et al.*, 1991; Simms *et al.*, 1987). These pure CheA and CheR proteins were then used for this *in vitro* study. After confirming that the CheA methylation reaction does occur *in vitro*, kinetic studies were used to further explore the nature of this reaction. Furthermore, the methylation rates of Tar, phosphorylated CheA and unphosphorylated CheA were compared. Based on my

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Figure 11. Homologous sites between K1 and CheA

The underlined amino acid sequence represents regions of homology with the K1 peptide. Double dots denote identical residues whereas single dots indicate evolutionary similarity between the amino acids.

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Locus Number	Amino acid sequence	% Identity
STYTAR	NTDLSSRTEQQASALEETAASMEQLTATVK	100
	::. ...... :::. .... ::..::	
STYCHEA	RIVLSRLKANEVDLLEEELGNLATLTDVVK	33
	::. .. ... :::. . .. ::..::	
ECOCHEA	RIILSPLKAGEVDLLEEELGHLTTLTDVVK	33

STYTAR: *S. typhimurium Tar* (*Tar* K1 peptide)

STYCHEA: *S. typhimurium cheA*

ECOCHEA: *E. coli cheA*

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observations, it seems that the methylation site(s) in CheA is (are) embedded and less accessible to CheR. The embedded methylation site(s) might become more accessible under certain conditions.

Protease digestion and site-directed mutagenesis were performed to examine the methylation site of CheA. My results show that both Glu^{182} and Glu^{183} in CheA protein were involved in CheA methylation.

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Experimental Procedures

Chemicals

AdoMet was obtained from Boehringer Mannheim. [γ-^{32}P]ATP (specific activity, 5000 Ci/mmol) and S-adenosyl-L-[methyl-^{3}H] methionine (85 mCi/mmol) were obtained from Dupont New England Nuclear Research Products (Boston, MA). Scintillation fluid and autofluor were purchased from National Diagnostic Inc. Kodak X-Omat AR film was used for fluorography and was purchased from Eastman Kodak Company, Rochester, NY. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, DNA polymerase I Klenow fragment, and M13 17-mer reverse universal primer were purchased from New England Biolabs, Inc. (Beverly, MA) or Boehringer-Mannheim Biochemical (Indianapolis, IN) and were used according to the suppliers' recommendations. Deoxynucleotides were from Pharmacia Biotech (Uppsala, Sweden). The synthetic isoaspartyl peptide, H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu, was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Oligonucleotides used for mutagenesis and sequencing were synthesized by the Cyclone™ Plus DNA Synthesizer from Millipore Inc. (Milford, MA). Chameleon™ double-stranded site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). Magic™ Megapreps DNA Purification System was from Promega (Madison, WI). Anti-CheA antibody was prepared from pure CheA by Pocono Rabbit Farm, Pocono, PA. Ammonium sulfate (utrapure grade) was obtained from ICN Biomedicals, Inc. (Aurora, OH). V8 protease, Arg-C protease and DEAE-cellulose were obtained from Sigma (St. Louis, MO). Acrylamide, SDS, and Bio-Gel P-60 were from Bio-Rad (Richmond, CA). Molecular weight standards (phosphorylase a, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin

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inhibitor, 21,000; and lysozyme, 14,000) were obtained from Pharmacia (Piscataway, NJ). All other chemicals used were obtained from commercial sources and were of the highest purity available.

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

Methods

Preparation of Competent Cells

Bacterial cells were grown to $OD_{600} = 0.4$. The cells were then chilled thoroughly on ice for at least 15-20 min, centrifuged at 2,000 x *g* for 15 min at 4 ^{0}C. The pellet was resuspended in 50 ml cold 50 mM $MgCl_2$ and then centrifuged at 2,000 x *g* for 15 min at 4 ^{0}C. The pellet was resuspended in 50 ml cold 50 mM $CaCl_2$ and incubated for 20 min on ice. The suspension was centrifuged again at 2,000 x *g* for 15 min at 4 ^{0}C and the pellet was resuspended in 5 ml cold 50 mM $CaCl_2$. These competent cells were stable for up to three days if stored at 4 ^{0}C.

Transformation of Plasmid into *E. coli* Cells

Plasmid DNA, 2 to 10 ng, was incubated with 200 µl of competent *E. coli* cells for 45 min on ice and shaken gently every 15 min. The cells were placed in a water bath at 42 ^{0}C for 90 sec to heat-shock the cells, then transferred to an ice bath for 10 min. LB

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Table 1.

	Relevant characteristics	Source
E. coli strains		
RP 4080	methyltransferase-deficient	J. S. Parkinson/ Goy *et al.*, 1978
RP 437	wild type for chemotaxis	J. S. Parkinson Parkinson et al., 1982
RZ1032	*ung⁻ dut⁻*	D. E. Koshland, Jr./ Smith, 1985
XL mutS⁻	mutS⁻ *ung⁺*	Stratagene, La Jolla, CA/ Smith, 1985
Plasmids		
pWK 3-55.2	*Tar* expression vector	D. E. Koshland, Jr./ Simms *et al.*, 1987
pMO4	*che*A expression vector	J. Stock
pME3	*che*B expression vector	J. Stock
pME43	*che*R expression vector	J. Stock

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medium, 1 ml, was then added to the cells, and the mixture incubated for 1 hr at 37 ^{0}C. One hundred μl of the transformed transformation mixture was then spread on LB plates containing 50 μg/ml ampicillin and the cells grown overnight at 37 ^{0}C.

Purification of CheA Protein

E. coli HB101 containing the *cheA* expression vector pMO4 (a gift from Prof. Jeffrey Stock, Princeton University, Princeton, New Jersey) was used for expression of the enzyme. The cells were grown at 37^{0}C overnight in 4 liters of LB broth supplemented with ampicillin (50 μg/ml) and IPTG (50 μg/ml) with vigorous aeration. The cells were chilled for 5 min at 0 ^{0}C, and harvested by centrifugation. The pellet was washed and resuspended in 5 ml of TEDG 20/PMSF buffer (50 mM Tris-HCl buffer, pH 7.5 containing 0.5 mM EDTA, 2 mM DTT, 20% glycerol and 50 mM PMSF) per g, wet weight of cells and sonicated for 12 min (3 x 4 min). All operations were performed at 4 ^{0}C. The lysate was ultracentrifuged at 100,000 x *g* for 90 min to remove cell debris. CheA was purified as described earlier (Hess *et al.*, 1991) with the following modifications. CheA was precipitated from the cell lysate by addition of ammonium sulfate to 35% saturation (19.4 g/100 ml), followed by centrifugation at 16,000 x *g* for 30 min, resuspension of the pellet in TEDG (50 mM Tris-HCl buffer, pH 7.5 containing 0.5 mM EDTA, 2 mM DTT, and 10% glycerol) buffer and dialysis against TEDG buffer to remove salt. The dialyzed CheA preparation was loaded onto a 25 ml (1.5 cm x 20 cm) Affi-Gel Blue affinity column (100-200 mesh, BioRad, Richmond, CA) equilibrated with TEDG buffer. The column was washed with two to three column volumes of TEDG and the bound CheA was eluted using a 0.25 M to 1 M NaCl/TEDG buffer gradient developed

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over 10 column volumes. Fractions containing CheA were pooled, precipitated by the addition of ammonium sulfate brought to 45% saturation (25.8 g/100 ml), centrifuged, resuspended, and dialyzed against TEDG buffer to remove salt as described above. The dialyzed CheA was loaded onto a 75 ml (2.5 cm x 50 cm) DEAE-cellulose column equilibrated with TEDG buffer. The column was washed with TEDG buffer and the bound CheA was eluted using a 0.05 M to 0.6 M NaCl/TEDG gradient developed over 5 column volumes. Eluted CheA showed only one band of molecular weight 73 kDa when subjected to SDS-polyacrylamide gel electrophoresis (Fig. 13). However, two bands of molecular weight 73 and 64 kDa, corresponding to $CheA_L$ and $CheA_S$ (Hess *et al.*, 1987; Wolfe *et at.*, 1993), were observed during some purifications.

Purification of CheR Protein

The CheR methyltransferase was purified as described earlier (Simms *et al.*, 1987) with some modifications. *E. coli* HB101 containing the *che*R over-expression vector pME43 was used for expression of the enzyme. The cells were grown overnight in 4 liters of LB broth (supplemented with ampicillin 50 μg/ml and IPTG 50 μg/ml) at 37 ^{0}C with vigorous aeration. The cells were chilled for 5 min at 4 ^{0}C, and harvested by centrifugation. The pellet was washed, and resuspended in 5 ml of 0.01 M potassium phosphate/PMSF buffer (0.01 M potassium phosphate, 1.0 mM EDTA, 1.0 mM β-mercaptoethanol, 50 mM PMSF, pH 7.0) per g, wet weight, of cells. The resuspended

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Figure 12. 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified CheA and CheR: The gel was stained with Coomassie Blue. Lane 1, standard proteins with sizes noted at left. Lane 2, purified CheA. Lane 3, purified CheR.

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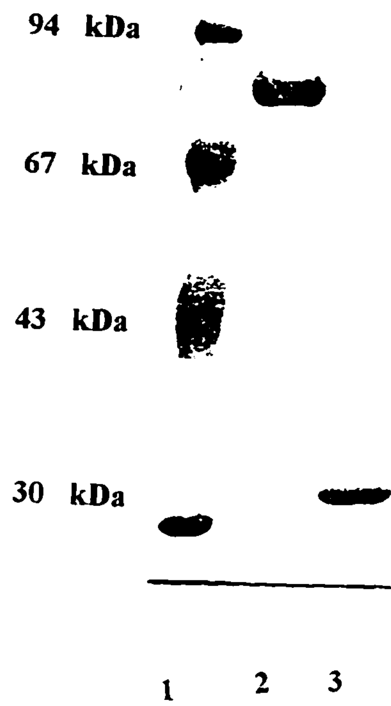

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cells were sonicated for 12 min (3 x 4 min) using a Branson Model 200 sonifier. All operations were performed at 4 ^{0}C. The lysate was centrifuged at 16,000 x *g* for 15 min, and the supernatant was further centrifuged for 60 min at 100,000 x *g*. One fiftieth volume of 1.0 M $MnCl_2$ was added dropwise to the supernatant while keeping the pH constant at 7.1 with 2.0 M Tris base. After stirring for 30 min and standing for 15 min, the suspension was centrifuged for 20 min at 8000 x *g*. The pellet was discarded and EDTA and β-mercaptoethanol in the supernatant were each adjusted to 0.01 M. CheR was precipitated by addition of ammonium sulfate to 42.3% saturation (29.6 g/100 ml), centrifuged, resuspended, and dialyzed against 0.01 M potassium phosphate/PMSF buffer as described previously. The dialyzed solution was diluted 1:10 with 0.02 M Tris buffer pH 8.0, 1.0 mM EDTA, 1.0 mM β-mercaptoethanol, and applied to a DEAE-cellulose column. The flow-through, which contained the methyltransferase, was collected. The flow-through, adjusted to pH 6.6 by adding 5.5 M HCl, placed on a 5.0 cm x 50 cm CM cellulose column (200 ml) and the bound CheR eluted using a gradient of 55 to 70 mM NaCl in 0.01M potassium phosphate buffer pH 7.0, 1.0 mM EDTA, 1.0 mM β-mercaptoethanol. The purified CheR showed one band when subjected to SDS-polyacrylamide gel electrophoresis (Fig. 12).

Purification of CheB protein

The CheB methylesterase was purified as described earlier (Simms *et al.*, 1985) with the following modifications. *E. coli* HB101 cells containing the CheB expression vector pME3 was used for expression of the enzyme. Cells were grown in 4 liters of LB broth to a density of approximately 2 x 10^9 cells/ml at 37 ^{0}C, harvested , and washed by

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resuspension in 10 ml of 0.1 M potassium phosphate/PMSF buffer (0.10 M potassium phosphate, 1.0 mM EDTA, 1.0 mM β-mercaptoethanol, 50 mM PMSF, pH 7.0) per g, wet weight, cells. The suspension was centrifuged for 20 min at 10,000 x *g*, resuspended in 3 ml of 0.1 M potassium phosphate buffer/gram wet weight of cells, and disrupted in 50-ml aliquots using a Branson Model 200 sonifier for 12 min (3 x 4 min). The resulting lysate was centrifuged for 15 min at 17,000 x *g*, and the supernatant further clarified by centrifugation for 1 h at 100,000 x *g*. This crude extract was brought to 42.3% saturation with respect to ammonium sulfate by adding 29.6 g of finely divided ammonium sulfate/100 ml of solution. The pH was maintained at 7.0 by the addition of 1 M NH_4OH. The solution was kept on ice and stirred for 30 min and then centrifuged for 20 min at 13,000 x *g*. The precipitate was redissolved in a minimal volume of 0.1 M potassium phosphate buffer and then dialyzed for 5 h against 2 x 4 liters of 0.1 M potassium phosphate/PMSF buffer, pH 7.0. This dialyzed ammonium sulfate fraction was diluted 1:10 (v/v) with Tris buffer (20 mM Tris-HCl, 1.0 mM EDTA, 1.0 mM β-mercaptoethanol, pH 8.5), and applied to an 8 x 24 cm DEAE-cellulose column (800 ml). The column was then washed with 1.5 liters of Tris buffer, and the flow through, which contained the esterase, was immediately supplemented with 0.10 M NaCl and adjusted to pH 7.0 with 1 M HCl. Esterase was precipitated from this solution by adding 0.49 g/ml ammonium sulfate (70% saturation), stirring for 60 min and centrifuging for 20 min at 13,000 x *g*. The precipitate was dissolved in a minimal volume of phosphate buffer, and applied to a 2.8 x 90 cm Bio-Gel P-60 molecular sieve column (200 ml) which had been equilibrated in 0.1 M potassium phosphate/PMSF buffer. The column was eluted with 0.1 M potassium

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phosphate/PMSF. The purified CheB was then subjected to SDS-polyacrylamide gel electrophoresis. Two bands of molecular weight 21 and 37 kDa were observed (Simms *et al.*, 1985).

Preparation of Membrane Receptor

The Tar receptor used in our studies was produced in an *E. coli* methyltransferase-deficient strain, RP4080 (J. S. Parkinson, University of Utah) carrying the *S. typhimurium tar* gene on a multicopy plasmid pWK3-55.2 (obtained from Dr. Jeffrey Stock, Princeton University) (Simms *et al.*, 1987). This strain was grown in 4 liters of LB broth supplemented with 50 μg/ml ampicillin at 30 ^{0}C overnight until the culture attained a density of 2 x 10^9 cells/ml. Unless stated elsewhere, all procedures were performed at 4^{0}C.

