COMPARISON OF THE MECHANISM OF TRANSMEMBRANE SIGNALING IN BACTERIAL CHEMORECEPTORS AND SENSOR KINASES

A Dissertation

by

SCOTT MICHAEL WARD

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

August 2005

Major Subject: Microbiology

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ABSTRACT

Comparison of the Mechanism of Transmembrane Signaling in Bacterial Chemoreceptors and Sensor Kinases. (August 2005)
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Membrane-bound receptors transmit information from the cell exterior to the cell interior. Bacterial receptors capable of transmitting this information include sensor kinases, which control gene expression via response regulators, and methyl-accepting chemotaxis proteins (MCPs), which control rotation of the flagellar motor. These receptors, which have a similar general architecture and function, are predicted to share similar mechanisms of transmembrane signaling. The majority of such work has been conducted on MCPs. Our goal is to extend this work to the closely related sensor kinases by creating functional hybrid transducers. I show that a chimeric protein (Nart) that joins the periplasmic, ligand-binding domain of the sensor kinase NarX (nitrate/nitrite sensor) to the cytoplasmic signaling domain of the chemoreceptor Tar is capable of modulating flagellar rotation in response to both nitrate and nitrite. Consistent with the properties of NarX, our Nart elicits a stronger response to nitrate than to nitrite. Introduction of mutations into a highly conserved periplasmic region affects Nart signaling in a fashion that is consistent with the effects seen in NarX. I also present the first example of a substitution in a presumed ligand-binding domain that confers a reverse-signal phenotype for both nitrate and nitrite

in Nart. These results support the hypothesis that the key aspects of transmembrane signaling are closely similar in homodimeric bacterial chemoreceptors and sensor kinases.

DEDICATION

I dedicate this thesis to my mother, Gloria Ward, for the many years she spent teaching me the value of education and fostering my love of science. Without her, I would not be the person that I am today.

ACKNOWLEDGEMENTS

I would like to thank:

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CHAPTER I

INTRODUCTION

Transmembrane signaling overview

The enteric bacterium *Escherichia coli* is incapable of exerting large changes on its surroundings. The bacteria instead depend on their ability to detect changes in the environment and to modify their structure, physiology, and behavior accordingly. These changes are triggered by the transmission of external stimuli to the internal machinery of the cell. Thus, a cell can determine its position in a host, utilize a preferred carbon source, or move towards a more favorable environment. Some of this information is relayed across the cell membrane by receptor proteins that contain a periplasmic input domain connected via a transmembrane region to a cytoplasmic signaling domain. Two major types of transmembrane receptors are found: sensor kinases and chemoreceptors.

Sensor kinases are elements of two-component systems that detect external conditions and transmit this information to internal response regulators that control transcription. These pathways allow the cell to make metabolic and structural changes in response to external conditions. Two-component systems are responsible, among many other things, for porin regulation, nitrate detection, and expression of virulence factors. Since the output for most two-component systems is transcription, the induced changes occur on a time scale of minutes.

This dissertation follows the style and format of the journal *Molecular Microbiology*.

Cells also react to external stimuli using rotary motor-driven flagellar filaments. Cell locomotion is coupled to the ability to detect changes in the environment by transmembrane chemoreceptors. The interaction of ligands with these proteins elicits a signal that causes the flagellar motor to change its rotational bias, resulting in a net motion towards improving environmental conditions. By coupling flagellar rotation to external detection systems, the chemotaxis circuit allows cells to move towards better environmental conditions. This behavior occurs on a time scale of seconds.

In summary, bacteria respond to changes in their surrounding by moving towards more favorable environments and by expressing genes that increase survival in those environments. The cells convert receptor input into appropriate output responses, allowing both directional change and adaptation to fluctuating conditions. It is not surprising to find both similarities and differences between these two types of transmembrane receptors.

Bacterial chemotaxis

Chemotaxis in *E. coli* is a model system for signal transduction (see Stock & Surette (1996) for a review of chemotaxis). The chemical environment is monitored by transmembrane proteins known as chemoreceptors. Information is transmitted from the receptors to the flagellar motor using a phospho-relay system of cytoplasmic chemotaxis (Che) proteins. The binding of phosphorylated CheY to the flagellar motor enhances clockwise (CW) rotation. By modulating rotation of the flagellar motor, the cell is able to move towards more favorable environmental conditions.

