INTRADIMER AND INTERDIMER METHYLATION RESPONSE BY BACTERIAL CHEMORECEPTORS TO ATTRACTANT STIMULUS

A Dissertation

by

ARJAN FRANK BORMANS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2005

Major Subject: Microbiology

INTRADIMER AND INTERDIMER METHYLATION RESPONSE BY BACTERIAL CHEMORECEPTORS TO ATTRACTANT STIMULUS

A Dissertation

by

ARJAN FRANK BORMANS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Michael D. Manson
Committee Members,	Deborah Bell-Pedersen
	Andreas Holzenberg
	Deborah A. Siegele
	Ryland F. Young
Head of Department,	Vincent M. Cassone

December 2005

Major Subject: Microbiology

ABSTRACT

Intradimer and Interdimer Methylation Response by Bacterial Chemoreceptors to Attractant Stimulus. (December 2005)

Arjan Frank Bormans, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Michael D. Manson

This study focuses on the mechanism of transmembrane signaling by Tar, the aspartate chemoreceptor of *Escherichia coli*. Like other bacterial chemoreceptors, Tar localizes to the cell membrane and relays information about the external chemical environment through the membrane to a cytoplasmic signaling domain. The output of the signaling domain controls the directional bias of the rotary flagellar motors of the cell. Net movement of a cell in a chemical gradient involves temporal comparison of the current concentration with the concentration in the recent (a few seconds) past. The current concentration is measured as the percent occupancy of the extracellular ligand-binding domain of the receptor, and the past is represented by the extent of covalent methylation of four conserved glutamyl residues in the cytoplasmic domain. Under steady-state conditions, the methylation level corresponds to ligand occupancy. Tar is a dimer, and much evidence suggests that dimers associate into trimers of dimers. Higher-order arrays of receptors form in the presence of the cytoplasmic proteins CheA and CheW. The conformational change generated by ligand binding is transmitted through the membrane by one subunit of a dimer. To examine whether this initially asymmetric signal becomes symmetric within the cytoplasmic domain, I examined aspartate-induced

adaptive methylation of the two subunits of mutant Tar receptor heterodimers. In the presence of CheA and CheW, adaptive methylation after addition of aspartate was symmetric, but in their absence, although the level of methylation increased, the rates were different for the two subunits. I also found that cross-talk, at the level of adaptive methylation, occurs between different receptor types even in the absence of CheA and CheW. These results provide support for the idea that a tight association of receptor dimers within trimers of dimers allows for an actively signaling receptor to affect the methylation state, and thus presumably the signaling state, of receptors within a trimer that are not bound to an attractant ligand.

ACKNOWLEDGEMENTS

I would like to thank the following people: Michael Manson, my friend and mentor, and his much better half Lily Bartoszek, for their time and great conversation throughout the years; my committee, for their guidance and advice (A special thanks to Deborah Bell-Pedersen and Deborah Siegele for their frank discussions and extra attention to detail); the “Mike Manson Friends”, whose camaraderie, knowledge, and patience are almost without limit; my parents, whose support was without end but whose comments as to the completion of this degree increased with time; and my wife Connie and daughter Hunter, the only reasons I do not want to be in the lab 24 hours a day.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

E. coli is a Gram-negative, rod-shaped, enteric bacterium. A typical cell is 2-4 μm long and less than 1 μm wide. Locomotion is powered by the rotation of 4-7 left-handed helical filaments, each of which is driven by a reversible, proton-powered motor at its base. Counterclockwise (CCW) rotation leads to formation of a helical bundle of filaments that propels the cell at up to 40 μm/sec (77) in a gently curved path termed a run (13). The flagellar bundle can form at either end of the cell. Thus, a swimming bacterium has no permanent "head" or "tail" (15). Clockwise (CW) rotation of the motor dissociates the filament bundle and causes a rapid, active reorientation of the cell, known as a tumble. These two modes of motility are shown in Figure 1A.

In a uniform environment, *E. coli* alternates between runs of a few seconds and tumbles of a fraction of a second (14). The result is a random walk in three dimensions (Fig. 1B). In a gradient of a chemical sensed as an attractant, the cell biases the random walk to migrate up the gradient. In a gradient of a chemical sensed as a repellent, the cell biases the random walk to migrate down the gradient. In either case, the frequency of tumbling is suppressed when the cell moves in the "favorable" direction (Fig. 1B) because the switch to CW flagellar rotation is inhibited. Since the new direction taken by a cell following a tumble is still random, the pattern of movement in a spatial gradient of an attractant or a repellent is called a "biased random walk."

A cell monitors its chemical environment by continuously sampling and relaying information about the external conditions into the cell. In many cases, the monitoring is

---

This dissertation follows the style of the Journal of Bacteriology.

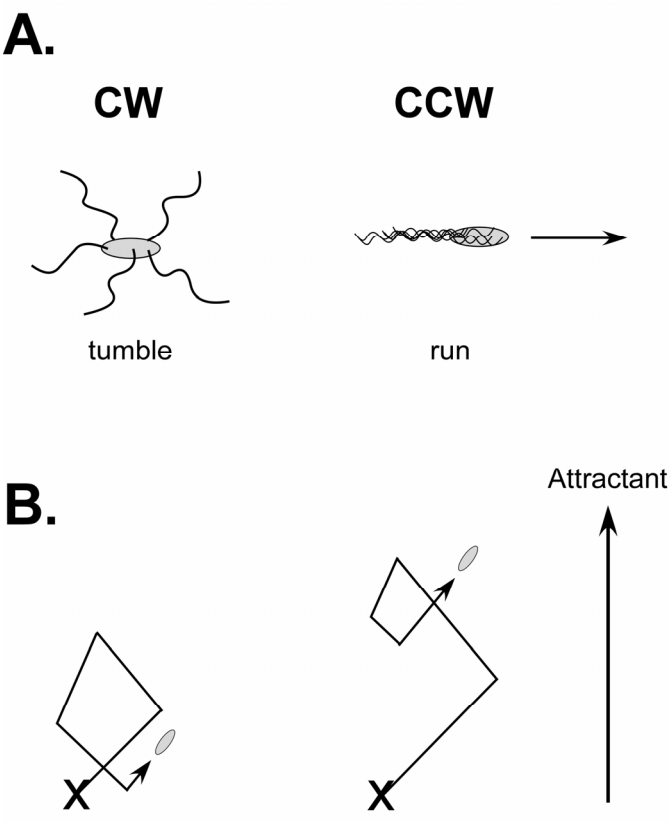

Figure 1. Run and tumble motion of *E. coli* in the absence or presence of a chemical gradient.

A) In *E. coli*, clockwise (CW) flagellar rotation causes a cell to tumble, whereas counter-clockwise (CCW) flagellar rotation leads to a smooth swim (run). B) A cell in an isotropic chemical environment swims in a series of runs (straight arrows) and tumbles (inflection points) that describes a three-dimensional random walk. Suppression of CW rotation when traveling up an attractant gradient, or down a repellent gradient, results in a biasing of the random walk that allows net movement towards a higher concentration of an attractant or a lower concentration of a repellent.

the job of receptors that span the cell membrane. In *E. coli*, these receptors typically consist of a periplasmic input domain that is connected to a cytoplasmic signaling domain via a transmembrane region. Many of these proteins, including the receptors involved in chemotaxis, belong to two-component regulatory systems. Whereas the output of chemoreceptors is cell motility, most such proteins are sensor kinases that control gene expression by changing the phosphorylation levels, and hence activity, of cytoplasmic response regulators. A few examples of two-component sensor kinases are: PhoP and PhoQ, whose activity is modulated by divalent cations, among other things; NarQ and NarX, which monitor the presence of nitrate and nitrite in the environment; EnvZ, which is involved in adaptation to external osmolarity. Each of these kinases has one or more cognate response regulators.

Sensor kinases have an intrinsic autophosphorylation activity directed at a highly conserved histidyl residue that serves as a reaction intermediate in phosphorylation of the cognate response regulator(s). Chemoreceptors themselves are not enzymes but rather function as regulatory subunits of a soluble kinase known as CheA, which is coupled to the receptor via another soluble protein, CheW.

Chemoreceptors and their ligands

There are four known transmembrane chemoreceptors in *E.coli*; Tsr, Tar, Trg, and Tap (Table 1). They detect a variety of stimuli, including changes in pH (126), temperature (75), and the levels of nutrients, including amino acids (78), sugars (3) and di- and tripeptides (76). Tsr and Tar are high abundance receptors, with copy numbers of around 3,000 and 1,500 per cell, respectively (28, 58). Either Tsr or Tar can support

Table 1: The four MCPs of *E.coli* and their ligands

Receptor	Attractants	Repellents
High abundance		
Tsr	Serine	Leucine
Tar	Aspartate, Maltose	Ni, Co
Low abundance		
Trg	Ribose, Galactose	
Tap	Dipeptides	

run-tumble motility and chemotaxis to its respective ligands when it is present as the sole chemoreceptor in a cell. Tsr mediates attractant chemotaxis toward serine through direct interaction with the ligand (78, 114). Tar mediates chemotaxis toward aspartate and maltose. Aspartate bind to Tar directly (78, 79, 81), whereas maltose first binds to the periplasmic maltose-binding protein (MBP; 22, 47), which then interacts with Tar.

Trg and Tap are low abundance chemoreceptors, and their copy number per cell is one-tenth or less that of Tsr (50, 110). Trg and Tap do not support run-tumble motility or chemotaxis by themselves, even when they are expressed at the same level as Tsr (35). Trg mediates chemotaxis toward ribose, galactose, and glucose via interaction with the ribose-binding protein (RBP) and galactose/glucose-binding protein (GGBP), respectively (4, 48). Tap mediates chemotaxis toward di- and tripeptides via interaction with the dipeptide-binding protein (DBP; 1, 76, 91).

The chemoreceptor homolog Aer, which contains tightly bound flavin adenine dinucleotide (FAD; 16), mediates chemotaxis to oxygen by sensing the redox potential of the cell (16, 122). Unlike the four canonical chemoreceptors, Aer has no functional sites

of covalent methylation (17) and it is therefore not considered a methyl-accepting chemotaxis protein (MCP), as the others are.

The components of the chemotaxis signal transduction pathway

The signal transduction pathway of bacterial chemotaxis involves the chemoreceptors and six cytoplasmic chemotaxis proteins: CheA, CheW, CheY, CheZ, CheB, and CheR (Figure 2). CheA is the histidine protein kinase (43) that modulates the activity of the response regulators CheY and CheB. CheA and the coupling factor CheW form a ternary complex with the receptors. The autophosphorylation activity of CheA increases 50 to 100 fold in such complexes formed with Tsr or Tar, respectively (19). Phosphorylated CheY readily transfers its phosphoryl group to the small cytoplasmic protein CheY, and phospho-CheY binds to the FliM component of the switch component of the flagellar motor to increase the probability of CW rotation (130). An increase in CheA activity, therefore, heightens the probability of CW rotation, whereas a decrease in CheA activity lowers the probability of CW rotation.

When a chemoreceptor binds an attractant ligand, it inhibits CheA activity below the basal level (86). The half-life of phospho-CheY is only about 10 seconds (117), and this rate is greatly accelerated in the presence of CheZ, which is a phospho-CheY phosphatase (51). Thus, rapid decreases in phospho-CheY levels can be achieved after cells are exposed to attractants.

An important aspect of chemotaxis is that cells respond to temporal changes in attractant or repellent concentrations, not to their ambient levels (14, 73). This capability is conferred by the adaptation system, which consists of the constitutive CheR

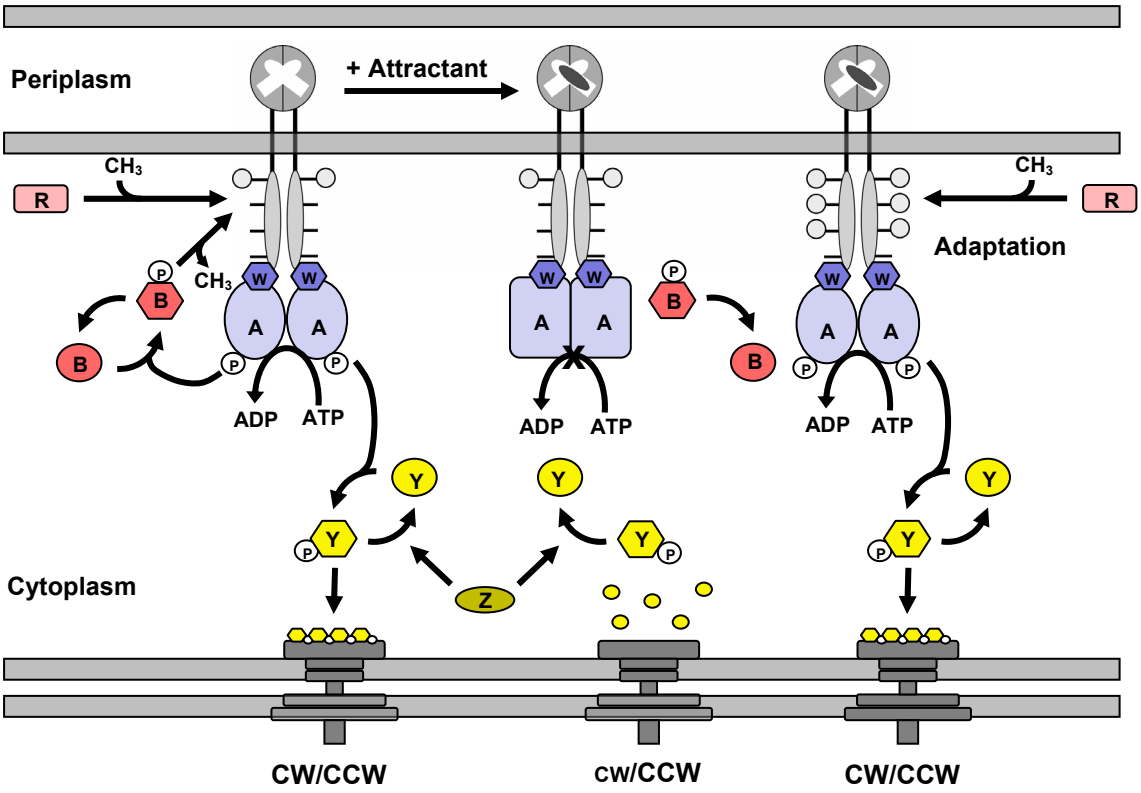

Figure 2. Chemotaxis circuit of *E. coli*.

Chemotactic circuit of *E. coli*. The baseline activity of the receptors in stimulating CheA activity produces an amount of phospho-CheY that results in alternating CW and CCW rotation of the flagellar motors. Attractants bind to their cognate receptors and inhibit their stimulation of CheA. The result is a decrease in the amount of phospho-CheY that is made, and phospho-CheY that is already present is rapidly dephosphorylated by CheZ. The reduction in intracellular phospho-CheY lowers the frequency of CCW to CW switching of the flagella and increases the mean duration of runs. The system is reset by increased methylation of the receptors due to a transient decrease in phospho-CheB, the active form of the methylesterase, and conversion of an attractant bound receptor into a better substrate for methylation by the constitutively active CheR methyltransferase.

methyltransferase (115) and the regulated CheB methylesterase (118). CheR, utilizing S-adenosylmethionine as a methyl group donor (115), can methylate four or five conserved glutamyl residues in the cytoplasmic portion of the chemoreceptors (33, 49, 123). Phospho-CheB, generated by phosphoryl-group transfer from CheA, removes these methyl groups. The interplay of the activities of CheR and CheB is responsible for adaptation during chemotaxis.

The molecular mechanism of adaptation

Cells detect spatial gradients of attractants and repellents by making temporal comparisons of concentration along their swimming paths. This ability requires both an essentially instantaneous measurement of concentration in the vicinity of the cell and a transient memory of concentration in the very recent past. The memory consists of the level of covalent methylation of the conserved glutamyl residues that are methylated by CheR and demethylated by CheB. The resetting of methylation levels in response to ligand occupancy is called adaptation.

An increase in methylation counteracts the signal inhibitory signal generated by attractant binding to reset the receptor-coupled CheA kinase activity to pre-stimulus levels. Under constant conditions, therefore, the methylation level of a particular receptor type reflects its current ligand occupancy. Repellent ligands increase the ability of a receptor to stimulate CheA activity, and adaptation to repellents correspondingly involves lowering the level of methylation of the cognate receptor.

When Tar, Tsr and Trg (and presumably Tap) are translated and first assembled into the cell membrane, some of their methylation sites are occupied by glutaminyl residues

(54, 89, 124, 125). In the case of Tar, two of the four sites are occupied by glutaminyl residues to create the QEQE form of the receptor ($Tar_{\mathbf{QEQE}}$). This form of Tar is in a signaling state midway between fully methylated and fully demethylated. Phospho-CheB, in addition to being a methylesterase, is a deamidase that converts $Tar_{\mathbf{QEQE}}$ into $Tar_{\mathbf{EEEE}}$, which then enters the bulk pool of receptors that are undergoing methylation and demethylation.

An attractant ligand affects receptor methylation in two ways. First, it decreases the ability of its cognate receptor to stimulate the CheA kinase, which in turn reduces production of the tumble regulator phospho-CheY and the active form of the methylesterase, phospho-CheB. Since CheR constitutively methylates the receptors, the net effect of decreased methylesterase activity is to increase the level of methylation of all of the receptors, whether they are bound to the attractant or not (55). Second, the receptor, in the conformation it assumes upon ligand binding, becomes a better substrate for CheR (114). This latter effect maintains the level of methylation of the attractant-bound receptor higher then compared to the non-attractant bound receptor even after adaptation is complete and CheA has returned to its pre-stimulus activity.

Chemoreceptor structure

E. coli chemoreceptors exist as homodimers in the absence and presence of ligands (82) and are composed of four distinct domains (Fig. 3): 1) a periplasmic ligand-binding domain; 2) a transmembrane domain consisting of two membrane-spanning helices; 3) a HAMP linker domain; and 4) a cytoplasmic signaling and adaptation domain. The crystal structures of the periplasmic domain of Tar from *Salmonella enterica var Typhimurium*

(52, 79, 102, 134, 135) and *E.coli* (21, 27) reveal that each subunit of the dimer consists of a 4-helix bundle. The binding pocket for aspartate is at the dimer interface. Residues R64, R69', and R73' (where the prime denotes residues from the second subunit) form a positively charged binding pocket for L-aspartate. R64 interacts with the α-carboxyl group, and R69' and R73' interact with the γ-carboxyl group of the aspartyl side chain (132). Residues Y149, Q152, and T154 interact directly with the α-amino group of aspartate, and Y149 also interacts, via bound water molecules, with the α-carboxyl and γ-carboxyl groups (79; Fig. 4). *E. coli* Tar, unlike *Salmonella* Tar, also interacts with MBP in its closed, ligand-bound form (22, 30, 60). Missense mutations in *E. coli tar* that affect maltose sensing selectively alter residues in the loop regions between helices 1 and 2 and helices 3 and 4 (39) at the membrane-distal apex of the periplasmic domain. Residue substitutions in Tar and MBP that disrupt maltose chemotaxis were used to develop a computer-generated docking model for the interaction of the two proteins (136).

Transmembrane signaling

The conformational change induced by ligand binding must propagate across the membrane to affect the conformation of the cytoplasmic signaling domain. Chemical crosslinking studies show that introduction of a disulfide bond between the first transmembrane (TM1) helices of the Trg dimer has little effect on ribose taxis, whereas crosslinking TM1 with the second transmembrane region (TM2) of one subunit eliminated ribose taxis (66). The inference drawn was that ligand binding to a receptor involves a conformational change that requires movement of TM1 relative to TM2. The manner of aspartate binding to Tar suggests that displacement of helix 4 relative to

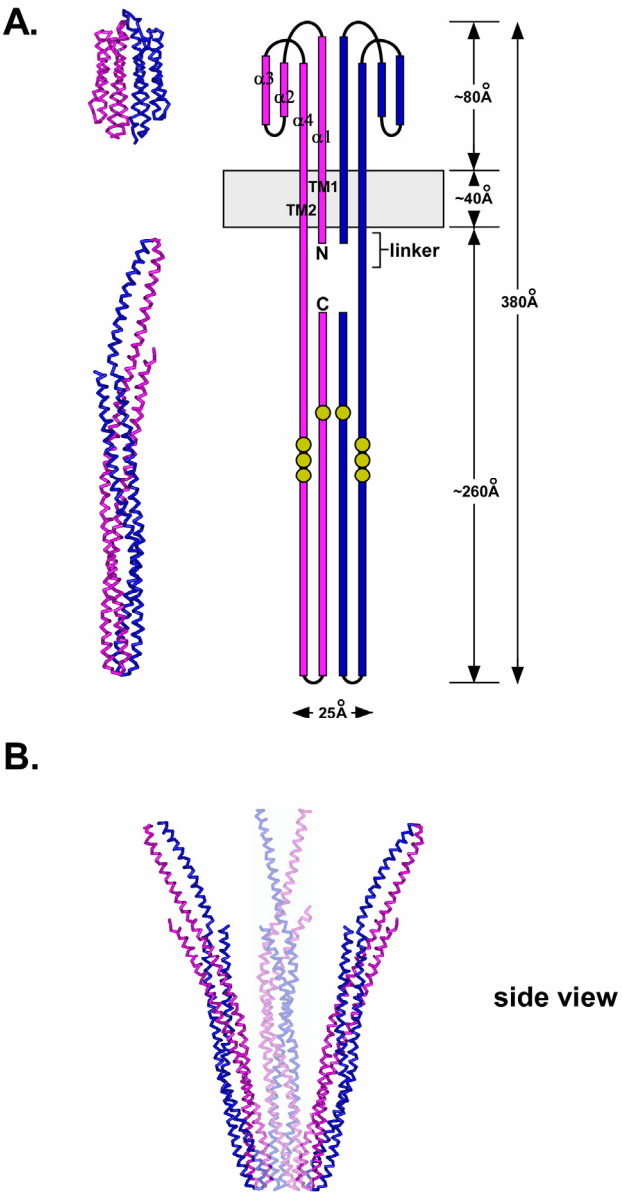

Figure 3. Chemoreceptor structure.

A) Model of a chemoreceptor dimer based on the crystal structure of the periplasmic domain of Tar and the cytoplasmic domain of Tsr. The structures of the transmembrane and HAMP linker domains have not been determined. B) Schematic of the crystal structure of the cytoplasmic domain of Tsr_{QQQQ}. Residues 286-526 are resolved. The crystal structure revealed that receptor dimers form a trimer of dimers, which is thought to be a form in which intact receptors associate in the cell.

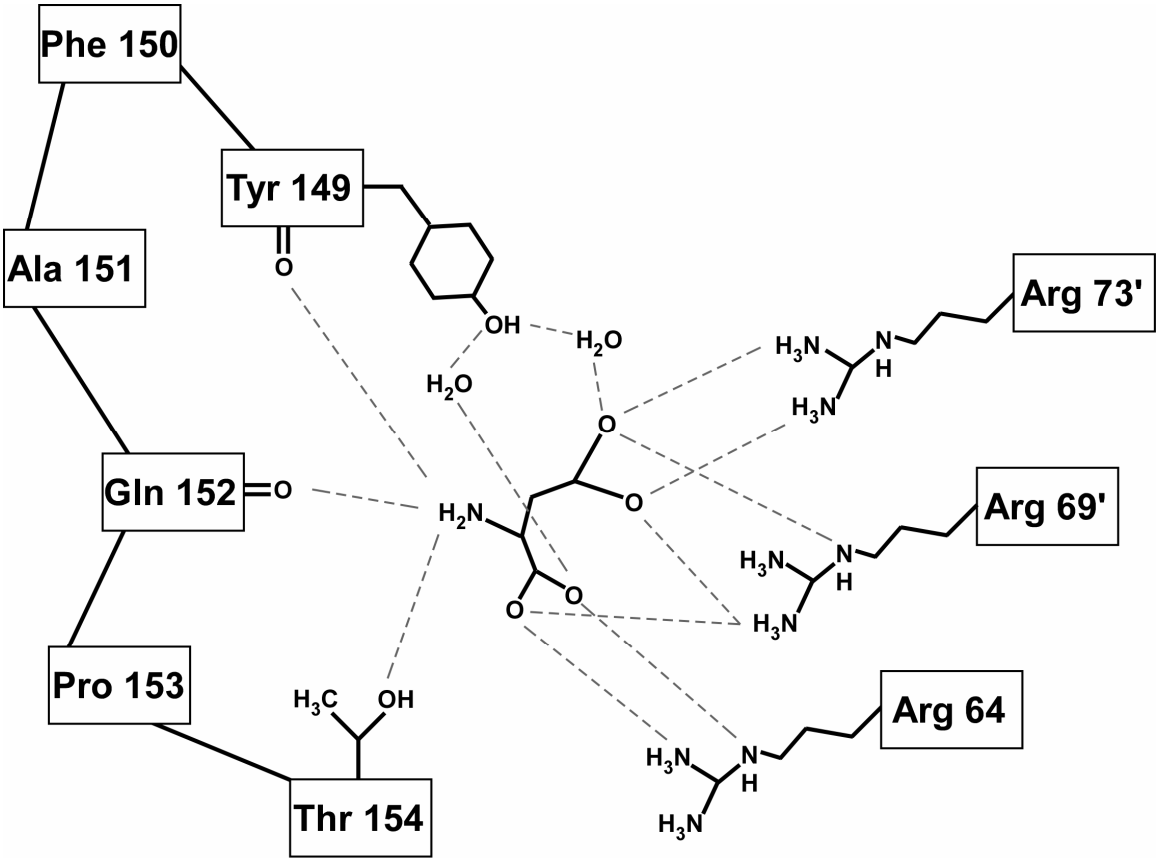

Figure 4. Aspartate binding pocket of *E. coli* Tar.

One of the two aspartate-binding pockets found at the dimer interface of the periplasmic domain of Tar. Residues Arg-64, Tyr-149, Gln-152, and Thr-154 of one subunit form the "major" binding half-site and hydrogen bond, directly or through a water molecule, with the α-amino and α-carboxyl groups of L-aspartate. Residues Arg-69' and Arg-73' of the opposing subunit form the "minor" binding half-site and interact with the γ-carboxyl group in the aspartate side chain. Arg-69' also forms a hydrogen bond with the α-carboxyl group, and Tyr-149 interacts via bound water with the γ-carboxyl group. Thus, these two residues contribute to both the "major" and "minor" binding half-sites.

helix 1 of the periplasmic domain extends to TM2 and beyond into the cytoplasmic domain. A currently favored model proposes that ligand binding facilitates a downward piston-like movement of helix 4/TM2 relative to helix 1/TM1 (26, 92, 93). In this view, TM1 contributes to signaling by serving as an anchor within the cell membrane.

Recent experiments have repositioned TM2 relative to the membrane by moving Trp residues that normally sit at the membrane/cytoplasm interfacial zone (31). They support the notion that a downward movement (*i.e.*, into the cytoplasm) biases the receptor toward an "off" state with respect to CheA stimulation, mimicking an attractant response. Conversely, upward movement of TM2 biases the receptor toward an "on" state, mimicking a repellent response.

The role of the HAMP linker domain in signaling

The linker is a region of about 45 residues between TM2 and the first methylation region and signaling domain. The linker belongs to a family of sequence motifs known as HAMP domain (10, 132), which typically contain two amphipathic helices termed ASI (amphipathic sequence I) and ASII (amphipathic sequence II). HAMP linkers are found in both chemoreceptors and homodimeric sensor kinases. Functional chimeric proteins can be made by joining different chemoreceptors and kinases in the HAMP linker region (35, 61, 128, 129). ASI is postulated to interact with the membrane and with ASII. Small deletions within ASI can lead to constitutive activity of a sensor kinase, whereas certain small deletions in ASII reverse the response such that activating ligands become inhibitory, and vice versa (9). In Tsr substitutions at position 235 in ASI result in a "lock-on" phenotype which fails to stimulate the CheA kinase (5). Cysteine-scanning

mutagenesis and subsequent crosslinking analysis with *Salmonella* Tar revealed some interaction between the helices of ASI with ASI' and a strong interaction between the helices of ASII with ASII' (25). Cysteine substitutions at residues 217 and 220 in ASI and 246, 250, and 258 in ASII inhibited kinase activation *in vitro*, whereas cysteine substitutions in at residues 253 and 254 resulted in increased activation of the kinase. These aforementioned substitutions are located along the proposed packing face of ASI and ASII (25).

The cytoplasmic signaling domain

The cytoplasmic domain of a Tsr dimer consists of a four helix bundle (56). Given the high sequence conservation in this region of chemoreceptors (62), Tar, Trg, and Tap are presumably organized in the same way. The distal, and most highly conserved, portion of the cytoplasmic portion of the receptors interacts with CheW and CheA to control the activity of the latter. Tar contains four known methylation sites. Residues 295, 302, and 309 are located in methylation helix 1 (MH1), which is N-terminal to the signaling domain. The fourth site is residue 491 in methylation helix 2 (MH2), which is C-terminal to the signaling domain. The first and third sites (positions 295 and 309) are synthesized as glutaminyl residues and must be deamidated by phospho-CheB before methylation by CheR can occur (54). In an unstimulated *E. coli* cell there are about 0.5 methyl groups per Tar monomer, whereas saturating concentrations of aspartate result in about 2 methyl groups per Tar monomer (125). Higher levels of methylation can be reached with concurrent or subsequent addition of maltose (83).

The C-terminus of Tsr and Tar ends in a pentapeptide sequence (NWETF) to which CheR binds (133). Many residue substitutions in this region decrease the methylation level of the affected receptor, and suppressors of these mutations causing these methylation defects generate an internal xWxxF motif (106). Thus, the NWETF sequence apparently need not be located at the extreme C-terminus. Trg and Tap lack the NWETF motif and, as a consequence, are inefficiently methylated *in vivo* when they are expressed by themselves (36, 90).