These cells were harvested by centrifugation at 12,000 x *g* for 20 min and washed with 0.1 M sodium phosphate buffer, pH 7.0, containing 1.0 mM EDTA. The pellet, 15 g, was resuspended in 30 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 1.0 mM EDTA and subjected to sonication for 12 min (3 x 4 min) in a Sonics and Material Vibra-Cell. Unbroken cells and large debris were removed by centrifugation at 15,000 x *g* for 15 min, and the supernatant further processed by ultracentrifugation for 60 min at 100,000 x *g*. The pellet was resuspended, using a glass homogenizer, in 2.5 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 1.0 mM EDTA, washed twice, and the membrane stored in 0.5 ml aliquots at -80^{0}C. This membrane preparation containing Tar was used as the substrate for the CheR methyltransferse assay.

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Preparation of [^{3}H]- labeled Methylated Membrane Receptor

E. coli membranes containing *S. typhiurium* aspartate receptors (Tar), 2.5 ml of the above homogenate, were incubated with 500 μl of 1.0 mCi/ml [^{3}H]AdoMet and 2.5 ml of the purified CheR methyltransferase . After incubation at 37 ^{0}C for 30 min, 5-6 volumes of 0.1 M sodium phosphate buffer, pH 7.0, containing 1.0 mM EDTA was added and the diluted receptors were centrifuged for 1 h at 100,000 x *g*. The pellet was washed further with 10 mM potassium phosphate buffer, pH 7.0, containing 1.0 mM EDTA and 2.0 M potassium chloride. The final pellet was resuspended by homogenization in 6.5 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 1.0 mM EDTA and stored as aliquots at -80^{0}C.

Methyltransferase Activity

Methylation of different protein substrates were assayed (Simms *et al.*, 1987) by incubating CheR, [^{3}H]AdoMet (85 mCi/mmole), and different protein substrates in a total volume of 100 μl. At different time intervals , a 15 μl aliquot of the assay mixture was removed and placed on 3 MM paper which was then plunged into 10% trichloroacetic acid at 25 ^{0}C. The paper squares were washed once with 10% trichloroacetic acid, twice with methanol, air-dried. The vapor-phase equilibrium procedure (Stock *et al.*, 1984) was used to quantitate the methyl ester groups. After washing, the 3 MM paper for each sample was placed in 1.5 ml microcentrifuge tubes, and 0.2 ml of 1.0 M NaOH added. The tubes were then placed in 10 ml vials with 2.5 ml of Ecoscint scintillation fluid. The vials were incubated overnight at 25 ^{0}C without shaking (to avoid introducing scintillation fluid in the

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microcentrifuge tube) and the amount of [^{3}H] methanol that had diffused into the scintillation fluid was counted in a Packard Tri-carb 1500 liquid scintillation analyzer and each value is the average of two determination (the standard deviation is about 10%).

Autophosphorylation Assay

The autophosphorylation assay was performed as described by Hess *et al.* (1991). Purified CheA in TEDG buffer was autophosphorylated by incubating it in the presence of [γ-^{32}P]ATP (specific activity, 5000 Ci/mmol), 5 mM $MgCl_2$, and 50 mM KCl. After incubation for 30 min at room temperature, the reaction was terminated by the addition of 700 µl of 5% (wt/vol) ice-cold trichloroacetic acid containing 1% sodium pyrophosphate and then the preparation was placed on ice for 20 min prior to centrifugation for 10 min in a microcentrifuge. The resulting pellet was resuspended in SDS sample buffer (0.125 M Tris/pH 6.8, 4% SDS, 20% sucrose, 10% (v/v) 2-mercaptoethanol, 0.02% Bromophenol Blue) and subjected to SDS-polyacrylamide gel electrophoresis and fluorography analysis.

Incorporation of label into proteins was also analyzed by trichloroacetic acid precipitation onto Whatman GF/C glass fiber filter discs. Reactions were terminated by spotting the preparations onto the discs which were immediately plunged into ice-cold 10% trichloroacetic acid containing 1% sodium pyrophosphate. The discs were washed three times for 30 min each in ice-cold 10% trichloroacetic acid containing 1% sodium pyrophosphate. The discs were then washed briefly in ethanol, dried, and placed into 10 ml vials with 2.5 ml of Ecoscint scintillation fluid and the vials were counted in a Packard Tri-carb 1500 liquid scintillation analyzer and each value is the average of two determinations.

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Esterase activity

Esterase activity was assayed as described previously (Simms *et al.*, 1985) using *E. coli* membranes containing [^{3}H] methyl-labeled *S. typhimurium* aspartate receptors prepared from an *E. coli* methyltransferase deficient strain, RP 4080. The esterase was mixed with 100 μg of [^{3}H] labeled membranes in a total volume of 100 μl and the reaction mixture was incubated at 30 ^{0}C for 5 min. After 5 min, 20 μl samples were removed, and the reaction was quenched with 20 μl of 5 M acetic acid , and the amount of [^{3}H] methanol produced was determined (Simms *et al.*, 1985). [^{3}H] methanol was determined by a modification of the vapor phase equilibrium method as described previously (Simms *et al.*, 1985). Eppendorf tubes were placed in 10 ml liquid scintilation vials with 2.5 ml of Ecoscint scintillation fluid, incubated overnight at 25 ^{0}C, and assayed in a Packard Tri-carb 1500 liquid scintillation analyzer. The total amount of [^{3}H] methylester substrate in the membranes was estimated by measuring the amount of [^{3}H] methanol produced when 20 μl of membranes was incubated with 200 μl of 5 M NaOH.

Estimation of Protein

Protein concentrations were estimated by the method of BioRad (Richmond, CA) using BSA as the standard.

SDS- Polyacrylamide Gel Electrophoresis and Fluorography

The methylated and phosphorylated proteins were identified using SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie Blue, and the

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labeled proteins were analyzed by fluorography as described earlier (Subbaramaiah *et al.*, 1992). In the case of [^{3}H] labeled samples, the gel was soaked in Autofluor for 1 hour, dried, and exposed to Kodak X-Omat AR film at -80^{0}C.

V8 Protease Cleavage of Methylated CheA Protein

V8 protease was used to cleave the methylated CheA protein at glutamic acid residues. Cleavage was carried out essentially as described earlier (Subbaramaiah *et al*, 1992). Ninety μg of [^{3}H]-labeled methylated protein was incubated with 10 μg of V8 protease at room temperature for 48 hr in 300 μl 50 mM ammonium bicarbonate buffer, pH 8.0 containing 0.1% SDS. Complete digestion of [^{3}H] labeled methylated CheA will release Glu^{182} and Glu^{183} as free amino acids. The digest was analyzed by thin layer chromatography as described below.

Thin Layer Chromatography

Thin layer chromatography was performed by following the procedure described earlier (Subbaramaiah *et al.*, 1992). After the digestion of radio-labeled CheA by V8 protease, the mixture was spotted on a TLC (Cellulose-DEAE sheet, 250 micron) sheet, and the sheet was developed using butanol : acetic acid : water (4 : 1 : 1) as the solvent system. The TLC plate was dried and 1 cm^2 aliquots were removed and placed in 10 ml vials with 2.5 ml of Ecoscint scintillation fluid. The amount of [^{3}H] in each vials were counted in a Packard Tri-carb 1500 liquid scintillation analyzer.

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Digestion of Methylated CheA Using Clostripain

Cleavage at arginine residues by clostripain (endoprotease Arg-C) was carried out essentially as described earlier (Bousfield *et al.*, 1988). One hundred µg of methylated protein was digested with 10 µg of endoprotease Arg-C at room temperature for 48 hours in 100 µl of 0.1 M ammonium bicarbonate pH 8.5 containing 2.5 mM DTT. The cleaved products were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described before.

Oligonucleotide-directed Mutagenesis of the *che*A gene

Oligonucleotide-directed site-specific mutagenesis was used to create mutations in the *cheA* gene using the double-stranded plasmid method as described by Markvardsen *et al.*, 1995 with minor modifications. Several steps are involved in the procedure and are illustrated in Figures 13.

(a) Uracil incorporation in the template DNA:

The plasmid pMO4 was transformed into the *E. coli ung*$^-$ *dut*$^-$ strain RZ1032 transformants (Step 1 of Figure 14) selected and grown in LB medium supplemented with 6 µg/ml uridine (Sigma Chemical, St. Louis, MO) and 50 µg/ml ampicillin. In this step, uracil will become incorporated into the plasmid DNA. The uracil incorporated pMO4 DNA was

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Figure 13. The strategy for oligonucleotide-site direct mutagenesis

This strategy is modified from the methods described by Markvardsen *et al.*, 1995 and the Chameleon™ double-stranded, site-directed mutagenesis kit instruction manual (Stratagene, La Jolla, CA).

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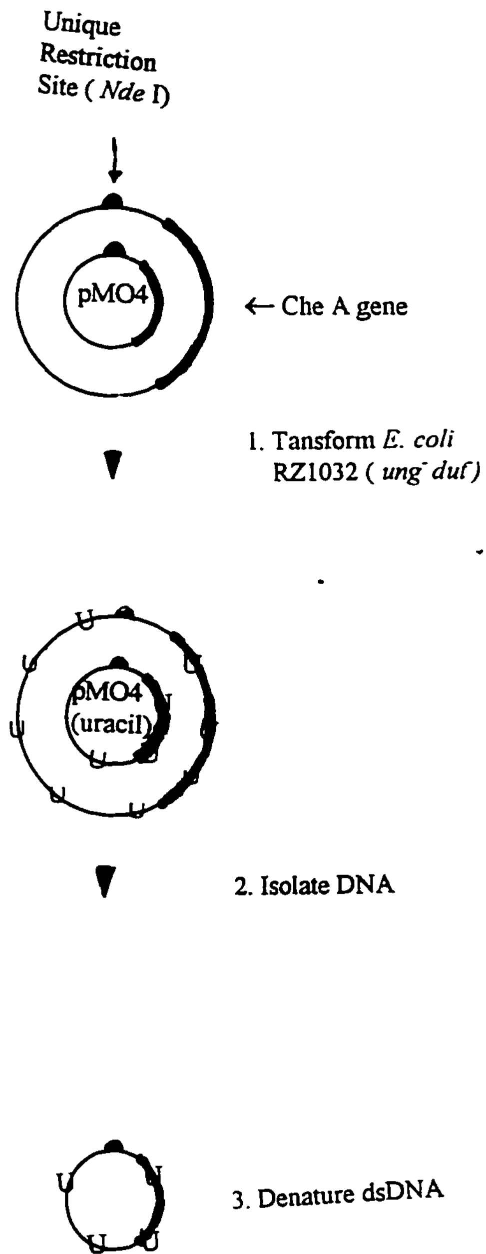

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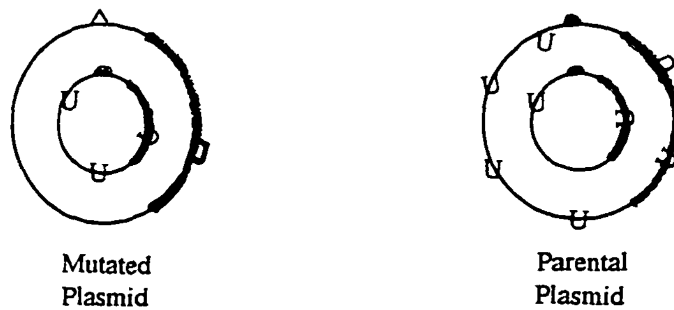
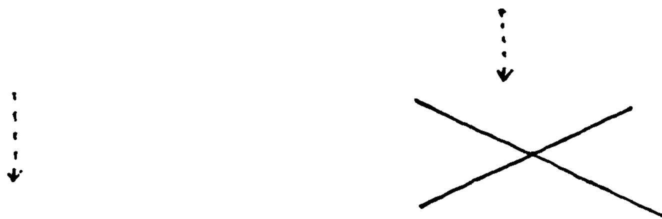
4. Anneal Primers

5. Synthesize second strand
with T4 DNA polymerase and
seal gaps with T4 DNA ligase



6. Transform *mutS E. coli* (*ung*$^+$)



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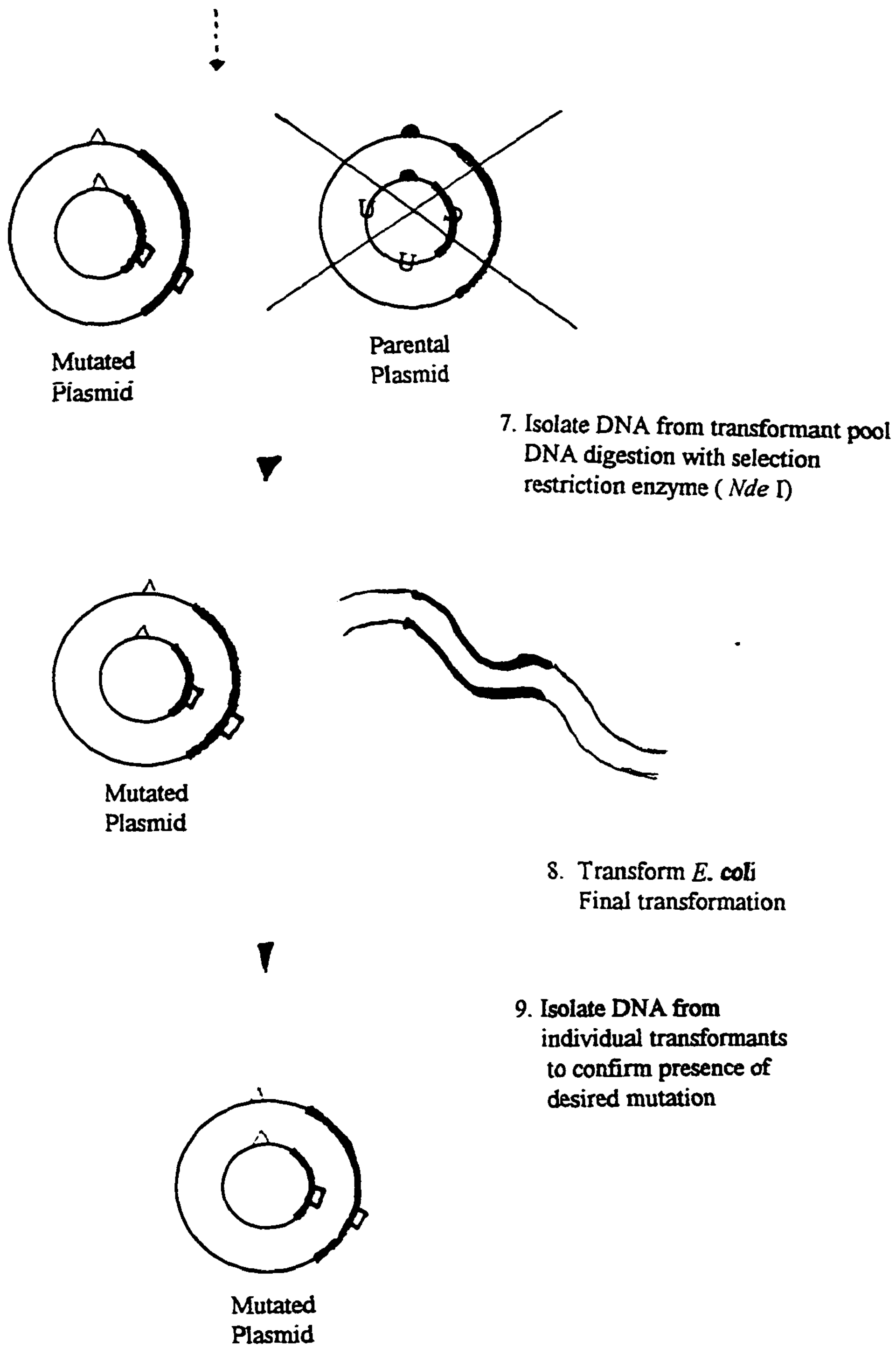

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used as a template strand. Uracil-containing pMO4 was purified by the Magic™ Megapreps DNA Purification System (Promega, Madison, WI) as recommended by the manufacturer.