Motile *E. coli* cells alternate between two types of motion. The default behavior is to move in a smooth-swimming pattern (run) in which the cell travels in a gently curved path (Berg and Brown, 1972). These runs are punctuated by abrupt changes in direction (tumbles) that are caused by brief reversals in the direction of flagellar rotation (Berg and Brown, 1972). After a tumble, the cell again runs. Alternating runs and tumbles result in a three-dimensional random walk (Fig. 1).

Chemoreceptors detect both attractant and repellent ligands, collectively known as chemoeffectors. The cells compare the current concentration of chemoeffectors to the concentration 2-3 seconds earlier (McNabb and Koshland, 1972; Berg and Brown, 1974; Segall *et al.*, 1986). If the environment is improving, because of an increasing concentration of attractant or a decreasing level of repellent, the probability of tumbling decreases, and runs in the favorable direction are extended. This temporal, as opposed to spatial (one end of the cell relative to the other), sensing mechanism allows even relatively shallow gradients to be detected.

The output of chemotaxis is the modulation of flagellar rotation. The default counter-clockwise (CCW) rotation (Clegg and Koshland, 1984) allows the rigid left-handed flagellar filaments to coalesce into a helical bundle, resulting in a run. Reversal to clockwise (CW) rotation causes the helical bundle to fall apart, and the cell tumbles. When all motors switch back to CCW rotation, the flagellar bundle reforms, and the cell is randomly oriented for its next run.