For some of the experiments in this study I replaced the QEQE codons at the methylation sites with codons for Ala (to make $Tar_{\mathbf{AAAA}}$) or Asp (to make $Tar_{\mathbf{DDDD}}$). These substitutions were chosen because Ala, like Gln or methylated Glu, is electrically neutral and because Asp, like Glu, is negatively charged. Furthermore, neither Ala nor Asp is a substrate for CheR. Not surprisingly, increasing the number of the methylation sites occupied by Asp decreases the rate of methylation of the remaining Glu residues (103).

Previous work with $Tar_{\mathbf{AAAA}}$ and $Tar_{\mathbf{DDDD}}$ focused on their behavior as thermosensors. $Tar_{\mathbf{QEQE}}$ cannot sense temperature, but fully unmethylated Tar ($Tar_{\mathbf{EEEE}}$) is a warmth sensor, and the fully methylated form ($Tar_{\mathbf{E^m_E^m_E^m_E^m}}$) is a cold sensor (84). $Tar_{\mathbf{AAAA}}$, in the presence of aspartate, was also a cold sensor (87), and $Tar_{\mathbf{DDDD}}$ functioned as a warmth sensor, although, because of the inability of $Tar_{\mathbf{DDDD}}$ to change its methylation state, the addition of saturating levels of aspartate did not convert $Tar_{\mathbf{DDDD}}$ into a cold sensor (88). $Tar_{\mathbf{AAAA}}$ is not a mimic for $Tar_{\mathbf{E^m_E^m_E^m_E^m}}$ but because

Tar_{AAAA} functions as a thermosensor and responds to the addition of aspartate it must be able to modulate the kinase and therefore was a good candidate for our experiments.

In my study, I was interested in determining how the presence of a subunit of one of these altered forms of Tar in a dimer affects the methylation of its partner subunit that has normal methylation sites.

Detailed description of *E. coli* Tar

E. coli Tar is unique among known chemoreceptors in its ability to interact with a small attractant ligand (L-aspartate) and a periplasmic binding protein (MBP). A number of mutations have been identified that block aspartate taxis but not maltose taxis or that block maltose taxis but not aspartate taxis (39). Aspartate binds at the dimer interface at one of two rotationally symmetric sites (79). Extremely strong negative cooperativity creates a phenomenon called "half-of-sites" binding (18). MBP interacts asymmetrically with each subunit of the Tar dimer (40), with the N-terminal domain of MBP in contact with one Tar monomer and the C-terminal domain of MBP in contact with the other subunit (47).

The conformational change in Tar associated with ligand binding is poorly understood, but several lines of evidence suggest that the change occurs predominantly in one subunit of the dimer. Replacements of Arg-69 or Arg-73 with almost any other residues destroy the ability of Tar to interact normally with the γ-carboxyl group of L-aspartate. Replacements at Arg-64, Tyr-149, or Thr-154 interfere with recognition of the α-carboxyl and α-amino groups of aspartate. These two sets of residues are present in the opposing subunits (see Fig. 4). Because of the relative number and strength of contacts

involved, Asp-64, Tyr-149, and Thr-154 delineate the "major" binding half-site, and Arg-69 and Arg-73 delineate the "minor" binding half-site. Residue substitutions that sufficiently disrupt either half-site eliminate aspartate taxis (132).

When two mutant Tar receptors, one with a substitution in the "major" binding half-site and one with a substitution in the "minor" binding half-site, are coexpressed in a cell lacking all other chemoreceptors, they can form heterodimers that are competent to support aspartate chemotaxis. In such complemented dimers, one of the two aspartate binding sites is doubly inactivated and one is intact (Fig. 5A). Because of the strong negative cooperativity discussed earlier, this situation is quite similar to what happens with a wild-type Tar dimer. The difference, of course, is that aspartate can bind only in one of the two orientations possible with the wild type.

When two complementing Tar proteins are expressed, one of which is full length and the other of which is truncated near the end of the HAMP linker, aspartate taxis is still observed *if* the full-length subunit has its "major" binding half-site intact (Fig. 5B and 5C; 42, 121). That is, the signal is asymmetric and is carried through the subunit that recognizes the α-amino and α-carboxyl groups of aspartate. The interactions with Tyr-149 and Thr-154 are especially critical, since these two residues are in helix 4, which connects to TM2.

Wild-type Tar can respond to aspartate in the presence of saturating concentrations of maltose, and it can respond to maltose in the presence of saturating concentrations of aspartate (41). As noted above, the binding of MBP to Tar is also asymmetric, mutations that affect different half-site sites complement, and there is also a "major" binding half-site and a "minor" binding half-site for MBP (40). Only in complemented heterodimers,

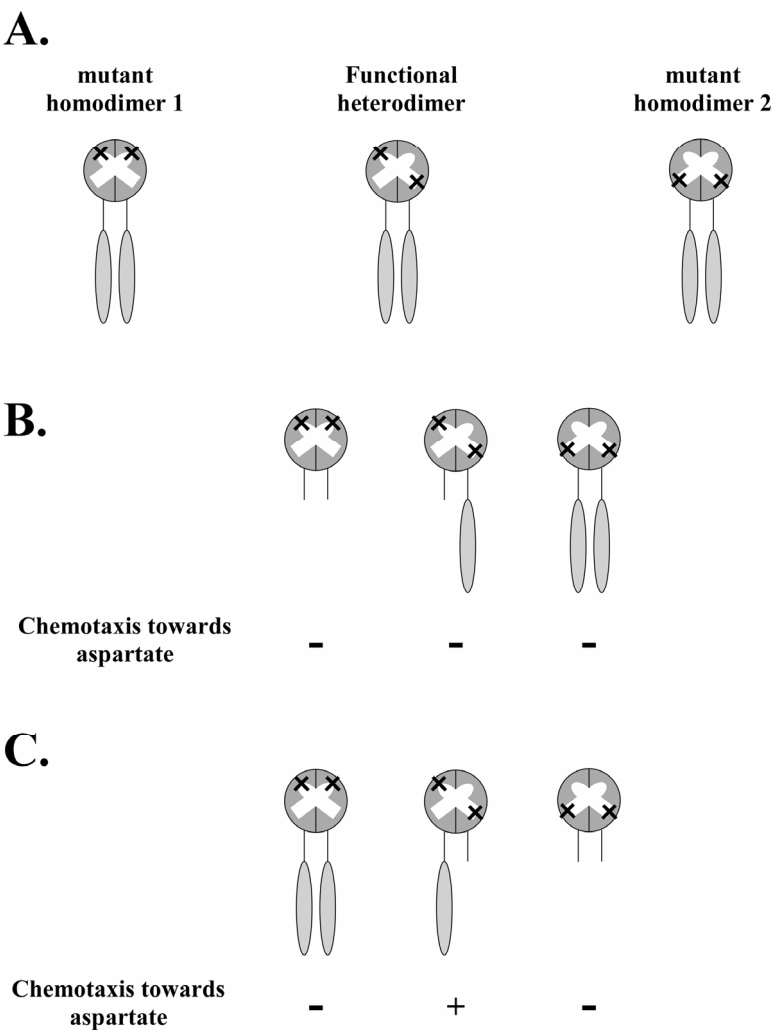

Figure 5. The formation of a functional heterodimer and asymmetric signaling.

A) Dimers that can form with two mutant Tar proteins, each with a different binding half-site intact. The "major" binding half-site is rectangular and the "minor" binding half-site is rounded. Only the heterodimer can mediate aspartate chemotaxis. B) Dimers that can form with full-length Tar with its "major" binding half-site disrupted and a C-terminally truncated Tar subunit with its "minor" binding half-site disrupted. None of the three dimers supports aspartate chemotaxis. C) Dimers that can form with full-length Tar with its "minor" binding-half-site disrupted and a C-terminally truncated Tar with its "major" binding half-site disrupted. The heterodimer supports good aspartate chemotaxis.

in which the aspartate and MBP binding half-sites are in different subunits of a heterodimer, are capable of signaling concurrently to aspartate and MBP (Fig. 6). The conclusion is that each of the two ligands, aspartate and MBP, uses the signaling capacity of a different subunit. It is noteworthy that *Salmonella* Tar, which does not mediate maltose taxis and presumably does not interact effectively with MBP, exhibits a less-extreme negative cooperativity. The first aspartate binds with a K_d of about 0.1 μM, and the second binds with a K_d of about 2 μM (18). In this case, the signaling capacity of the dimer is used to provide a high-affinity aspartate-binding site and a lower-affinity aspartate-binding site to expand the dynamic range of aspartate sensing.

Experimental rationale and research goals

The research described in this dissertation addresses the question of how the cytoplasmic domain of the *E. coli* Tar chemoreceptor responds to conformational changes induced by ligand binding to the periplasmic domain of the receptor. Tar was the chemoreceptor of choice for three reasons. First, the strong negative cooperativity that leads to "half-of-sites" binding of aspartate by wild-type Tar means that Tar heterodimers with only one intact aspartate binding site are still likely to behave much like the native protein. Second, the *in vivo* and *in vitro* signaling properties of *E. coli* Tar have been extensively studied. Third, a number of mutations that alter the aspartate binding site have been identified and their affects on receptor function have been characterized.

The primary experimental approach in this work is to monitor changes in the cytoplasmic domain that result from adaptive methylation in response to aspartate binding. The behavior of the two subunits of a receptor heterodimer can be determined by

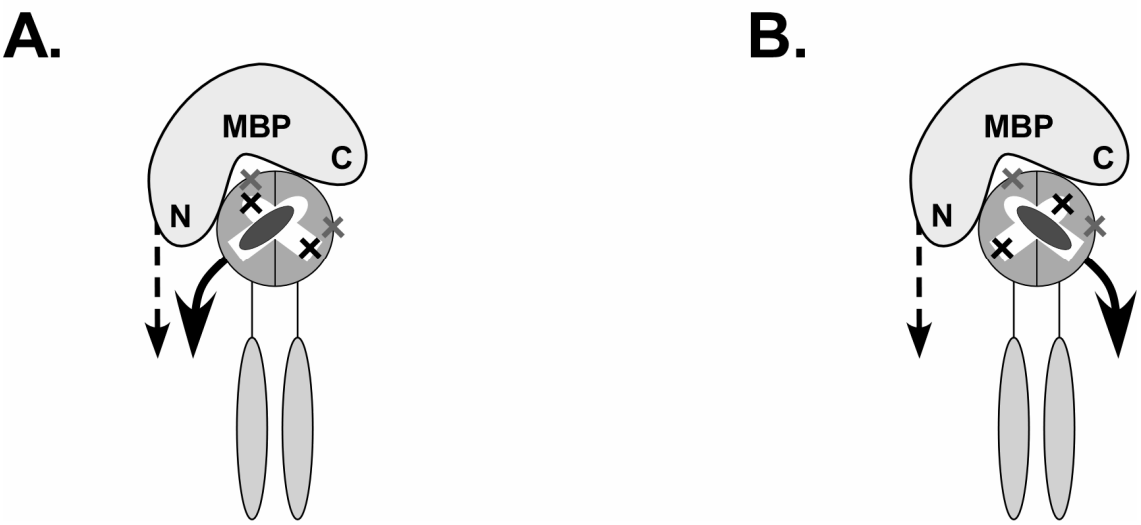

Figure 6. “Same-side” and “opposite-side” signaling.

Schematic representation of “same-side” and “opposite-side” signaling during concurrent aspartate and maltose chemotaxis. Tar subunits with double residue substitutions, one disrupting aspartate taxis and one disrupting maltose taxis, can be coexpressed in cells. Mutations affecting maltose taxis also can be divided into ones that disrupt a “major” binding half-site for MBP, which interacts with the N-terminal domain of MBP, and a “minor” binding half-site, which interacts with the C-terminal domain of MBP. Binding mutations specific to aspartate binding are indicated with a black X whereas those mutations specific for MBP binding are grey. A) Coexpression of two mutant Tar proteins in which the “major” binding half-sites for aspartate and MBP are intact in the same subunit. Although the heterodimers can support either maltose or aspartate chemotaxis, they cannot support maltose taxis in the presence of a saturating concentration of aspartate. B) Coexpression of two mutant Tar proteins in which the “major” binding half-sites for aspartate and MBP are intact on opposite subunits. In this case, the heterodimer can mediate maltose taxis in the presence of a saturating concentration of aspartate.

placing a distinguishing epitope tag on one subunit. In practice, I found that a 14-residue sequence that serves as a recognition site for the anti-V5 antibody was nearly ideal for my purpose. When this sequence was attached to the C-terminus of Tar by a 7-residue flexible linker it proved to disturb aspartate chemotaxis very little, if at all. In particular, it did not significantly alter methylation, which was not a given considering that the binding site for CheR, the NWETF pentapeptide, is at the C-terminus of Tar and thus immediately precedes the linker and V5 tag.

Visualization of the five different methylated forms of Tar (from $Tar_{\mathbf{EEEE}}$ to $Tar_{\mathbf{E^m E^m E^m E^m}}$) was achieved by immunoblotting with anti-V5 antibody after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). During SDS-PAGE, $Tar_{\mathbf{EEEE}}$ migrates slowest and $Tar_{\mathbf{E^m E^m E^m E^m}}$ migrates fastest, with the forms of Tar with intermediate levels of methylation migrating at intermediate rates. In a 12% acrylamide gel, Tar in each of its five possible methylation states forms a distinct band. Furthermore, external calibration is possible using V5-tagged $Tar_{\mathbf{EEEE}}$, $Tar_{\mathbf{QEQE}}$, and $Tar_{\mathbf{QQQQ}}$. The latter two proteins have the same mobility's as $Tar_{\mathbf{E^m EE^m E}}$ and $Tar_{\mathbf{E^m E^m E^m E^m}}$, respectively.

The choice of the proper residues in the "major" and "minor" binding half-sites was crucial. In previous experiments on asymmetric signaling conducted by our group, T154I and R73K, respectively, had been employed (42). However, I found that the T154I protein became methylated after addition of high concentrations of aspartate, and I therefore switched to T154P, which has been described as having a more extreme phenotype (67, 121). Fortunately, the T154P protein did not become methylated at any

aspartate concentration I tested. Similarly, the R73K substitution was discarded in favor of R69G, which provided a cleaner aspartate-taxis-negative phenotype, although the R69G protein does show some level of adaptive methylation at the highest concentrations of aspartate.

Both *in vivo* and *in vitro* assays were utilized to determine the properties of these two mutant proteins expressed singly and in combination. As expected, when expressed together they complemented one another, presumably by forming heterodimers, and supported normal chemotaxis. As discussed above, in such heterodimers the subunit with the "major" binding half-site intact is the signaling subunit. By expressing the V5-tagged form of Tar R69G with non-tagged Tar T154P, and vice versa, I could monitor the methylation of the signaling or non-signaling subunit, albeit in a separate experiment. This basic approach was used to conduct the following investigations.

Chapter II: Conversion of asymmetry to symmetry during transmembrane signaling by the Tar chemoreceptor

This phase of the work focused on the interactions between the two subunits of a heterodimer in the presence of the full chemotaxis signaling pathway. The asymmetry observed in signaling raised the question of whether the asymmetric signal in the periplasmic domain persists in the cytoplasmic domain. Based on the equal extent and rate of methylation of the signaling and non-signaling subunits of a Tar R69G/Tar T154P heterodimer, the answer is a resounding "no." An asymmetric signal in the periplasm becomes a symmetric signal in the cytoplasm. My favored hypothesis, presented as a model in Chapter II, is that the conversion occurs through an interaction of the two

HAMP linker domains of the heterodimer. The *in vitro* kinase activity and inhibition curves presented in this chapter were performed by Roger Draheim and Run-zhi Lai.

Chapter III: Neither CheA nor CheW is required for conversion of asymmetry to symmetry during transmembrane signaling by *E. coli* Tar

This portion of the research extends the work described in Chapter II to ask whether the conversion of asymmetry to symmetry requires an intact chemotactic signaling pathway. CheA and CheW facilitate the polar localization of the chemoreceptors and are absolutely needed for their association in tight clusters (Fig. 7; 74, 112). In the absence of CheA, levels of phospho-CheB are very low, methylesterase activity is correspondingly low, and the chemoreceptors have a much higher baseline level of methylation (~2 methyl groups per receptor monomer as opposed to ~0.5). However, additional methylation is still observed after the addition of aspartate. The clear result was that even in the absence of CheA and CheW and the virtual absence of phospho-CheB, both the signaling and non-signaling subunits became methylated. However, in this situation the rates and final extents of methylation were no longer equal under all conditions. It also became clear from these experiments that the C-terminal V5 epitope tag could, under these conditions, affect the signaling properties of the receptors *in vivo*. A model that attempts to reconcile the different findings is the culmination of Chapter III. The *in vitro* kinase activity and inhibition curves presented in this chapter were performed by Roger Draheim and Run-zhi Lai.

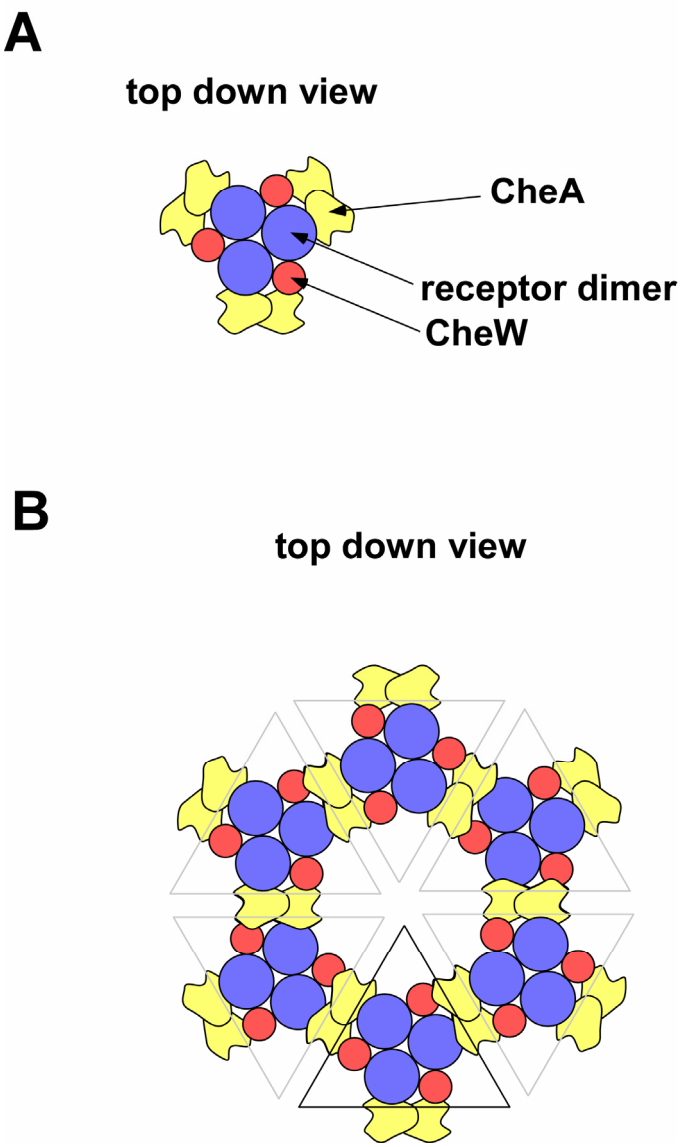

Figure 7. Higher order organization of the receptors in the presence of cytoplasmic proteins CheA and CheW.

Trimers of receptor dimers interact with CheW and CheA at the distal tips of their cytoplasmic domains resulting in lattices of linked receptor trimers. Top down view of receptor trimer (A) and receptor lattice (B).

Chapter IV: Cross-talk between Tar and Tsr in the absence of the receptor/CheA/CheW ternary signaling complex

In the last set of experiments described in this dissertation I investigated interactions between two different receptors, Tar and Tsr, which should not be able to dimerize with one another. The primary question was whether, in the absence of CheA and CheW, and thus in the absence of tight receptor clusters, these receptors still communicate, as they clearly do in the presence of CheA and CheW (6, 63). The question is not nonsensical, since chemical cross-linking studies (119) suggest that, even in the absence of CheA and CheW, Tar and Tsr are in close physical proximity, perhaps in mixed trimers of dimers. The experimental paradigm was to assess the levels and rates of adaptive methylation of a V5-tagged receptor of one type in the presence of another receptor. Previous work has shown that only the receptor that is bound to ligand retains a higher methylation state after adaptation is complete (98, 114). We decided to revisit this problem since our methylation assay is more sensitive and versatile than previous assays utilizing [^{3}H-methyl]-radiolabeled proteins, which detect only methylated receptors. Moreover, we wanted to test whether symmetric methylation within a dimer is affected by having either cognate or heterologous dimers present with it in the putative trimers of dimers. The novel finding was that rather extensive and rapid methylation of non-cognate receptors does occur. This result demands a re-examination of how assemblies of receptors communicate during chemotactic signaling in ways that may significantly affect the final integrated output response.

CHAPTER II

CONVERSION OF ASYMMETRY TO SYMMETRY DURING TRANSMEMBRANE SIGNALING BY THE *E. COLI* TAR CHEMORECEPTOR DIMER

Summary

The Tar protein of *Escherichia coli* is a transmembrane homodimer that serves as the chemoreceptor for aspartate and maltose. Aspartate binds directly to Tar at the dimer interface near the apex of the periplasmic domain. The conformational change associated with aspartate binding is propagated through the periplasmic domain of one subunit of the Tar dimer. This observation raises the question of whether the asymmetry of signaling is maintained in the cytoplasmic domain of the dimer. To address this problem, we monitored aspartate-induced adaptive methylation. Two mutant Tar proteins, each of which has a different half of the aspartate-binding site intact, were co-expressed in cells. Only the heterodimers that form can bind aspartate, and only the periplasmic domain in which the "major" aspartate-binding half-site is intact can transmit the conformational change associated with ligand binding. By placing a C-terminal V5-epitope tag on one or the other of the mutant Tar proteins, we could distinguish which subunits in the heterodimers change their methylation state. We could also test whether the covalent-modification state of one subunit affects that of the other, by replacing the glutamyl residues at the methylation sites in one mutant protein with alanyl residues. Our conclusion is that, although the initial periplasmic signal is asymmetric, both cytoplasmic domains of a heterodimer become methylated. Our interpretation of this finding is that an asymmetric signal in the periplasmic domain of a Tar dimer manifests itself as a

symmetric signal in the cytoplasmic domain. We propose that this conversion of asymmetry to symmetry may occur through interactions between the HAMP linker domains of the two subunits.

Introduction

The Tar chemoreceptor of *E. coli* mediates attractant responses to L-aspartate and maltose and repellent responses to certain divalent metal ions, including Ni^{2+} and Co^{2+}. It also detects changes in pH (126) and temperature (75, 85). Tar is a homodimer (82) that is thought to associate as a trimer of dimers (56). In the presence of the soluble cytoplasmic proteins CheA and CheW, Tar and the other chemoreceptors assemble into higher-order structures that are usually located at a cell pole (74, 112). For reviews of chemoreceptor structure and function, see references 34 and 120.

Aspartate binds directly to Tar at the interface of the two subunits near the apex of the periplasmic domain. Each dimer contains two rotationally symmetric binding sites (79) that exhibit strong negative cooperativity (18). Each binding site consists of different residues from the opposing subunits. Aspartate hydrogen-bonds with the side chains of Arg-64, Tyr-149, and Thr-154 and with the backbone carbonyl of Gln-152 in the "major" binding half-site of one subunit and with the side chains of Arg-69' and Arg-73' in the "minor" binding half-site of the other subunit (134). Maltose, in contrast, first binds to the periplasmic maltose-binding protein (MBP). Maltose stabilizes the closed form of MBP (96), which interacts with Tar (136).

A current model for signal propagation proposes that binding of an attractant in the periplasmic domain initiates a small (~1.6 Å) downward movement of helix 4 (93). The

second transmembrane region (TM2) is an extension of helix 4. This transmembrane signal is propagated, in an unknown manner, through the HAMP linker domain (10, 131) that immediately follows TM2 to regulate the activity of the cytoplasmic signaling domain.

The signaling domain of Tar associates with the histidine kinase CheA and the coupling factor CheW to control the activity of CheA. In the absence of an attractant, the receptors stimulate the autophosphorylation activity of CheA. CheA donates the phosphoryl group to one of two response regulators, CheY or CheB. Phospho-CheY binds to FliM in the switch component of the flagellar motor (24) to increase the probability of CW flagellar rotation (29, 100), which promotes tumbling. Phospho-CheY is a substrate for the CheZ phosphatase. In the presence of an attractant, the cognate receptor inhibits, rather than stimulates, the activity of CheA. Combined with the activity of CheZ, this reduction of CheA activity rapidly decreases the intracellular level of phospho-CheY to suppress tumbling.

Sensing of spatial gradients is accomplished by a temporal mechanism that requires a memory of a few seconds to compare past and present conditions. The memory consists of reversible covalent methylation and demethylation of four glutamyl residues located in the cytoplasmic domain of the receptor. More of these residues become methylated after attractants bind, and fewer are methylated when repellents are present. Methylation and demethylation counteract the signals generated by ligands to reset CheA activity to pre-stimulus levels. Under steady-state conditions, the methylation level reflects the mean ligand occupancy of a receptor.

CheR and CheB mediate adaptation. CheR is a constitutively active methyltransferase that uses S-adenosylmethionine as a methyl-group donor to generate glutamyl methyl esters (108). CheB is both a methylesterase and deamidase (57, 127). It removes amide groups from the glutaminyl residues present at the first and third methylation sites in the newly translated Tar protein, and it also removes the methyl groups added by CheR. Methylesterase activity increases about 100-fold when CheB is phosphorylated (8). Thus, decreased CheA activity lowers the level of phospho-CheB, leading to a net increase in methylation of the receptors. Right after an attractant stimulus, the decrease in phospho-CheB increases methylation of both the cognate and non-cognate receptors (55, 99). However, since the affinity of CheR for the methylation sites is affected by ligand-induced changes in the conformation of the receptor, when adaptation is complete the attractant-bound receptor retains a higher level of methylation (114).

Tar homodimers in which either half of the aspartate-binding pocket is defective cannot mediate chemotaxis to aspartate (39). However, when two mutant Tar proteins, each of which is defective in a different binding half-site, are coexpressed, they can form a heterodimer in which one functional binding site remains (42, 121). Under these conditions, signaling in response to aspartate is asymmetric, occurring only through the subunit in which the "major" binding half-site is intact. Binding by MBP is also asymmetric (40). A Tar dimer occupied by aspartate can mediate maltose taxis only if the signals to aspartate and maltose are propagated through different subunits of the dimer (41), a result consistent with asymmetric signaling.

We have performed *in vivo* and *in vitro* assays to determine whether the asymmetric, ligand-induced conformational changes in the periplasmic domain of Tar result in a

symmetric change within the cytoplasmic domain. Our results demonstrate that aspartate causes equal methylation of each subunit of a heterodimer. The pattern of methylation does not depend on the presence of functional methylation sites in both subunits. We propose that the conformational change associated with aspartate binding becomes symmetric within the conserved HAMP linker (10, 131) to evoke an equivalent response from the cytoplasmic domain of each subunit of the dimer. A similar conclusion was reached for the genetically engineered Tez1 protein, in which the periplasmic and transmembrane domains of Tar are fused to the HAMP linker and cytoplasmic domain of the osmosensor EnvZ (137). The Tez1 chimera, like Tar, functions as a homodimer. The similarity in the results obtained with Tar and Tez1 strongly suggest that conversion of asymmetry to symmetry, perhaps within the HAMP linker, is a property shared by chemoreceptors and transmembrane histidine kinases.

Materials and methods

Bacterial strains and plasmids. Strain SW02 (*thr*$^+$ *eda*$^+$ *tsr7021 trg::*Tn*10*[Kan^r] Δ[*tar-tap5201*]) is derived from the chemotactically wild-type strain RP437 (94). Strain RP3098 (111) is a Δ(*flhD-flhB*) derivative of strain RP437 that produces neither flagellar nor chemotaxis proteins.

Plasmid pMK113, a derivative of pBR322, contains the *E. coli tar* gene expressed from a mutant *meche* operon promoter that decreases transcription several fold (39). Plasmid pRBB16, a pACYC184 derivative, encodes *E. coli* Tar expressed from the native *meche* promoter. The difference in expression levels from the two promoters compensates reasonably well for the different copy numbers of the two plasmids. Plasmid

pMK113CV5 was constructed via PCR extension of the 3' end of *tar* to introduce a seven-residue flexible linker (GGSSAAG) and a fourteen-residue C-terminal V5-epitope tag (GKPIPNPLLGLDST). Plasmid pRBB164A was constructed by PCR amplification of the gene encoding $Tar_{\mathbf{AAAA}}$ from plasmid pRA126 (87) and insertion of the amplified gene into plasmid pRBB16, using *Nde*I and *Eco*RI restriction sites. Mutations introducing the R69G or T154P substitution into the products of plasmid-borne *tar* genes were made via QuickChange site-directed mutagenesis (Stratagene).

Capillary assay. We used a modified version of the classical protocol of Adler (2). Overnight cultures were grown at 32°C in tryptone broth (TB; 80) containing 25 μg/ml ampicillin and 7.5 μg/ml tetracycline. These cultures were then diluted 1:100 in 15 ml of the same medium and grown to OD_{590nm} of 0.6. Cells were harvested by 5 min centrifugation at 1000g and washed three times with 10 ml of chemotaxis buffer (10mM potassium phosphate [pH 7.0], 0.1 mM EDTA, and 0.01 mM L-methionine). Cells were gently resuspended between washes. The final 10 ml resuspension was allowed to sit at room temperature for 10 min to allow clumps of cells to settle, after which the top 4 ml were removed for use in the assay. The cells in this aliquot were diluted to a final OD_{590nm} of 0.1. This high cell density was adopted because of the relatively poor motility of the plasmid-bearing SW02 cells grown in the presence of antibiotics. The assay was performed on a humidified slide warmer at 32°C. Cells entering the capillary tubes were harvested after 45 min and diluted for colony counts on Luria broth (LB) agar (80).