(b) Phosphorylation of synthetic oligonucleotides:

Phosphorylation of oligonucleotides was performed as described previously (Zoller *et al.*, 1983). The reaction mixture contained in a 50-μl volume: 1000 pmol of the indicated oligonucleotide, 0.33 mM ATP, 100 mM Tris-HCl (pH 8) 10 mM $MgCl_2$, 10 mM DTT, and 40 units of T4 polynucleotide kinase. After 45 min at 37^0C, the reaction was terminated by heating at 70^0C for 10 min.

(c) Annealing, extension and ligation of template oligonucleotides:

Site-directed mutagenesis were performed by using the Chameleon™ Double-Stranded, Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) as recommended by the manufacturer with the following modifications.

1) Annealing the primers to the DNA:

Two oligonucleotide primers— a mutagenic primer (oligo-56 or oligo-57, Figure 14) and a selection switch primer (Trans oligo *Nde* I/*Nco* I , Figure 14) (Clontech, Palo Alto, CA) were simultaneously annealed to the double-stranded target plasmid pMO4 (uracil) DNA (Step 4 of Figure 13). By using the mutagenic primer oligo-56, Glu^{182} in CheA could be converted to Asp^{182}, and oligo-57 could change the Glu^{182} to Ala^{182} (Figure 14).

The uracil incorporated plasmid, 0.25 pmole, was mixed with 25 pmole of the mutagenic primer, 25 pmole of the selection primer and 2 μl of 10x mutagenesis buffer to

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Fig. 14. DNA sequences of oligo-56, oligo-57 and Trans oligo *Nde I/Nco* I

(A) oligo-56: 3' ---- TA GAC GAA CTT CTG CTC AAC CCG TTA G--5'
^{182}Asp

The underlined sequences represent base pairs changed during mutagenesis.

(B) oligo-57: 3' ---- TA GAC GAA CTT CGT CTC AAC CCG TTA G--5'
^{182}Ala

The underlined sequences represent base pairs changed during mutagenesis.

(C) Trans oligo *Nde I/Nco* I:

5' ----GAGTGCACCAT**GG**GCGGTGGTGAAAT---3'

The bold sequences represent base pairs changed during mutagenesis; the underlined portions of the sequences represent the second restriction enzyme sites (after site conversion)

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a final volume of 20 µl. The mixture was placed in a boiling water bath for 10 min (Step 3 of Figure 13) and then immediately placed on ice for 10 min. The tubes were then incubated at 55 ^{0}C for 1 hr, and then at room temperature for 30 min (Step 4 of Figure 13).

2) Extending the primers and ligating the new strands:

The nucleotide solution, 7 µl, was mixed with 3 µl of freshly diluted 1:10 enzyme solution. The tubes were incubated at 37 ^{0}C for 2 hr, and then at 15 ^{0}C overnight. In this step, the primers in the reactions were extended with T7 DNA polymerase and the new strands were ligated with T4 DNA ligase (Step 5 of Figure 13).

(d) Transformation of *E. coli* competent cells (XL mutS$^-$) and isolation of mutant hybrids:

The mutated plasmids were then transformed into *E. coli* competent cells (XL mutS$^-$ *ung*$^+$, from Stratagene, La Jolla, CA) and grown overnight at 37 ^{0}C in 3 ml LB medium supplemented with 50 µg/ml ampicillin (Step 6 of Figure 13). The *E. coli* mutS$^-$ bacterial strain is deficient in DNA repair strand selection, therefore, at this point, the parent DNA should fail to replicate and most of the plasmids that replicate should be the mutant hybrids.

(e) Recovery of the DNA and restriction enzyme digestion:

Miniprep plasmid DNA was isolated from these overnight cultures by the Magic™ Megapreps DNA Purification System (Promega, Madison, WI) as recommended by the manufacturer and then subjected to *Nde* I restriction enzyme digestion (Step 7 of Figure 13). By using the selection primer, Trans oligo *Nde* I / *Nco* I, the *Nde* I side was changed to *Nco* I site. Therefore, the mutant plasmids could be further enriched by digestion of the

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DNA with restriction enzyme *Nde* I. This step is try to increase the efficiency of mutagenesis.

(f) Transformation into *E. coli* competent cells:

The DNA digested with *Nde* I restriction enzyme was then transformed into *E. coli* XL1-Blue competent cells provided according to the manufacturer (Stratagene, La Jolla, CA). The cells were plated out on LB-ampicillin agar plates (Step 8 of Figure 13). There were about 15 colonies for each set. Finally, the plasmids from each colony were prepared and subjected to DNA sequencing to verify the mutation (Step 9 of Figure 13).

Immune precipitates between CheA antibody and CheA form mutant strains

The immune precipitate occurs not only in solution but also in an agar matrix. The double-immunodiffusion (Ouchterlony) method of gel diffusion was used in this study (Burrell and Mascoli, 1970). When antigen and antibody diffuse from wells toward one another in agar, a visible line of precipitation will form. This simple technique is an effective qualitative tool for determining the relationship of antigen-antibody systems. A layer of 1% melted agar in plastic petri plate was prepared by pouring 5 ml of clarified diffusion agar into it. After hardening, holes were cut into the agar to form hollow wells. The wells were then filled with the designated reagents into the appropriate wells and the plate incubated with humidification at 37 ^{0}C overnight for development of precipitation.

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Results and Discussion

Our computer analysis of the GenBank database indicated the presence of a 27 amino acid region from Leu^{169} to Lys^{195} in CheA which shared a 33% identity and more than 85% evolutionary similarity with the methylation region, termed K1, of the chemoreceptor, Tar (Figure 11). This analysis also showed that CheA contained an identical glutamic acid that is methylated in Tar by CheR methyltransferase. These findings prompted me to investigate whether CheA could be methylated by CheR.

Purification of CheR and CheA proteins

CheA was purified from *E. coli* cells using the over-expression vector pMO4 (Hess *et al.*, 1991) as described in "Methods". SDS-polyacrylamide gel electrophoresis (Figure 12) and autophophoryation assay was performed to determine this enzyme's purity and activity. The purified protein which migrated as a 71 kDa on SDS-polyacrylamide gel (Figure 12, lane 2) contained autophosphorylation activity (Data not shown).

Similarly, *E. coli* HB101 containing the CheR over-expression vector pME43 was used for expression of CheR methyltransferase, and the enzyme was purified as described in "Methods". SDS-polyacrylamide gel electrophoresis (Figure 12, lane 3) and the methylation assay were performed to determine this enzyme's purity and activity. As shown in Figure 12, purified CheR migrates as a single band at a position of 31 kDa as expected.

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Methylation reaction of CheA *in vitro*

Methylation experiments were performed with purified CheR methyltransferase, S-adenosyl-L-[methyl-^{3}H] methionine (AdoMet), and different substrates. BSA, an isoaspartyl peptide and an ovalbumin were used in the methylation studies as controls.

As observed by others (Springer and Koshland, 1977 and Clarke *et al.*, 1980), CheR appeared to be completely specific for the methyl-accepting chemotaxis receptors. CheR does not catalyze the methylation of a variety of soluble proteins which are the methyl-accepting substrates for eucaryotic methyltransferase (Clarke, 1985). When equal or more amounts of BSA, ovalbumin, and isoaspartyl peptides were incubated as the substrates of CheR methyltransferase, the extents of methylation observed as a function of time were found to be negligible (Figure 15). Likewise when CheR or CheA was incubated with AdoMet separately, no methylation reactions were observed (Data not shown).

However, when CheA was incubated with CheR methyltransferase and AdoMet, the extent of methylation increased with time (Figure 15). After 24 hours incubation, the radioactivity of CheA reached up to 20,000 cpm and the counts for the control proteins were less than 100 cpm. These results confirm our prediction that, the CheA protein could be methylated by CheR. Moreover, the [^{3}H]-labeled methylated CheA could be visualized after it was subjected to SDS-polyacrylamide gel electrophoresis and autoradiography (Figure 16). These results showed that CheR methyltransferase could specifically methylate CheA protein *in vitro* and the methyl group on CheA was stable.

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Figure 15. Extent of methylation toward different substrates:

Purified CheA (•), 10 µg, was incubated with 10 µl of [H^3] AdoMet (85 mCi/ mmole) and 10 µg of purified CheR in a total volume of 100 µl for the indicated times at 30 ^{0}C. In the control experiments BSA (□) (10 µg), ovalbumin (o) (10 µg), and isoaspartyl peptide (▲) (10 µg) were used. The extent of methylation was measured as described in " Methods".

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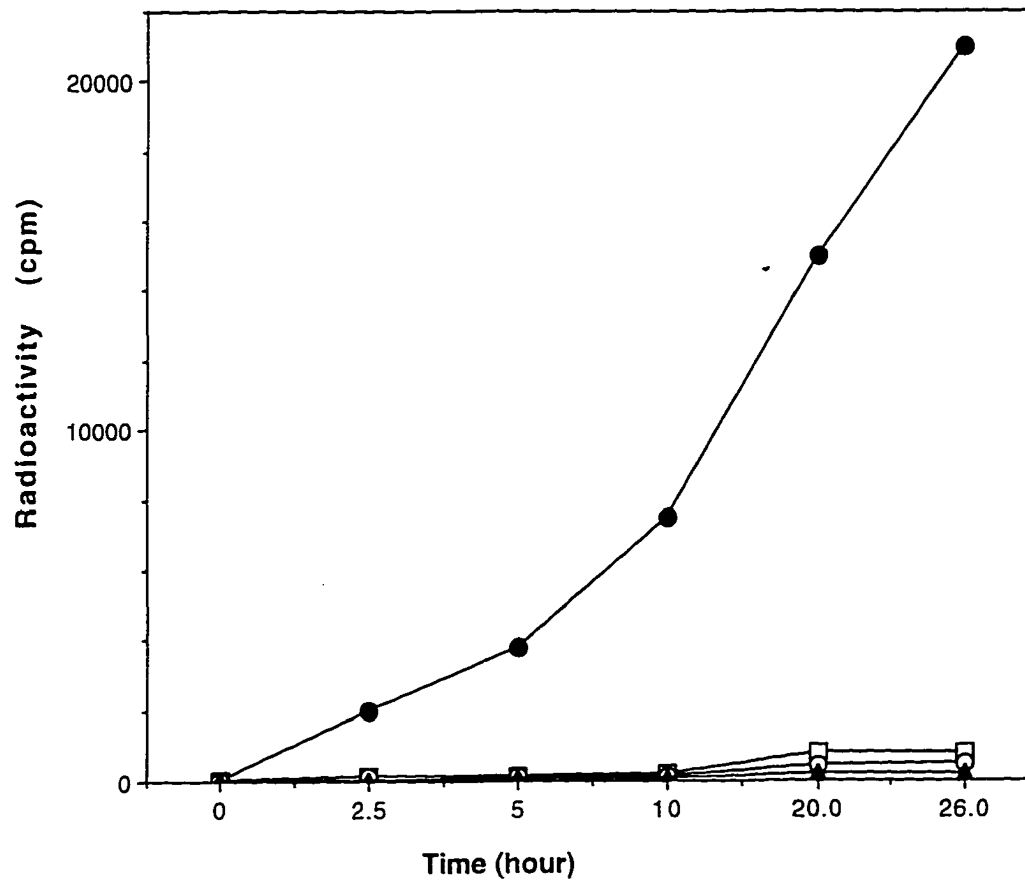

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Figure 16. Fluorography of [3H]- labeled methylation of CheA

SDS-polyacrylamide gel electrophoresis was performed as described in "Methods". For fluorographic analysis, the gel was soaked in Autofluor for 1 hour, dried, and exposed to Kodak X-Omat AR film at -80^{0}C. Lane 1, shows the standard marker proteins, 94 and 67 kDa respectively; Lane 2, shows methylated $CheA_L$; Lane 3, shows methylated BSA (as a control); lane 4, shows methylated $CheA_L$ & $CheA_S$ (Wolfe *et al.* 1993).

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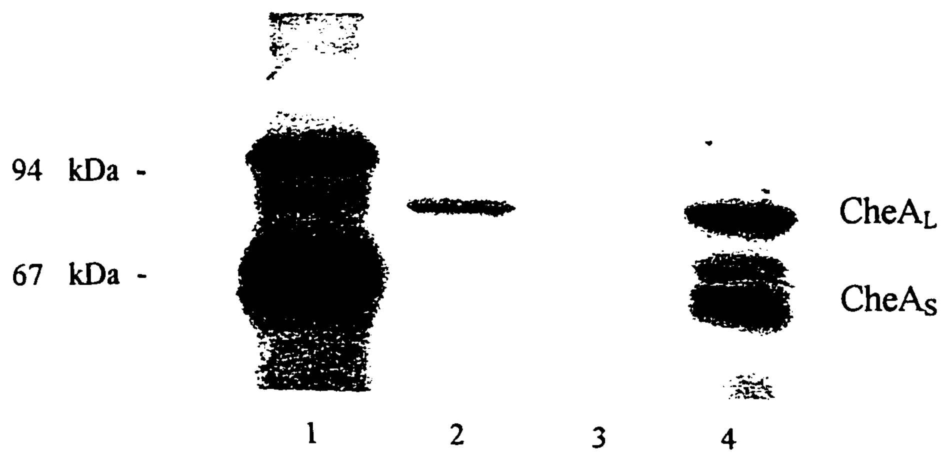

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Analysis of the nature of CheA methylation

The level of methylation was found to rise with increase in amounts of CheR, CheA, and AdoMet respectively (Figures 17 A, B, C). These results further support the conclusion that CheA methylation is an enzymatic reaction and is methylated by CheR, the methyltransferase.

The nature of the reaction were further investigated. The effects of temperature, pH, and ionic strength of the medium upon the CheA methylation reactions were investigated. Like other enzymatic reactions, the level of methylation accelerated when the reaction temperature was increased (Figure 18). pH changes also had some effect on CheA methylation reaction. The reaction rates were dramatically decreased when the reaction was performed in 50 mM Tris-HCl at a pH above 8.0 (Figure 19). This methylation reaction was found to be sensitive to the ionic strength of the medium. The reaction activity dramatically increased as the ionic strength decreased (Figures 20 A, B, C). These data further support the conclusion that CheA methylation is a specific enzymatic reaction.