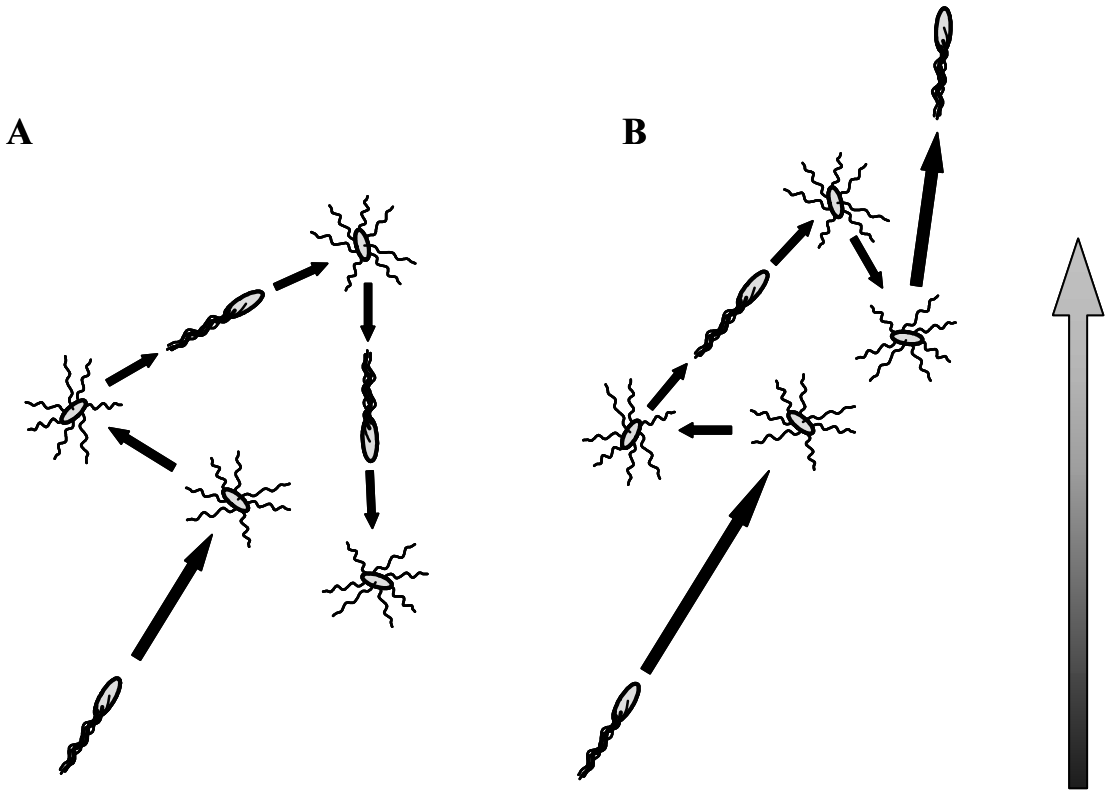

Fig. 1. Motion of *E. coli* in the absence (A) or presence (B) of a chemical gradient. Runs result from CCW motor rotation, which allows the flagellar filaments to coalesce into a left-handed helical bundle and propel the cell forward. When the motor switches rotation from CCW to CW, the flagellar bundle falls apart, which causes the cell to tumble. Alternation of these two modes of locomotion facilitates movement of the cell in its environment. In (A), absence of a chemoeffector gradient results in random movement of the cell. The large arrow (B) represents an improving chemical gradient (increasing attractant or decreasing repellent). Longer runs are seen as the cell travels in the favorable direction.

Chemotactic signaling pathway

The interplay of the Che proteins that are involved in the chemotaxis circuit is shown in Fig. 2. Chemoreceptors embedded in the cell membrane provide sensory input from their periplasmic ligand-binding domains. Ligand binding initiates a conformational change in the periplasmic region that propagates through the inner membrane to the cytoplasmic signaling domain (Milligan and Koshland, 1991; Tatsuno *et al.*, 1996; Gardina and Manson, 1996). The autophosphorylation activity of the core component in the signaling pathway, the CheA kinase, is affected by changes in the cytoplasmic domain of the receptor. Binding of an attractant inhibits the ability of the receptor to stimulate CheA activity (Borkovitch and Simon, 1990). Binding of a repellent activates autophosphorylation (Borkovich *et al.*, 1989; Hess *et al.,* 1988; Stock *et al.*, 1988). Phosphorylated CheY (phospho-CheY), the product of phosphoryl group transfer from CheA, binds to the switch complex of the motor to promote CW flagellar rotation.

The default directional rotation in the absence of Che proteins is CCW (Clegg and Koshland, 1984; Wolfe *et al.*, 1987). Phospho-CheY binds to FliM, one of three proteins in the motor-switch complex, to promote CW rotation (Ravid *et al.*, 1986; Welch *et al.,* 1993). As attractant concentrations rise, CheA activity is inhibited, and the level of phospho-CheY decreases (Alon *et al.,* 1998; Cluzel *et al.,* 2000). Lower levels of phospho-CheY promote CCW motor rotation, and cells become more smooth-swimming. Repellents cause the level of phospho-CheY to rise, promoting CW rotation and increasing tumbling. In practice, cells react to decreasing repellent concentrations by decreasing tumbles, thereby increasing run lengths.