Tethered-cell assay. Cells were grown as for the capillary assay. The cells in 10 ml of a late-logarithmic TB culture (OD_{590nm} of 0.6) were sheared in a 50 ml stainless steel cup of a Waring blender, using the low-speed setting. Blending was done in eight

repetitions of bursts of 7 sec interspersed with breaks of 13 sec to prevent overheating of the sample. Sheared cells were pelleted at top speed in a table-top centrifuge for 5 min and washed twice with 5 ml of tethering buffer (10mM potassium phosphate [pH 7.0], 0.1 M NaCl, 0.01 mM EDTA, 0.02 mM L-methionine, 20 mM sodium D-lactate, 200 μg/ml chloramphenicol). The pellet was resuspended in 2.5 ml of tethering buffer. Tethered cells were prepared and assayed for chemotactic responses at room temperature (22-24^{o}C) as described previously (128). The responses of at least 20 cells were averaged for each strain tested.

Determination of the *in vivo* methylation state of Tar. Our assay was based on a published method (129). Overnight cultures of strain SW02 (deleted for the four chemoreceptor genes: *tsr*, *tar*, *tap* and *trg*) containing two compatible plasmids encoding different mutant versions of Tar were grown as for the capillary assay. Cells were harvested by centrifugation, washed thrice with 10 ml of chemotaxis buffer, and finally resuspended in 5 ml of chemotaxis buffer containing 10 mM sodium D-lactate and 200 μg/ml chloramphenicol. The OD_{590nm} was adjusted to 0.4, and 1 ml aliquots were transferred to 10 ml scintillation vials and incubated, with shaking, at 32^{o}C for 40 min. Aspartate was added at concentrations ranging from 0.01 to 10 mM, and the cells were incubated for an additional 20 min. Control reactions received an equal volume of buffer. Reactions were terminated by addition of 100 μl ice-cold 100% trichloracetic acid (TCA), followed by incubation on ice for 15 min. Proteins were pelleted by centrifugation for 15 min in a microcentrifuge, washed with 0.5ml cold 1% TCA, and subsequently washed with 0.5 ml acetone. The samples were allowed to dry overnight at room temperature and then resuspended in 100 μl 2X SDS-loading buffer. A 20 μl

aliquot of each sample was loaded onto a 7.5% SDS gel. Following electrophoresis, the proteins were transferred to nitrocellulose filters (0.2 μm pore size) and subjected to immunoblotting using commercial antibody against the V5 epitope (Invitrogen) and goat-anti-mouse secondary antibody conjugated with alkaline phosphatase (GAM-AP; Bio-Rad) to visualize the bands.

Time-course methylation assay. Methylation assays were carried out as above, with the following modifications. The final resuspension of washed cells at an OD_{590nm} of 0.4 was placed in a 125 ml Erlenmeyer flask in a 32^{o}C water bath and agitated by vigorous swirling. L-aspartate was added to 1 mM, and 1 ml aliquots were removed at various times and added to 1.5 ml Eppendorf tubes pre-loaded with 100 μl of 100% TCA and immediately put on ice. The samples were prepared for immunoblotting as described above. The immunoblots were scanned, and the densities of the bands were analyzed with the program ImageJ (http://rsb.info.nih.gov/ij/). First, the cumulative density of all the bands in a lane was measured. Then, the density of the individual bands was determined, and the relative density of each band as a fraction of the total density was calculated. The individual bands were assigned a methylation state by comparing them to a TarV5 standard comprised of three different covalent modification states: $Tar_{\mathbf{EEEE}}$, $Tar_{\mathbf{QEQE}}$, and $Tar_{\mathbf{QQQQ}}$. $Tar_{\mathbf{EEEE}}$ migrates slowest, $Tar_{\mathbf{QEQE}}$ migrates at an intermediate rate, and $Tar_{\mathbf{QQQQ}}$ moves fastest. The total mean population level of methylation at each time point was calculated from Equation 1:

$$M_t = (M_1 + 2M_2 + 3M_3 + 4M_4)/R_t \qquad \text{(Equation 1)}$$

where M_t is the mean number of methyl groups per receptor, M_1 is the density of the band corresponding to the singly methylated receptor, M_2 is the density of the band corresponding to the doubly methylated receptor, M_3 is the density of the band corresponding to the triply methylated receptor, M_4 is the density of the band corresponding to the quadruply methylated receptor, and R_t is the sum of the band densities, including the unmethylated form M_0.

***In vitro* receptor-coupled CheA kinase assays with inner membranes containing overexpressed Tar**. Cells were grown, inner membranes prepared, and *in vitro* receptor-coupled CheA activities determined exactly as described previously (31). Production of radiolabeled phospho-CheY was linear over 20 sec and was directly proportional to the amount of Tar over a range from 5 pmol to 40 pmol. We used 20 pmol Tar in each reaction because this value is in the middle of the linear range, allowing us to measure increases or decreases in phospho-CheY production accurately. Analysis of the titration curves for aspartate inhibition was performed according to Bornhorst and Falke (20) as modified by Draheim *et al*. (31).

Results

Aspartate-dependent methylation and inhibition of CheA kinase activity are impaired in Tar proteins with altered aspartate-binding sites. The residue substitutions T154P and R69G disrupt the "major" and "minor" aspartate-binding half-sites, respectively (Fig. 8A). When V5-tagged Tar R69G (TarRGV5) and Tar R69G were coexpressed as the only methyl-accepting chemoreceptors in strain SW02 (receptor gene-

deleted) cells, the basal methylation level of TarRGV5 was higher than that of either V5-tagged wild-type Tar (TarV5) expressed with wild-type Tar or V5-tagged Tar T154P (TarTPV5) expressed with Tar T154P (Fig. 8B).

Aspartate at 0.1 mM was sufficient to produce the greatest degree of methylation of TarV5 that was observed. The approximately five-fold higher-than-normal ratio of receptor to CheR may have decreased the final level of methylation after adaptation was complete. No concentration of aspartate, up to 10 mM, led to any detectable increase in methylation of TarTPV5. Although 1 mM aspartate did not increase methylation of TarRGV5, a modest increase in methylation was seen with 10 mM aspartate. However, even at 10 mM aspartate, TarRGV5 was still significantly less methylated than wild-type Tar at 0.1 mM aspartate. We therefore used 1 mM aspartate in subsequent experiments because it did not cause a detectable increase in methylation of either TarRGV5 or TarTPV5 expressed by itself.

TarV5 supported excellent aspartate chemotaxis in the capillary assay (Fig. 8C). Co-expression of Tar with TarV5 decreased the peak accumulation by about 40%, probably because the total amount of receptor increased, thereby leading to a less favorable stoichiometry relative to the soluble, chromosomally expressed Che proteins. Neither TarRGV5 nor TarTPV5 coexpressed with their non-tagged counterparts supported significant accumulation in capillaries containing up to 100 mM aspartate.

To establish whether defective aspartate taxis was due to decreased ligand binding by the mutant proteins or decreased ability to stimulate CheA, we performed *in vitro* receptor-coupled kinase assays. Both mutant receptors stimulated CheA activity 60-70 percent as well as wild-type Tar (Fig. 9A). This activity is consistent with the ability of

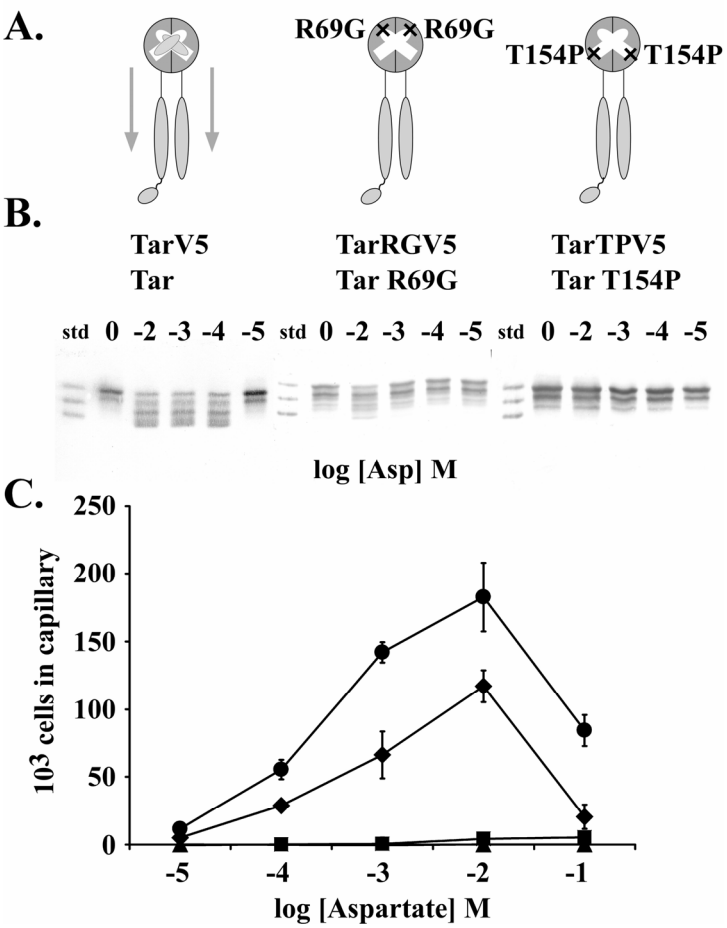

Figure 8. Properties of V5-tagged wild-type and mutant Tar proteins *in vivo*.

A) Representation of V5-tagged Tar/normal Tar heterodimers. Gray ovals represent aspartate molecules bound in one or the other binding pocket. The strong negative cooperativity of aspartate binding dictates that only one binding pocket will be occupied. The R69G substitution affects the "minor" binding pocket (round) and the T154P affects the "major" binding pocket (rectangular). Either substitution interferes with aspartate binding. B) *In vivo* methylation assays, using SW02 cells expressing V5-tagged Tar and untagged Tar containing the same substitutions in the binding pocket. The tagged and non-tagged proteins were expressed in roughly equal amounts from compatible plasmids. The primary antibody used in immunoblotting was specific for the V5 epitope. The standards, shown in the leftmost lane of the gel for each protein set, contain equal amounts of the V5-tagged forms of $Tar_{\mathbf{EEEE}}$, $Tar_{\mathbf{QEQE}}$, and $Tar_{\mathbf{QQQQ}}$. C) Capillary assays carried out with SW02 cells expressing V5-tagged Tar only (TarV5, circles), TarV5 and wild-type Tar (diamonds), TarRGV5 and Tar R69G (squares), and TarTPV5 and Tar T154P (triangles). The assays were done in duplicate and repeated three times. The error bars represent the standard deviation of the mean, with n = 6.

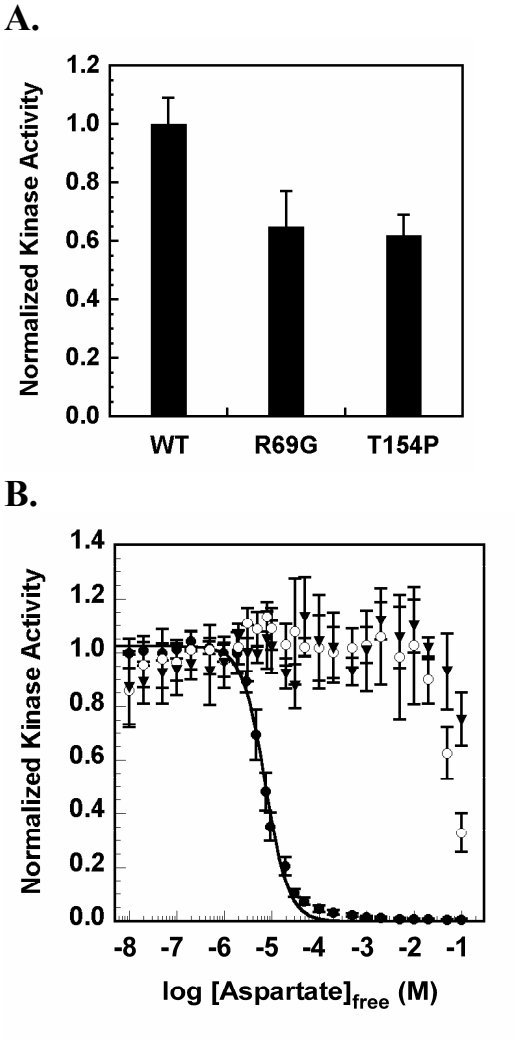

Figure 9. Normalized kinase activity and K_i curves for wild type Tar and individual binding pocket mutations.

Tar R69G and Tar T154P stimulate CheA activity *in vitro* but are poorly inhibited by aspartate. A) *In vitro* CheA kinase activity for wild-type Tar and mutant Tar homodimers containing a substitution in the aspartate-binding site. The kinase activity was normalized to 1 for wild-type Tar and was 0.65 +/- 0.12 for Tar R69G and 0.61 +/- 0.07 for Tar T154P. The error bars represent the standard deviation of the mean, with n = 3. B) Inhibition by aspartate over the range of 0.01 μM to 100 mM for wild-type Tar (filled circles), Tar R69G (open circles), and Tar T154P (filled triangles). The aspartate K_i for wild-type Tar was 7±1 μM. Half-maximal inhibition of Tar R69G required >10 mM aspartate. Half-maximal inhibition of Tar T154P required more than 100 mM aspartate and was not achieved in our experiment. The error bars represent the standard deviation of the mean, with n = 3.

either of these mutant proteins to support essentially normal run-tumble motility in strain SW02 (data not shown).

The mutant receptors were defective in their ability to inhibit CheA activity in the presence of aspartate. Stimulation by wild-type Tar was almost totally abrogated by 0.1 mM aspartate, with an apparent K_i for aspartate of 7±1 μM. Half-maximal inhibition by Tar R69G required >10 mM aspartate, 1000 fold more than for wild-type Tar. No significant inhibition by Tar T154P was seen at aspartate concentrations <100 mM, and half-maximal inhibition was never achieved with this mutant receptor (Fig. 9B). We conclude that the impaired chemotaxis in strains expressing either Tar R69G or Tar T154P as their sole chemoreceptor is primarily the result of their failure to bind aspartate at physiologically relevant concentrations.

We note that all *in vitro* assays were performed with the non-V5-tagged forms of the proteins. Although the V5 tag did not interfere with CheA stimulation, it substantially increased the concentration of aspartate that was required to inhibit CheA activity in the receptor-coupled assay with wild-type Tar (R. R. Draheim, unpublished results). The mechanism by which the V5 tag interferes with attractant inhibition of CheA stimulation *in vitro* without disrupting aspartate chemotaxis *in vivo* (Fig. 8C) is currently under investigation. The vast majority of the V5-tagged receptors in both the *in vivo* and *in vitro* assays retain the epitope (A. F. Bormans, unpublished results), so proteolytic removal of the C-terminal extension *in vivo* is probably not the explanation.

The R69G and T154P proteins complement one another for aspartate chemotaxis. The R69G and T154P proteins, when coexpressed in a cell, can form heterodimers that have only one functional binding site for aspartate (Fig. 10A). Previous

work (41, 42) has shown that in such heterodimers the R69G polypeptide, in which the "major" binding half-site is intact, is the subunit that communicates the signal associated with aspartate binding. We thus refer to it as the "signaling" subunit.

The V5-tag enabled us to assess the methylation state of individual subunits within the heterodimer, since either the signaling subunit or the non-signaling subunit can bear the epitope label. Regardless of which mutant protein contained the V5 tag, they complemented in the capillary assay to support normal responses to aspartate (Fig. 10C). Immunoblotting revealed that either the signaling subunit or the non-signaling subunit became equally methylated in response to aspartate binding (Fig 10B). Thus, the orientation in which aspartate binds relative to the V5-tagged subunit did not affect the final methylation state of the tagged subunit.

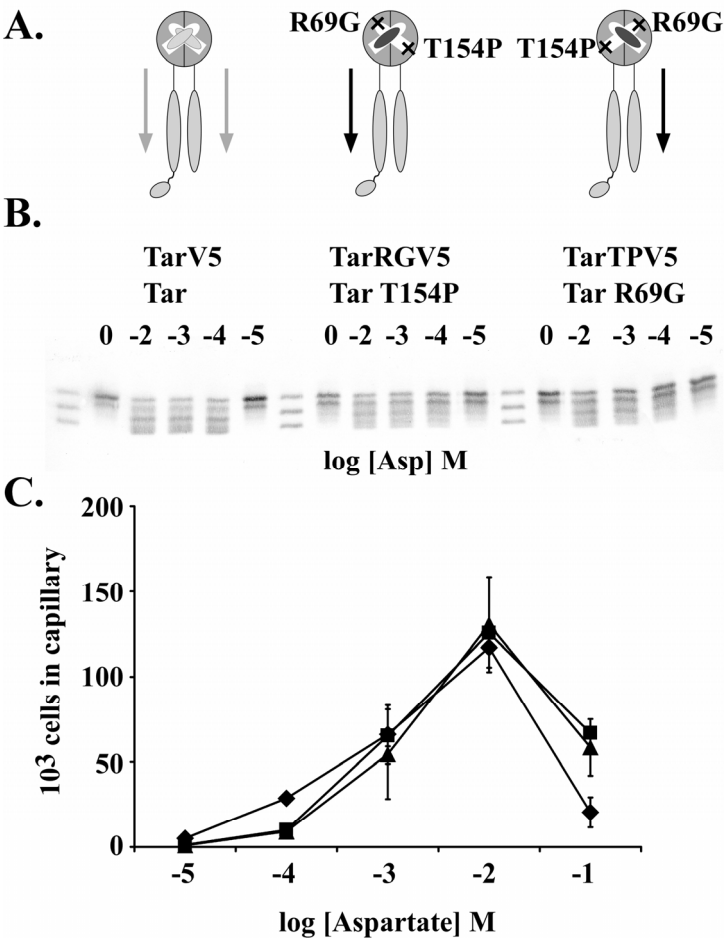

Figure 10. Properties of coexpressed, complementing V5-tagged mutant Tar and Tar proteins *in vivo*.

A) Representation of functional heterodimers present when complementing mutant Tar subunits are coexpressed. Each heterodimer contains one functional aspartate-binding site and can therefore bind one aspartate molecule (black oval). Also, each heterodimer contains one V5-tagged subunit. B) *In vivo* methylation assays with strains expressing different combinations of mutant Tar subunits. Standards are as in Figure 8B. C) Capillary assays conducted with SW02 cells expressing TarV5 coexpressed with wild-type Tar (diamonds), TarRGV5 coexpressed with Tar T154P (squares), and TarTPV5 co-expressed with Tar R69G (triangles). The assays were done in duplicate and repeated three times. The error bars represent the standard deviation of the mean, with n = 6.

The covalent modification state of one Tar subunit does not determine the methylation state of the opposing subunit. Equal methylation of both cytoplasmic domains of a heterodimer that binds aspartate asymmetrically could, in principle, occur through one or more of at least three mechanisms (Fig. 11): A) The conformational change propagated in the signaling subunit could remain asymmetric in the cytoplasmic domain, initially causing methylation only of the signaling subunit. Methylation of the signaling subunit then would induce methylation of the non-signaling subunit. B) The signal could travel through one subunit in the periplasmic domain but subsequently be converted into a symmetric signal in the HAMP linker region, leading to methylation of both subunits. C) Signaling in one subunit of a dimer could remain asymmetric but increase the probability of all subunits within a local area becoming methylated by virtue of transmethylation (65, 70).

Alternatives A and B can be distinguished by replacing the glutamyl residues at the methylation sites in one mutant subunit with residues whose modification state does not change. We chose alanyl residues because the properties of Tar with the Glu to Ala replacements ($Tar_{\mathbf{AAAA}}$) have been characterized and shown to respond to both changes in temperature and the addition of aspartate (87). $Tar_{\mathbf{AAAA}}$ had about 80% of the CheA-stimulating ability of $Tar_{\mathbf{QEQE}}$ in the *in vitro* receptor-coupled CheA kinase assay (Fig. 12A). The apparent $K_{\mathbf{i}}$ for $Tar_{\mathbf{AAAA}}$ was 30±1 μM, which is four-fold higher than the 7±1 μM $K_{\mathbf{i}}$ measured with $Tar_{\mathbf{QEQE}}$ (Fig. 12B). For comparison, the *in vitro* CheA-stimulating activities of $Tar_{\mathbf{EEEE}}$, $Tar_{\mathbf{DDDD}}$, and $Tar_{\mathbf{QQQQ}}$, assayed under the same conditions, were 5%, 80%, and 200% of the activity of $Tar_{\mathbf{QEQE}}$, and the corresponding aspartate $K_{\mathbf{i}}$

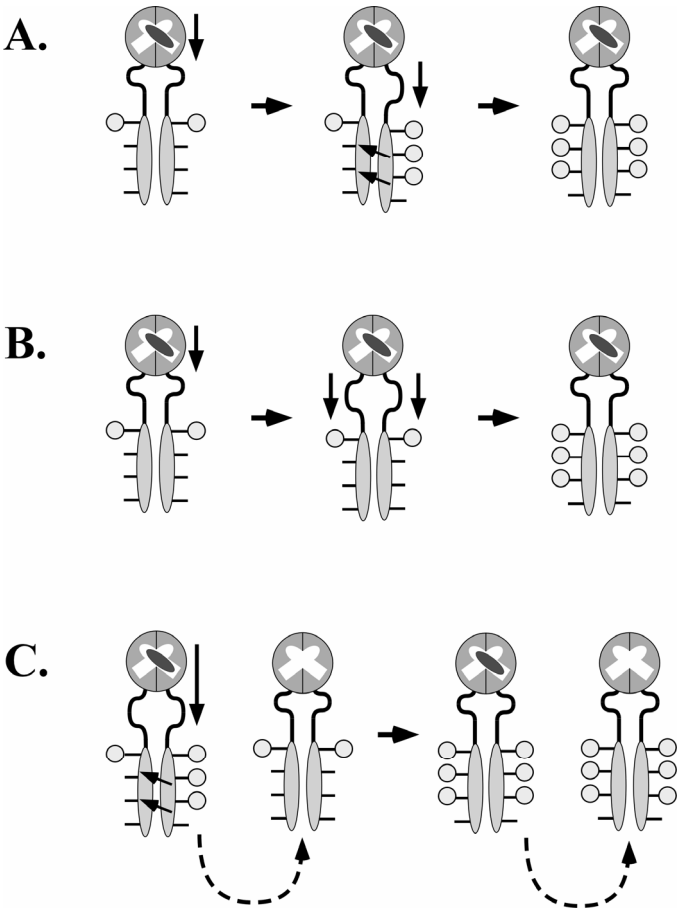

Figure 11. Three models to explain symmetric methylation in response to an asymmetric signal.

A) The asymmetric signal propagates to the cytoplasmic domain and causes that subunit to become methylated. Methylation is then induced in the non-signaling subunit. B) The asymmetric signal is converted to a symmetric signal before it reaches the signaling and adaptation domains of the dimer. C) A signal carried by one subunit of an asymmetrically signaling dimer leads to methylation of closely associated dimers. The solid arrows indicate events occurring within a dimer and the dashed arrows indicate events occurring between dimers.

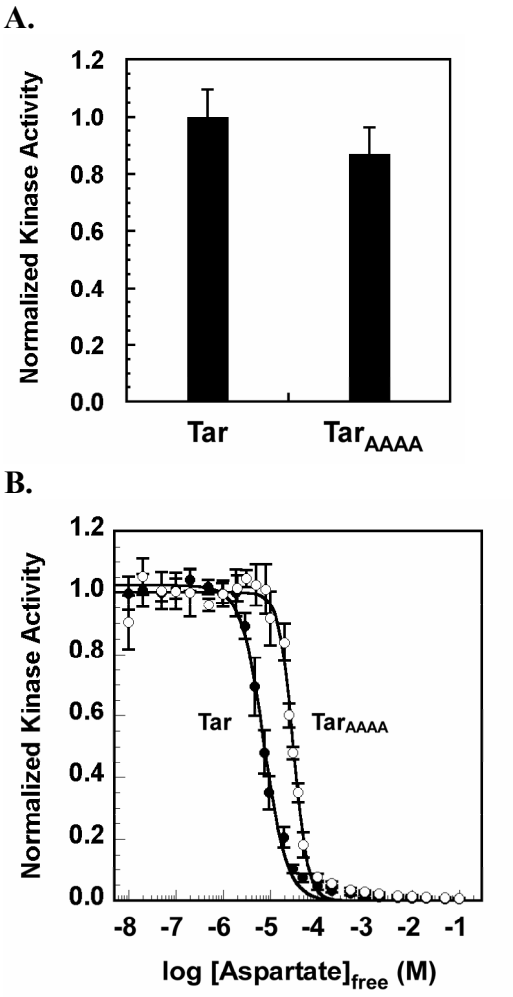

Figure 12. Normalized kinase activity and K_i curves for wild type Tar and Tar_{AAAA}.

Tar_{AAAA} stimulates CheA activity *in vitro* and can be inhibited by aspartate. A) *In vitro* receptor-coupled CheA activity assays with wild-type Tar and Tar_{AAAA}. The error bars represent the standard deviation of the mean, with n = 3. B) Aspartate inhibition of receptor-coupled CheA activities over a range of aspartate concentrations from 0.01μm to 100mM for wild-type Tar (filled circles) and Tar_{AAAA} (open circles). Calculated K_i values were 7±1 μM for wild-type Tar and 30 ± 1 μM for Tar_{AAAA}. The error bars represent the standard deviation of the mean, with n = 3.

values were 0.3±0.1 μM, 4±1 μM, and 350±50 μM (R. R. Draheim and R.-Z. Lai, unpublished results). Thus, in the receptor-coupled CheA assay,$Tar_{\mathbf{AAAA}}$ behaves more like $Tar_{\mathbf{DDDD}}$ or $Tar_{\mathbf{QEQE}}$ than like either $Tar_{\mathbf{EEEE}}$ or $Tar_{\mathbf{QQQQ}}$.

When the Ala substitutions were introduced into the non-V5-tagged partner in the *in vivo* complementation experiments (Fig. 13A), they affected two aspects of aspartate chemotaxis in capillary assays (Fig. 13C). First, the maximal accumulation was seen in capillaries containing 1 mM rather than 10 mM aspartate. Second, the numbers of cells at the peak were only 50% and 30% of the peak accumulation of wild-type cells, respectively, when the non-signaling subunit and the signaling subunit contained the four Ala residues.

The *in vivo* methylation data show that a mutant V5-tagged subunit paired with a complementing mutant $Tar_{\mathbf{AAAA}}$ subunit has a normal basal level of methylation in the absence of aspartate (Fig. 13B; compare with Fig. 10B). Making the reasonable assumption that a significant fraction of the V5-tagged monomers are present in heterodimers with a $Tar_{\mathbf{AAAA}}$ partner, this result suggests that having Ala residues at the methylation sites in one subunit of the heterodimer did not alter the basal level of methylation of the opposing subunit significantly. The final aspartate-adapted levels of methylation were also similar for V5-tagged signaling and non-signaling subunits when they were paired with subunits with glutamyl residues or alanyl residues at the positions that are normally methylated. These results argue against option A, although they do not preclude a difference in the rate of methylation between the signaling and non-signaling subunits (see below). They also suggest that the defects in chemotaxis seen with cells

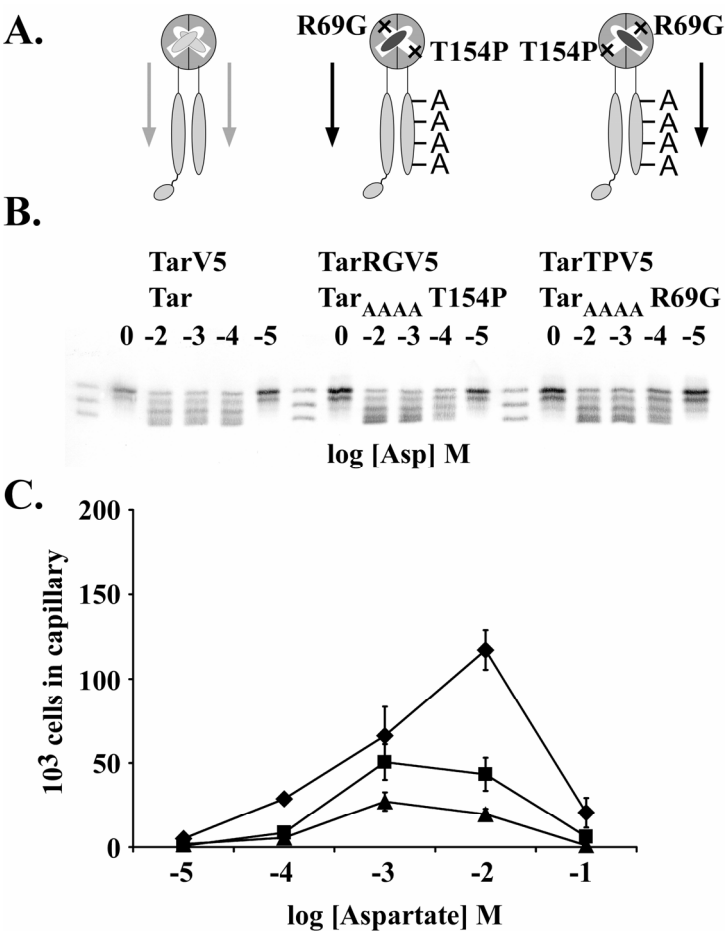

Figure 13. Properties of V5-tagged mutant Tar proteins coexpressed with complementing mutant Tar$_{\mathbf{AAAA}}$ proteins.