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Figure 17A. Dependence of CheA methylation on the concentration of CheR

Purified CheA, 20 μg, was incubated with 10 μl of [H^3] AdoMet (85 mCi/ mmole) and different amounts of purified CheR, in a total volume of 100 μl of 10 mM Tris buffer pH 7.9 at 30 ^{0}C. At the indicated times, 15 μl of the reaction mixture was removed and the extent of methylation was measured as described in "Methods".

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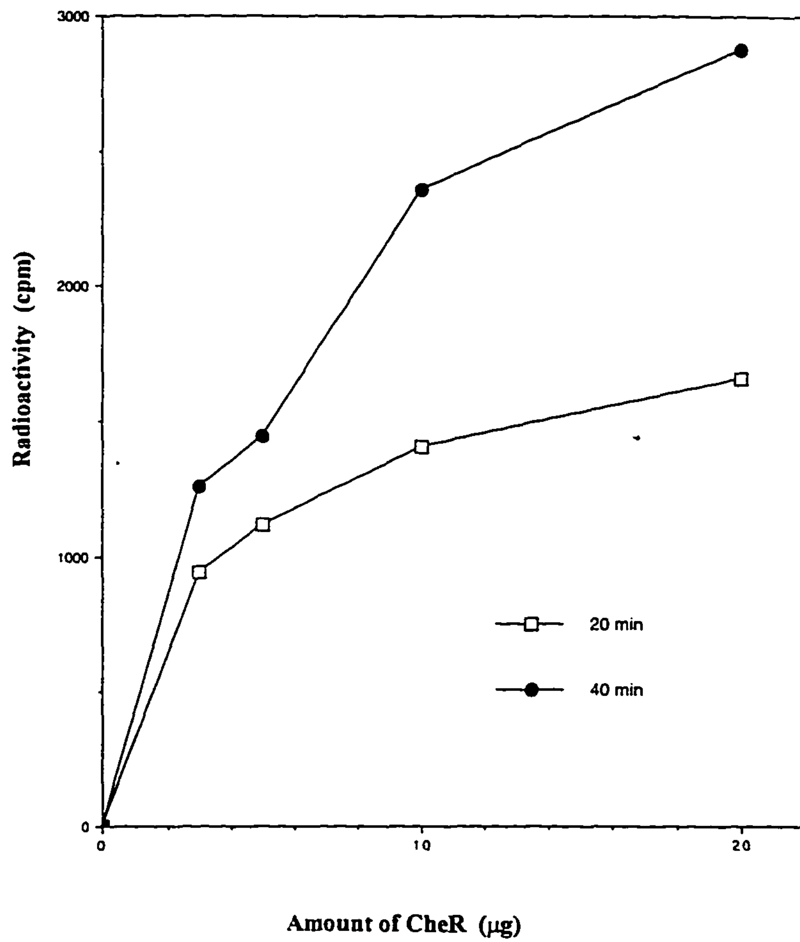

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Figure 17B. Dependence of CheA methylation on the concentration of CheA

Different amounts of CheA, were incubated with 10 μg of CheR and 10 μl of $[H^3]$ AdoMet (85 mCi/ mmole) at 30 ^{0}C in 10 mM Tris-HCl pH 7.9 in a total volume of 100 μl for a period of 30 min. At the end of incubation period the extent of methylation was measured as described in " Methods".

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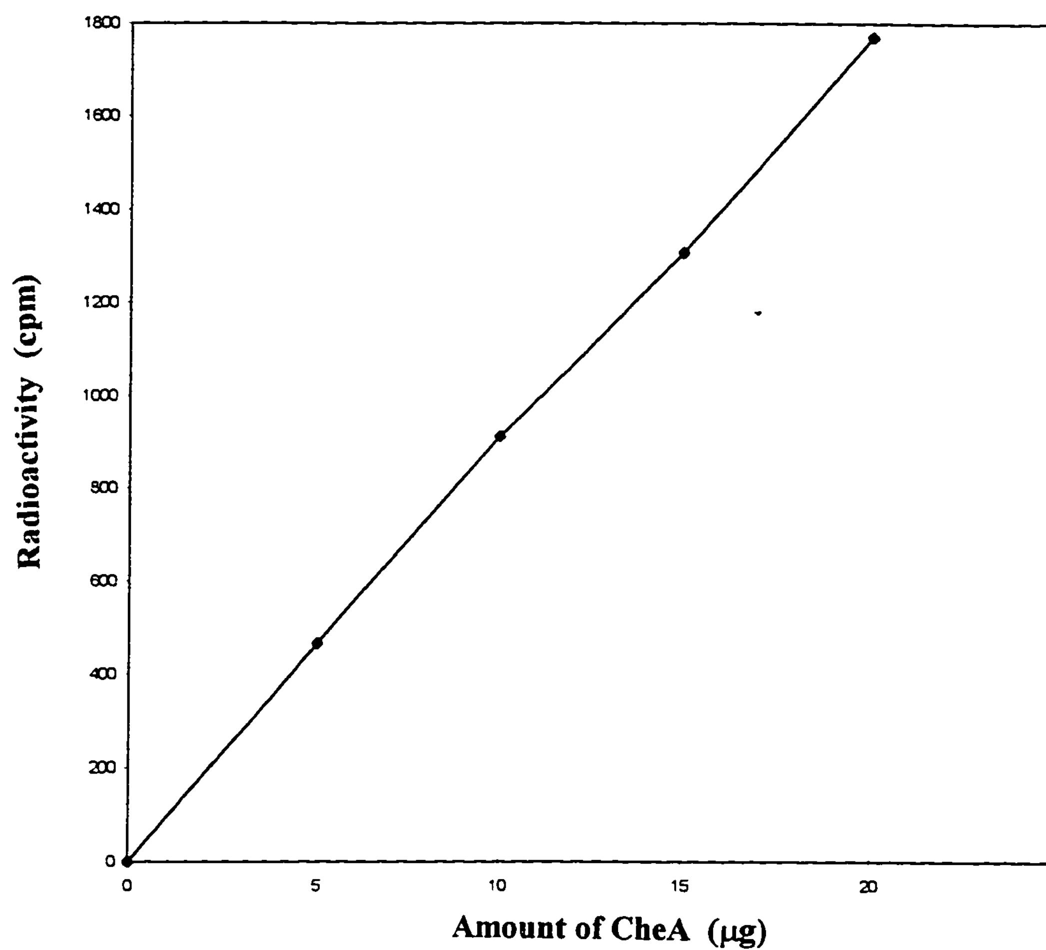

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Figure 17C. Dependence of CheA methylation on concentration of AdoMet

CheA protein, 20 μg, was incubated for 30 min at 10 mM Tris buffer pH 7.9, 30 ^{0}C in the presence of purified CheR methylase (10 μg) and varying concentration of [H^3] AdoMet (85 mCi/ mmole) in a total volume of 100 μl. The assay was performed as described in "Methods".

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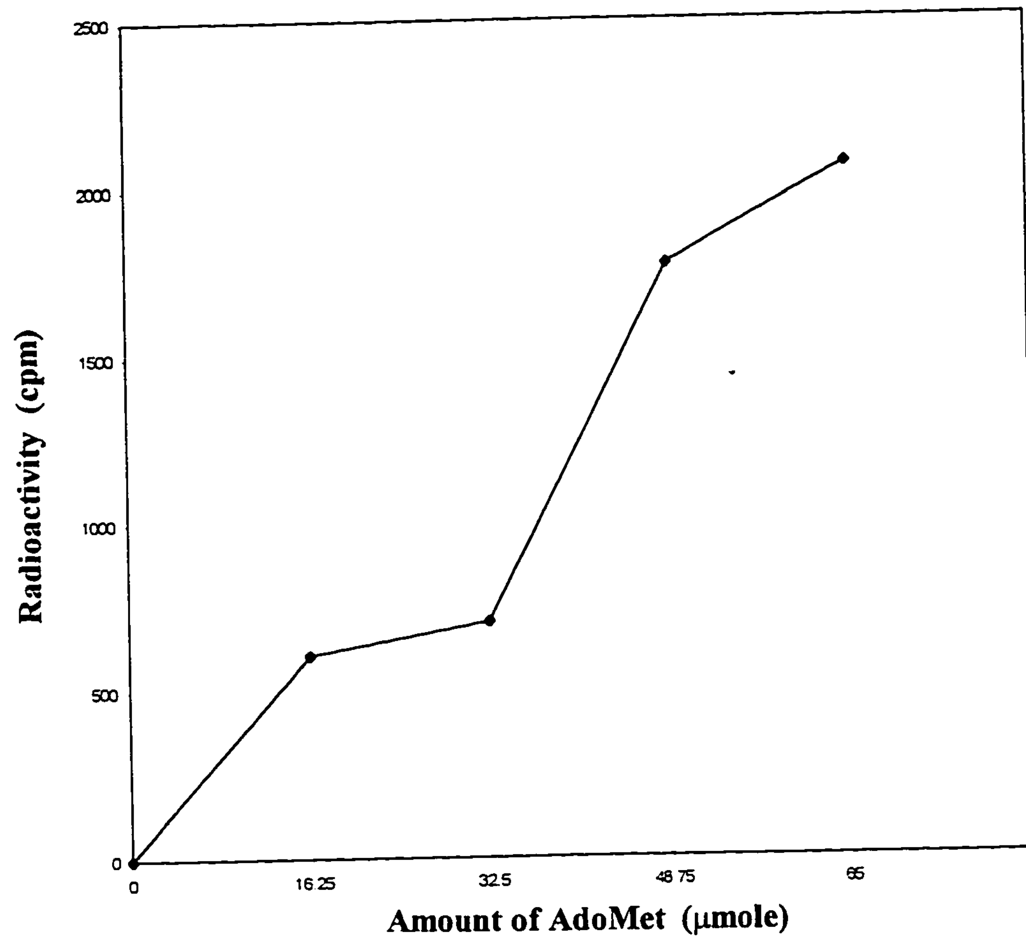

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Figure 18. Effect of temperature on CheA methylation reaction:

CheA (10 μg) incubated with CheR (6 μg) and 10 μl of [H^3] AdoMet (85 mCi/ mmole) in 0.1 M potassium phosphate buffer, pH 7.0, in a total volume of 100 μl for a period of 60 min at either 30 ^{0}C (o) or 37 ^{0}C (•). The extent of methylation was measured as described in " Methods".

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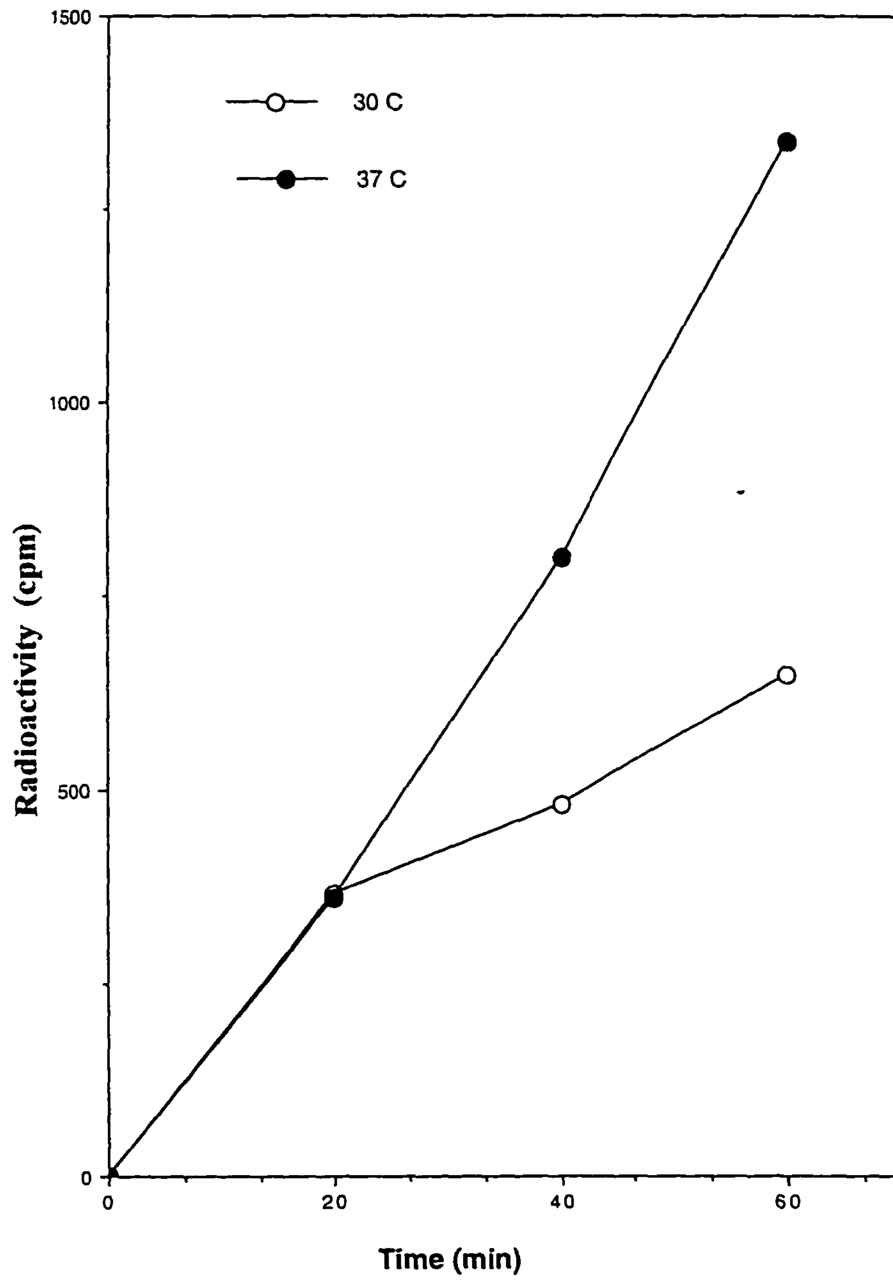

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Figure 19. Effect of pH on the rate of CheA methylation:

CheA (10 μg) was incubated with CheR (6 μg) and 10 μl of [H^3] AdoMet (85 mCi/ mmole) for a period of 60 min in a total volume of 100 μl of different buffers. The extent of methylation was measured as described in " Methods".