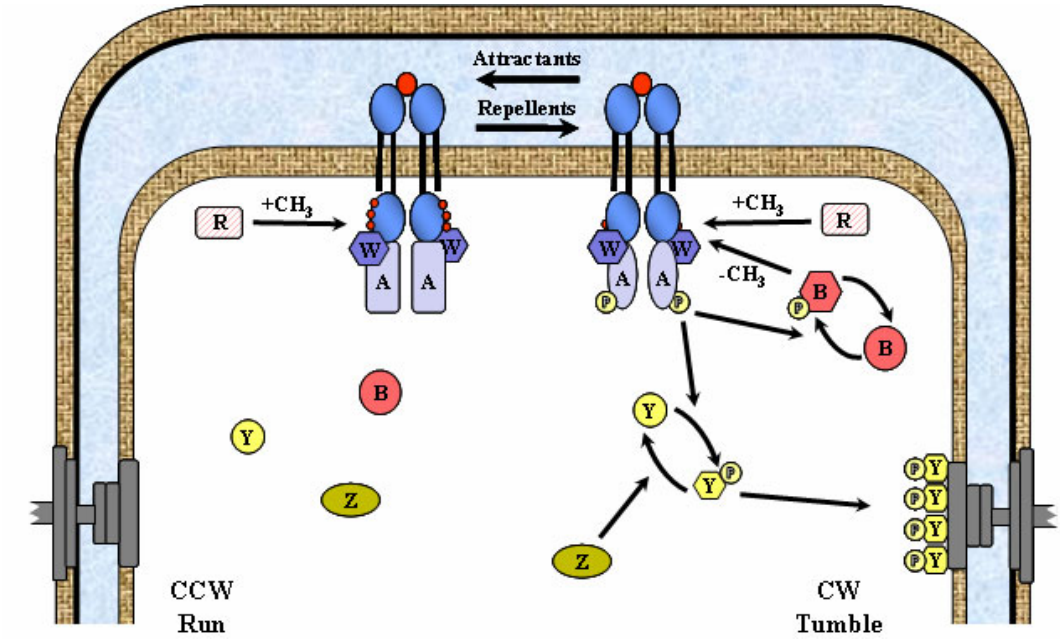

Fig. 2. Chemotaxis circuit of *E. coli.* Signal detection occurs via homodimeric chemoreceptors. Detection of attractants or repellents, respectively, inhibits or stimulates CheA autophosphorylation. CheA is coupled to the receptor by CheW. A phosphorylated CheA (CheA~P) can donate a phosphoryl group to either CheY or CheB. The default counterclockwise (CCW) rotation results in a run. Interaction of CheY~P with switch components of the flagellar motor promotes clockwise (CW) rotation, which leads to tumbling. CheZ facilitates the restoration of a basal signaling state by promoting dephophorylation of CheY~P. Adaptation is modulated by the competing activities of the CheR methyltransferase and the CheB methylesterase. When phosphorylated by CheA, CheB~P antagonizes the action of CheR by removing methyl groups.

Several other Che proteins are required for the function of the chemotaxis circuit. CheW appears to stabilize the interaction between chemoreceptors and CheA (Gegner *et al.*, 1992). The phophatase CheZ greatly accelerates the conversion of phospho-CheY back to inactive CheY (Hess *et al.,* 1988). Adaptation in the chemotaxis pathway depends on two proteins, CheR and CheB. The CheR methyltransferase covalently modifies the chemoreceptors, and phospho-CheB, also generated by phosphoryl group transfer from CheA, removes these methyl groups. Receptor methylation and demethylation reset the receptor, allowing cells to adapt to the current ligand concentration and return to the basal signaling state.

Chemoreceptors

The four known *E. coli* chemoreceptors (Tsr, Tar, Tap, and Trg) have been divided into two classes, major and minor, based on their relative activities and abundances within the cell (Boyd *et al*., 1983; Krikos *et al.*, 1983; Bollinger *et al.*, 1984; Bibikov *et al.*, 1997). The major (or high-abundance) receptors are serine-sensing Tsr and aspartate-sensing Tar, which occur in about 3000 and 1500, respectively, monomer copies per cell (Koman *et al.*, 1979; Clarke and Koshland, 1979). The minor transducers, which occur at 10-fold lower levels, comprise galactose, glucose, and ribose-sensing Trg and dipeptide/tripeptide-sensing Tap.

Both the major and minor transducers function as homodimers within the inner membrane (Milligan and Koshland, 1988). Each monomer consists of an N-terminal transmembrane helix (TM 1), a periplasmic ligand-recognition domain, a second

transmembrane helix (TM2), a linker region, and a cytoplasmic signaling and adaptation domain (Fig. 3; Krikos *et al.*, 1985; Aravind and Ponting, 1999; Williams and Stewart, 1999). The cytoplasmic domain contains four or five glutamyl residues that are targets for methylation by the CheR methyltransferase, which is why the chemoreceptors are alternatively referred to as methyl-accepting chemotaxis proteins (MCPs; Kort *et al.,* 1975)

Mutational analyses and sequence similarities suggest that the four receptors share similar overall structure (Wolff and Parkinson, 1988; Lee *et al.*, 1988; Gardina *et al.*, 1992; Park and Hazelbauer, 1986). The periplasmic domains contain a four-helix bundle in which the final helix (α4) extends though the membrane (as TM2) to connect with the linker region (Bowie *et al.,* 1995; Pakula and Simon, 1992; Lee *et al.*, 1994). Current evidence suggests that ligand-initiated axial movement of helix α4 communicates ligand binding to the cytoplasmic domain (Chervitz and Falke, 1996; Umemura *et al.*, 1998; Ottemann *et al.,* 1999). Exchanges of periplasmic domains between various receptors has been used to create functional hybrid proteins, indicating that signaling mechanisms are conserved in these chimeras. For example, a receptor containing the periplasmic and linker regions of Tar connected to the cytoplasmic region of Tsr produced a receptor with the ligand-recognition properties of Tar and the signaling characteristics of Tsr (Krikos *et al.*, 1985). Similar results were found upon combining the periplasmic region of the minor receptor Tap with the cytoplasmic region of the major receptor Tar. This hybrid signaled in response to Tap ligands, but, unlike Tap, could function as the sole receptor in the cell (Weerasuriya *et al.*, 1998). Hybrid