A) Representation of heterodimers present when complementing Tar subunits, one of which is Tar$_{\mathbf{AAAA}}$, are coexpressed. One intact aspartate-binding site and one methylation-competent V5-tagged subunit are present in each heterodimer. B) *In vivo* methylation assays with SW02 cells expressing the combinations of Tar proteins shown in 6A. Standards are as in Figure 8B. C) Capillary assays of TarV5 coexpressed with Tar (diamonds), TarRGV5 coexpressed with Tar$_{\mathbf{AAAA}}$ T154P (squares), and TarTPV5 co-expressed with Tar$_{\mathbf{AAAA}}$ R69G (triangles). The assays were done in duplicate and repeated three times. The error bars represent the standard deviation of the mean, with n = 6.

expressing the Tar_{AAAA} subunit are due to the reduced adaptive capacity of heterodimers with only one methylatable subunit rather than to any gross change in their signaling behavior.

The adaptation times of cells expressing Tar_{AAAA} are longer. A tethered cell assay was performed to examine directly whether cells expressing Tar_{AAAA} as one of two complementing subunits were slower to adapt to temporal changes in aspartate concentration than are cells expressing Tar_{QEQE}. Somewhat surprisingly, cells expressing only Tar_{AAAA} exhibited normal run/tumble patterns while swimming and no significant change in the swimming behavior was seen when Tar_{AAAA} was coexpressed with wild-type Tar (data not shown).

When tethered cells expressing either TarRGV5 with Tar T154P or TarTPV5 with Tar R69G were exposed to 1 mM aspartate, exclusively CCW rotation ensued and was maintained for 7-8 min. In a parallel experiment, in which the non-V5-tagged mutant subunit was Tar_{AAAA}, the mean response time was 10-11 min. This 50% increase in adaptation time could explain the decrement in chemotaxis seen in the capillary assay (Fig. 13C). Since all of the adaptation times are less than 20 min, we became convinced that the banding patterns we observed 20 min after addition of 1 mM aspartate reflect the steady-state adapted level of methylation.

Rates of methylation are similar for the signaling and non-signaling subunits. The methylation experiments described thus far did not explore the kinetics of methylation. We therefore examined the time course of methylation of the V5-tagged subunits in heterodimers for 40 min after the addition of 1 mM aspartate. The increase in

methylation was linear for 7 min for all combinations of proteins examined, whether the non-tagged subunit had wild-type methylation sites (Fig. 14A) or had Ala residues introduced at the methylation sites (Fig. 14B).

Rates of methylation during the first 7 min, expressed as the number of methyl groups added per min per receptor monomer, are given in Table 2. The heterodimers in which both the V5-tagged and non-tagged subunits can be methylated exhibited rates of 0.09 methyl groups per min (for TarRGV5 coexpressed with Tar T154P) and 0.06 methyl groups per min (for TarTPV5 coexpressed with Tar R69G). These values are substantially lower than the rate of 0.12 methyl groups per min for TarV5 coexpressed with Tar. The net change in methyl groups per monomer after 40 min was also lower for TarRGV5 coexpressed with Tar T154P (1.0 methyl groups) and TarTPV5 coexpressed with Tar R69G (0.8 methyl groups) than for TarV5 coexpressed with wild-type Tar (2.0 methyl groups).

The initial rates of methylation for TarRGV5 coexpressed with $Tar_{\mathbf{AAAA}}$ T154P and TarTPV5 coexpressed with $Tar_{\mathbf{AAAA}}$ R69G were 0.13 and 0.11 methyl groups per min, respectively, values quite similar to those obtained with TarV5 coexpressed with wild-type Tar. The total numbers of methyl groups added over 40 min were also similar. Thus, there is no evidence for differential rates of methylation for signaling and non-signaling subunits of a Tar heterodimer with only one intact aspartate-binding site. These data are consistent with the idea that symmetric methylation of the two subunits of a receptor dimer that binds aspartate asymmetrically does not depend upon methylation of the signaling subunit inducing methylation of the non-signaling subunit.

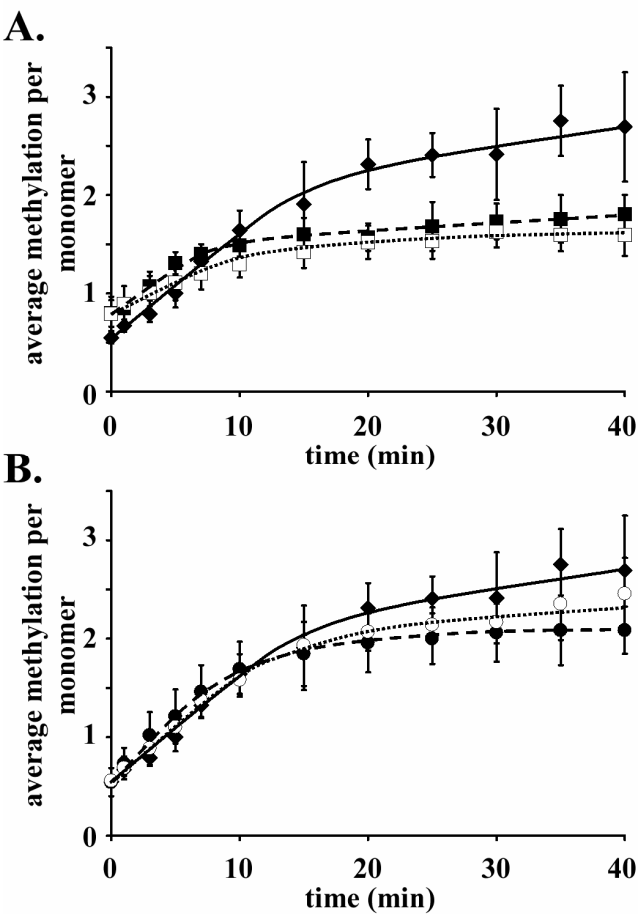

Figure 14. Time-course methylation assays carried out with different combinations of coexpressed Tar proteins.

A) TarV5 coexpressed with Tar (filled diamonds, solid line), TarRGV5 coexpressed with Tar T154P (filled squares, dashed line), and TarTPV5 coexpressed with Tar R69G (open squares, dotted line). B) TarV5 co-expressed with Tar (filled diamonds, solid line), TarRGV5 coexpressed with Tar_{AAAA} T154P (filled circles, dashed line), and TarTPV5 coexpressed with Tar_{AAAA} R69G (open circles, dotted line). In each case, the increase in methylation was linear for at least 7 min after the addition of 1 mM aspartate.

Table 2. Methylation rates of complementing Tar proteins

Tar proteins present	Increase in methyl groups per minute [a]
TarV5 / Tar	0.12 ± 0.01
TarRGV5 / Tar T154P	0.09 ± 0.01
TarTPV5 / Tar R69G	0.06 ± 0.01
TarRGV5 / Tar_{AAAA} T154P	0.13 ± 0.02
TarTPV5 / Tar_{AAAA} R69G	0.11 ± 0.01

[a] Methylation rates are expressed as the increase in the number of methyl groups per monomer per min during the first 7 min after the addition of 1 mM aspartate. The rates for each complement were determined in three separate assays. The mean rate and standard deviation are shown. The data are taken from Figure 7.

Discussion

A heterodimer formed between two complementing mutant Tar monomers has only one functional aspartate-binding site (Fig. 10A). An *E. coli* wild-type Tar homodimer also binds only one molecule of aspartate because of strong negative cooperativity (18). Thus, the complemented heterodimer and wild-type Tar should behave very similarly except at the very highest concentrations of aspartate, at which both sites of the wild-type dimer might be occupied. Previous studies utilizing complementing mutant Tar proteins have focused on asymmetry in transmembrane signaling (41, 42, 121). In this study, we

queried whether the adaptive methylation that occurs in response to aspartate binding is asymmetric or symmetric.

We first measured the levels of steady-state methylation in each subunit of mutant Tar heterodimers after adaptation to aspartate. We distinguished the two subunits of complementing heterodimers by tagging one with a fourteen-residue long V5 epitope fused at the native C-terminus of Tar via a seven-residue flexible linker (see Materials and methods for details). These studies demonstrated that the V5-tagged versions of wild-type Tar and the coexpressed Tar R69G and T154P mutant proteins had essentially normal pre-stimulus methylation levels.

We envisioned three possible responses to an asymmetric transmembrane signal. 1) The signaling subunit could achieve a higher steady-state level of methylation. 2) The non-signaling subunit could achieve a higher steady-state level of methylation. 3) The two subunits could be methylated equally. The third alternative is correct (Fig. 10B). The lower level of methylated mutant receptors relative to wild-type Tar may be due to the existence of homodimers of the mutant receptors (Fig. 8 and Fig. 14A), which will not bind aspartate, and which will therefore not increase their level of methylation.

Symmetric steady-state methylation in aspartate-saturated receptors can potentially occur by several different mechanisms, which are illustrated in Figure 11. The initial methylation could be asymmetric, with the more highly methylated subunit then inducing methylation of its partner (Fig. 11A). We believe this possibility unlikely based on two lines of evidence. First, when either the signaling or non-signaling Tar subunit contained alanyl residues at the four positions at which methylation normally occurs ($Tar_{\mathbf{AAAA}}$; 87), the V5-tagged partner subunit was methylated to the same degree as when it was co-

expressed with a complementing mutant protein with normal methylation sites. The alanyl residues of Tar_{AAAA} cannot be methylated, so this result suggests that changes in methylation of one subunit are not required for changes in methylation of the opposing subunit. Second, the methylation rates of the non-signaling and signaling subunits were similar, whether the normal methylation sites were intact (Fig. 14A; Table 2) or in the Tar_{AAAA} configuration (Fig. 14B; Table 2) in the non-tagged subunit.

With this first possibility eliminated, the alternatives presented in Figure 11B and 11C remain. Option B suggests that the asymmetric signal in the periplasmic domain is converted into a symmetric signal in the cytoplasmic domain. Option C hinges upon the phenomenon of transmethylation. Transient methylation of non-signaling receptors has been observed *in vivo* (99), and CheR bound to one receptor monomer can methylate both its partner subunit within the dimer and subunits in adjacent dimers (65, 70). Thus, it is possible that the signal induced by aspartate binding is transmitted to only one cytoplasmic domain of the heterodimer but that this signal is then propagated among the cytoplasmic domains of a larger assemblage of receptor dimers, thereby leading to the methylation of all subunits.

Option C runs into immediate difficulty. Several published reports indicate that Tar and Tsr form mixed trimers of dimers (6, 119). If so, then option C predicts that Tsr should also achieve a higher steady-state level of methylation after adaptation to saturating concentrations of aspartate, but that is not the case (46, 99, 114). Also, we find that some Tar polypeptides, which we think are mutant homodimers that cannot bind aspartate, do not increase their level of methylation after addition of aspartate to cells expressing two complementing mutant forms of Tar (Fig. 10B and Fig. 14A). Therefore,

we favor option B, which postulates that the signal associated with attractant binding directly affects both subunits of a Tar dimer.

The conformational change associated with ligand binding is propagated through the membrane by one subunit of the dimer (42, 121). This change then alters the interaction of the two HAMP linker domains of the dimer to generate a conformation change in both cytoplasmic domains. A similar interpretation was reached by Zhu and Inouye (137) in their study of signaling by Tar-EnvZ chimeras. How can this idea be reconciled with earlier reports (42) that a Tar heterodimer with only one full-length cytoplasmic domain signals in response to aspartate binding only if the "major" binding site is intact in the full-length subunit?

We have no definitive answer, but we note that the truncated subunit in the heterodimers examined in the earlier work of our group (42) terminates six residues before the predicted end of the second amphipathic helix (ASII) of the linker (131). The absence of these six residues could weaken interactions between the two HAMP linkers of the heterodimer. In that case, the HAMP linkers from the full-length subunits of two neighboring heterodimers could interact, so that the functional unit would actually be a receptor tetramer. Each of the two subunits contributing to a cytoplasmic dimer that interacts with CheA and CheW would be connected to a different aspartate-binding periplasmic dimer. We suspect that the activity of Tar-EnvZ heterodimers with one Tar HAMP linker and one EnvZ HAMP linker per dimer (137) may also function by having homologous linkers from adjacent dimers interact with each other. Chemical crosslinking experiments could be employed to test this hypothesis.

Although the observation is somewhat tangential to the main argument presented here, a notable feature of the behavior of $Tar_{\mathbf{AAAA}}$ was that, in the *in vitro* receptor-coupled CheA assay, it behaved more like $Tar_{\mathbf{QEQE}}$ than like $Tar_{\mathbf{QQQQ}}$. Furthermore, $Tar_{\mathbf{DDDD}}$, in which the methylation sites are occupied by negatively charged aspartyl residues, also behaved more like $Tar_{\mathbf{QEQE}}$ than like $Tar_{\mathbf{EEEE}}$ in this assay. Thus, it is clear that both the charge and the size of the residues at the methylation sites have profound effects on both the intrinsic activity of the receptors in stimulating CheA and in the ability of the activity to be inhibited by an attractant ligand. This point clearly deserves more intensive and extensive investigation.

The experiments described here were performed with cells that contain the full arsenal of Che proteins. We note that CheA and CheW, which may tie trimers of dimers together to form a higher-order assemblage of receptors (74), could have a profound effect on the receptor signaling mechanism. For example, Li and Weis (69) found that the covalent modification state of the Tsr receptor had a large effect on the serine $K_{\mathbf{i}}$ of the Tsr/CheA/CheW ternary complex. In contrast, Dunten and Koshland (32) found that covalent modification had little effect on the aspartate K_d of inner membrane enriched Tar. It will be informative to examine whether, in the absence of CheA and CheW, Tar still induces symmetric methylation in response to asymmetric ligand binding.

CHAPTER III

NEITHER CHEA NOR CHEW IS REQUIRED FOR CONVERSION OF ASYMMETRY TO SYMMETRY DURING TRANSMEMBRANE SIGNALING BY THE *E. COLI* TAR CHEMORECEPTOR DIMER

Summary

Within ternary signaling complexes containing CheA and CheW, the asymmetric signal generated by aspartate binding to the periplasmic domain of a Tar homodimer results in symmetric methylation of the two subunits in the cytoplasmic domain. This result leaves open the question of whether symmetric methylation requires the intervention of CheA and/or CheW. In Tar R69G / Tar T154P heterodimers, the subunit containing the R69G substitution is the "signaling" subunit within the periplasm. Using a C-terminal V5 epitope tag to distinguish between the two subunits of such heterodimers, we have shown both subunits become methylated in response to aspartate addition even in the absence of CheA and CheW. Unlike the situation in ternary complexes, however, the non-signaling (T154P) subunit was methylated more quickly. This difference disappeared when the non-tagged subunit in a heterodimer had its four methylatable glutamyl residues replaced with alanyl residues. Thus, the prior state of covalent modification affects the communication between Tar subunits within a dimer that occurs in response to aspartate binding when Tar is not complexed with CheA and CheW.

Introduction

Bacterial chemoreceptors are the sensory components of a signal transduction pathway that controls the activity of the CheA kinase and thereby the levels of the

phosphorylated response regulator proteins CheY and CheB. Phosphorylated CheY (CheY-P) binds to FliM in the switch complex of the flagellar motor. The number of FliM proteins occupied by CheY-P determines the clockwise/counterclockwise (CW/CCW) bias of the flagellar motor, with more CheY-P leading to more CW rotation, and hence more tumbling by the cell. (For a review of chemotaxis in *E. coli* and *Salmonella*, see reference 120).

The countervailing activities of CheR, a constitutively active methyltransferase, and CheB, a methylesterase activated through phosphorylation by CheA, control the methylation level of the chemoreceptors, which are also known as methyl-accepting chemotaxis proteins (MCPs). Four conserved glutamyl residues are the targets for methylation. Two of these positions contain glutaminyl residues in the initial translation product and are subsequently deamidated by CheB-P. Binding of attractant ligands inhibits the CheW-dependent ability of a receptor to stimulate CheA activity, and an attractant-induced increase in the level of methylation of the cognate receptor then restores its ability to stimulate CheA and results in adaptation to the initial attractant stimulus.

The Tar chemoreceptor is a homodimeric protein (82) in which each subunit comprises a periplasmic ligand binding domain, a transmembrane (TM) domain composed of two hydrophobic α-helices (TM1 and TM2), and a cytoplasmic signaling domain that is joined to TM2 via a HAMP linker (10, 131). In the absence of any other chemotaxis proteins, the basic unit of organization of the receptors is a trimer of dimers (56, 119). The addition of the soluble cytoplasmic proteins CheW and CheA leads to the formation of polarly localized clusters of receptors (74).

Tar is the receptor responsible for chemotaxis towards aspartate and maltose. Aspartate binds to Tar in one of two rotationally symmetric binding sites across the dimer interface (79) that exhibit strong negative cooperativity (18). Maltose chemotaxis requires an intermediary binding protein that binds to maltose which then interacts across the top of the Tar periplasmic domain.

The signal associated with ligand binding is thought to be a small (~1.5 Å) downward movement of helix 4 of the periplasmic domain, which is propagated through TM2 and the linker to the cytoplasmic signaling domain, which then down regulates the activity of the CheA kinase. Because of the initial asymmetry in the signal, aspartate sensing only requires one of the two aspartate-binding sites. Functional Tar heterodimers can be generated by coexpressing proteins in each of which a different half of the aspartate-binding pocket is disrupted (39). Such heterodimers transmit the conformational signal through the subunit in which the majority binding half-site, containing residues Arg-64 in helix 1 and Tyr-149, Gln-152 and Thr-154 in helix 4, is intact (42, 121).

We have recently shown (Chapter II) that this initially asymmetric signal leads to methylation of the cytoplasmic domains of both the signaling and non-signaling subunit when the chemotaxis signaling pathway is intact, *i.e.*, in cells containing all six of the cytoplasmic Che proteins (A, W, R, B, Y and Z). It was unclear, however, to what extent incorporation of the heterodimeric Tar into the receptor patch (74), which has been shown to contain all six of the Che proteins, was responsible for the conversion of the asymmetric periplasmic signal (aspartate binding) into a symmetric cytoplasmic response (methylation).

Here, we report that even in the absence of CheA and CheW, which are required for

formation of the receptor patch, the asymmetric binding of aspartate leads to methylation of both subunits of the heterodimer. However, unlike the situation in the patch, the signaling and non-signaling subunits become methylated at different rates. When one of the subunits in the heterodimer contains alanyl residues instead of methylatable glutamyl residues, the difference between the rates of methylation of the signaling and non-signaling subunits disappeared. However, when the glutamyl residues in one subunit of the heterodimer were replaced with negatively charged but non-methylatable aspartyl residues, the asymmetry in methylation rates between the signaling and non-signaling subunits was still observed. Furthermore, under these conditions both the signaling and non-signaling subunits were methylated more quickly than when both subunits contained glutamyl residues. These data are discussed in terms of a model for transmembrane signaling and the subsequent interactions between the two subunits of a receptor dimer and the three dimers that make up the putative trimer of receptor dimers (56).

Materials and methods

Bacterial strains and plasmids. Strain SW02 (*thr+ eda+ tsr7021 trg::Tn10 Kan Δtar-tap5201*) is a derivative of strain RP437 (94). Strain RP3098 (111) lacks four transducers (Tsr, Tap, Tar, Trg) as well as all Che proteins. Strain AB2898 is a derivative of RP2898 (*ΔcheA-cheW-tar-tap*) transduced with P1 lysate from SW02 making AB2898 (eda^+ Δ*tsr7021* Δ*cheA-cheW-tar-tap*) Plasmid pMK113, a derivative of pBR322, contains the *E. coli tar* gene expressed from a mutant *meche* (*tar tap cheR cheB cheY cheZ*) operon promoter with a somewhat decreased efficiency of transcription initiation (39). Plasmid pRBB16, a pACYC184 derivative, contains *E.coli tar* expressed from the

native *meche* promoter. The difference in expression levels of the two promoters compensates for the difference in copy number of the two plasmids used. Plasmid pMK113CV5 was constructed via a PCR based extension of the 3' end of tar with a seven amino acid linker (GGSSAAG) and a C-terminal V5 epitope tag (GKPIPNPLLGLDST). Plasmid pRBB164A was cloned from pRA126 (87) via PCR amplification and inserted into pRBB16 using the NdeI and EcoRI restriction sites. Plasmid pRBB164D was cloned from pNI156 (88) via PCR amplification and inserted into pRBB16 using the NdeI and EcoRI restriction sites. Plasmid missense mutations encoding the R69G and T154P were made using site-directed mutagenesis (Strategene).

Tethered-cell assay. Overnight cultures were grown at 32^{o}C in tryptone broth (TB; 80) containing 25 μg/ml ampicillin and 7.5 μg/ml tetracycline. These cultures were then diluted 1:100 in 15 ml of the same medium and grown to OD_{590nm} of 0.6. Ten ml of the cells were sheared in a 50 ml stainless steel cup of a Waring blender using the low-speed setting for 8 repetitions of 7 sec bursts of blending interspersed with 13 sec breaks to prevent overheating of the sample. Sheared cells were pelleted at the highest speed setting of a table-top centrifuge for 5 min and washed twice with 5 ml of tethering buffer (10mM potassium phosphate [pH 7.0], 0.1 M NaCl, 0.01 mM EDTA, 0.02 mM L-methionine, 20 mM sodium D-lactate, and 200 μg/ml chloramphenicol). The final pellet was resuspended in 2.5 ml tethering buffer. Tethered cells were then prepared and assayed for chemotactic responses at room temperature (22-24^{o}C) as described previously (128). The responses of at least 20 cells were averaged for each strain tested.

Determination of the *in vivo* methylation state of Tar. Our assay was based on a published method (129). Overnight cultures of strain SW02 (deleted for the four

chemoreceptor genes *tsr*, *tar*, *tap* and *trg*) containing two compatible plasmids encoding different mutant versions of Tar were grown as for the capillary assay. Cells were harvested by centrifugation, washed three times with 10 ml of chemotaxis buffer, and finally resuspended in 5 ml of chemotaxis buffer containing 10 mM sodium D-lactate and 200 μg/ml chloramphenicol. The OD_{590nm} was adjusted to 0.4, and one ml aliquots were transferred to 10 ml scintillation vials and incubated with shaking at 32^{o}C for 40 min. Aspartate was then added at concentrations from 0.01 to 10 mM, and the cells were incubated for an additional 20 min. Control reactions received an equal volume of buffer. Reactions were terminated by addition of 100 μl ice-cold 100% trichloracetic acid (TCA), followed by incubation on ice for 15 min. Proteins were pelleted by centrifugation for 15 min in a microcentrifuge, washed with 0.5ml cold 1% TCA, and subsequently washed with 0.5 ml acetone. The samples were allowed to dry overnight at room temperature and then resuspended in 200 μl 2X SDS-loading buffer. A 20 μl aliquot of each sample was loaded onto a 7.5% SDS gel.

Following electrophoresis, the proteins were transferred to 0.2 micron nitrocellulose and subjected to immunoblotting and visual detection by antibody against the V5 epitope (Invitrogen), using GAM-AP (BioRad) as the secondary antibody. The immunoblots were then scanned into a computer and the densities of the bands analyzed with the program ImageJ (http://rsb.info.nih.gov/ij/). First, the total density of all the bands a particular lane was measured. Then the individual bands were analyzed, and the relative density of each band as a fraction of the total density was calculated. The individual bands were assigned a methylation state by comparing them to a TarV5 standard comprise of three different covalent modification states: EEEE, QEQE, and the fully

amidated QQQQ. The EEEE species migrates slowest, the QEQE form migrates at an intermediate rate, and the QQQQ form moves fastest. The total mean population level of methylation at each time point was calculated from Equation 1:

$$M_t = (M_1 + 2M_2 + 3M_3 + 4M_4)/R_t \qquad \text{(Equation 1)}$$

where Mt is the mean total number of methyl groups per total number of receptors present, M_1 is the band-density of the singly methylated species, M_2 is the band-density of the doubly methylated species, M_3 is the band-density of the triply methylated species, M_4 is the band-density of the quadruply methylated species, and R_t is the sum of the receptor band-densities, including the unmethylated form M_0.

Time-course methylation assay. The methylation assays were carried out as above, with the following modifications. The final resuspension of washed cells at an OD_{590nm} of 0.4 was placed in a 125 ml Erlenmeyer flask in a 32^{o}C water bath and agitated by vigorous swirling. L-aspartate was added to 1 mM, and 1 ml aliquots were removed at various times and added to 1.5 ml Eppendorf tubes pre-loaded with 100 μl of 100% TCA and put on ice. The samples were then prepared for immunoblotting and densitometric scanning as described above.

***In vitro* receptor-coupled CheA kinase assays with inner membranes containing overexpressed Tar**. Cells were grown, inner membranes prepared, and receptor-coupled CheA activities determined *in vitro* exactly as described previously (31). Production of radiolabeled CheY-P was linear over 20 sec and directly proportional to the amount of Tar over a range from 5 pmol to 40 pmol. We chose to use 20 pmol Tar in each reaction because this value is in the middle of the linear range, allowing us to measure increases or decreases in CheY-P production accurately. Analysis of the titration curves for

aspartate inhibition was performed according to Bornhorst and Falke (20) with the modifications of Draheim *et al.* (31).

Results

In vivo methylation patterns of Tar in the absence of CheA and CheW.

Chemoreceptors cluster tightly in patches at the cell pole in the presence of CheA and CheW, whereas in their absence polar localization of receptors still occurs but tight clustering is impaired. Since clustering should affect accessibility of the receptors to the adaptation proteins CheR and CheB, we decided to compare the baseline methylation state of Tar in the presence and absence of CheA and CheW. Different methylated forms of TarV5, which carries a 14-residue V5-epitope tag and 7-residue flexible glycine-rich linker inserted immediately in behind the C-terminal NWETF pentapeptide that binds CheR, were visualized with anti-V5 antibody. Two things became immediately apparent when TarV5 was coexpressed with wild-type Tar in strain SW02 (Δ*tar-tap*, Δ*tsr*, Δ*trg*) and strain AB2898 (Δ*tsr* Δ*cheA-cheW-tar-tap*). First, because of the absence of receptor-stimulated CheA kinase activity in strain AB2898, there is presumably no phospho-CheB, and therefore little methylesterase activity. As expected, basal methylation levels are correspondingly higher in strain AB2898 than in strain SW02: 0.55 ± 0.06 methyl groups per monomer (CH_3/monomer) in the former strain and 2.17 ± 0.01 CH_3/monomer in the latter (Fig 15A). Second, the rate of methylation after addition of 1 mM aspartate decreased from 0.11 ± 0.02 methyl groups added per minute per monomer (CH_3/min) in strain SW02 to 0.037 ± 0.001 CH_3/min in strain AB2898 (Fig. 15A). The rate of

methylation in strain SW02 was linear for 7 min, whereas in strain AB2898 it was linear for at least 20 min.

The R69G and T154P substitutions target residues in the minor and major aspartate-binding half-sites of Tar, respectively. When the V5-tagged and non-tagged forms of these proteins were coexpressed, they had basal methylation levels of 2.22 ± 0.04 and 1.94 ± 0.01 CH_3/monomer, respectively. As anticipated, neither of these proteins showed a substantial increase in methylation 0.003 ±0.004 and 0.003 ±0.001 CH_3/min, respectively upon addition of aspartate (Fig. 15A).

R69G and T154P Tar complement and support methylation of both subunits of the heterodimer. Previous work (42, 121) demonstrated that heterodimers containing mutant proteins with alterations in the major and minor aspartate-binding half-sites conduct the transmembrane signal through the subunit that contains the intact major binding site (Thr-154 in our case). We have also previously shown that both TarV5 R69G (TarRGV5) coexpressed with Tar T154P and TarV5 T154P (TarTPV5) coexpressed with Tar R69G show increased methylation *in vivo* in the presence of CheA and CheW after addition of aspartate (Chapter II). Our conclusion was that both the signaling and non-signaling subunit of such a heterodimer become methylated equally well, leading us to propose that an asymmetric transmembrane signal is converted into a symmetric signal in the cytoplasmic domain of Tar.

We repeated this experiment in the absence of CheA and CheW (Fig. 15B). TarRGV5 coexpressed with Tar T154P had an initial methylation level of 2.06 ± 0.03 CH_3/monomer and TarTPV5 coexpressed with Tar R69G had an initial methylation level of 2.09 ± 0.14 CH_3/monomer (Fig. 15B). These values are nearly identical to the

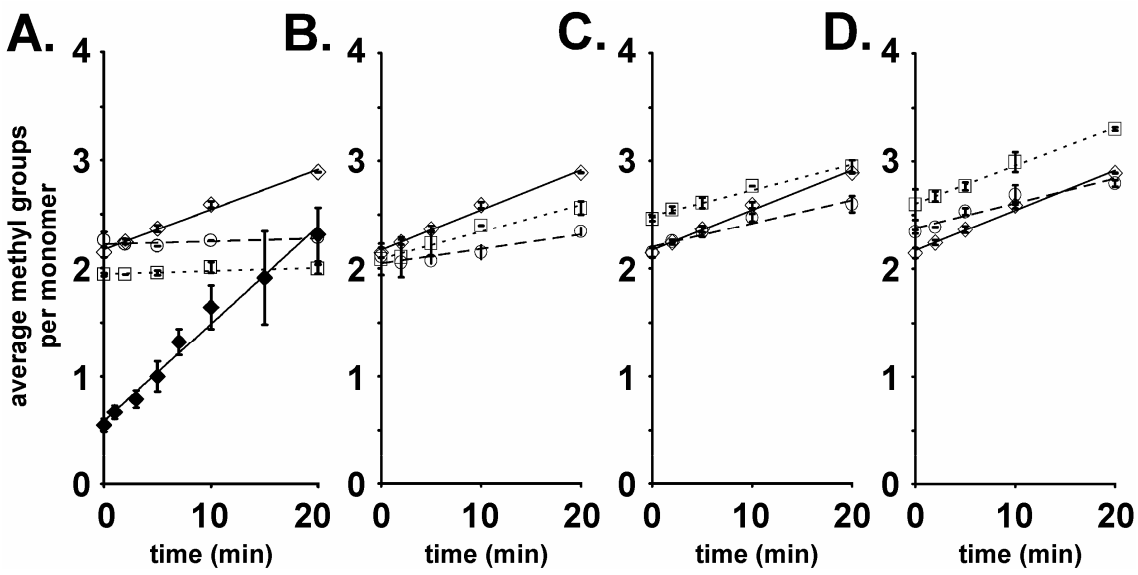

Figure 15. *In vivo* methylation assay of complementing TarV5 and Tar mutants in the absence of ternary complex.