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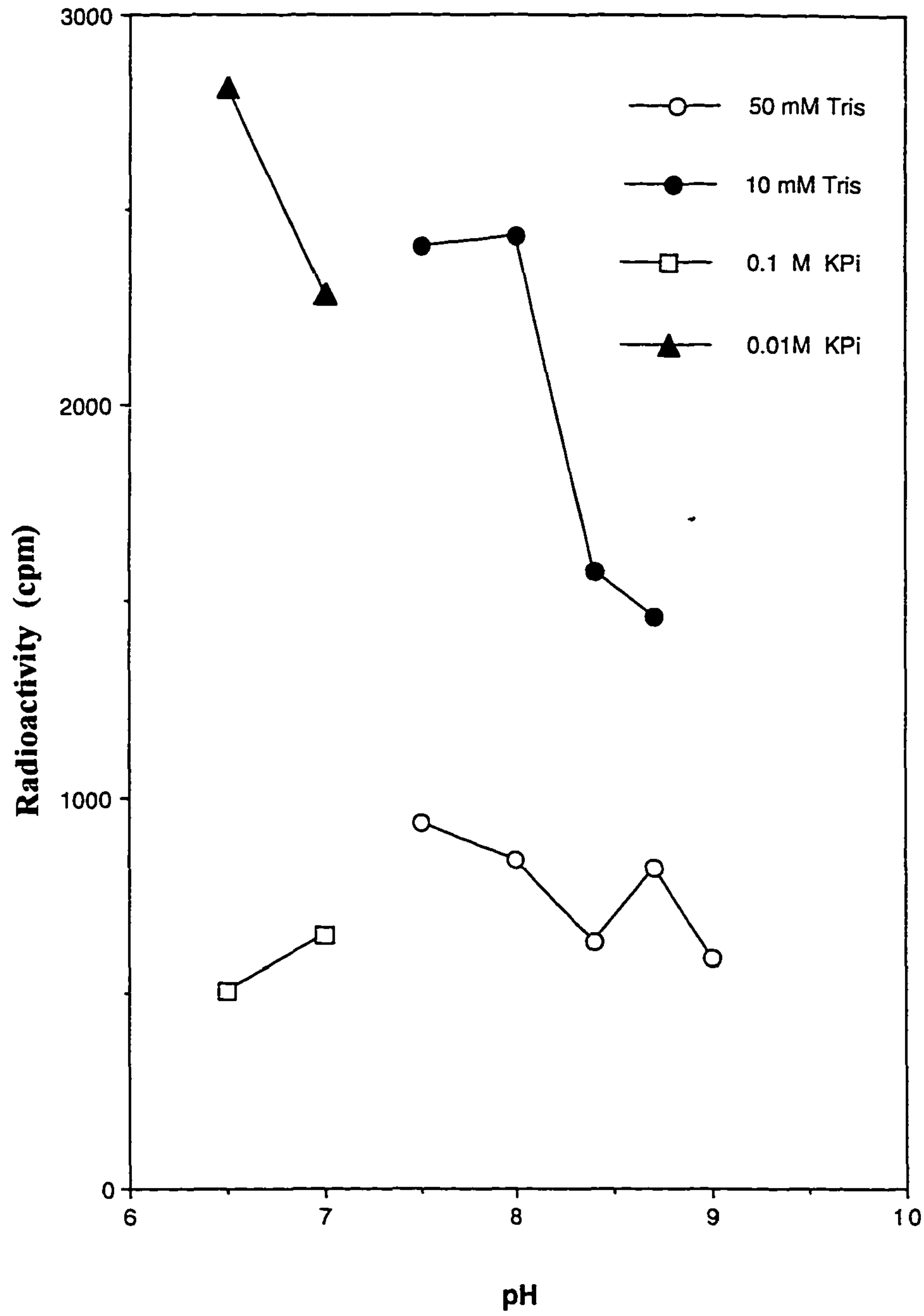

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Figures 20 A, B, C. Effect of ionic strength on the rate of CheA methylation reaction:

CheA (10 μg) was incubated with CheR (6 μg) and 10 μl of [H^3] AdoMet (85 mCi/ mmole) at 30 ^{0}C for a period of 60 min in a total volume of 100 μl of different media as indicated in the abscissa. The extent of methylation was measured as described in "Methods". (A) in 0.01 M (▲), 0.02 M (■) and 0.1 M (♦) potassium phosphate buffer, pH 7.0, (B) in 10 mM (o) and 50 mM (•) Tris-HCl buffer, pH 8.0. (c) for 60 min of reaction time, the effect of ionic strength of Tris-HCl (•) and potassium phosphate buffer (■) were plotted.

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Figure 20A.

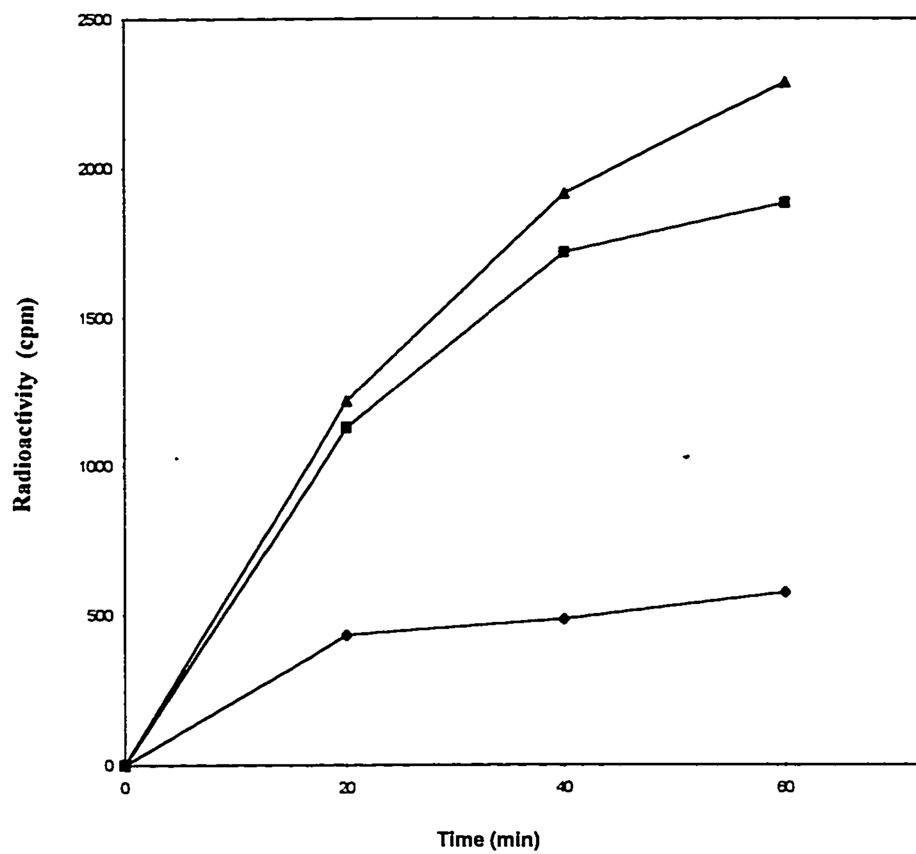

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Figure 20B.

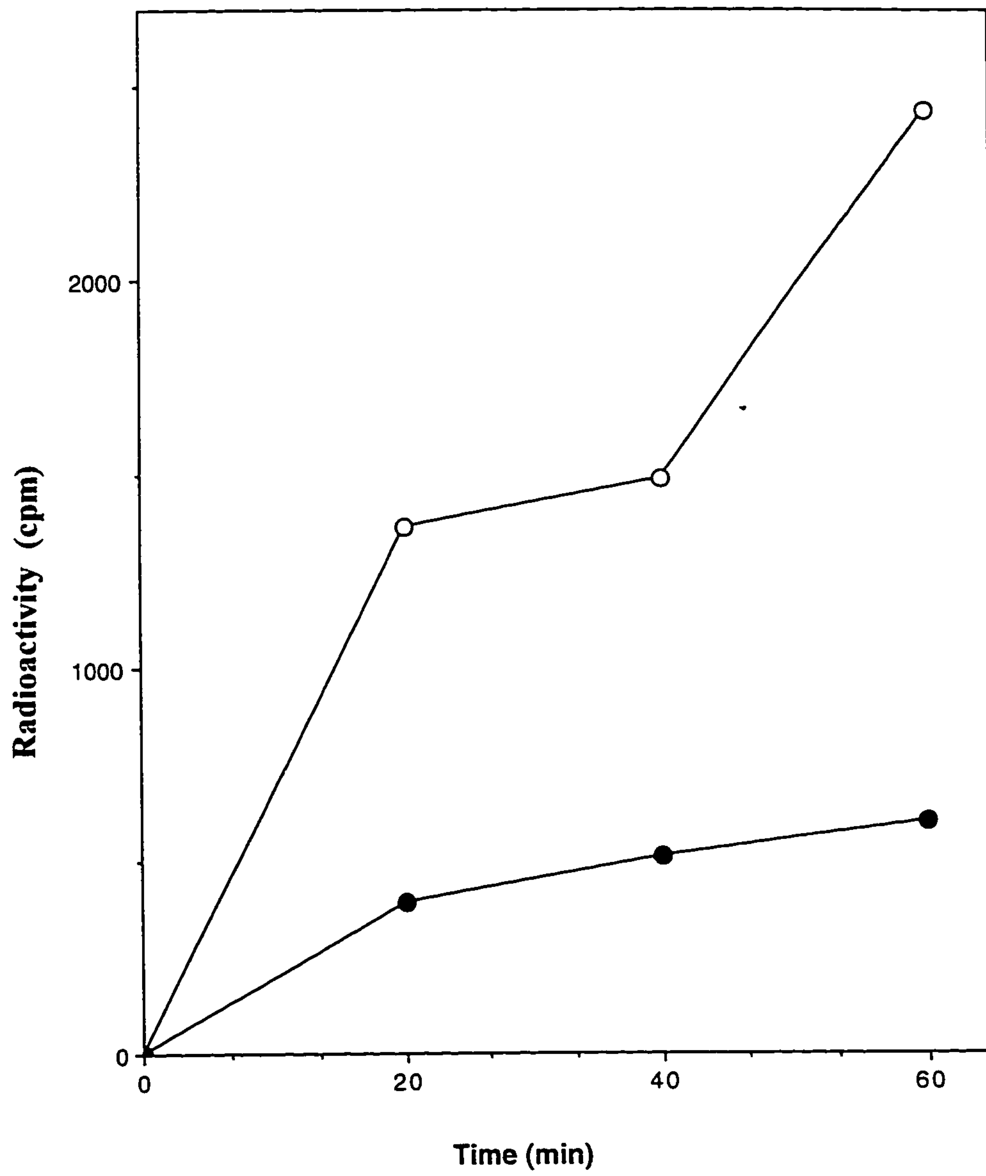

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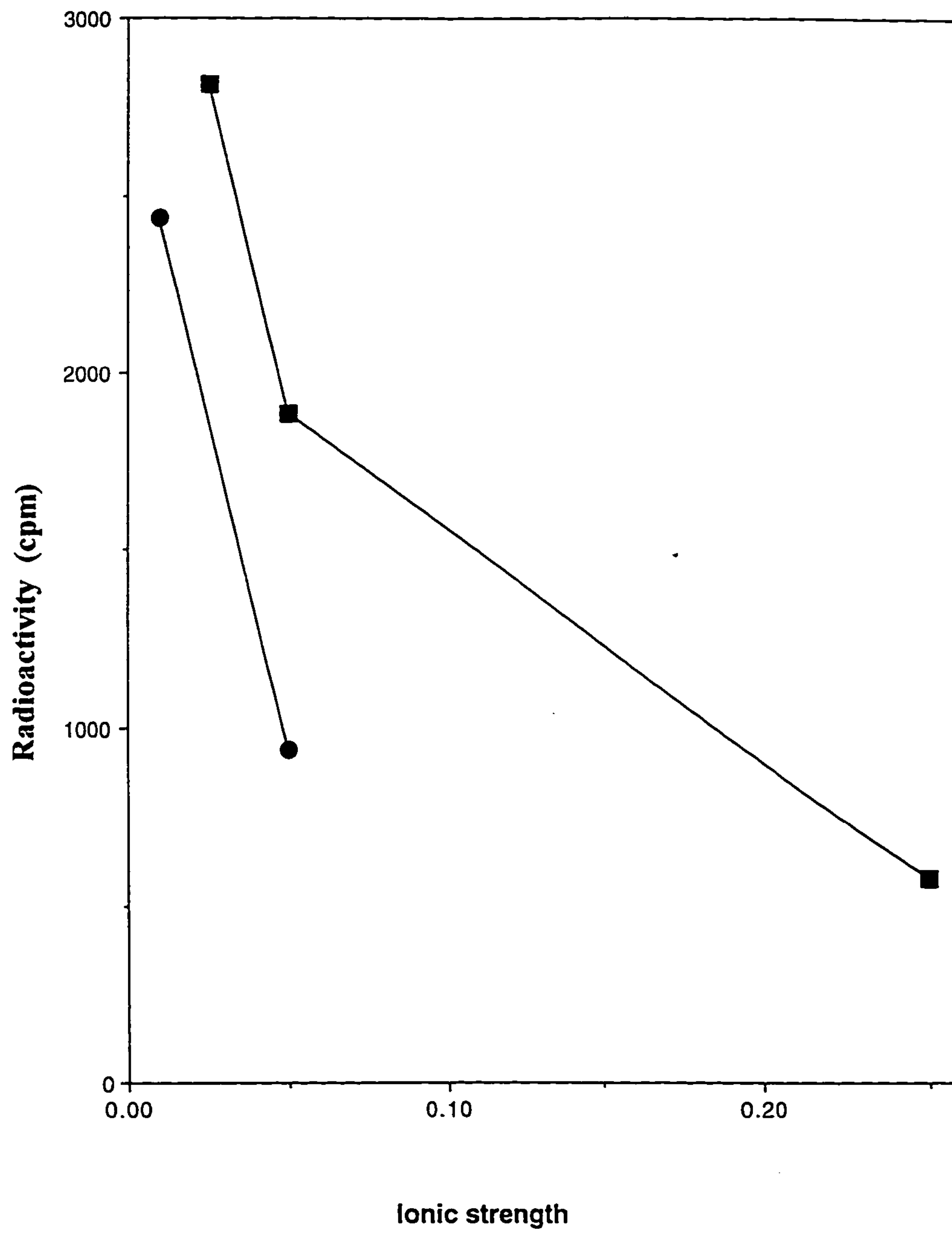
Figure 20C.



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Effect of autophosphorylation on CheA methylation

CheA is the central regulator in the bacterial chemotaxis pathway. It was earlier reported that CheA plays a key role both in the initial excitation response to stimuli as well as subsequent events associated with the adaptation process. This is because CheA is an autophosphokinase and it can transfer its phosphoryl group to CheY and CheB and regulate their activities. There is no direct evidence to date of any direct role for CheR in the excitation process. However, we have found that CheA has a methylation site homologous to the K1 peptide of Tar. We have also shown that CheA can be methylated specifically by CheR *in vitro*. In order to further explore the role of the methylation on CheA, the effect of autophosphorylation on CheA methylation was performed. The methylation rates between phosphorylated CheA and unphosphorylated CheA were compared. I found, within 2 hours incubation time, the methylation rate of phosphorylated CheA increased about two fold to that of the unphosphorylated CheA (Figure 21).