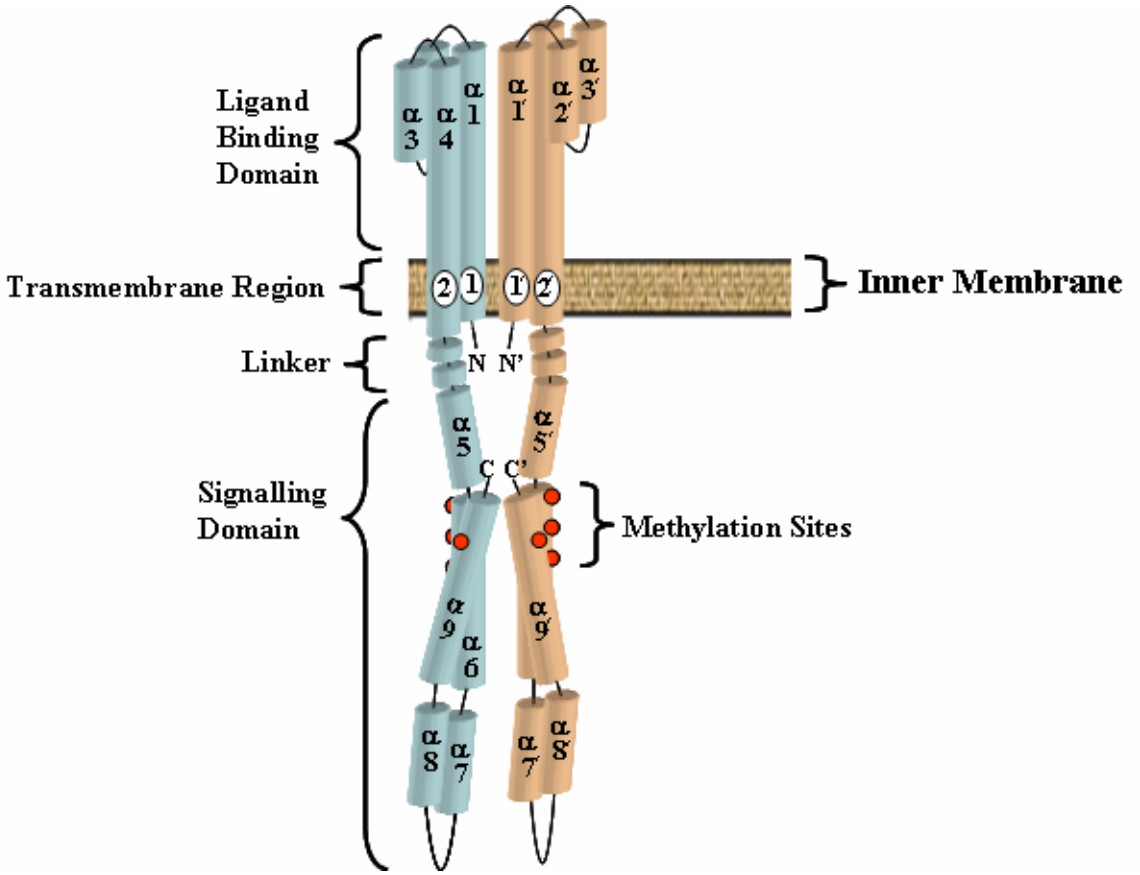

Fig. 3. A chemoreceptor dimer in *E.coli*. Each monomer contains nine helices (teal), α1–α9, and passes through the inner membrane twice (TM1 and TM2, ovals). Components of the second subunit (beige) are labeled α1'–α9', TM1' and TM2'. The N-terminal ligand-binding domain connects to the C-terminal signaling domain via a conserved HAMP linker domain. Four methylatable glutamyl residues (red circles), which are involved in adaptation, are located in helices α6 (α6') and α9 (α9').

constructs linking the periplasmic domain of Trg to the cytoplasmic domain of Tsr produced similar results (Feng *et al.,* 1997).