A) *In vivo* methylation in response to 1mM aspartate of coexpressed TarV5 and Tar proteins in the presence (closed diamonds) and absence (open diamonds) of CheA and CheW. Coexpressed TarRGV5 / Tar R69G (open circles) and TarTPV5 / Tar T154P (open squares) did not increase their methylation to 1 mM aspartate. B) Methylation rate observed with coexpressed complementing binding mutations to 1mM aspartate. The rate of the signaling subunit (open circles, dashed line) and non-signaling subunit (open squares, dotted line) are markedly different from each other and less than the co-expressed TarV5 and Tar (open diamonds, solid line). C) *In vivo* methylation rate of complementing Tar subunits, one of which is $Tar_{\mathbf{AAAA}}$, are coexpressed. The rate of the signaling subunit (open circles, dashed line) and non-signaling subunit (open squares, dotted line) are similar but less than the coexpressed TarV5 and Tar (open diamonds, solid line). D) *In vivo* time course methylation assay of complementing Tar subunits, one of which is $Tar_{\mathbf{DDDD}}$, are coexpressed. Methylation rates of the signaling (open circles, dashed line) and non-signaling (open squares, dotted line are different from each other. The rates of the non-signaling subunit and TarV5 coexpressed with Tar (open diamonds, solid line) are nearly identical with a difference noted in initial methylation levels.

value of 2.17 ± 0.01 CH_3/monomer for TarV5 coexpressed with wild-type Tar (Fig. 15). However, the behavior after addition of 1 mM aspartate was quite different for the three sets of proteins. The methylation rates were 0.037 ± 0.001 CH_3/min for TarV5 / Tar, 0.025 ± 0.006 CH_3/min for TarTPV5 / Tar R69G, and 0.013 ± 0.002 CH_3/min for TarRGV5 / Tar T154P. The intriguing result was that, in the heterodimers, the non-signaling subunit had an aspartate-induced methylation rate nearly twice that of the signaling subunit. The opposite relationship was observed when these same heterodimers were examined in the *in vivo* methylation assay in the presence of CheA and CheW (Chapter II). TarTPV5 / Tar R69G had an aspartate-induced methylation rate of 0.06 ± 0.01 CH_3/min and TarRGV5 / Tar T154P had an aspartate-induced methylation rate of 0.09 ± 0.01 CH_3/min. Note, however, that the rates in the presence of CheA and CheW are still significantly higher than in their absence.

Alanine substitutions at methylation sites of the non-tagged monomer do not perturb methylation of the V5-tagged subunit. We previously found that the methylation of TarTPV5 in the presence of CheA and CheW occurred normally when the four glutamyl residues in a complementing Tar R69G subunit were replaced with alanyl residues to create the $Tar_{\mathbf{AAAA}}$ protein (Chapter II). That study was conducted to see if aspartate-induced methylation of the non-signaling (T154P) subunit depends on a change the methylation state of the opposing, signaling subunit. To see if this same behavior is observed in the absence of CheA and CheW, we repeated the experiment using strain AB2898. The basal methylation levels of the two subunits were somewhat different: 2.20 ± 0.03 CH_3/monomer for TarRGV5 and 2.49 ± 0.01 CH_3/monomer for TarTPV5 (Fig.

15C). However, the methylation rates after the addition of 1 mM aspartate were very similar: 0.022 ± 0.004 CH_3/min for TarRGV5 and 0.024 ± 0.004 CH_3/min for TarTPV5 (Fig. 15C). The rates for both proteins approach the rate of 0.025 ± 0.006 CH_3/min seen with TarTPV5 / Tar R69G when both subunits contained glutamyl residues at the methylation sites and are nearly double the rate of 0.013 ± 0.002 CH_3/min seen with TarRGV5 / Tar T154P under those conditions (Fig. 15B).

Effect of aspartate-for-glutamate substitutions at the methylation sites of the non-epitope-tagged subunit. Using a Tar variant, in which the methylation sites were substituted with alanyl residues ($Tar_{\mathbf{AAAA}}$), as the complementing subunit eliminated the difference between the rates of aspartate-stimulated methylation of the signaling and non-signaling V5-tagged mutant subunits. This result raised the question of whether the removal of methylation sites or the introduction of four uncharged residues at the methylation sites was responsible for this effect. To address this problem, we generated versions of strain AB2898 that contain either Tar RGV5 or Tar TPV5 together with the complementing mutant Tar protein with aspartyl residues at the four methylation sites ($Tar_{\mathbf{DDDD}}$). $Tar_{\mathbf{DDDD}}$ has the same overall charge as $Tar_{\mathbf{EEEE}}$, but its aspartyl residues are not substrates for methylation by CheR (104).

The intrinsic activity of $Tar_{\mathbf{DDDD}}$ was tested in the *in vitro* receptor-coupled CheA kinase activity assay. Wild-type $Tar_{\mathbf{QEQE}}$, $Tar_{\mathbf{AAAA}}$ and $Tar_{\mathbf{EEEE}}$ were also assayed as controls (Fig. 16A). The surprising result was that $Tar_{\mathbf{DDDD}}$ had an activity in the absence of aspartate that was nearly as high as that of $Tar_{\mathbf{QEQE}}$, about the same as that of $Tar_{\mathbf{AAAA}}$, and much higher than that of $Tar_{\mathbf{EEEE}}$. Thus, aspartyl residues are clearly not fully equivalent to glutamyl residues at the methylation sites even in the absence of

methylation. Ligand-inhibition curves revealed an IC50 for aspartate of 0.3 ± 0.1 μM for Tar_{EEEE}, of 4 ± 1 μM for Tar_{DDDD}, of 8 ± 2 μM for Tar_{QEQE}, and of 30 ± 5 μM for Tar_{AAAA} (Fig. 16B). Thus, Tar_{DDDD} behaves more like Tar_{QEQE} than like Tar_{EEEE} by this criterion as well. We used the non-tagged proteins in these assays, since we showed earlier that, although the V5 tag does not interfere with basal CheA-stimulating activity, it prevents aspartate-dependent inhibition of that activity *in vitro* (Chapter II).

Mutant Tar_{DDDD} complements poorly for chemotaxis but supports methylation of a V5-tagged non-signaling subunit. A Tar_{AAAA} variant with one aspartate-binding half-site disrupted by either the R69G or T154P substitution allows reasonably good accumulation of SW02 cells in aspartate-containing capillaries when it is coexpressed with the complementary V5-tagged mutant Tar (Chapter II). We therefore decided to employ the capillary assay (3) to examine whether the Tar_{DDDD} R69G and T154P proteins can function in the same way. The result was striking: there was no accumulation above background levels for SW02 cells expressing any combination of a Tar_{DDDD} protein with a V5-tagged partner, even when Tar_{DDDD} with intact aspartate-binding half-sites was co-expressed with TarV5 with intact aspartate-binding half-sites. TarV5 by itself or co-expressed with wild-type Tar supports an excellent response to aspartate in the capillary assay (A. F. Bormans, unpublished results).

To discover the root of the problem, we tethered SW02 cells expressing Tar_{DDDD}, as their sole chemoreceptor. All of the spinning cells were locked in CCW rotation, which corresponds to incessant smooth swimming. However, coexpression of TarV5 restored some CW rotation, although the cells were still CCW biased relative to cells expressing TarV5 alone. This ability to reverse rotational direction allowed us to look at the

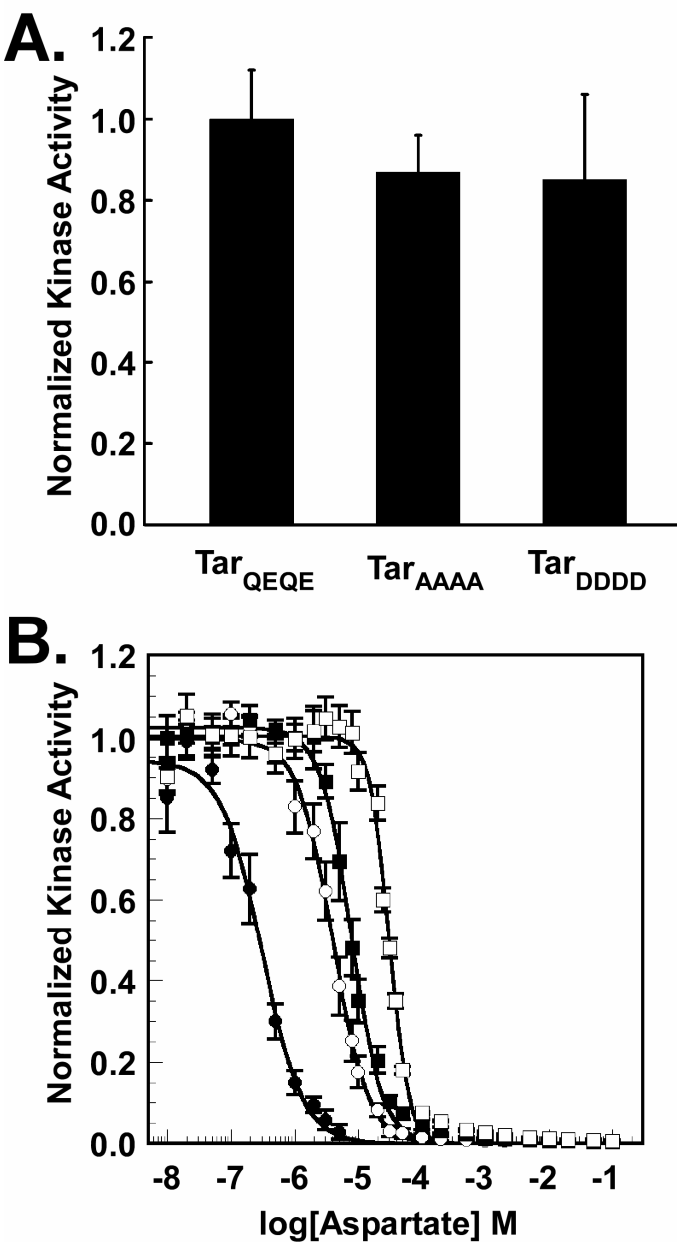

Figure 16. Normalized kinase activity and inhibition curves for wild type Tar, Tar_{AAAA}, and Tar_{DDDD}.

A) *In vitro* CheA kinase activity assay for wild-type Tar, Tar_{AAAA} and Tar_{DDDD} construct. B) Kinase inhibition curves for wild-type (closed squares), Tar_{AAAA} (open squares), Tar_{DDDD} (open circles), and Tar_{EEEE} (closed circles). Calculated IC50 values were 8 ± 1, 30 ± 1, 4 ± 1and 0.03 ± 0.1 μM respectively.

temporal responses to aspartate of cells expressing Tar_{DDDD} variants with V5-tagged Tar variants. We found that the adaptation times of SW02 cells coexpressing Tar_{DDDD} R69G / TarTPV5 or Tar_{DDDD} T154P / TarRGV5 were very much extended, lengthening from 7-8 min for the Tar R69G / TarTPV5 and Tar T154P / TarRGV5 pairings to about 30 min (Fig. 17C).

The basal *in vivo* methylation level of either V5-tagged mutant Tar protein in the presence of its complementary partner was significantly greater than for TarV5 expressed together with wild-type Tar. The level of methylation of the V5-tagged subunit 20 min after addition of various concentrations of aspartate was also greater in SW02 cells expressing either Tar_{DDDD} R69G or Tar_{DDDD} T154P (Fig. 17B).

Complementation by a Tar_{DDDD} binding-pocket variant confers different methylation rates to signaling and non-signaling V5-tagged subunits in the absence of CheA and CheW. The V5-tagged component in the coexpressed protein pairs TarRGV5 / Tar_{DDDD} T154P and TarTPV5 / Tar_{DDDD} R69G in AB2898 cells had similar basal methylation levels of 2.38 ± 0.02 CH_3/monomer and 2.61 ± 0.08 CH_3/monomer, respectively (Fig. 15D). The corresponding methylation rates after addition of 1 mM aspartate were 0.023 ± 0.002 CH_3/min and 0.035 ± 0.007 CH_3/min (Fig. 15D). Thus, complementation by a Tar_{DDDD} binding-site mutant, like complementation by a Tar_{QEQE} binding-site mutant, leads to more rapid methylation of non-signaling (T154P) TarV5 than of signaling (R69G) TarV5 in the absence on CheA and CheW. It is noteworthy that these aspartate-induced methylation rates are higher than those seen with Tar_{QEQE} variants, which were 0.013 ± 0.002 CH_3/min for TarRGV5 / Tar T154P and 0.025 ± 0.006 CH_3/min for TarTPV5 / Tar R69G.

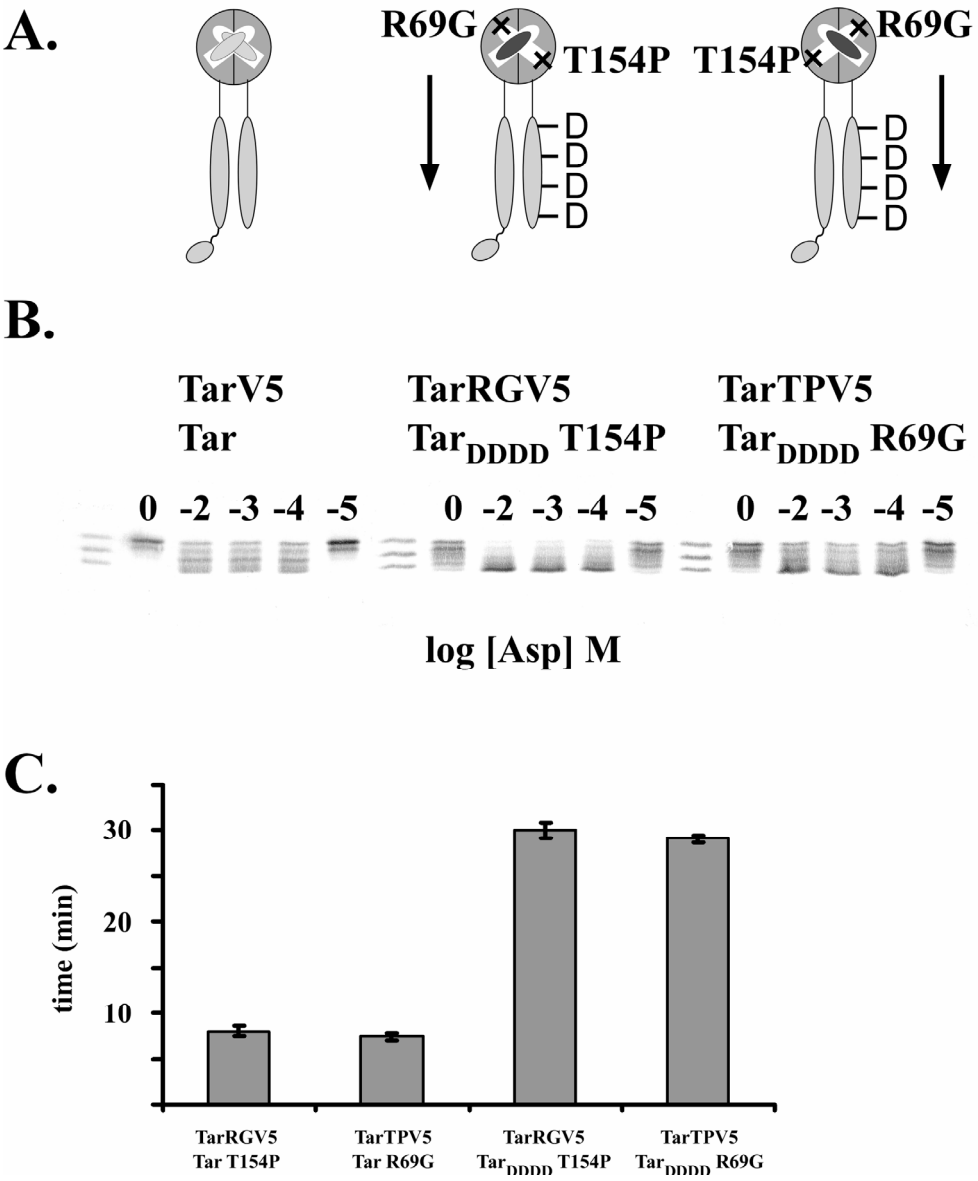

Figure 17. Properties of coexpressed, complementing V5-tagged mutant Tar and Tar_{DDDD} proteins *in vivo*.

A) Model representing the functional heterodimers present in the assay. B) *In vivo* methylation assay allowing an adaptation time of 20 minutes to each concentration of aspartate shown in strain SW02. C) Tethering assay in SW02 of the complementing mutations with half the methylation sites substituted with aspartate as compared to wild-type. Adaptation times are upon the addition of 1mM aspartate.

Pattern in unstimulated and aspartate-induced methylation in the absence of CheA and CheW. The *in vivo* basal methylation levels of the V5-tagged subunits in AB2898 cells varied over a relatively small range, from a low of 2.06 CH_3/monomer with TarRGV5 / Tar T154P to a high of 2.60 CH_3/monomer with Tar TPV5/ $Tar_{\mathbf{DDDD}}$ R69G. Although the differences are not statistically robust, the tendency was for TarV5 paired with $Tar_{\mathbf{QEQE}}$ to have the lowest basal level of methylation, TarV5 paired with $Tar_{\mathbf{AAAA}}$ to have an intermediate basal level, and TarV5 paired with $Tar_{\mathbf{DDDD}}$ to have the highest basal level. In contrast, the rates of methylation differed widely. The data seem to fall into three categories: high, medium and low. Figure 18 shows the aspartate-induced methylation rates normalized to the highest rate observed, which was with the combination of TarV5 / Tar. In this case, all four methylation sites are available in each of the two proteins and both aspartate-binding pockets in the three possible dimers (TarV5 / TarV5, Tar / Tar, and TarV5 / Tar) are intact. The methylation rate of 0.037 induced by 1 mM aspartate in AB2898 cells expressing these two proteins was set to 100% and defined the high-rate group, which also includes TarTPV5 / $Tar_{\mathbf{DDDD}}$ R69G. The medium-rate group, with normalized rates of 60-70%, comprises TarTPV5 / $Tar_{\mathbf{QEQE}}$ R69G, TarRGV5 / $Tar_{\mathbf{AAAA}}$ T154P, TarTPV5 / $Tar_{\mathbf{AAAA}}$ R69G, and TarRGV5 / $Tar_{\mathbf{DDDD}}$ T154P. The low-rate group, with a normalized rate of 35%, consists solely of TarRGV5 / $Tar_{\mathbf{QEQE}}$ T154P. Thus, the TarV5-based monomer increased its methylation level in response to 1 mM aspartate for every combination of binding-site substitutions and configurations of methylation sites in its heterodimer partner. However, the rate of increase varied over a threefold range based on whether the V5-tagged protein was the

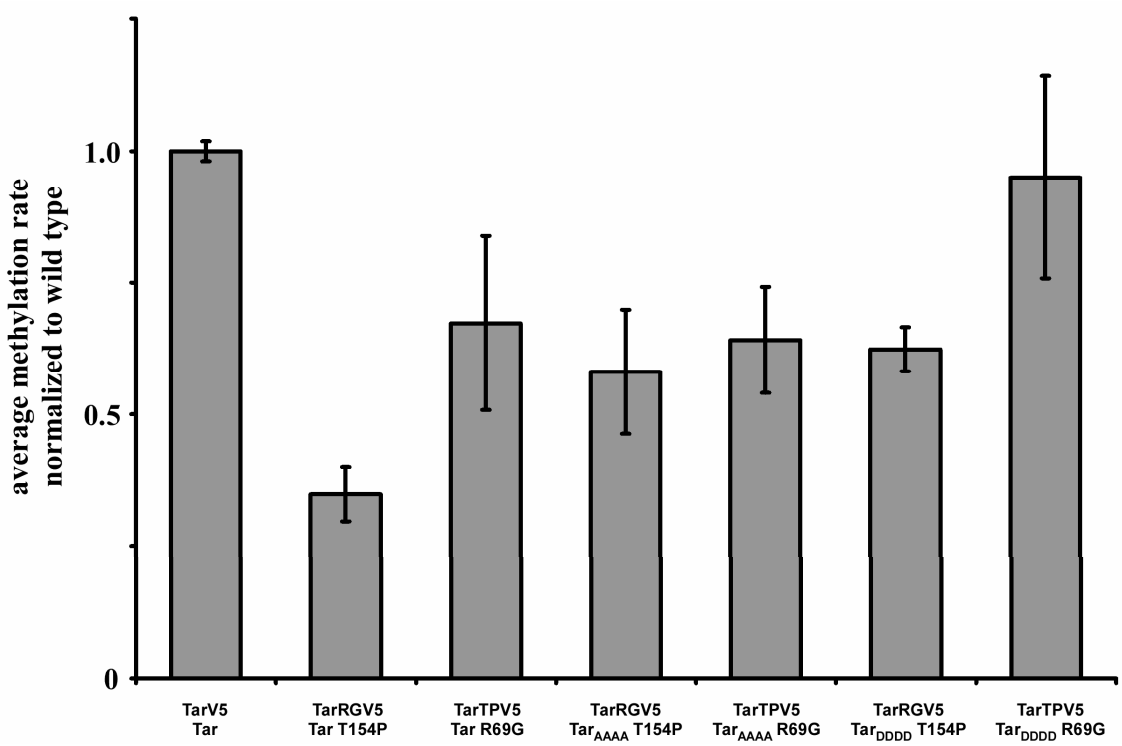

Figure 18. Summation of the methylation rates observed for the various functional heterodimers normalized to the coexpressed TarV5 and Tar.

signaling or non-signaling subunit and on the state of covalent modification at the methylation sites of it partner.

Discussion

Previous experiments had shown symmetrical methylation in response to asymmetric signal but the limitation of that experiment was the feedback from the fluctuation of activated response regulator CheB. This problem was solved by removing the CheA kinase responsible for its activation. Removal of the kinase CheA and CheW also has an additional effect, that of patch disruption. The receptors in these proteins absence are still polarly located but are no longer clustered. Therefore an additional item of communication has been removed by denying the potential ability for trimers of dimers to interact and crosstalk with each other via the coupling protein CheW and CheA. The functional signaling subunits in our experiments are most likely composed in trimers of dimers. We cannot exclude the possibility that crosstalk can occur within trimers of dimers as we do know that interdimer and intradimer mechanisms exist for sharing the localization capability of the c-terminally located CheR binding site of high abundance transducer (65, 70).

The removal of the two cytoplasmic proteins that make up the ternary complex reduces but does not hinder the ability of ligand to cause methylation of the cytoplasmic domain of Tar (Fig. 15A) and agrees with previous results (99). A strength of our assay is the ability to assess the initial methylation levels of the monomer. These levels were also much higher as compared to the presence of CheA and CheW due to the lack of activated

CheB. Complementation experiments with a functional heterodimer in which the methylation levels of the signaling and non-signaling were assessed showed a marked difference in the rate of methylation. First, both subunits were methylated with the addition of 1mM aspartate therefore both subunits must see the conformational change associated with ligand binding. Contrary to our expectations that signaling should remain symmetric an additional surprise was that the rate of the signaling subunit was less than that of the non-signaling subunit (Fig. 15B). Potential explanations ranged from the effects of the c-terminal V5 tag on the localization of CheR and its ability to preferentially methylate the opposite subunit in the dimer to the effects of the binding mutant itself that were previously not apparent in the presence of CheW and CheA. A third possibility was that of the methylation sites themselves had an effect and therefore we used the complementing mutants in conjunction with the alanine substituted methylation sites to see if any effect was made by those sites. Interestingly the rates became similar with both signaling and non-signaling subunits exhibiting about 60% the methylation observed for our wild-type (Fig. 15C, Fig. 18). We thought we were on to something here and therefore we used another methylation site substituted Tar protein this time using the negatively charged aspartate to mimic the charge associated with glutamates at that position. Electrostatic interactions among the cytoplasmic domains of the Tar dimer are thought to play a crucial role in CheA kinase regulation (116). Since the complementing binding mutants with normal and with half sites replaced with alanines worked for chemotaxis we first wanted to see if the $Tar_{\mathbf{DDDD}}$ construct could support chemotaxis in a strain containing the necessary Che proteins. The resulting heterodimers were unable to support chemotaxis in a capillary assay. The lack of

chemotactic ability was best shown by its extremely long adaptation time in a tethering assay (Fig. 17C). Nevertheless increasing methylation to increasing attractant concentration was observed in the *in vivo* methylation assay and therefore we went ahead with the experiment in the strain lacking CheW and CheA. Here we found the same pattern of lower methylation rate for the signaling subunit and that the rates of methylation were increased for both the signaling and non-signaling subunit relative to the complementing mutations with normal methylation sites and (Fig. 15D, Fig. 18). An explanation of the differences in initial levels of methylation and methylation rates observed between TarV5 and Tar resides with the effects of the V5 epitope tag located at the C-terminus. Extensions of Tar are known to affect the rates of methylation, demethylation, and deamidation (64). Furthermore, *in vitro* assays reconstitution assays with the V5 tagged Tar cannot be inhibited with the addition of aspartate (R. R. Draheim, unpublished results) which is similar to *in vitro* experiments performed with a c-terminally truncated serine receptor (R.-Z. Lai, unpublished results). We propose that the positively charged C-terminus of Tar interacts with negative charges associated within the HAMP linker region (Fig. 19A). When ligand binding occurs a conformational change is propagated to the HAMP domain resulting in the release of the C-terminus of Tar. The CheR/CheB c-terminal binding motif is now free to interact with CheR which when bound achieves optimal positioning at the sites of methylation. The V5 tag prevents the association of the HAMP domain with the c-terminal tail in the absence of ligand and therefore the internal CheR/CheB binding site is exposed allowing for a greater local concentration of CheR which results in a higher initial level of methylation (Fig. 19B).

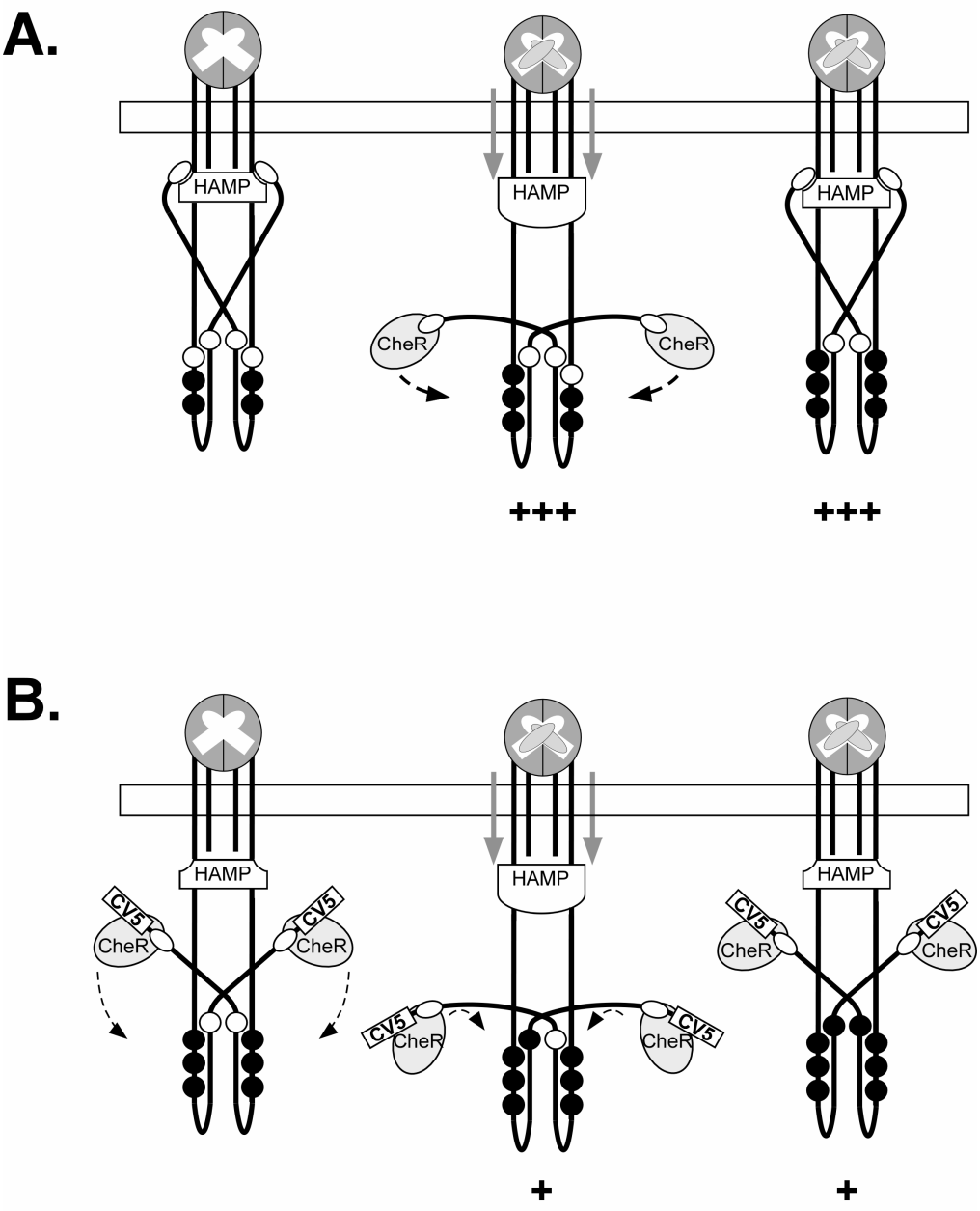

Figure 19. Model for the interaction of the c-terminal tail of the Tar receptor with the HAMP domain.