Comparison of the methylation rate of Tar and CheA proteins

CheR methyltransferase has been shown to be involved in the adaptation pathway of bacterial chemotaxis system where it exerts its effect by modulating the sensitivities of the MCPs. To date there is no report about any other methylation reaction involved in this signal transduction pathway. In this project, CheA, the central regulator in this pathway, was shown to be able to become methylated *in vitro*.

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Figure 21. Effect of phosphorylation on CheA methylation:

CheA was incubated at room temperature with 0.1 mM ATP, 5 mM $MgCl_2$, 50 mM KCl and 50 mM Tris-HCl (pH 7.5) for 1 hr. ATP was removed by dialysis. The phosphorylated CheA protein (7.5 μg) (♦) was methylated in the presence of 10 μg of CheR methylase and 10 μl of [^{3}H]AdoMet (85 mCi/mmole) in a total volume of 100 μl at 30 ^{0}C. At the indicated times, 15 μl aliquots were removed and the level of methylation was measured as described earlier. An equal amount of unphosphorylated CheA (7.5 μg) (□) was used as a control.

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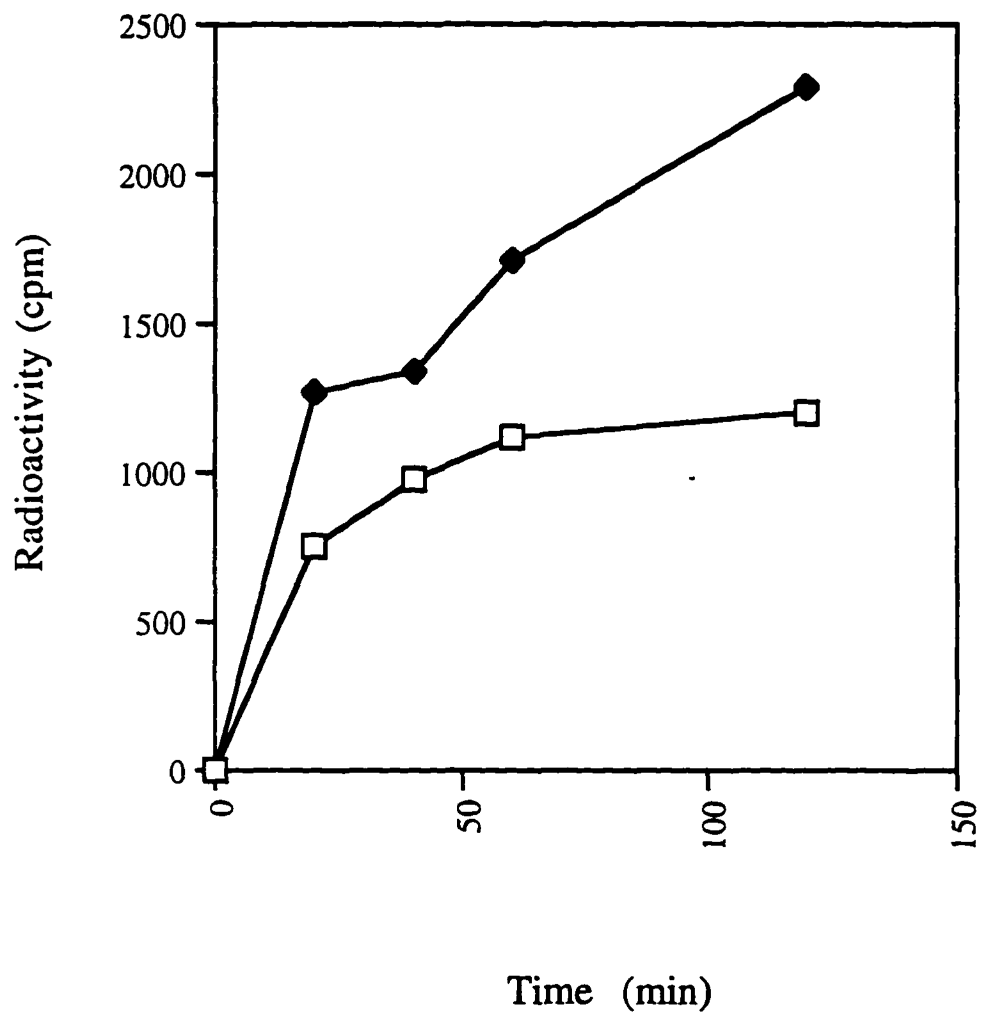

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To determine the role of CheA methylation, a comparative analysis of methylation reactions of similar amounts of Tar, CheA and phosphorylated CheA was performed. As shown in Figure 22, the methylation of Tar rapidly increased within the first two hours, and after that the reaction were increased slowly with increasing time. The methylation rates of both phosphorylated CheA and unphosphorylated CheA were found to be slowly increased with increasing time.

CheR is a soluble globular protein that binds tightly to MCPs (K_d ~1 μM, Stock *et al.*, 1994). CheR has been shown to methylate only specific glutamate residues on MCPs (Kleene *et al.*, 1977 and Werf *et al.*, 1977), and not to methylate proteins other than the MCPs (Stock *et al.*, 1984, Springer *et al.*, 1977b, and Clarke *et al.*, 1980). When the *in vitro* methylation rate of Tar membrane, in the absence of attractant, was analyzed (Figure 22), it was found that the reaction was very fast in the first 20 min (1300 cpm/min). The reaction rate then became slower (~145-170 cpm/min). After two hours incubation, the reaction progressed at a much lower rate (4 cpm/min). During the first two hours incubation, about 54% of the methylation sites were methylated. At the end of twenty hours incubation, approximately 66% of the methylation sites were methylated.

As reported before (Terwilliger *et al.*, 1986a,b) there are four methylation sites on Tar. Because of protein folding, some of these methylation sites may be embedded and not accessible. CheR methylates each of the four sites in Tar with quite different intrinsic rates (relative rates 1.0, 0.63, 0.09, and 0.02, Terwilliger *et al.*, 1986a,b). However, after

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Figure 22. Comparison of methylation rate on Tar and CheA proteins

At 30 ^{0}C in a total volume of 100 μl of 10 mM Tris buffer pH 7.9, 100 pmole of unphosphorylated (□) and phosphorylated CheA (♦), 7.3 μg, and equal pmole of Tar (o), 6 μg, were incubated with 10 μl of [H^3] AdoMet (85 mCi/ mmole) and 500 pmole of purified CheR, respectively. At the indicated times, 15 μl of the reaction mixture was removed and the extent of methylation was measured as described in "Methods".

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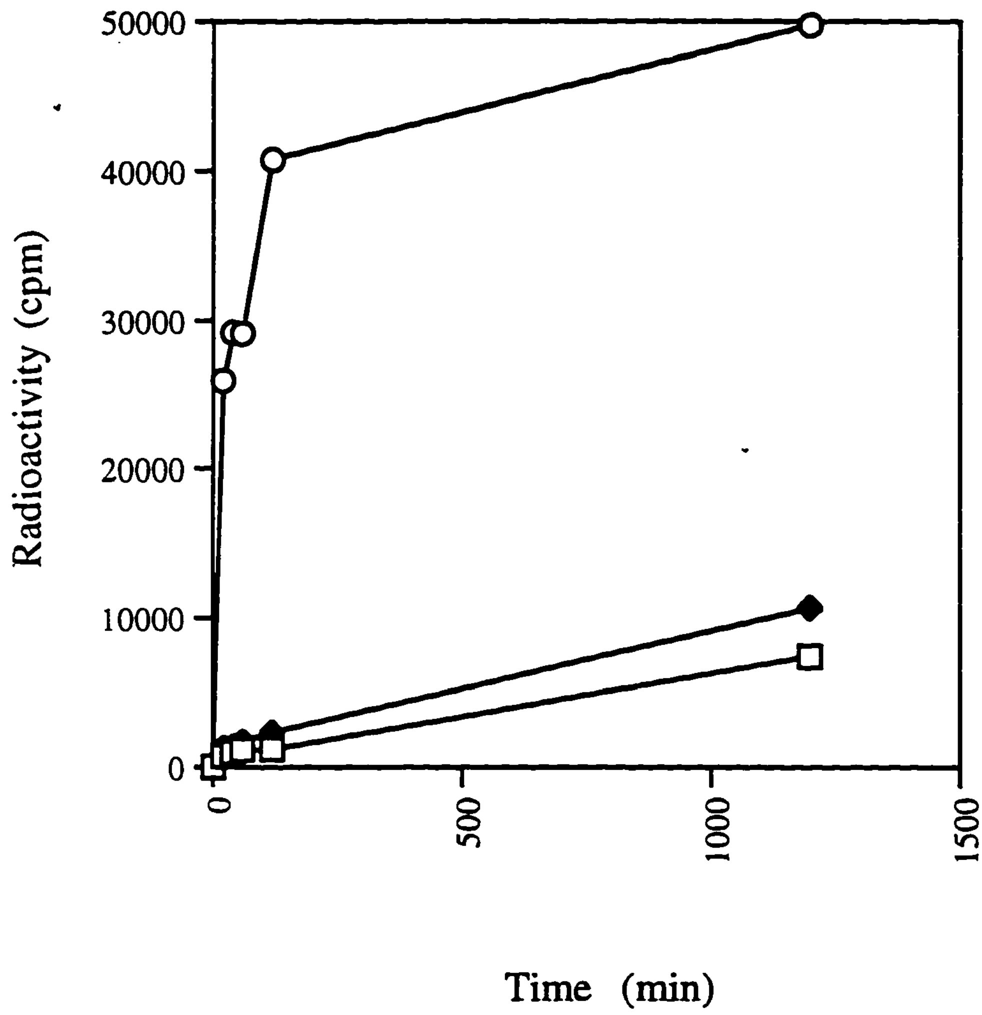

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binding an attractant, the receptor undergoes a conformational change which causes all the methylation sites to be exposed and easier to be methylated. Therefore, when Tar is incubated without attractant (Figure 22), the methylation rate is very high for the first 20 min. This is probably because the highly exposed sites are modified. Hereafter, the methylation rate drops and could continue at a slow rate for overnight, since there are still available, although less accessible, methylation sites in Tar (Figure 22).

Under the same reaction conditions, the methylation rates of both phosphorylated CheA and unphosphorylated CheA were found to slowly increase with time. During the first two hours incubation (Figure 22), approximately 6% of the methylation sites of CheA were methylated and at the end of twenty hours incubation, approximately 39% of the methylation sites of CheA were methylated. Therefore, it is possible that the methylation sites in CheA are embedded and is inaccessible as was observed for some of the sites in Tar. Moreover, the methylation rate of phosphorylated CheA is about two times faster than that of the unphosphorylated CheA (Figure 22). During the first two hours incubation, approximately 12% of the methylation sites of phosphorylated CheA were methylated and at the end of twenty hours incubation, approximately 56% of the sites were methylated. Therefore, one hypothesis is proposed— The methylation site(s) in CheA is (are) embedded and is (are) inaccessible as was observed for some of the sites in Tar. However, some circumstance, phosphorylation of CheA for example, may cause a conformational change in CheA that exposes the methylation site(s) and makes the methylation site(s) more accessible to CheR.

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Esterase activity

From our computer analysis, the amino acid region from Leu^{169} to Lys^{195} in CheA is highly homologous to the K1 site of the chemoreceptor, Tar. CheA contains an identical glutamic acid (Glu^{182}) that is methylated in Tar and this residue appears to be methylated in CheA. CheB methylesterase catalyzes the removal of the methyl groups from methylated Tar (Springer *et al.*, 1979) and seems to be the most likely candidate to demethylate the methylated CheA. Our results showed that about 10% of the [^{3}H] labeled methyl group of methylated CheA was not stable in the present of glacial acetic acid and was lost from the [^{3}H] labeled methylated CheA in the first 15 min incubation (Table 2). The remaining 90% of [^{3}H]-labeled CheA methyl groups were stable under the same condition. CheB had esterase activity toward methylated Tar, but had little or no activity towards methylated CheA (Table 2). This finding indicates that the methylation site is not exactly the same between the K1 peptide and this homologous site of CheA.

Protease digestion to confirm the location of CheA methylation site

In order to confirm that the methylated site in CheA is located at Glu^{182} as predicted from our homology studies, protease digestion of [^{3}H] labeled methylated CheA was performed.

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Table 2. Demethylation of CheA by CheB:

In a total volume of 20 μl, different amounts of purified CheB, as shown in the table, were incubated with 16630 cpm of [^{3}H] labeled methylated CheA at 30 ^{0}C. After the indicated times, 20 μl of glacial acetic acid were added to quench the reaction. The esterase activity were then measured as described in " Methods".

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CheB	0 min	15 min	30 min	overnight
4.0 μg	0 cpm	1326 cpm	1328 cpm	1363 cpm
25 μg	0 cpm	1327 cpm	1251 cpm	1459 cpm
0 μl	0 cpm	1548 cpm	1452 cpm	1184 cpm

** 10 ug of CheB can remove up to 22310 cpm of methyl group from Tar after 10 min incubation time.

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Clostripain (endoprotease Arg-C), which cleaves at Arg-X bonds, was used to cleave the $[^3H]$-labeled methylated CheA. Based on the amino acid sequence of CheA, there are thirty five Arg-C cutting sites (Table 3). Therefore, if the CheA methylation site really falls in that highly homologues region (Leu^{169} to Lys^{195}), a complete digestion of $[^3H]$-labeled methylated CheA with Arg-C should produce a radiolabeled 10 kDa fragment from Leu^{172} to Arg^{269} (Table 3). The cleaved products of such a digestion were analyzed by SDS-PAGE and fluorography, and as expected, a radio-labeled fragment with a molecular weight of approximately of 10 kDa was observed (Figure 23). This confirms that the methylated site of CheA is located in the region Leu^{172} to Arg^{269}.

Staphylococcal protease V8, which cuts at the carboxyl end of a glutamic acid (except Glu-Pro), was used to digest $[^3H]$ labeled methylated CheA. There are three glutamic acid residues, Glu^{181}, Glu^{182}, and Glu^{183} which are located in the region that is suspected to be methylated by CheR. Complete digestion of $[^3H]$ labeled methylated CheA by the V8 protease should release Glu^{182} and Glu^{183} as free amino acids. The digest mixture was subjected to thin layer chromatography and the results are shown in Figure 24. More than 73% of the label was recovered, and 45% of the recovered label migrated with an equivalent mobility to methylated L-Glu (Sigma, St. Louis, MO). Recovery of label near the origin probably indicates incomplete digestion of CheA. Based on these data, it appears that the CheA methylation site is located between Lys^{174} to Lys^{195} as we predicted (Figure 23) and at least one of either Glu^{182} or Glu^{183} could be methylated.

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Table 3. Predicted fragments to be produced by clostripain cleavage of CheA

Clostripain (endoprotease Arg-C) cleaves at Arg-X bonds. Based on the amino acid sequence of CheA, clostripain have thirty five cutting positions in CheA protein. A complete digestion of CheA with clostripain will produce thirty six fragments. The fragment from Leu^{172} to Arg^{269} , which contains the fragment from Leu^{169} to Lys^{195} is about 10 kDa.

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Position in sequence		Length	Weight (Da)
Leu- 172	to Arg- 269	98 aa	10096
Gly- 79	to Arg- 124	46 aa	5326
Val- 1	to Arg- 45	45 aa	5220
Ile- 447	to Arg- 448	42 aa	4433
Gly- 573	to Arg- 607	35 aa	3803
Gln- 125	to Arg- 160	36 aa	3698
Leu- 410	to Arg- 444	35 aa	3578
Ala- 46	to Arg- 77	32 aa	3430
Asp-359	to Arg- 386	28 aa	3000
Lys- 633	to Arg- 662	30 aa	2935
Val- 283	to Arg- 308	26 aa	2867
Tyr- 609	to Arg- 632	24 aa	2800
Asn- 498	to Arg- 519	22 aa	2371
Ser- 309	to Arg- 329	21 aa	2325
Val- 536	to Arg- 555	20 aa	2184
Ile- 520	to Arg- 535	16 aa	1725
Asn- 397	to Arg- 409	13 aa	1526
Glu- 556	to Arg- 567	12 aa	1322
Met- 343	to Arg- 352	10 aa	1291
Asp- 333	to Arg- 342	10 aa	1177
Ile- 387	to Arg- 396	10 aa	1176
Gly- 489	to Arg- 497	9 aa	960
Asp- 161	to Arg- 166	6 aa	747
Glu- 277	to Arg- 282	6 aa	692
Val- 568	to Arg- 572	5 aa	615

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-Table 3 continued-

Glu- 270	to Arg- 274	5 aa	600
Ile- 167	to Arg- 171	5 aa	587
Met- 666	to Ala- 671	6 aa	577
Glu- 663	to Arg- 665	3 aa	431
Ple- 353	to Arg- 355	3 aa	418
Leu- 356	to Arg- 358	3 aa	386
Asn- 330	to Arg- 332	3 aa	359
Glu- 445	to Arg- 446	2 aa	303
Glu- 275	to Arg- 276	2 aa	303

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Figure 23. SDS-PAGE and fluorography of [^{3}H]- labeled methylated CheA digested with clostripain: 100 μg of [^{3}H] labeled methylated CheA was digested with 10 μg of endoprotease Arg-C at room temperature for 48 hours in 100 μl of 0.1 M ammonium bicarbonate (pH 8.5) 2.5 mM DTT. The cleaved products were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described in "Methods".
Lane 1 & 2, standard proteins of molecular masses 30, 21.5, 12.5, 6.5 kDa respectively; Lane 3 & 4, methylated CheA digested with clostripain.

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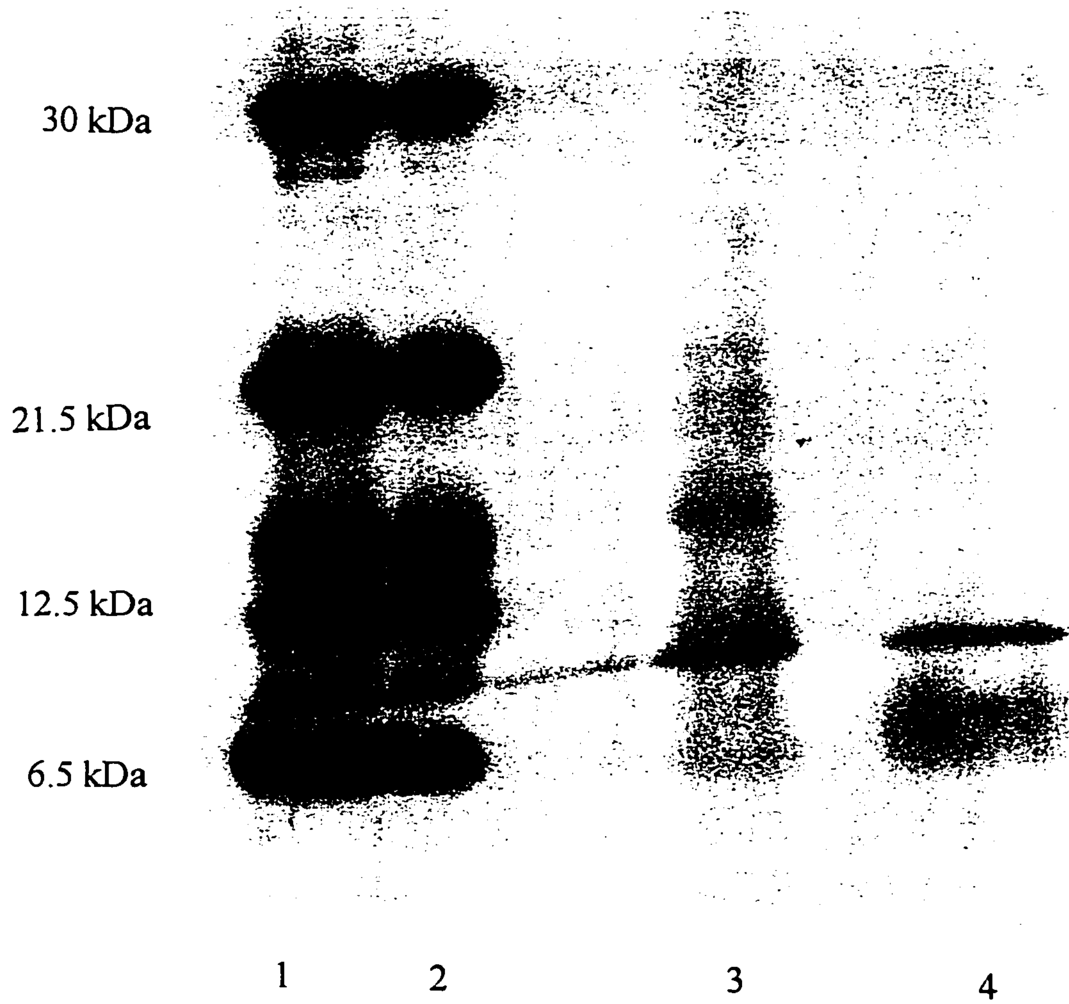

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Figure 24. Radioactivity migration pattern of V8 protease digest of [^{3}H]- labeled methylated CheA on TLC: Thin layer chromatographic analysis of the [^{3}H]-labeled methylated CheA after V8 protease digestion. Ninety μg of [^{3}H]-labeled methylated protein was digested with 10 μg of V8 protease at 37^{0}C for 12 hours as described in "Methods". The digestion products were subjected to thin layer chromatography using butanol : acetic acid : water (4 : 1 : 1) as solvent and methylated L-Glu (Sigma, St. Louis, MO) was used as a standard. The TLC plate was dried, sprayed with ninhydrin, and heated. Blue or purple spots, each indicating the presence of an amino acid, would be visualized. Each 1 cm^2 aliquot from the lane of V8 protease digest of [^{3}H]- labeled methylated CheA was removed and added to 2.5 ml of Ecoscint scintillation fluid. The amount of [^{3}H] in each vial was counted in a Packard Tri-carb 1500 liquid scintillation analyzer. The distance on the x axis indicates the migration of the digest from the point of application. The peak "a" migrated at the same position as methylated L-Glu.

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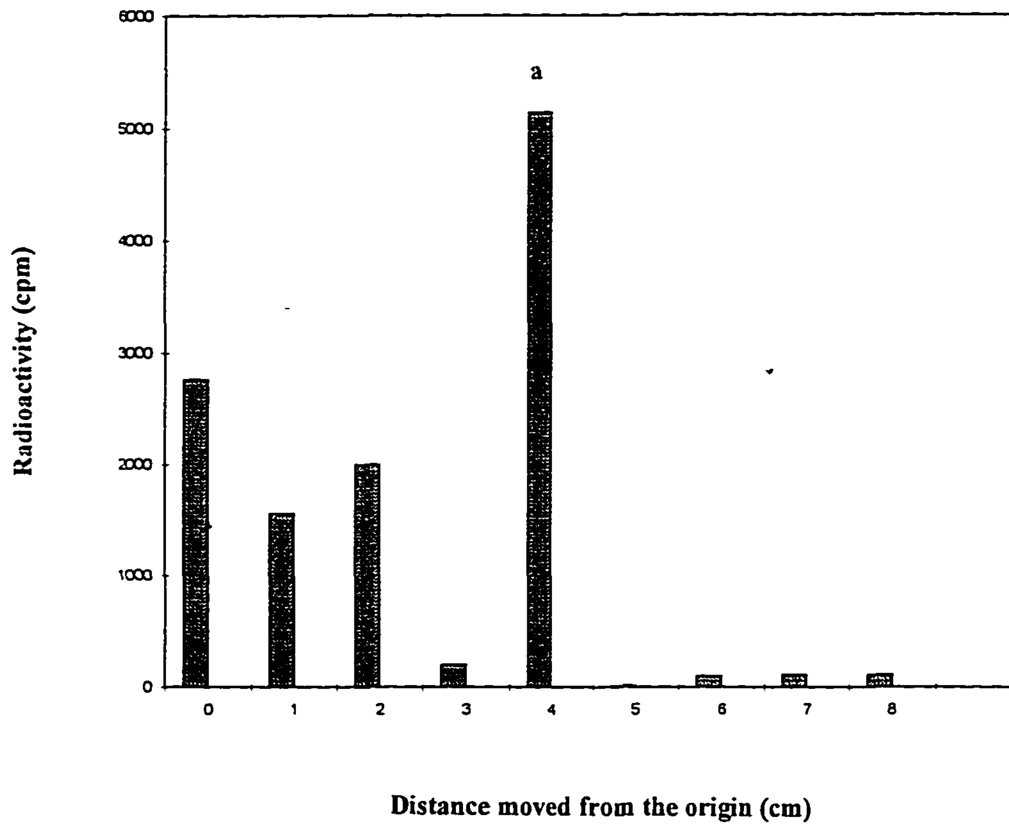

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Oligonucleotide-directed mutagenesis of the *cheA* gene

Inspection of the sequences of the K1 or R1 peptides (Figure 10), indicates that the conserved methylation site is always the second of two adjacent glutamates (Boy *et al.*, 1983). Therefore, I predicted the amino acid, Glu^{182} of CheA (Leu^{180} Glu^{181} Glu^{182} Glu^{183}) is the methylation site and this was corroborated by the protease digestion studies. To further confirm this prediction, oligonucleotide-directed site mutagenesis was performed.

The most commonly used methods for oligonucleotide-directed mutagenesis employ either single-stranded M13 or double-stranded plasmids. Because of the unavailability of appropriate restriction sites for subcloning the CheA gene from pMO4 into the M13 vector, the double-stranded plasmid method was used in our mutagenesis study (Vlasuk *et al.*, 1983). However, this method normally gives a low yield in the number of mutants. Fortunately, several modifications have been made recently which improve the efficiency of the mutagenesis. A modification of that reported by Markvardsen *et al.*, 1995 was performed in my study as described in "Methods" and the strategy was illustrated in Figure 13.

Plasmid pMO4 was used as the source of the CheA gene. I planned to create two mutant strains, one strain with the change of Glu^{182} to Asp^{182}, the other Glu^{182} to Ala^{182} (Figure 14). Two different oligonucleotides, oligo-56 and oligo-57 (Figure 14), were synthesized and were used as mutagenic primers in the oligonucleotide side-direct mutagenesis experiments. However, some problems were encountered. DNA sequencing

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shows that mutant strains, $CheA_{Asp}$ & $CheA_{Lys}$, were obtained (Figure 25). In the $CheA_{Asp}$ strain, the Glu^{182} was successfully replaced with Asp^{182}, whereas in the $CheA_{Lys}$ strain, the primer changed Glu^{183} Leu^{184} to Lys^{184} Met^{184} instead of Glu^{182} to Ala^{182} (Figure 25).

Purification and characterization of mutant CheA proteins

CheA derived from CheA mutant strains, $CheA_{Asp}$ and $CheA_{Lys}$, were purified as described in "Methods". These proteins were shown to migrate similarly as wild type CheA protein on SDS-polyacryamide gel (Figure 26), and, as expected, contained the autophosphokinase and phosphate transfer activities (Data not shown). Moreover, these mutant proteins cross-reacted with rabbit anti-wild type CheA antibodies and formed immune precipitates in an agar matrix (Figure 27). These results indicated that the replacement of Glu^{182} to Asp^{182} (mutant $CheA_{Asp}$) and Glu^{183} Lys^{184} to Lys^{183} Met^{184} (mutant $CheA_{Lys}$) did not affect protein size or function.

Comparison of methylation of wild type and mutant CheA proteins

The rate of methylation of the two mutant proteins, $CheA_{Asp}$ & $CheA_{Lys}$, were compared to the wild type CheA protein. As shown in Figure 28, the methylation rate of the $CheA_{Asp}$ protein was about 50% of the wild type CheA protein. The methylation rate of $CheA_{Lys}$ protein decreased to 20% of the wild type CheA protein (Figure 29). Thus, unexpectedly both mutant proteins retained some capacity to be methylated and this

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Figure 25. Protein and DNA Sequences:

(A) shows the CheA protein sequence from Asp^{178} to Lys^{187}. (B) is the DNA sequence of both strains of that region. (C) By using the oligo-56, mutant strain $CheA_{Asp}$ was obtained in which Glu^{182} was replaced with Asp^{182}. (D) By using the oligo-57, mutant strain $CheA_{Lys}$ was obtained in which Glu^{183} Leu^{184} was replaced with Lys^{183} Met^{184}.

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(A) Protein sequence --------Asp Leu Leu Glu Glu^{182} Glu^{183} Leu Gly Asp Leu---
(wild type CheA)

(B) DNA sequence 5' ---^{533}AT CTG CTT GAA GAA GAG TTG GGC AAT CT---3'
(wild type CheA) 3' ---- TA GAC GAA CTT CTT CTC AAC CCG TTA GA---5'

(C) DNA sequence 3' ---- TA GAC GAA CTT CTG CTC AAC CCG TTA G--5'
($CheA_{Asp}$) ^{182}Asp

(D) DNA sequence 3' ---- TA GAC GAA CTT CTT TTT TAC CCG TTA G--5'
($CheA_{Lys}$) ^{183}Lys ^{184}Met

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Figure 26. Fluorography of [^{32}P]-labeled CheA proteins from wild type, CheA$_{Asp}$ and CheA$_{Lys}$ strains:

CheA derived from each strain were purified as described in "Methods". These proteins were then subject to autophosphorylation assays and SDS-polyacrylamide gel electrophoresis as described in "Methods". Lane 1, shows the standard marker proteins, 94 and 67 kDa respectively; Lane 2, shows phosphorylated wild type CheA; Land 3, shows phosphorylated CheA$_{Asp}$; Lane 4, shows phosphorylated CheA$_{Lys}$.