The presence of the V5 tag at the C-terminus increase the local concentration of CheR leading to an increased initial level of methylation but subsequently interferes with optimal methylesterase activity. The four methylation sites are represented with white circles. Black circles represent added methyl groups. Pluses indicate the rate of methylation.

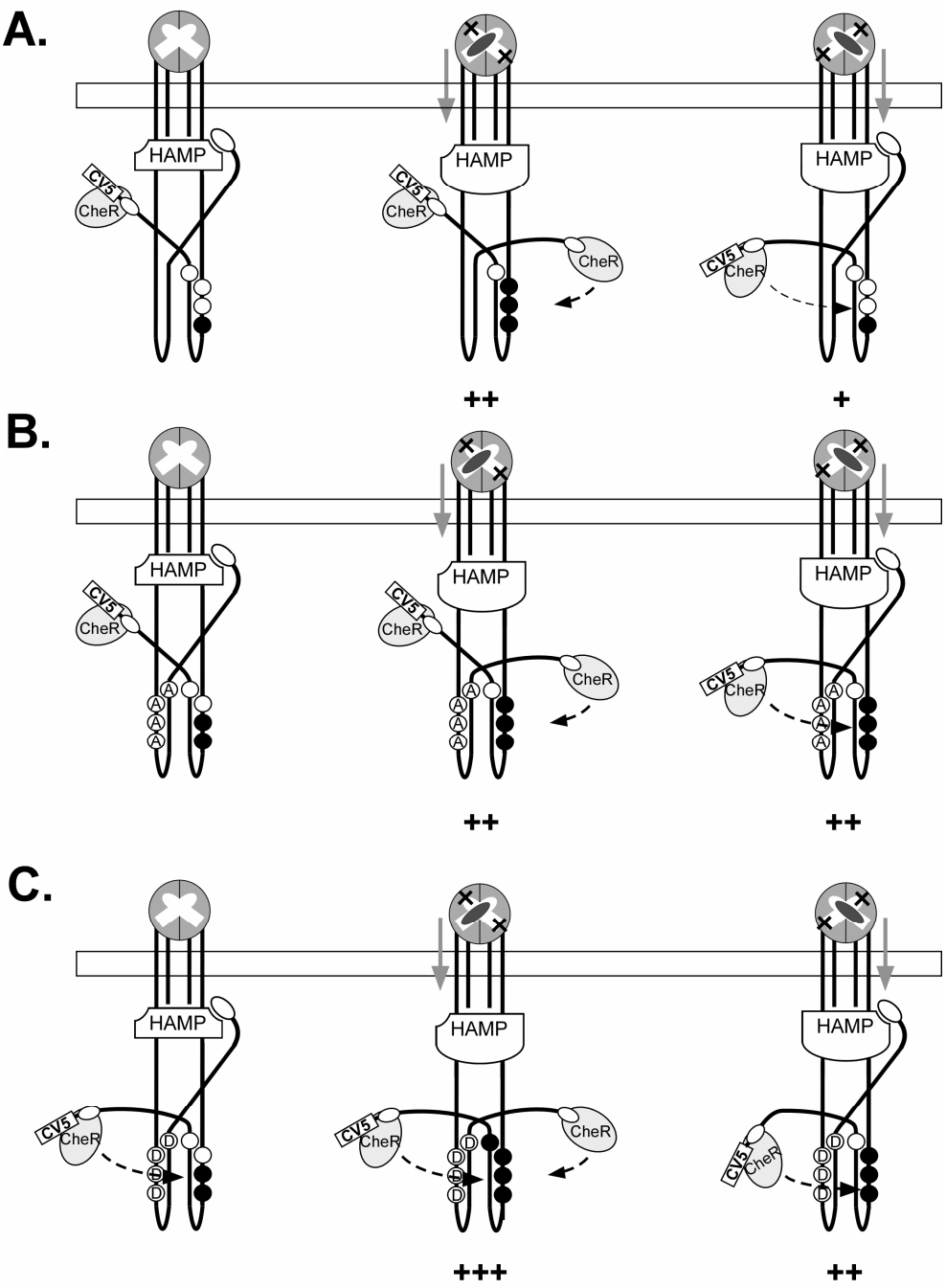

Figure 20. Model explaining the differences in initial methylation level and methylation rates observed between various functional Tar heterodimers.

The four methylation sites are represented with white circles. Black circles represent added methyl groups. Pluses indicate the rate of methylation.

The ability of CheR to achieve an optimal positioning and function at the methylation sites is perturbed by the presence of the V5 tag due to the reduced rates observed when TarV5 is solely expressed (data not shown). In this context the differences in the rates of methylation of the functional heterodimers can be explained. The differences in the rates of methylation observed for wild-type, alanyl, and aspartyl substituted methylation sites can be attributed to the electrostatic charge associated with the adaptation region. Both the wild-type and aspartyl substituted methylation sites have negative charge associated at this region which allows for an increased presence of CheR binding. The more negatively charged aspartyl residues might enhance the interaction of the CheR with the functional heterodimer resulting in higher methylation rates then that of wild-type. The alanyl substituted methylation sites have no enhancement of CheR binding and therefore have methylation rates that are indistinguishable between signaling and non-signaling subunits. (Fig. 20; 116). We therefore conclude that both cytoplasmic signaling domains respond to asymmetric signal and that charges in the cytoplasmic domain play a significant role in intradimer signaling.

CHAPTER IV

COMMUNICATION AMONG *E. COLI* CHEMORECEPTORS IN THE ABSENCE OF RECEPTOR/CHEA/CHEW COMPLEXES

Summary

In *E.coli*, chemoreceptors cluster at the cell poles in higher-order structures that require the CheA kinase and the CheW coupling factor for their formation. During a chemotactic response to an attractant, the receptors undergo reversible covalent modification through methylation at specific glutamyl residues in their cytoplasmic domains. Crosstalk during adaptation refers to the phenomena whereby receptors that do not bind a particular ligand become transiently methylated in response to a ligand that binds to a different receptor. This effect has generally been attributed to a decrease in the amount of the active, phosphorylated form of the methylesterase CheB, which removes methyl groups that are added by the constitutively active CheR methyltransferase. Since CheB is phosphorylated by CheA, cells lacking CheA or CheW cannot form phospho-CheB. To assess whether receptors still communicate during adaptation in the absence of phospho-CheB, we set up a system in which we could easily distinguish signaling and non-signaling receptors by tagging one of them with a C-terminal V5 epitope tag. Here, we report that crosstalk during adaptation still occurs in the absence of CheW and CheA. Furthermore, the methylation rate for a non-signaling receptor increased when it had a different cytoplasmic domain than the signaling receptor. We conclude that both the nature of the cytoplasmic domain of a non-signaling receptor and the covalent modification state of a signaling receptor control the crosstalk that occurs among receptors in the absence of CheA, CheW, and phospho-CheB. We suggest that this

process also plays a significant role within cells that have a full complement of chemotaxis machinery.

Introduction

Chemoreceptors that span the cell membrane of *E. coli* allow the bacteria to respond to attractants and repellents (3, 75, 78, 85, 126). The receptors exist as homodimers (82). The ability to swim up an attractant gradient depends upon adaptation to the chemical environment sensed a few seconds earlier. In the case of the aspartate receptor Tar, this adaptation is achieved by methylation and demethylation of four glutamyl residues in the cytoplasmic domain of each receptor monomer (34, 120).

Under steady-state conditions, the methylation level of a receptor reflects its percent ligand occupancy averaged over time (59, 107, 113, 114). Thus, adaptive methylation serves as a primitive, short-term memory that allows detection of spatial gradients by a process of temporal comparison. Methylation and demethylation of the receptor are carried out by the cytoplasmic proteins CheR, a constitutive methyltransferase (108), and CheB, a methylesterase that is activated 100 fold by phosphorylation of a conserved aspartyl residue in its N-terminal regulatory domain (8). CheB is also a deamidase that removes the amide groups of the glutaminyl residues that are originally present at the first and third methylation sites to convert them into glutaminyl residues (127). Since CheB is phosphorylated by the CheA kinase, the absence of CheA and or the CheA/receptor coupling factor CheW not only drastically decreases the amount of phospho-CheY resulting in smooth-swimming, non-chemotactic cells, but also leads to an increase in methylation due to the deficit in phospho-CheB.

The transient decrease in phospho-CheB that accompanies an attractant response by wild-type cells causes a transient increase in the methylation level of both the actively signally receptors and non-cognate receptors (55, 99). The affinity of the methylation sites of an attractant-bound receptor for CheR also increases (98). As a result, when adaptation is complete the ligand-bound receptor still retains a higher level of methylation (114), even though the steady-state level of phospho-CheB is restored.

CheR and CheB have both been reported to interact with the NWETF pentapeptide sequence located at the extreme C-terminus of Tar and the serine receptor Tsr (12, 133), although CheR binding occurs with much higher affinity (11). Such binding should increase the local concentration of these proteins within the receptor patch. It has also been shown that CheR bound to the NWETF motif of one subunit in a dimer can methylate the other subunit or, indeed, even subunits of another dimer during *in vitro* methylation in the absence of CheA and CheW (65, 70).

The association of receptors, CheA and CheW is referred to as the ternary complex (86) and has been considered the basic signaling component of chemotaxis (44, 101). However, it has recently become clear that receptor homodimers come together to form a trimer of dimers that can contain dimers of different receptor types (56, 119). In the presence of CheA and CheW, receptors can form higher-order polar patches of receptors (72, 74). Shimizu *et al* (105) suggested that these patches consist of trimers of dimers tied together by CheW and CheA.

Here, we look at adaptive methylation in cells that lack CheA and CheW, within which the highest order specific association of receptors could be the trimer of dimers. By labeling one receptor type with a C-terminal V5 epitope tag, we could distinguish the

behavior of cognate and non-cognate receptors after the addition of an attractant specific for one receptor. Our results indicate that crosstalk during adaptive methylation still occurs in the absence of CheA and CheW. Methylation of the non-cognate receptor is also better than methylation of a mutant cognate receptor that is unable to respond to a ligand, suggesting that interactions among heterologous dimers within the trimer are more robust. Finally, substitution of the glutamyl residues at the methylation sites with non-modifiable alanyl residues does not prevent that receptor from increasing the methylation level of a non-cognate receptor whereas aspartyl substitutions at the methylation sites negated the interaction between signaling and non-signaling receptors.

Materials and methods

Bacterial strains and plasmids. Strain AB2898 is a derivative of RP2898 (*ΔcheA-cheW-tar-tap*; 95) transduced with P1 lysate from SW02 (*thr+ eda+ tsr7021 trg::Tn10 Kan Δtar-tap5201*) a derivative of RP437 (94) making AB2898 (*eda+ tsr7021 ΔcheA-cheW-tar-tap*). Plasmid pMK113, a derivative of pBR322, contains the E. coli. Tar gene and a mutant *meche* promoter with decreased expression (39). Plasmid pRBB16, a pACYC184 derivative, contains *E.coli* Tar expressed by the native *meche* promoter. The difference in expression levels of the two promoters compensates for the difference in copy number of the two plasmids used. Plasmid pMK113CV5 was constructed via a PCR based extension of the 3' end of tar with a seven amino acid linker (GGSSAAG) and a C-terminal V5 epitope tag (GKPIPNPLLGLDST). Plasmid pMK113Tsr and pMK113TsrCV5 were constructed by PCR amplification of the Tsr with primers that that included the n-terminal mutant *meche* promoter from pMK113 and c-terminal primers

with and without the V5 epitope tag. The resulting DNA was then cut with BamHI and HindIII and cloned into pMK113H. Plasmid pMK113Tsar and pMK113TsarCV5 were cloned from pMK113TsrCV5 and pMK113/pMK113CV5 using the restriction enzymes BamHI and NdeI resulting in a chimeric receptor with the n-terminal Tsr receptor residues and the Tar c-terminal residues from Tar residue 254 onward. Tsr is more abundant than Tar in wild-type cells (28) due to differences in their respective promoters, therefore similar amounts of receptor protein were maintained through the sole use of the mutant *meche* (Tar) promoter on all pMK plasmid constructs. Plasmid pRBB164A was cloned from pRA126 (87) via PCR amplification and inserted into pRBB16 using the NdeI and EcoRI restriction sites. Plasmid pRBB164D was cloned from pNI156 (88) via PCR amplification and inserted into pRBB16 using the NdeI and EcoRI restriction sites.

Determination of the *in vivo* methylation state of Tar. Our assay was based on a published method (129). Overnight cultures of strain AB2898 (deleted for three chemoreceptor genes *tsr*, *tar*, *tap* and cytoplasmic chemotaxis proteins *cheW* and *cheA*) containing two compatible plasmids encoding different mutant versions of Tar were grown to stationary phase. Cells were diluted 1:100 into fresh TB containing 25ug/ml ampicillin or 5ug/ml tetracycline depending on the plasmids and grown to OD_{590nm} of 0.6. Cells were harvested by centrifugation, washed three times with 10 ml of chemotaxis buffer, and finally resuspended in 5 ml of chemotaxis buffer containing 10 mM sodium D-lactate and 200 μg/ml chloramphenicol. The final resuspension of washed cells at an OD_{590nm} of 0.4 was placed in a 125 ml Erlenmeyer flask in a 32^{o}C water bath and agitated by vigorous swirling for 30 min. L-aspartate was added to 1 mM, and 1 ml aliquots were removed at various times and added to 1.5 ml Eppendorf tubes pre-loaded with 100 μl of

100% TCA and then immediately put on ice for 15 min. Proteins were pelleted by centrifugation for 15 min in a microcentrifuge, washed with 0.5ml cold 1% TCA, and subsequently washed with 0.5 ml acetone. The samples were allowed to dry overnight at room temperature and then resuspended in 200 µl 2X SDS-loading buffer. A 20 µl aliquot of each sample was loaded onto a 7.5% SDS gel.

Following electrophoresis, the proteins were transferred to 0.2 micron nitrocellulose and subjected to immunoblotting and visual detection by antibody against the V5 epitope (Invitrogen), using GAM-AP (BioRad) as the secondary antibody. The immunoblots were then scanned into a computer and the densities of the bands analyzed with the program ImageJ (http://rsb.info.nih.gov/ij/). First, the total density of all the bands a particular lane was measured. Then the individual bands were analyzed, and the relative density of each band as a fraction of the total density was calculated. The individual bands were assigned a methylation state by comparing them to a TarV5 standard comprise of three different covalent modification states: EEEE, QEQE, and the fully amidated QQQQ. The EEEE species migrates slowest, the QEQE form migrates at an intermediate rate, and the QQQQ form moves fastest. The total mean population level of methylation at each time point was calculated from Equation 1:

$$M_t = (M_1 + 2M_2 + 3M_3 + 4M_4)/R_t \qquad \text{(Equation 1)}$$

where Mt is the mean total number of methyl groups per total number of receptors present, M_1 is the band-density of the singly methylated species, M_2 is the band-density of the doubly methylated species, M_3 is the band-density of the triply methylated species, M_4 is the band-density of the quadruply methylated species, and R_t is the sum of the receptor band-densities. The band representing the unmethylated form M_0 was not

observed when proteins were expressed in the absence of CheA and CheW but this is most probably due to the loss of the phosphorylated and thus activated form of CheB resulting in a higher initial methylation state of the receptor. The V5 tagged Tsr (TsrV5) and the V5 tagged hybrid protein Tsar (TsarV5) were analyzed the same way. It should be noted that Tsr had a maximum of 3 bands visible as observed in other works (68). Methyl group incorporation was found to primarily occur at methylation site residues 304 and 493 whereas the contribution of residue 503 is minimal (97). Therefore the calculations were modified somewhat to give the slowest migrating band a value of one and the fastest migrating band a value of three. Since TsarV5 has the cytoplasmic domain of Tar four methylation bands were observed in the immunoblots and calculations were performed as described for Tar.

Results

Attractant dependent methylation to specific ligands occurs in the absence of coupling proteins CheA and CheW. A response to attractant addition is mediated through its cognate receptor. Three receptor proteins were expressed individually in a strain lacking three receptors Tsr, Tar, and Tap and the coupling proteins CheW and CheA. The proteins assayed were the aspartate receptor Tar, the serine receptor Tsr, and a chimeric protein Tsar which is a hybrid transducer with the periplasmic domain of Tsr and the cytoplasmic domain of Tar joined at the NdeI position located at residue 254 in Tar. The hybrid protein was used due the better resolution of individual methylation bands as opposed to that of the Tsr methylation pattern which resulted in only 3 distinct bands (see Materials and methods). The three receptors assayed all had a V5 tag attached

to the C-terminus via a 7 amino acid linker. The methylation rates observed for Tar were 3 to 5 fold lower than in the presence of CheW and CheA (data not shown). Tar responded to the addition of 1mM aspartate with a methylation rate of 0.016 methyl groups per min while Tsr and Tsar did not (Fig. 21A). The response to 1mM serine was only observed in Tsr and Tsar with methylation rates of 0.026 and 0.023 methyl groups per min respectively (Fig. 21B).

In the absence of CheA and CheW of the five epitope tag negatively affects the rate of methylation. In a strain containing a full complement of chemotaxis proteins the c-terminal V5 tag did not significantly interfere with the ability of Tar to perform effective chemotaxis (data not shown). Methylation assays performed where TarV5 was expressed in the absence of other receptors showed a slightly higher level of initial methylation than when it was expressed with Tar with a non modified C-terminus (Fig. 21). There are two questions in our current assay. 1) Does amount of receptor protein affect the methylation rate? 2) Does the presence of the V5 tag interfere with the binding of CheR and/or CheB? Coexpressing the proteins from compatible plasmids effectively doubles the amount of receptors in the cell. There is a low copy number of both CheR and CheR present in any given cell. In a cell the average number of CheB and CheR are 1:0.625 or 280 to 180 copies per cell (71). The doubling of receptors could titrate out the effective methylation and demethylation of the receptors. Additionally, with the removal of CheW and CheA the CheB remains unphosphorylated and thus does not demethylate the protein as rapidly. The difference in activity of phosphorylated to unphosphorylated CheB is 100 fold (7). The binding of CheB is unaffected by its phosphorylation state (11). Doubling the amount of V5 tagged protein did not change the methylation rate

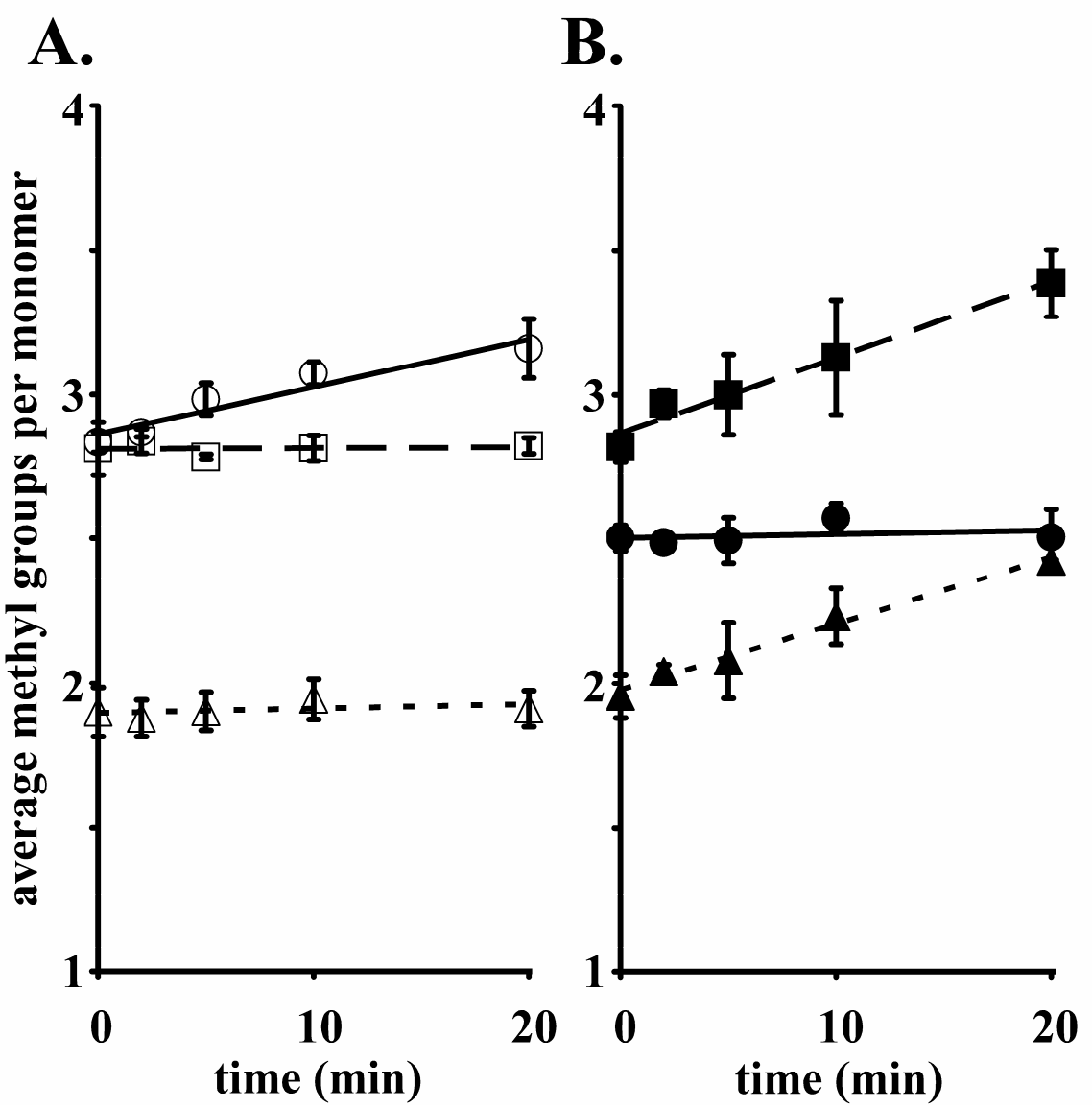

Figure 21. *In vivo* methylation assay of solely expressed receptors.

A) Response to 1mM aspartate for TarV5 (open circles), TsrV5 (open squares), and TsarV5 (open triangles). B) Response to 1mM serine for TarV5 (closed circles), TsrV5 (closed squares), and TsarV5 (closed triangles).

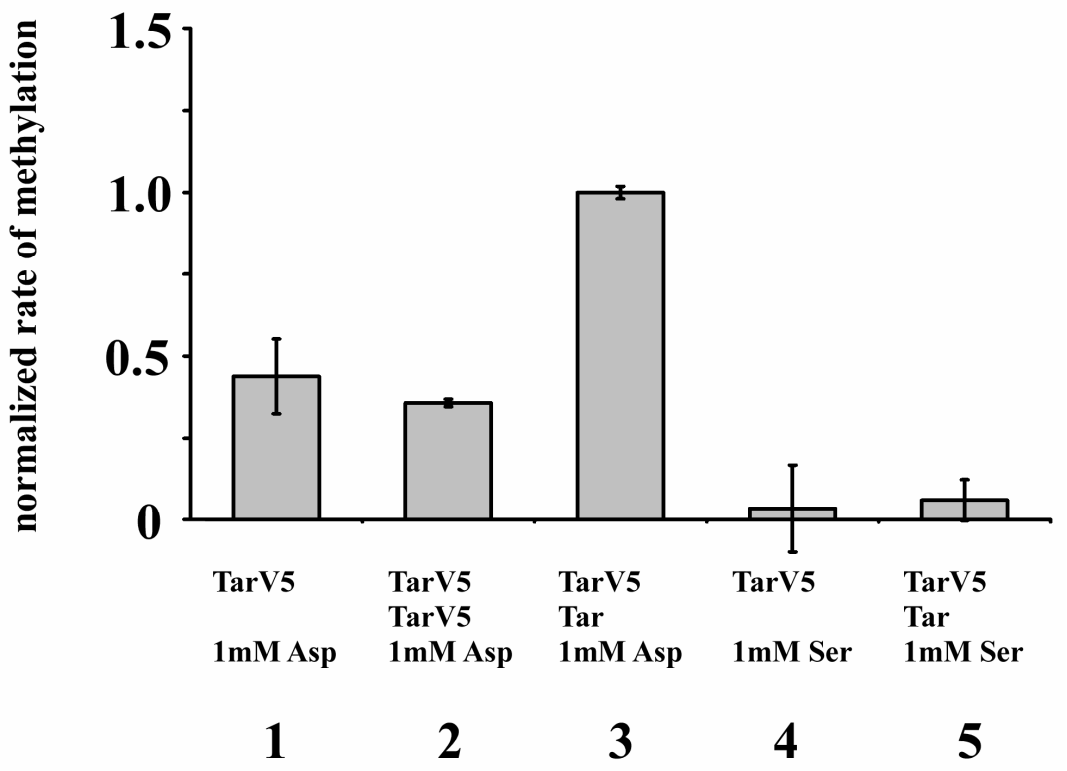

Figure 22. The effects of the addition of the V5 epitope tag at the C-terminus of Tar.

Methylation rates of single and coexpressed receptors were normalized to TarV5 expressed with Tar.

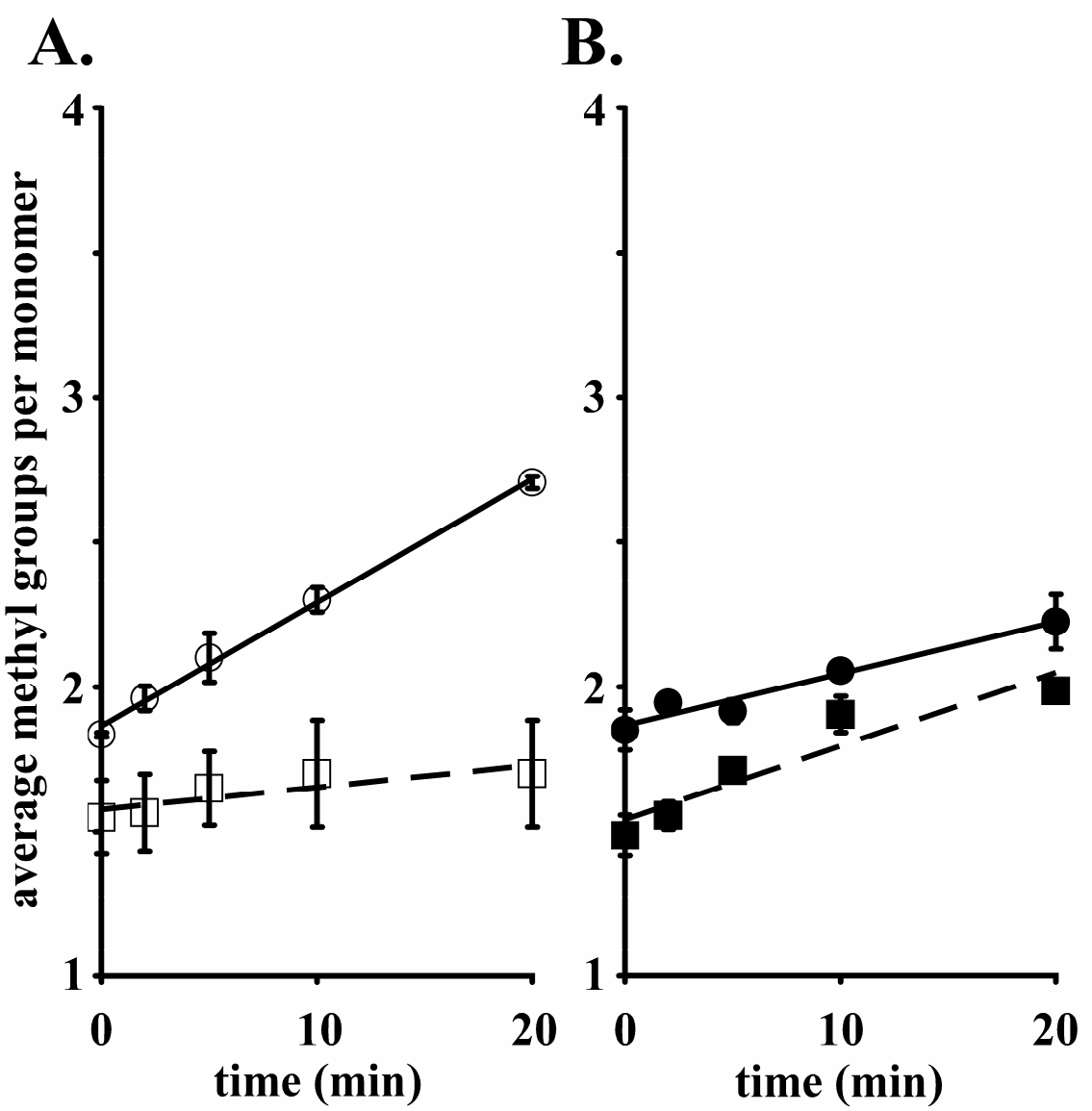

Figure 23. Communication between the two major transducers Tar and Tsr.

Tar and Tsr are coexpressed. The symbols represent only the receptor that has the V5 tag. A) Response to 1mM aspartate as shown by the increase in the average methyl groups observed per monomer of coexpressed TarV5 / Tsr (open circles) and TsrV5 / Tar (open squares). B) The response to 1mM serine for TarV5 / Tsr (closed circles) and TsrV5 / Tar (closed squares).

(Fig. 22 lane 1 and 2). The coexpression of the TarV5 protein with a Tar protein with a native C-terminus resulted in a greater increase in methylation rate in response to 1mM aspartate (Fig. 22 lane 3). No change in methylation rates were observed for the response to 1mM serine (Fig. 22 lane 4 and 5). Due to the differences in methylation rates observed and the higher initial methylation state of the V5 tagged protein (data not shown) we conclude that CheR and/or CheB binding is affected by the C-terminal V5 tag which is consistent with recent results involving peptide additions to the C-terminus of Tar and Tsr (64).

Interaction among receptors with different cytoplasmic domains. Tar and Tsr are the major transducers in E. coli and can function when expressed as the sole receptor in a cell. However, coexpression of Tar with Tsr results in an increase in the chemotactic response to either receptors cognate attractant. This synergy might be due to the differences in the cytoplasmic domains. We wanted to find out if methylation rates were affected in a similar way and therefore we examined the methylation rates of alternately V5 tagged Tar or Tsr when coexpressed. When coexpressed with Tsr, the methylation rates of TarV5 increased to both aspartate and serine. The methylation rates were 0.043 +/- 0.001 and 0.018 +/- 0.007 methyl groups per min after the addition of aspartate and serine, respectively (Fig. 23). Similarly the methylation rate of TsrV5 increased in the presence of both aspartate (0.0078 +/- 0.003 methyl groups per min) and serine (0.025 +/- 0.001 methyl groups per min; Fig. 23).

Interaction among similar cytoplasmic domains. Our initial use of the Tsar hybrid protein was to increase the sensitivity of our assay because the different methylated forms of Tsar separate better than those of Tsr itself. An added benefit was that the cytoplasmic

domains of Tar and Tsar were now the same from residue 254 onward which allowed us to ask whether the interaction of similar cytoplasmic domains increases or decreases the effective crosstalk observed. The rate of methylation of TarV5 after the addition of 1mM aspartate was identical when coexpressed with either Tsar or wild-type Tar (0.037 +/- 0.001 methyl groups per min) (Fig. 24). The addition of aspartate also lead to a slight increase in the methylation of the V5 tagged Tsar (TsarV5) from almost no response (Fig. 21A) to a rate of 0.0075 +/- 0.002 (Fig. 24A). The methylation rate in response to 1mM serine for the TarV5 was 0.017 +/- 0.004 methyl groups per min whereas the TsarV5 became methylated at a rate of 0.035 +/- 0.004 methyl groups per min (Fig. 24B).

The covalent modification state of the Tar receptor dimer alters the methylation rates when different cytoplasmic domains are present. To test whether or not the covalent modification state of the receptor was important for the interaction between different receptors the 4 methylation sites of the Tar protein were substituted with either uncharged alanines ($Tar_{\mathbf{AAAA}}$) or negatively charged aspartates ($Tar_{\mathbf{DDDD}}$). These mutant Tar proteins were then coexpressed with either TsrV5 or TsarV5. The rate of methylation the V5 tagged proteins after the addition of 1mM aspartate was assessed (Fig. 25). When the cytoplasmic domains of the two receptors are different (Tar and TsrV5), the substitution of the methylation sites in Tar with alanines almost doubled the methylation rate of the TsrV5 compared to the rate when coexpressed with the wild-type Tar. The methylation rate of TsrV5 decreased by a factor of 2 when it was coexpressed with $Tar_{\mathbf{DDDD}}$ (Fig. 25A). When receptors with similar cytoplasmic domains (Tar and TsarV5) were coexpressed a different pattern was seen. The rate of methylation of TsarV5 was the same when coexpressed with either the wild-type Tar or the mutant

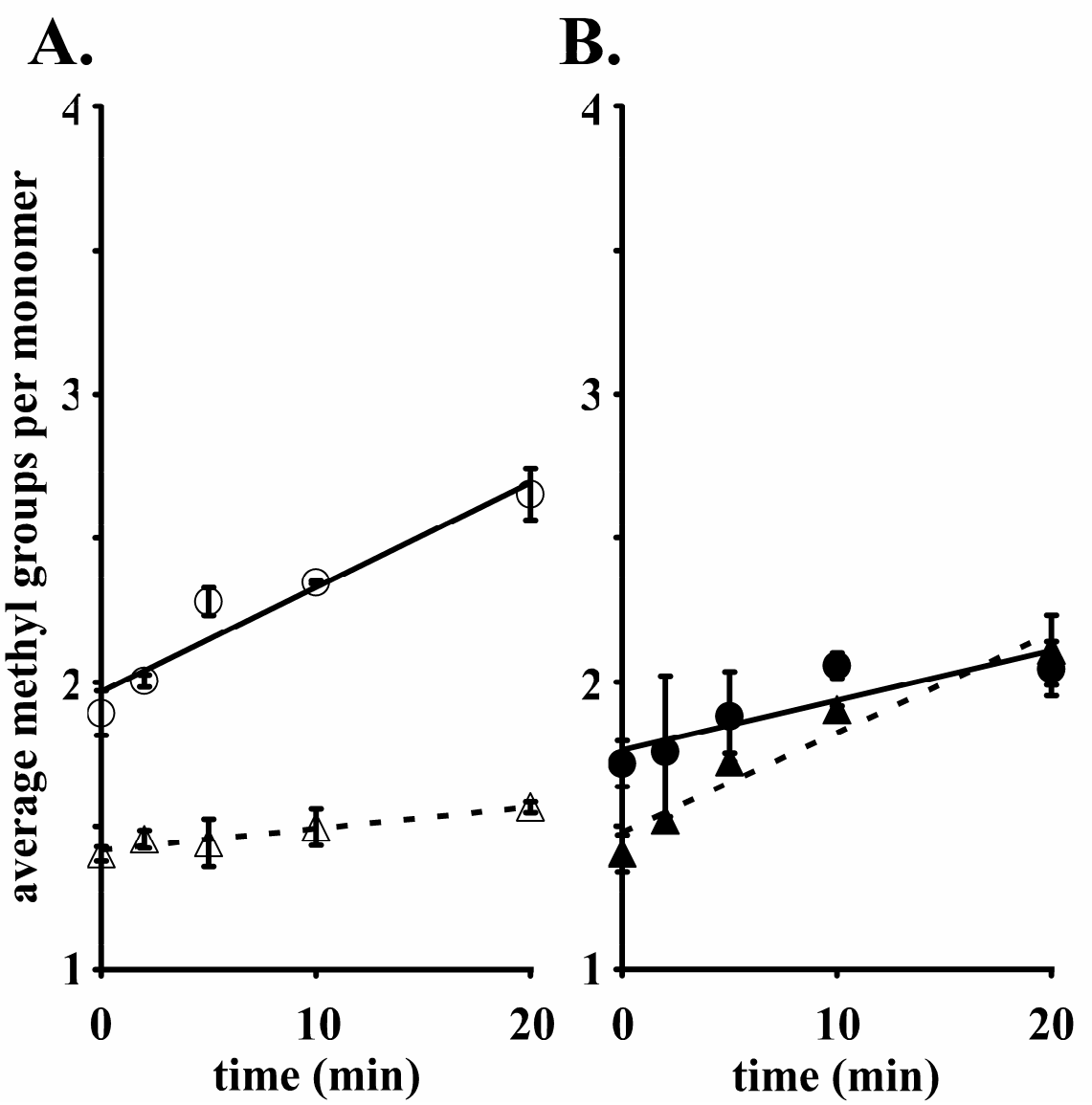

Figure 24. Communication between the major transducer Tar and the hybrid receptor Tsar.

Tsar has the ligand binding domain of Tsr and the signaling domain of Tar. A) Response to 1mM aspartate as shown by the increase in the average methyl groups observed per monomer of coexpressed TarV5 / Tsar (open circles) and TsarV5 / Tar (open triangles). B) The response to 1mM serine for TarV5 / Tsr (closed circles) and TsrV5 / Tar (closed squares).

$Tar_{\mathbf{AAAA}}$ (Fig. 25B). The rate of methylation of TsarV5 decreased 3 fold when coexpressed with $Tar_{\mathbf{DDDD}}$, similar to the results seen for TsrV5.

Discussion

The higher order structure observed in the polarly located receptor patch is used to explain phenomena ranging from the sensitivity of the chemotaxis system to low levels of ligand to the functional interactions of the major and minor transducers (23, 53). The current model is that the patch is made of mixed trimers of dimers (119). Additional evidence supports the notion that the trimer of dimers is a functional building block of the patch that differs only with the CheA bound trimer in the spacing between the tips of the cytoplasmic domains located between helix 4 and 5 as denoted in the crystal structure of the serine receptor Tsr (38, 56).

In this paper we addressed what we consider the building block of the polar patch, the trimer dimers, in the absence of CheW and CheA in order to see if close interactions with a ligand bound receptor leads to methylation of a non-ligand bound receptor. In setting up our experiments we used three receptor proteins, Tar, Tsr, and Tsar (a hybrid protein containing the periplasmic domain of Tsr and the cytoplasmic domain of Tar). V5 epitope tags were added to the C-terminus of these receptors in order to separate the similarly sized receptors and assess their individual methylation rates via immuno-detection. Each of these receptor proteins, when individually expressed in the cell was able to become methylated in response to its specific attractant ligand and did not exhibit a significant response to non-cognate ligands (Fig. 21).

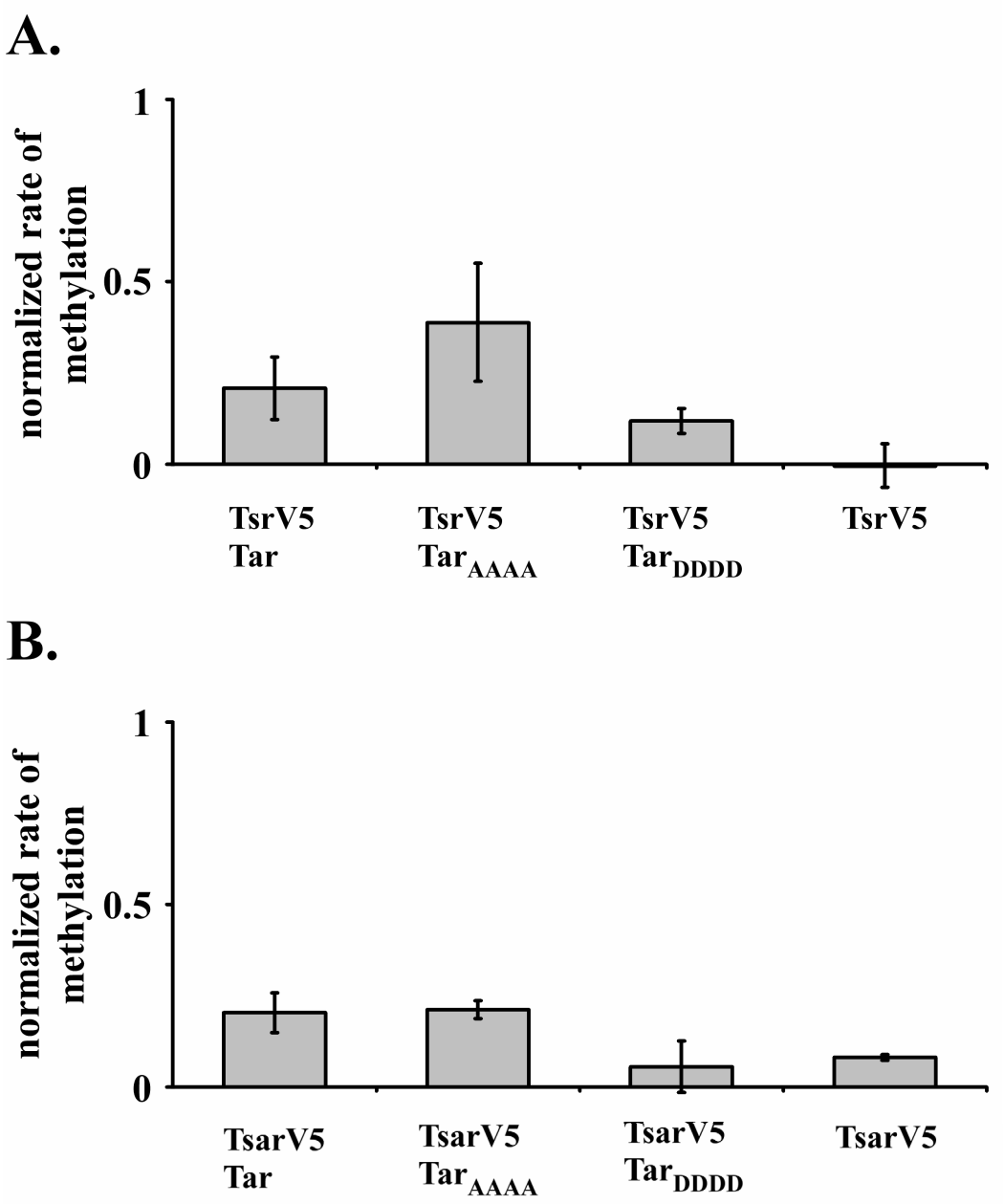

Figure 25. Comparison of methylation rates to 1mM aspartate of non-ligand bound receptors due to the binding and signaling of various Tar receptor constructs.

A) Relative methylation rates of V5 tagged Tsr normalized to TarV5 coexpressed with Tar. B) Relative methylation rates of V5 tagged Tsar normalized to TarV5 coexpressed with Tar.

In order to assess the ability of receptors to interact with other receptors during a signaling event, two receptors were coexpressed with only one receptor containing the V5 tag. Since two receptors are expressed and only one has the free C-terminal pentapeptide sequence responsible for binding the methyltransferase CheR and methylesterase CheB it was necessary to detect any effects that the V5 tag might have on receptor methylation and demethylation. Coexpression of TarV5 with a wild-type Tar resulted in a 2-fold increase in the rate of methylation in response to aspartate (Fig. 22 lanes 1 and 3). There was no increase in methylation rate in response to serine (Fig. 22 lanes 4 and 5). This two fold increase in methylation is not due to the doubling of receptor protein in the cell (Fig. 22 compare lanes 1 and 2). In addition, when the TarV5 was the only receptor in the cell the initial methylation levels were higher than when it was coexpressed with a receptor with a non-modified C-terminus (data not shown) which supports the perception of normal CheR function and abnormal CheB function. The C-terminal NWETF sequence seems only to increase the local concentration of the CheR protein (133), whereas the CheB interaction with the NWETF sequence in the receptor leads to an allosteric enhancement of demethylation activity (11). Therefore we assumed that CheB binding and/or activity is somehow influenced by the presence of the V5 tag.

Interdimer interactions of different cytoplasmic domains occurred when coexpressing Tar with Tsr and led to a greater level of methylation of the non-signaling receptor (Fig. 23, Table 3) than if the cytoplasmic domains were similar (Fig. 24, Table 3) as they were with Tar and Tsar. This supports data that receptors work better when a mixed complements of receptors are present (45) and provides additional evidence that receptors expressed at the same time form heterotrimers (6). Heterotrimers might predominate due

Table 3. Methylation rates of the V5 tagged receptor in the absence of CheA and CheW

Receptor proteins present	Normalized increase in methyl groups per min [a]	
	1mM Asp	1mM Ser
TarV5	0.43 ± 0.11	0.004 ± 0.14
TsrV5	0.01 ± 0.05	0.70 ± 0.11
TsarV5	0.08 ± 0.14	0.62 ± 0.05
TarV5 / TarV5	0.36 ± 0.03	
TarV5 / Tar	1.00 ± 0.03	0.06 ± 0.05
TarV5 / Tsr	1.16 ± 0.03	0.49 ± 0.19
TarV5 / Tsar	0.97 ± 0.03	0.46 ± 0.11
TsrV5 / Tar	0.21 ± 0.08	0.68 ± 0.03
TsrV5 / Tar_{AAAA}	0.38 ± 0.16	
TsrV5 / Tar_{DDDD}	0.12 ± 0.03	
TsarV5 / Tar	0.20 ± 0.05	0.95 ± 0.11
TsarV5 / Tar_{AAAA}	0.21 ± 0.03	
TsarV5 / Tar_{DDDD}	0.06 ± 0.08	

a. Increase in methyl groups per minute normalized to TarV5 / Tar for aspartate

to cytoplasmic domains of different receptors allowing for better packing at the cytoplasmic domains.

Finally the effects of the charges associated at the methylation sites of a signaling receptor on its ability to interact with a non-signaling receptor was assessed using alanine or aspartate substitutions at the methylation sites of the Tar receptor and coexpressing with either the V5 tagged Tsr or V5 tagged Tsar receptor. Interestingly, an increase in methylation of both Tsr and Tsar in response to aspartate was only observed when co-

expressed with the $Tar_{\mathbf{AAAA}}$ construct (Fig. 25, Table 3). The $Tar_{\mathbf{DDDD}}$ construct was unable to interact or perhaps form stable interactions within the trimer to elicit or share signal due to steric hindrances (116) imposed by the negatively charged aspartyl residues at the methylation sites. The most striking difference is seen again in the ability of Tar to interact with a cytoplasmic domain that is distinctly different then its own. The interaction with Tsr leads to a two fold increase in the methylation rate of TsrV5 in response to aspartate as compared to coexpressing TsrV5 with wild-type Tar (Fig. 25A, Table 3), whereas the same experiment with Tsar resulted in similar methylation rates as observed when coexpressing Tsar with wild-type Tar (Fig. 25B, Table 3). This suggests that interaction between different cytoplasmic domains is somewhat dictated by the covalent modification state with the least effect observed between similar cytoplasmic domains.

The structure and function of individual receptors in a higher order unit is an area of intense investigation. The data point to dimers of receptor cytoplasmic domains tightly associated into trimers (56), which can be arranged into higher order structures held together by CheA and CheW (105). EM images using purified Tar or Tsr cytoplasmic domains held together by a leucine zipper indicate a slightly larger spacing between the cytoplasmic domains of the receptor dimers in a trimer which is where CheW and CheA are thought to bind (37, 38). Combining these results together one can hypothesize that patch formation occurs in 4 steps: 1) receptor monomers form the dimer, the basic unit of any receptor; 2) dimers associate into stable trimers with close interaction of the cytoplasmic domains; 3) trimers of dimers migrate towards the poles of cells even in the absence of CheW and CheA (74, 109) due to the curvature constraints of trimer packing;

and 4) CheA and CheW interact with the cytoplasmic domains resulting in a separation of cytoplasmic domains and the formation of the functional patch. Thus our results may represent the effects ligands have on an intermediary precursor to the receptor patch. Due to the limiting the amount of CheA and CheW relative to receptor ($CheA_2$ 1: CheW 1.6: $receptor_2$ 3.4; 71) present in a cell expressing the full complement of chemotaxis proteins some of the receptors are not associated with CheA and CheW. Also, considering that *de novo* proteins synthesis produces receptors that are neutrally biased in terms of its adaptive state then the ability of the receptors to still interact with each other within the trimer could potentially prevent the biasing of a signal when becoming incorporated into the patch under conditions where extreme concentrations of attractant and repellants are present. This could be one additional step in the fine tuning of the bacterial response under conditions of which new proteins are becoming incorporated into a patch over time.

CHAPTER V

CONCLUSIONS

Discussion

The *E.coli* Tar chemoreceptor is a homodimeric protein that spans the cell membrane. It has two major soluble domains, each located on a different side of the membrane: the periplasmic ligand-binding domain and the cytoplasmic output domain. These two domains are joined by the second transmembrane helix (TM2) and the proceeding HAMP linker domain. Tar mediates chemotaxis toward L-aspartate and related amino acids; toward maltose, via the periplasmic maltose-binding protein (MBP); and away from certain divalent cations, most notably Ni^{2+} and Co^{2+}. Although the periplasmic domain contains two rotationally symmetric aspartate-binding sites at the subunit interface, in effect it binds only one aspartate molecule because of strong negative cooperativity.

Another convenient property of the ligand recognition mechanism of Tar is that the "major" and "minor" binding half-sites for one aspartate molecule are in opposing subunits. The signal elicited by aspartate binding is channeled primarily through the subunit of the homodimer that contains the "major" half-binding site for aspartate. This asymmetry in signaling, coupled with the strong negative cooperativity for aspartate, allows aspartate-occupied Tar to respond to another ligand, MBP. Therefore, two mutant proteins, each with a defect in a different half-site, can be coexpressed in a cell and form a functional heterodimer with one intact aspartate-binding site. In such heterodimers the signaling subunit can be specified as the one in which the "major" binding half-site is unaltered.

Tar, like all chemoreceptors, functions in a ternary complex that also includes the CheA histidine kinase and the CheW coupling protein. Moreover, receptor homodimers interact to form trimers of dimers, which apparently can contain mixtures of different receptor types. Still higher levels of organization, in which extended lattices of receptors are joined together by CheA and CheW, also probably exist. Thus, it is clear that the functional units in which chemoreceptors localize within the cell are both structurally and functionally complex.

The fundamental question asked is how the asymmetric signal initiated by aspartate binding to the periplasmic domain of Tar is perceived by the cytoplasmic signaling domain. The tool used to assess this process was the adaptive methylation that occurs upon addition of aspartate to an *E. coli* cell. This methylation depends upon ligand-induced changes in the conformation of the cytoplasmic domain. Because the identity of the signaling and non-signaling subunits of a homodimer are known, it was possible to place a V5 epitope tag on either one of them and use immunoblotting to ask what the pattern of methylation level is before and after addition of aspartate.

In Chapter II, I investigated methylation within Tar heterodimers in the presence of a full complement of chemotaxis proteins and a normal chemotaxis signaling pathway. The key finding was that aspartate-induced adaptive methylation occurs to the same extent and nearly at the same rate in the cytoplasmic domains of both the signaling and non-signaling subunits. Furthermore, the methylation state of the non-tagged subunit did not determine the methylation state of the tagged subunit. I could make this distinction by converting the glutamyl residues at the methylation sites to non-methylatable alanyl residues ($Tar_{\mathbf{AAAA}}$) a manipulation that had little to no effect on the behavior of its tagged,

methylation-competent partner regardless of whether the latter was the signaling or non-signaling subunit. My interpretation of these results is that the aspartate-induced conformational change is asymmetric until it reaches the HAMP linker. I hypothesize that ligand-induced changes in the linker of the signaling subunit are communicated to the linker of the non-signaling subunit, causing them to act in concert to generate a symmetric signal in the cytoplasmic signaling and methylation regions.

In Chapter III, I address the mechanism of intradimer signaling in the absence of CheA and CheW. I found that both subunits of an asymmetrically signaling dimer still become methylated, but the rates of methylation were different for the signaling and non-signaling subunits. Interestingly, the signaling subunit was methylated more slowly than the non-signaling subunit. To investigate the basis for this difference, the methylation-site glutamyl residues were substituted either with electrically neutral alanyl residues ($Tar_{\mathbf{AAAA}}$) or negatively charged but non-methylatable aspartyl residues ($Tar_{\mathbf{DDDD}}$) in either the signaling or non-signaling subunits. The difference in methylation rates of the signaling and non-signaling subunits was maintained with aspartyl-substituted Tar, but alanyl-substituted Tar supported equal rates of methylation of its tagged partner whether is was the signaling or non-signaling subunit. My model to explain these curious results is incorporated into a figure in Chapter III.

In Chapter II and Chapter III I reported that $Tar_{\mathbf{AAAA}}$ and $Tar_{\mathbf{DDDD}}$ do not behave like $Tar_{\mathbf{QQQQ}}$ or $Tar_{\mathbf{EEEE}}$. The properties of $Tar_{\mathbf{AAAA}}$ been previously characterized in the context of thermotaxis (87), and it was expected that $Tar_{\mathbf{AAAA}}$ could still activate CheA kinase activity *in vitro*. On the other hand, the behavior of $Tar_{\mathbf{DDDD}}$ was very surprising. The only difference between an aspartyl and glutamyl residue is the deletion of one

methylene group. Therefore, glutamates extend their negatively charged R group 1.3 Å farther from the peptide backbone than an aspartyl residue. The dramatically higher stimulation of CheA by $Tar_{\mathbf{DDDD}}$ than by $Tar_{\mathbf{EEEE}}$ argues that there is likely to be a precise electrostatic interaction both within and among the dimers that are associated in trimers. $Tar_{\mathbf{DDDD}}$ did not block methylation of its partner subunit, which suggests that the conversion of asymmetry to symmetry is unperturbed by the negative charge of the aspartyl residues. This observation is easily reconciled with the idea that the conversion occurs within the HAMP linker, which is spatially distinct from the methylation sites.

In Chapter IV I explored interactions between different receptor dimers in the absence of CheA and CheW. Contrary to earlier reports, I found that crosstalk still occurs within mixed trimers of dimers. This result furthers our understanding of the interactions between receptor dimers that are closely associated in space, presumably because they are members of mixed trimers of dimers, or at least adjacent trimers of dimers. Interdimer crosstalk was greater between receptors that have wild-type methylation sites or electrically neutral residues replacing the glutamyl residues that normally occupy the methylation sites. The interdimer cross-talk was abolished when the glutamyl residues were substituted with aspartyl residues, implying that interaction among dimers is by the permanently negative charge of non-methylatable aspartyl residues. This result could mean that trimers of negatively charged dimers tend to disassociate or that methylation regions are physically separated by repulsive negative charge within a static trimer. One clear finding was that receptor dimers having cytoplasmic domains of the same type (from Tar or Tsr) were mare susceptible to methylation cross-talk than receptor dimers whose cytoplasmic domains were derived from a different receptor type.

Future experiments

The ability to distinguish between the methylation patterns of signaling and non-signaling subunits in mixed receptor arrays can be used to investigate the behavior of a single receptor dimer that senses multiple ligands. The most obvious example is the ability of *E. coli* Tar to mediate simultaneous responses to aspartate and maltose. Since the two subunits of a Tar dimer become equally methylated in response to binding of one aspartate molecule, adaptation to maltose seemingly must involve additional methylation of both subunits. In a wild-type cell, in which both aspartate and MBP can alternately bind to Tar in one or the other of two possible orientations, some level of interference is observed when one attractant is added after saturation with the other. This interference is abolished when mutations are used to force the two ligands to signal using opposing subunits. Conversely, it becomes nearly complete when both ligands must signal through the same subunit.

If cytoplasmic signaling is symmetric, what is responsible for this difference? I surmise that, even after adaptation to one ligand is complete, the HAMP linker of the signaling subunit is in a subtly different configuration that renders it unresponsive to additional ligand-induced conformational changes within that subunit. One way of testing this hypothesis is to look at methylation in double-mutant heterodimeric receptors that are already saturated with one attractant. After the second ligand is added, I predict that no additional methylation should be seen when the second ligand must signal through the same subunit as the first ("same-side" signaling). However, additional methylation of both subunits should be seen when the second ligand can signal through the other subunit ("opposite-side" signaling).

It will be informative to find out how the V5 tag interferes with the ability of aspartate to inhibit Tar-stimulated CheA kinase activity *in vitro*. V5-tagged Tar functions like wild-type Tar during aspartate taxis *in vivo*, and it must therefore be able to both activate the kinase and turn off that activation in response to aspartate within the cell. In the standard *in vitro* assay the CheR methyltransferase and the CheB methylesterase are both absent. Perhaps the V5 epitope tag interferes with CheA inhibition by interacting with the HAMP linker domain. *In vivo*, binding of CheR or CheB to the NWETF motif might prevent that interaction. If so, adding CheR and/or CheB to the *in vitro* reaction with V5-tagged Tar might restore the inhibitory effect of aspartate. I also propose to test *in vitro* whether having normal Tsr together with V5-tagged Tar in membranes allows aspartate to shut off Tar-stimulated CheA activity. This experiment is based on the known synergistic interaction of Tar and Tsr in mixed receptor populations (63).

The mechanism that leads to different methylation rates for the signaling and non-signaling subunits in the absence of CheA and CheW (Chapter III) and equal rates in their presence (Chapter II) remains to be determined. I believe the critical issue is the nature of "the effective signaling unit." In the latter situation, the signaling unit is minimally the ternary complex of receptor/CheA/CheW, whereas in the former case the signaling unit is probably a trimer of receptor dimers. The question is whether CheA activity is somehow responsible for the difference. One way to approach the problem is to maintain the ternary complex, but in an inactive form, by using a non-phosphorylating mutant form of CheA. If the methylation rates for the two subunits are equal under these conditions, then the role of the ternary complex in facilitating symmetric methylation is primarily structural, perhaps by maintaining a certain spatial relationship between the two

subunits of a dimer or among dimers within a larger lattice. If the rates are different, then the activity of CheA, perhaps because of its ability to phosphorylate and activate CheB methylesterase, is needed for the equal methylation of the signaling and non-signaling subunits that is observed with the intact system.

REFERENCES

1. **Abouhamad, W. N., M. Manson, M. M. Gibson, and C. F. Higgins.** 1991. Peptide transport and chemotaxis in Escherichia coli and Salmonella typhimurium: characterization of the dipeptide permease (Dpp) and the dipeptide-binding protein. Mol. Microbiol. **5:**1035-47.

2. **Adler, J.** 1973. A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by Escherichia coli. J Gen Microbiol **74:**77-91.

3. **Adler, J., G. L. Hazelbauer, and M. M. Dahl.** 1973. Chemotaxis toward sugars in Escherichia coli. J. Bacteriol. **115:**824-47.