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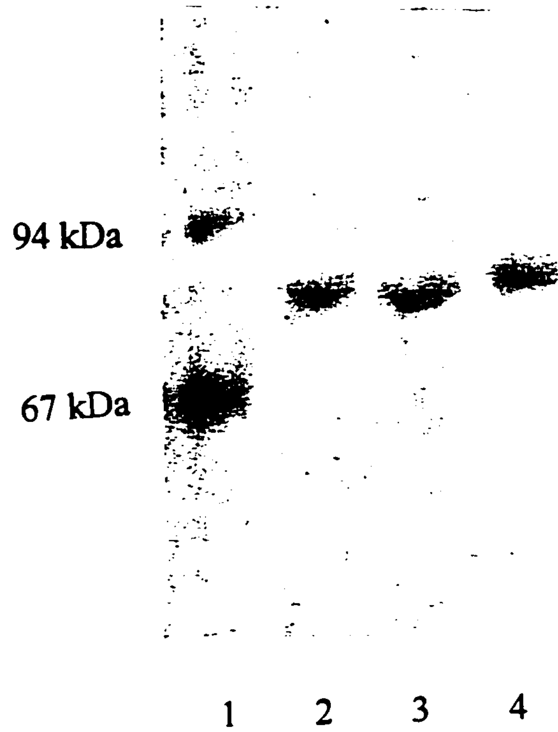

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Figure 27. Immunoprecipitation reactions of wild type CheA, $CheA_{Asp}$ and $CheA_{Lys}$ with anti-wild type CheA antibody:

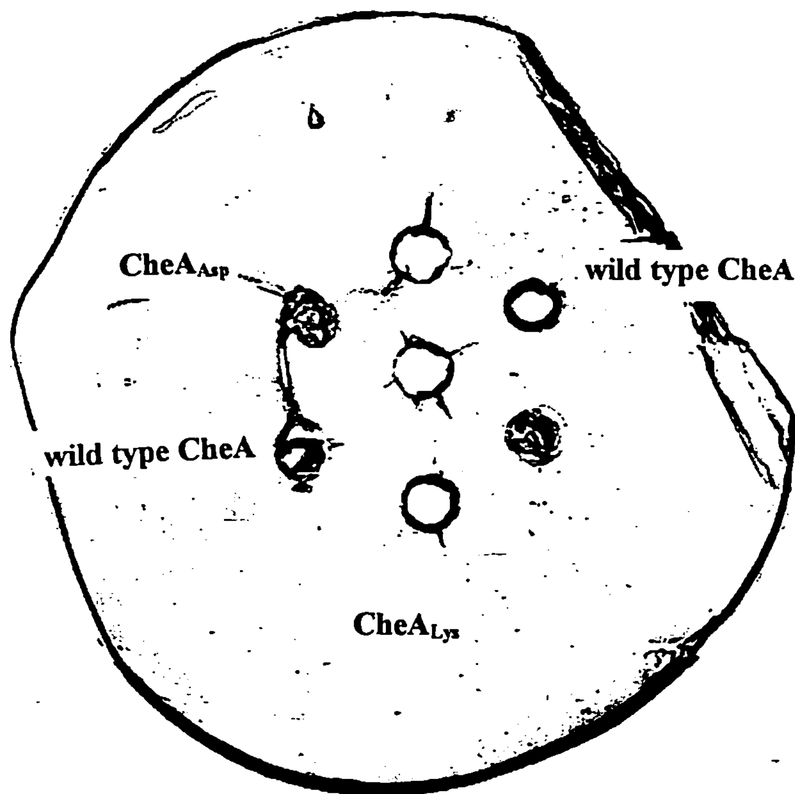

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Figure 28. Comparison of methylation rate between wild type CheA and $CheA_{Asp}$

Purified CheA (■), 2.5 μg or $CheA_{Asp}$ (♦), 2.5 μg, was incubated with purified CheR, 10 μg and 10 μl of [H^3] AdoMet (85 mCi/ mmole) in Tris-HCl, pH 7.9 at a total volume of 100 μl for a period of 2 hr at 30 ^{0}C. At the indicated times, 15 μl of the reaction mixture was removed and the extent of methylation was measured as descried in "Methods".

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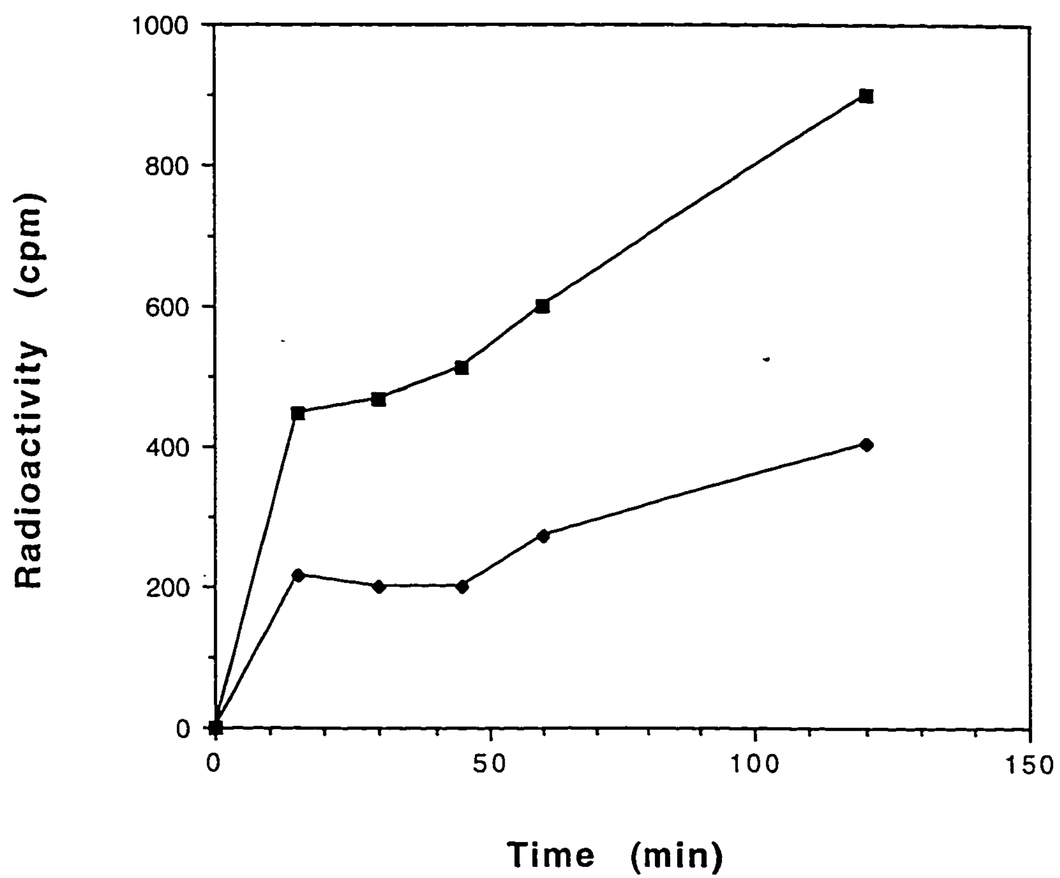

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Figure 29. Comparison of methylation rate between wild type CheA and $CheA_{Lys}$

Purified CheA (■), 20 μg or $CheA_{Lys}$ (♦), 20 μg, was incubated with purified CheR, 10 μg and 10 μl of [H^3] AdoMet (85 mCi/ mmole) in Tris-HCl, pH 7.9 at a total volume of 100 μl for a period of 2 hr at 30 ^{0}C. At the indicated times, 15 μl of the reaction mixture was removed and the extent of methylation was measured as descried in "Methods".

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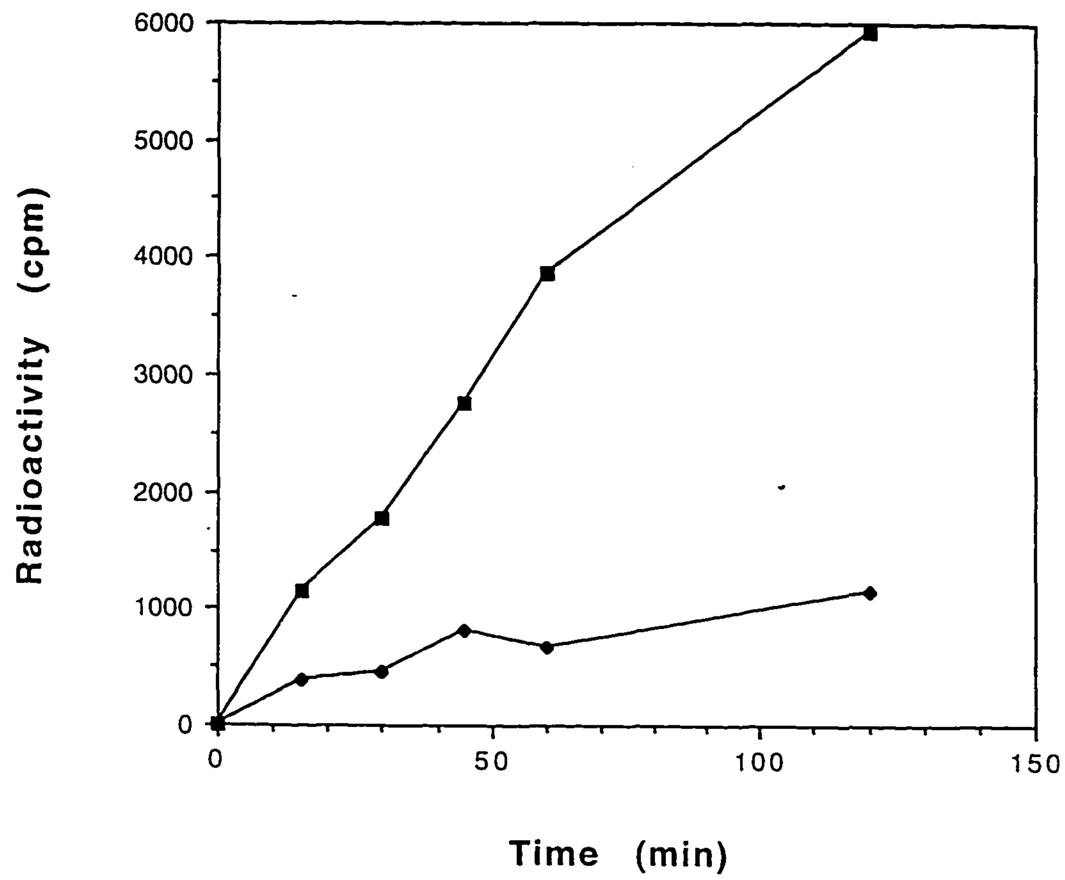

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might be because Asp also has the capacity of accepting a methyl group (Clarke, 1985) or might be because Glu^{182} is not the only methylation site. When Glu^{183} is changed to Lys^{183}, the amino acid residue is changed from positive to negative charge, the methylation rate decreased to 20% of the wild type CheA protein (Figure 29). The decrease in methylation rate might be because that the modified site is very close to the methylation site. This amino acid modification from positive to negative charge may interfere the protein-protein interaction or it may cause a conformational change in the CheA protein. It is also possible that Glu^{183} itself is the methylation site (or one of the methylation sites). Therefore, removal of this Glu^{183} could cause a decreased rate in methylation (Figure 29). Taken together, these results indicate that both Glu^{182} and Glu^{183} in CheA protein are involved in CheA methylation.

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Conclusions

The experiments described above showed that CheA could be methylated by CheR methyltransferase *in vitro* (Figure 15). Furthermore, the methyl group in methylated CheA is stable, since the $[^3H]$-labeled methylated CheA could be visualized when subjected to SDS-polyacrylamide gel and autoradiography (Figure 16). Thereafter, the nature of this reaction was further explored (Figures 17A, 17B, 17C, 18, 19, 20A, 20B and 20C) and shown to be enzymatic in nature. However, CheB does not have the methylesterase activity toward methylated CheA (Table 2). This finding might indicate that the methylation sites are not exactly the same between the K1 peptide and this homologous site of CheA.

The results of protease digestion of $[^3H]$- labeled methylated CheA by clostripain and V8 protease provide strong evidence that the CheA methylation site is located between Lys^{174} to Lys^{195} (Figure 23), and also at least, one of either Glu^{182} or Glu^{183} could be methylated (Figure 24). From the results of site-directed mutagenesis (Figure 13) both Glu^{182} and Glu^{183} (Figures 28 & 29) in CheA protein are involved in CheA methylation.

From the analysis of the methylation rate of CheA and Tar (Figure 22), it seems that the methylation site(s) in CheA may be embedded as is the case for some of the sites in Tar (Terwilliger *et al.*, 1986a,b). The embedded methylation site(s) in CheA could become more accessible to CheR. For example, after the phosphorylation, there might be a conformational change in CheA and this change might affect the rate of CheA methylation (Figure 21 & 22).

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CheA is the central regulator in the bacterial chemotaxis signal transduction system, and this is the first time CheA has been shown to be methylated *in vitro* by CheR. An important question might be asked as to whether this methylation reaction occurs *in vivo*? If so, why are there no reports to date about this, and is there any significance to this CheA methylation?

The *in vitro* CheA methylation could be increased dramatically in low ionic strength reaction medium, however, it is still a slow reaction (Figures 20 A & B). I also found that the methylation rate of CheA could be accelerated under some circumstances, such as when cheA is phosphorylation (Figure 21 & 22). However, the relative methylation rates for both phosphorylated and unphosphorylated CheA are small when compared with that of Tar (Figure 22). This indicates that CheA is not as good a substrate as Tar for CheR. Although, CheA methylation increases after autophosphorylation, the half-life of phosphorylated CheA *in vivo* is very short. Once CheA gets phosphorylated, it quickly transfers the high energy phosphate to CheY or CheB (Wylie *et al.*, 1988). It is also possible that the CheA methylation occurs only under certain stringent condition for the cell to survive. These might explain why there has been no report of CheA methylation *in vivo* to date.

Phosphorylation and methylation post-transcriptional modifications of cellular proteins in prokaryotic and eukaryotic systems are important for cellular coordination and control (Alex *et al.*, 1994; Wold, 1981). Although there are differences in the details of the underlying biochemistry, the basic principles of signal transduction may be similar in eukaryotes and prokaryotes. My research is the first to demonstrate that CheA kinase can

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be methylated and that the methylation rate is changed by phosphorylation. This is a very interesting finding, although the role of CheA methylation in living cells is still not clear. Some other experiments, especially those involved with recombination of mutant cheA *in vivo*, could be conduct to further elucidate the biophysical significance of the CheA methylation reaction. I believe that there is a certain interaction between the phosphorylation and methylation regulatory pathways. I feel that the *in vitro* CheA methylation by CheR might be an important observation. The results of my studies in this project might give evidence for this kind of interaction. This research might also have significance for understanding behavioral responses in higher organisms. It is hoped that my finding will lead more scientists to work in this direction.

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