4. **Aksamit, R. R., and D. E. Koshland, Jr.** 1974. Identification of the ribose binding protein as the receptor for ribose chemotaxis in Salmonella typhimurium. Biochemistry **13:**4473-8.

5. **Ames, P., and J. S. Parkinson.** 1988. Transmembrane signaling by bacterial chemoreceptors: E. coli transducers with locked signal output. Cell **55:**817-26.

6. **Ames, P., C. A. Studdert, R. H. Reiser, and J. S. Parkinson.** 2002. Collaborative signaling by mixed chemoreceptor teams in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. **99:**7060-5.

7. **Anand, G. S., P. N. Goudreau, and A. M. Stock.** 1998. Activation of methylesterase CheB: evidence of a dual role for the regulatory domain. Biochemistry **37:**14038-47.

8. **Anand, G. S., and A. M. Stock.** 2002. Kinetic basis for the stimulatory effect of phosphorylation on the methylesterase activity of CheB. Biochemistry **41:**6752-60.

9. **Appleman, J. A., and V. Stewart.** 2003. Mutational analysis of a conserved signal-transducing element: the HAMP linker of the Escherichia coli nitrate sensor NarX. J. Bacteriol. **185:**89-97.

10. **Aravind, L., and C. P. Ponting.** 1999. The cytoplasmic helical linker domain of receptor histidine kinase and methyl-accepting proteins is common to many prokaryotic signalling proteins. FEMS Microbiol. Lett. **176:**111-6.

11. **Barnakov, A. N., L. A. Barnakova, and G. L. Hazelbauer.** 2002. Allosteric enhancement of adaptational demethylation by a carboxyl-terminal sequence on chemoreceptors. J. Biol. Chem. **277:**42151-6.

12. **Barnakov, A. N., L. A. Barnakova, and G. L. Hazelbauer.** 1999. Efficient adaptational demethylation of chemoreceptors requires the same enzyme-docking site as efficient methylation. Proc. Natl. Acad. Sci. U. S. A. **96:**10667-72.

13. **Berg, H. C., and R. A. Anderson.** 1973. Bacteria swim by rotating their flagellar filaments. Nature **245:**380-2.

14. **Berg, H. C., and D. A. Brown.** 1972. Chemotaxis in Escherichia coli analysed by three-dimensional tracking. Nature **239:**500-4.

15. **Berg, H. C., and L. Turner.** 1995. Cells of Escherichia coli swim either end forward. Proc. Natl. Acad. Sci. U. S. A. **92:**477-9.

16. **Bibikov, S. I., R. Biran, K. E. Rudd, and J. S. Parkinson.** 1997. A signal transducer for aerotaxis in Escherichia coli. J. Bacteriol. **179:**4075-9.

17. **Bibikov, S. I., A. C. Miller, K. K. Gosink, and J. S. Parkinson.** 2004. Methylation-independent aerotaxis mediated by the Escherichia coli Aer protein. J. Bacteriol. **186:**3730-7.

18. **Biemann, H. P., and D. E. Koshland, Jr.** 1994. Aspartate receptors of Escherichia coli and Salmonella typhimurium bind ligand with negative and half-of-the-sites cooperativity. Biochemistry **33:**629-34.

19. **Borkovich, K. A., N. Kaplan, J. F. Hess, and M. I. Simon.** 1989. Transmembrane signal transduction in bacterial chemotaxis involves ligand-dependent activation of phosphate group transfer. Proc. Natl. Acad. Sci. U. S. A. **86:**1208-12.

20. **Bornhorst, J. A., and J. J. Falke.** 2000. Attractant regulation of the aspartate receptor-kinase complex: limited cooperative interactions between receptors and effects of the receptor modification state. Biochemistry **39:**9486-93.

21. **Bowie, J. U., A. A. Pakula, and M. I. Simon.** 1995. The three-dimensional structure of the aspartate receptor from *Escherichia coli*. Acta Crystallogr. D. **51:**145-154.

22. **Brass, J. M., and M. D. Manson.** 1984. Reconstitution of maltose chemotaxis in Escherichia coli by addition of maltose-binding protein to calcium-treated cells of maltose regulon mutants. J. Bacteriol. **157:**881-90.

23. **Bray, D., M. D. Levin, and C. J. Morton-Firth.** 1998. Receptor clustering as a cellular mechanism to control sensitivity. Nature **393:**85-8.

24. **Bren, A., and M. Eisenbach.** 1998. The N terminus of the flagellar switch protein, FliM, is the binding domain for the chemotactic response regulator, CheY. J. Mol. Biol. **278:**507-14.

25. **Butler, S. L., and J. J. Falke.** 1998. Cysteine and disulfide scanning reveals two amphiphilic helices in the linker region of the aspartate chemoreceptor. Biochemistry **37:**10746-56.

26. **Chervitz, S. A., and J. J. Falke.** 1996. Molecular mechanism of transmembrane signaling by the aspartate receptor: a model. Proc. Natl. Acad. Sci. U. S. A. **93:**2545-50.

27. **Chi, Y. I., H. Yokota, and S. H. Kim.** 1997. Apo structure of the ligand-binding domain of aspartate receptor from Escherichia coli and its comparison with ligand-bound or pseudoligand-bound structures. FEBS Lett. **414:**327-32.

28. **Clarke, S., and D. E. Koshland, Jr.** 1979. Membrane receptors for aspartate and serine in bacterial chemotaxis. J. Biol. Chem. **254:**9695-702.

29. **Cluzel, P., M. Surette, and S. Leibler.** 2000. An ultrasensitive bacterial motor revealed by monitoring signaling proteins in single cells. Science **287:**1652-5.

30. **Dahl, M. K., and M. D. Manson.** 1985. Interspecific reconstitution of maltose transport and chemotaxis in Escherichia coli with maltose-binding protein from various enteric bacteria. J. Bacteriol. **164:**1057-63.

31. **Draheim, R. R., A. F. Bormans, R. Z. Lai, and M. D. Manson.** 2005. Tryptophan residues flanking the second transmembrane helix (TM2) set the signaling state of the Tar chemoreceptor. Biochemistry **44:**1268-77.

32. **Dunten, P., and D. E. Koshland, Jr.** 1991. Tuning the responsiveness of a sensory receptor via covalent modification. J. Biol. Chem. **266:**1491-6.

33. **Engstrom, P., and G. L. Hazelbauer.** 1980. Multiple methylation of methyl-accepting chemotaxis proteins during adaptation of E. coli to chemical stimuli. Cell **20:**165-71.

34. **Falke, J. J., and G. L. Hazelbauer.** 2001. Transmembrane signaling in bacterial chemoreceptors. Trends Biochem. Sci. **26:**257-65.

35. **Feng, X., J. W. Baumgartner, and G. L. Hazelbauer.** 1997. High- and low-abundance chemoreceptors in Escherichia coli: differential activities associated with closely related cytoplasmic domains. J. Bacteriol. **179:**6714-20.

36. **Feng, X., A. A. Lilly, and G. L. Hazelbauer.** 1999. Enhanced function conferred on low-abundance chemoreceptor Trg by a methyltransferase-docking site. J. Bacteriol. **181:**3164-71.

37. **Francis, N. R., M. N. Levit, T. R. Shaikh, L. A. Melanson, J. B. Stock, and D. J. DeRosier.** 2002. Subunit organization in a soluble complex of tar, CheW, and CheA by electron microscopy. J. Biol. Chem. **277:**36755-9.

38. **Francis, N. R., P. M. Wolanin, J. B. Stock, D. J. Derosier, and D. R. Thomas.** 2004. Three-dimensional structure and organization of a receptor/signaling complex. Proc. Natl. Acad. Sci. U. S. A. **101:**17480-5.

39. **Gardina, P., C. Conway, M. Kossman, and M. Manson.** 1992. Aspartate and maltose-binding protein interact with adjacent sites in the Tar chemotactic signal transducer of Escherichia coli. J. Bacteriol. **174:**1528-36.

40. **Gardina, P. J., A. F. Bormans, M. A. Hawkins, J. W. Meeker, and M. D. Manson.** 1997. Maltose-binding protein interacts simultaneously and asymmetrically with both subunits of the Tar chemoreceptor. Mol. Microbiol. **23:**1181-91.

41. **Gardina, P. J., A. F. Bormans, and M. D. Manson.** 1998. A mechanism for simultaneous sensing of aspartate and maltose by the Tar chemoreceptor of Escherichia coli. Mol. Microbiol. **29:**1147-54.

42. **Gardina, P. J., and M. D. Manson.** 1996. Attractant signaling by an aspartate chemoreceptor dimer with a single cytoplasmic domain. Science **274:**425-6.

43. **Gegner, J. A., and F. W. Dahlquist.** 1991. Signal transduction in bacteria: CheW forms a reversible complex with the protein kinase CheA. Proc. Natl. Acad. Sci. U. S. A. **88:**750-4.

44. **Gegner, J. A., D. R. Graham, A. F. Roth, and F. W. Dahlquist.** 1992. Assembly of an MCP receptor, CheW, and kinase CheA complex in the bacterial chemotaxis signal transduction pathway. Cell **70:**975-82.

45. **Gestwicki, J. E., and L. L. Kiessling.** 2002. Inter-receptor communication through arrays of bacterial chemoreceptors. Nature **415:**81-4.

46. **Goy, M. F., M. S. Springer, and J. Adler.** 1977. Sensory transduction in Escherichia coli: role of a protein methylation reaction in sensory adaptation. Proc. Natl. Acad. Sci. U. S. A. **74:**4964-8.

47. **Hazelbauer, G. L.** 1975. Maltose chemoreceptor of Escherichia coli. J. Bacteriol. **122:**206-14.

48. **Hazelbauer, G. L., and J. Adler.** 1971. Role of the galactose binding protein in chemotaxis of Escherichia coli toward galactose. Nat. New Biol. **230:**101-4.

49. **Hazelbauer, G. L., and P. Engstrom.** 1981. Multiple forms of methyl-accepting chemotaxis proteins distinguished by a factor in addition to multiple methylation. J. Bacteriol. **145:**35-42.

50. **Hazelbauer, G. L., and S. Harayama.** 1983. Sensory transduction in bacterial chemotaxis. Int. Rev. Cytol. **81:**33-70.

51. **Hess, J. F., K. Oosawa, N. Kaplan, and M. I. Simon.** 1988. Phosphorylation of three proteins in the signaling pathway of bacterial chemotaxis. Cell **53:**79-87.

52. **Jancarik, J., W. G. Scott, D. L. Milligan, D. E. Koshland, Jr., and S. H. Kim.** 1991. Crystallization and preliminary X-ray diffraction study of the ligand-binding domain of the bacterial chemotaxis-mediating aspartate receptor of Salmonella typhimurium. J. Mol. Biol. **221:**31-4.

53. **Jasuja, R., Y. Lin, D. R. Trentham, and S. Khan.** 1999. Response tuning in bacterial chemotaxis. Proc. Natl. Acad. Sci. U. S. A. **96:**11346-51.

54. **Kehry, M. R., M. W. Bond, M. W. Hunkapiller, and F. W. Dahlquist.** 1983. Enzymatic deamidation of methyl-accepting chemotaxis proteins in Escherichia coli catalyzed by the cheB gene product. Proc. Natl. Acad. Sci. U. S. A. **80:**3599-603.

55. **Kehry, M. R., T. G. Doak, and F. W. Dahlquist.** 1985. Sensory adaptation in bacterial chemotaxis: regulation of demethylation. J. Bacteriol. **163:**983-90.

56. **Kim, K. K., H. Yokota, and S. H. Kim.** 1999. Four-helical-bundle structure of the cytoplasmic domain of a serine chemotaxis receptor. Nature **400:**787-92.

57. **Kleene, S. J., M. L. Toews, and J. Adler.** 1977. Isolation of glutamic acid methyl ester from an Escherichia coli membrane protein involved in chemotaxis. J. Biol. Chem. **252:**3214-8.

58. **Koman, A., S. Harayama, and G. L. Hazelbauer.** 1979. Relation of chemotactic response to the amount of receptor: evidence for different efficiencies of signal transduction. J. Bacteriol. **138:**739-47.

59. **Kondoh, H., C. B. Ball, and J. Adler.** 1979. Identification of a methyl-accepting chemotaxis protein for the ribose and galactose chemoreceptors of Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. **76:**260-4.

60. **Kossmann, M., C. Wolff, and M. D. Manson.** 1988. Maltose chemoreceptor of Escherichia coli: interaction of maltose-binding protein and the tar signal transducer. J. Bacteriol. **170:**4516-21.

61. **Krikos, A., M. P. Conley, A. Boyd, H. C. Berg, and M. I. Simon.** 1985. Chimeric chemosensory transducers of Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. **82:**1326-30.

62. **Krikos, A., N. Mutoh, A. Boyd, and M. I. Simon.** 1983. Sensory transducers of E. coli are composed of discrete structural and functional domains. Cell **33:**615-22.

63. **Lai, R. Z., J. M. Manson, A. F. Bormans, R. R. Draheim, N. T. Nguyen, and M. D. Manson.** 2005. Cooperative Signaling among Bacterial Chemoreceptors. Biochemistry **45:**in press.

64. **Lai, W. C., and G. L. Hazelbauer.** 2005. Carboxyl-terminal extensions beyond the conserved pentapeptide reduce rates of chemoreceptor adaptational modification. J. Bacteriol. **187:**5115-21.

65. **Le Moual, H., T. Quang, and D. E. Koshland, Jr.** 1997. Methylation of the Escherichia coli chemotaxis receptors: intra- and interdimer mechanisms. Biochemistry **36:**13441-8.

66. **Lee, G. F., M. R. Lebert, A. A. Lilly, and G. L. Hazelbauer.** 1995. Transmembrane signaling characterized in bacterial chemoreceptors by using sulfhydryl cross-linking *in vivo*. Proc. Natl. Acad. Sci. U. S. A. **92:**3391-5.

67. **Lee, L., and Y. Imae.** 1990. Role of threonine residue 154 in ligand recognition of the tar chemoreceptor in Escherichia coli. J. Bacteriol. **172:**377-82.

68. **Levit, M. N., and J. B. Stock.** 2002. Receptor methylation controls the magnitude of stimulus-response coupling in bacterial chemotaxis. J. Biol. Chem. **277:**36760-5.

69. **Li, G., and R. M. Weis.** 2000. Covalent modification regulates ligand binding to receptor complexes in the chemosensory system of Escherichia coli. Cell **100:**357-65.

70. **Li, J., G. Li, and R. M. Weis.** 1997. The serine chemoreceptor from Escherichia coli is methylated through an inter-dimer process. Biochemistry **36:**11851-7.

71. **Li, M., and G. L. Hazelbauer.** 2004. Cellular stoichiometry of the components of the chemotaxis signaling complex. J. Bacteriol. **186:**3687-94.

72. **Lybarger, S. R., and J. R. Maddock.** 2000. Differences in the polar clustering of the high- and low-abundance chemoreceptors of Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. **97:**8057-62.

73. **Macnab, R. M., and D. E. Koshland, Jr.** 1972. The gradient-sensing mechanism in bacterial chemotaxis. Proc. Natl. Acad. Sci. U. S. A. **69:**2509-12.

74. **Maddock, J. R., and L. Shapiro.** 1993. Polar location of the chemoreceptor complex in the Escherichia coli cell. Science **259:**1717-23.

75. **Maeda, K., Y. Imae, J. I. Shioi, and F. Oosawa.** 1976. Effect of temperature on motility and chemotaxis of Escherichia coli. J. Bacteriol. **127:**1039-46.

76. **Manson, M. D., V. Blank, G. Brade, and C. F. Higgins.** 1986. Peptide chemotaxis in E. coli involves the Tap signal transducer and the dipeptide permease. Nature **321:**253-6.

77. **Meister, M., G. Lowe, and H. C. Berg.** 1987. The proton flux through the bacterial flagellar motor. Cell **49:**643-50.

78. **Mesibov, R., and J. Adler.** 1972. Chemotaxis toward amino acids in Escherichia coli. J. Bacteriol. **112:**315-26.

79. **Milburn, M. V., G. G. Prive, D. L. Milligan, W. G. Scott, J. Yeh, J. Jancarik, D. E. Koshland, Jr., and S. H. Kim.** 1991. Three-dimensional structures of the ligand-binding domain of the bacterial aspartate receptor with and without a ligand. Science **254:**1342-7.

80. **Miller, J. H.** 1972. in *Experiments in Molecular Genetics* p 433, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

81. **Milligan, D. L., and D. E. Koshland, Jr.** 1993. Purification and characterization of the periplasmic domain of the aspartate chemoreceptor. J. Biol. Chem. **268:**19991-7.

82. **Milligan, D. L., and D. E. Koshland, Jr.** 1988. Site-directed cross-linking. Establishing the dimeric structure of the aspartate receptor of bacterial chemotaxis. J. Biol. Chem. **263:**6268-75.

83. **Mowbray, S. L., and D. E. Koshland, Jr.** 1987. Additive and independent responses in a single receptor: aspartate and maltose stimuli on the tar protein. Cell **50:**171-80.

84. **Nara, T., I. Kawagishi, S. Nishiyama, M. Homma, and Y. Imae.** 1996. Modulation of the thermosensing profile of the Escherichia coli aspartate receptor tar by covalent modification of its methyl-accepting sites. J. Biol. Chem. **271:**17932-6.

85. **Nara, T., L. Lee, and Y. Imae.** 1991. Thermosensing ability of Trg and Tap chemoreceptors in Escherichia coli. J. Bacteriol. **173:**1120-4.

86. **Ninfa, E. G., A. Stock, S. Mowbray, and J. Stock.** 1991. Reconstitution of the bacterial chemotaxis signal transduction system from purified components. J. Biol. Chem. **266:**9764-70.

87. **Nishiyama, S., T. Nara, M. Homma, Y. Imae, and I. Kawagishi.** 1997. Thermosensing properties of mutant aspartate chemoreceptors with methyl-accepting sites replaced singly or multiply by alanine. J. Bacteriol. **179:**6573-80.

88. **Nishiyama, S. I., T. Umemura, T. Nara, M. Homma, and I. Kawagishi.** 1999. Conversion of a bacterial warm sensor to a cold sensor by methylation of a single residue in the presence of an attractant. Mol. Microbiol. **32:**357-65.

89. **Nowlin, D. M., J. Bollinger, and G. L. Hazelbauer.** 1987. Sites of covalent modification in Trg, a sensory transducer of Escherichia coli. J. Biol. Chem. **262:**6039-45.

90. **Okumura, H., S. Nishiyama, A. Sasaki, M. Homma, and I. Kawagishi.** 1998. Chemotactic adaptation is altered by changes in the carboxy-terminal sequence conserved among the major methyl-accepting chemoreceptors. J. Bacteriol. **180:**1862-8.

91. **Olson, E. R., D. S. Dunyak, L. M. Jurss, and R. A. Poorman.** 1991. Identification and characterization of dppA, an Escherichia coli gene encoding a periplasmic dipeptide transport protein. J. Bacteriol. **173:**234-44.

92. **Ottemann, K. M., T. E. Thorgeirsson, A. F. Kolodziej, Y. K. Shin, and D. E. Koshland, Jr.** 1998. Direct measurement of small ligand-induced conformational changes in the aspartate chemoreceptor using EPR. Biochemistry **37:**7062-9.

93. **Ottemann, K. M., W. Xiao, Y. K. Shin, and D. E. Koshland, Jr.** 1999. A piston model for transmembrane signaling of the aspartate receptor. Science **285:**1751-4.

94. **Parkinson, J. S.** 1978. Complementation analysis and deletion mapping of Escherichia coli mutants defective in chemotaxis. J. Bacteriol. **135:**45-53.

95. **Parkinson, J. S., and S. E. Houts.** 1982. Isolation and behavior of Escherichia coli deletion mutants lacking chemotaxis functions. J. Bacteriol. **151:**106-13.

96. **Quiocho, F. A.** 1990. Atomic structures of periplasmic binding proteins and the high-affinity active transport systems in bacteria. Philos. Trans. R. Soc. Lond. B Biol. Sci. **326:**341-51; discussion 351-2.

97. **Rice, M. S., and F. W. Dahlquist.** 1991. Sites of deamidation and methylation in Tsr, a bacterial chemotaxis sensory transducer. J. Biol. Chem. **266:**9746-53.

98. **Russell, C. B., R. C. Stewart, and F. W. Dahlquist.** 1989. Control of transducer methylation levels in Escherichia coli: investigation of components essential for modulation of methylation and demethylation reactions. J. Bacteriol. **171:**3609-18.

99. **Sanders, D. A., and D. E. Koshland, Jr.** 1988. Receptor interactions through phosphorylation and methylation pathways in bacterial chemotaxis. Proc. Natl. Acad. Sci. U. S. A. **85:**8425-9.

100. **Scharf, B. E., K. A. Fahrner, L. Turner, and H. C. Berg.** 1998. Control of direction of flagellar rotation in bacterial chemotaxis. Proc. Natl. Acad. Sci. U. S. A. **95:**201-6.

101. **Schuster, S. C., R. V. Swanson, L. A. Alex, R. B. Bourret, and M. I. Simon.** 1993. Assembly and function of a quaternary signal transduction complex monitored by surface plasmon resonance. Nature **365:**343-7.

102. **Scott, W. G., D. L. Milligan, M. V. Milburn, G. G. Prive, J. Yeh, D. E. Koshland, Jr., and S. H. Kim.** 1993. Refined structures of the ligand-binding domain of the aspartate receptor from Salmonella typhimurium. J. Mol. Biol. **232:**555-73.

103. **Shapiro, M. J., I. Chakrabarti, and D. E. Koshland, Jr.** 1995. Contributions made by individual methylation sites of the Escherichia coli aspartate receptor to chemotactic behavior. Proc. Natl. Acad. Sci. U. S. A. **92:**1053-6.

104. **Shapiro, M. J., and D. E. Koshland, Jr.** 1994. Mutagenic studies of the interaction between the aspartate receptor and methyltransferase from Escherichia coli. J. Biol. Chem. **269:**11054-9.

105. **Shimizu, T. S., N. Le Novere, M. D. Levin, A. J. Beavil, B. J. Sutton, and D. Bray.** 2000. Molecular model of a lattice of signalling proteins involved in bacterial chemotaxis. Nat. Cell Biol. **2:**792-6.

106. **Shiomi, D., I. B. Zhulin, M. Homma, and I. Kawagishi.** 2002. Dual recognition of the bacterial chemoreceptor by chemotaxis-specific domains of the CheR methyltransferase. J. Biol. Chem. **277:**42325-33.

107. **Silverman, M., and M. Simon.** 1977. Chemotaxis in Escherichia coli: methylation of che gene products. Proc. Natl. Acad. Sci. U. S. A. **74:**3317-21.

108. **Simms, S. A., A. M. Stock, and J. B. Stock.** 1987. Purification and characterization of the S-adenosylmethionine:glutamyl methyltransferase that modifies membrane chemoreceptor proteins in bacteria. J. Biol. Chem. **262:**8537-43.

109. **Skidmore, J. M., D. D. Ellefson, B. P. McNamara, M. M. Couto, A. J. Wolfe, and J. R. Maddock.** 2000. Polar clustering of the chemoreceptor complex in Escherichia coli occurs in the absence of complete CheA function. J. Bacteriol. **182:**967-73.

110. **Slocum, M. K., and J. S. Parkinson.** 1983. Genetics of methyl-accepting chemotaxis proteins in Escherichia coli: organization of the tar region. J. Bacteriol. **155:**565-77.

111. **Smith, R. A., and J. S. Parkinson.** 1980. Overlapping genes at the cheA locus of Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. **77:**5370-4.

112. **Sourjik, V.** 2004. Receptor clustering and signal processing in E. coli chemotaxis. Trends Microbiol. **12:**569-76.

113. **Springer, M. S., M. F. Goy, and J. Adler.** 1979. Protein methylation in behavioural control mechanisms and in signal transduction. Nature **280:**279-84.

114. **Springer, M. S., M. F. Goy, and J. Adler.** 1977. Sensory transduction in Escherichia coli: two complementary pathways of information processing that involve methylated proteins. Proc. Natl. Acad. Sci. U. S. A. **74:**3312-6.

115. **Springer, W. R., and D. E. Koshland, Jr.** 1977. Identification of a protein methyltransferase as the cheR gene product in the bacterial sensing system. Proc. Natl. Acad. Sci. U. S. A. **74:**533-7.

116. **Starrett, D. J., and J. J. Falke.** 2005. Adaptation mechanism of the aspartate receptor: electrostatics of the adaptation subdomain play a key role in modulating kinase activity. Biochemistry **44:**1550-60.

117. **Stock, A. M., D. C. Wylie, J. M. Mottonen, A. N. Lupas, E. G. Ninfa, A. J. Ninfa, C. E. Schutt, and J. B. Stock.** 1988. Phosphoproteins involved in bacterial signal transduction. Cold Spring Harb Symp Quant Biol **53 Pt 1:**49-57.

118. **Stock, J. B., and D. E. Koshland, Jr.** 1978. A protein methylesterase involved in bacterial sensing. Proc. Natl. Acad. Sci. U. S. A. **75:**3659-63.

119. **Studdert, C. A., and J. S. Parkinson.** 2004. Crosslinking snapshots of bacterial chemoreceptor squads. Proc. Natl. Acad. Sci. U. S. A. **101:**2117-22.

120. **Surette, M. G., and J. B. Stock.** 1996. Role of alpha-helical coiled-coil interactions in receptor dimerization, signaling, and adaptation during bacterial chemotaxis. J. Biol. Chem. **271:**17966-73.

121. **Tatsuno, I., M. Homma, K. Oosawa, and I. Kawagishi.** 1996. Signaling by the Escherichia coli aspartate chemoreceptor Tar with a single cytoplasmic domain per dimer. Science **274:**423-5.

122. **Taylor, B. L., A. Rebbapragada, and M. S. Johnson.** 2001. The FAD-PAS domain as a sensor for behavioral responses in Escherichia coli. Antioxid Redox Signal **3:**867-79.

123. **Terwilliger, T. C., E. Bogonez, E. A. Wang, and D. E. Koshland, Jr.** 1983. Sites of methyl esterification on the aspartate receptor involved in bacterial chemotaxis. J. Biol. Chem. **258:**9608-11.

124. **Terwilliger, T. C., and D. E. Koshland, Jr.** 1984. Sites of methyl esterification and deamination on the aspartate receptor involved in chemotaxis. J. Biol. Chem. **259:**7719-25.

125. **Terwilliger, T. C., J. Y. Wang, and D. E. Koshland, Jr.** 1986. Kinetics of receptor modification. The multiply methylated aspartate receptors involved in bacterial chemotaxis. J. Biol. Chem. **261:**10814-20.

126. **Tso, W. W., and J. Adler.** 1974. Negative chemotaxis in Escherichia coli. J. Bacteriol. **118:**560-76.

127. **Van Der Werf, P., and D. E. Koshland, Jr.** 1977. Identification of a gamma-glutamyl methyl ester in bacterial membrane protein involved in chemotaxis. J. Biol. Chem. **252:**2793-5.

128. **Ward, S. M., A. Delgado, R. P. Gunsalus, and M. D. Manson.** 2002. A NarX-Tar chimera mediates repellent chemotaxis to nitrate and nitrite. Mol. Microbiol. **44:**709-19.

129. **Weerasuriya, S., B. M. Schneider, and M. D. Manson.** 1998. Chimeric chemoreceptors in Escherichia coli: signaling properties of Tar-Tap and Tap-Tar hybrids. J. Bacteriol. **180:**914-20.

130. **Welch, M., K. Oosawa, S. Aizawa, and M. Eisenbach.** 1993. Phosphorylation-dependent binding of a signal molecule to the flagellar switch of bacteria. Proc. Natl. Acad. Sci. U. S. A. **90:**8787-91.

131. **Williams, S. B., and V. Stewart.** 1999. Functional similarities among two-component sensors and methyl-accepting chemotaxis proteins suggest a role for linker region amphipathic helices in transmembrane signal transduction. Mol. Microbiol. **33:**1093-102.

132. **Wolff, C., and J. S. Parkinson.** 1988. Aspartate taxis mutants of the Escherichia coli tar chemoreceptor. J. Bacteriol. **170:**4509-15.

133. **Wu, J., J. Li, G. Li, D. G. Long, and R. M. Weis.** 1996. The receptor binding site for the methyltransferase of bacterial chemotaxis is distinct from the sites of methylation. Biochemistry **35:**4984-93.

134. **Yeh, J. I., H. P. Biemann, J. Pandit, D. E. Koshland, and S. H. Kim.** 1993. The three-dimensional structure of the ligand-binding domain of a wild-type bacterial chemotaxis receptor. Structural comparison to the cross-linked mutant forms and conformational changes upon ligand binding. J. Biol. Chem. **268:**9787-92.

135. **Yeh, J. I., H. P. Biemann, G. G. Prive, J. Pandit, D. E. Koshland, Jr., and S. H. Kim.** 1996. High-resolution structures of the ligand binding domain of the wild-type bacterial aspartate receptor. J. Mol. Biol. **262:**186-201.

136. **Zhang, Y., P. J. Gardina, A. S. Kuebler, H. S. Kang, J. A. Christopher, and M. D. Manson.** 1999. Model of maltose-binding protein/chemoreceptor complex supports intrasubunit signaling mechanism. Proc. Natl. Acad. Sci. U. S. A. **96:**939-44.

137. **Zhu, Y., and M. Inouye.** 2003. Analysis of the role of the EnvZ linker region in signal transduction using a chimeric Tar/EnvZ receptor protein, Tez1. J. Biol. Chem. **278:**22812-9.

VITA

Name:	Arjan Frank Bormans
Address:	6919 Chapel Meadow Lane, Richmond, TX 77649
Email Address:	abormans@gmail.com
Education:	B. S. (Microbiology), Texas A&M University Ph. D. (Microbiology), Texas A&M University