Both major and minor chemoreceptors sense ligands that interact with the periplasmic domain to initiate a signal. Although minor receptors share a similar overall topology with their major counterparts, they are incapable of relaying a functional signal in the absence of a major transducer (Koman *et al.*, 1979; Feng *et al.*, 1997; Weerasuriya *et al.*, 1998). Even when minor receptors are expressed at the same levels as major transducers they do not support a robust chemotactic response because they stimulate the CheA kinase inefficiently (Feng *et al.*, 1997; Weerasuriya *et al.*, 1998).

Tar binds aspartate at the dimer interface near the apex of the periplasmic domain (Mesibov and Adler, 1972; Milburn *et al.*, 1991; Milligan and Koshland, 1993). Tar responds to maltose as an attractant via its interaction with maltose-binding protein (MBP), which binds across the apex of the dimer (Hazelbauer, 1975; Brass and Manson, 1984; Zhang *et al.*, 1999). Repellent responses to the divalent cations Ni^{2+} and Co^{2+} are also transduced by Tar (Tso and Adler, 1974), although the binding sites for those ions are not known.

Serine and leucine bind to the other high-abundance chemoreceptor, Tsr, and mediate attractant and repellent responses, respectively (Mesibov and Adler, 1972; Springer *et al.*, 1977). The low-abundance transducer Tap responds to dipeptides via the dipeptide-binding protein (DppA; Manson *et al.*, 1986; Olson *et al.*, 1991; Adouhamad *et al.*, 1991). Trg responds to ribose via the ribose-binding protein (RBP) and to galactose/glucose via the galactose/glucose binding protein (GBP; Hazelbauer and

Adler, 1971).

Signaling by chemoreceptors is also affected by the adaptation machinery. The cytoplasmic region of each receptor contains four or five glutamyl residues that are targets for methylation by the CheR methyltransferase (Goy *et al*., 1977). The activity of CheR is antagonized by the CheB methylesterase, which when phosphorylated by phospho-CheA, removes methyl groups from the glutamyl-methyl ester residues (Lupas and Stock, 1989; Hess *et al*., 1988). Through these counteracting activities, the cells acquire a 'memory' of the environmental conditions that prevailed seconds earlier.

Without environmental stimulation, 1-2 glutamyl residues are methylated in steady state (Kort *et al.,* 1975; Goy *et al.,* 1977). When the activity of phospho-CheB decreases because of the inhibition of CheA activity, the methylation level of the receptor increases (Toews *et al.,* 1979; Kehry *et al.,* 1984). If repellents are detected by the receptor, increased CheA activity generates more phospho-CheB, thereby decreasing methylation of the receptor.

The cytoplasmic region of a high-abundance transducer transmits the signal received from the periplasmic region to the CheA kinase (Borkovich *et al*., 1989). In a chemically homogeneous environment, CheA activity is maintained at a level that supports baseline run-tumble motility. An increase in attractant or decrease in repellent concentration suppresses tumbling and results in smooth-swimming because levels of phospho-CheY transiently decrease (Borkovich and Simon, 1990).

Two-component system overview

Two-component systems contain one or more members from each of two protein families. The sensor kinase moiety typically receives the stimulus to trigger a signaling cascade that is received by a response regulator protein. Response regulators exist in either an active (typically phosphorylated) or an inactive conformation and mediate an adaptive response, usually by modifying gene expression. There are over 30 two-component sensor kinase response regulator pairs that have been identified in *E. coli.*

Most sensor kinases are membrane-spanning proteins that are predicted to function as homodimers. Typically, a periplasmic N-terminal input domain is joined to a C-terminal cytoplasmic transmitter domain by a transmembrane region and a HAMP (histidine kinase, adenylyl cyclase, MCP and phosphatase) linker. A chemical ligand or other stimulus modulates the activity of the kinase (Fig. 4).

Response regulators are cytoplasmic proteins that typically contain an N-terminal 'receiver' domain connected to a C-terminal output domain. The output domains often possess DNA-binding activity. Phosphorylation of a conserved Asp residue in the receiver domain alters DNA binding by the output domain to control transcription. Response regulators can either activate or repress transcription of their target genes. The receiver domains also catalyze hydrolysis of their own phosphoryl group, an activity that can be stimulated by their cognate sensor kinase acting as a phosphatase.

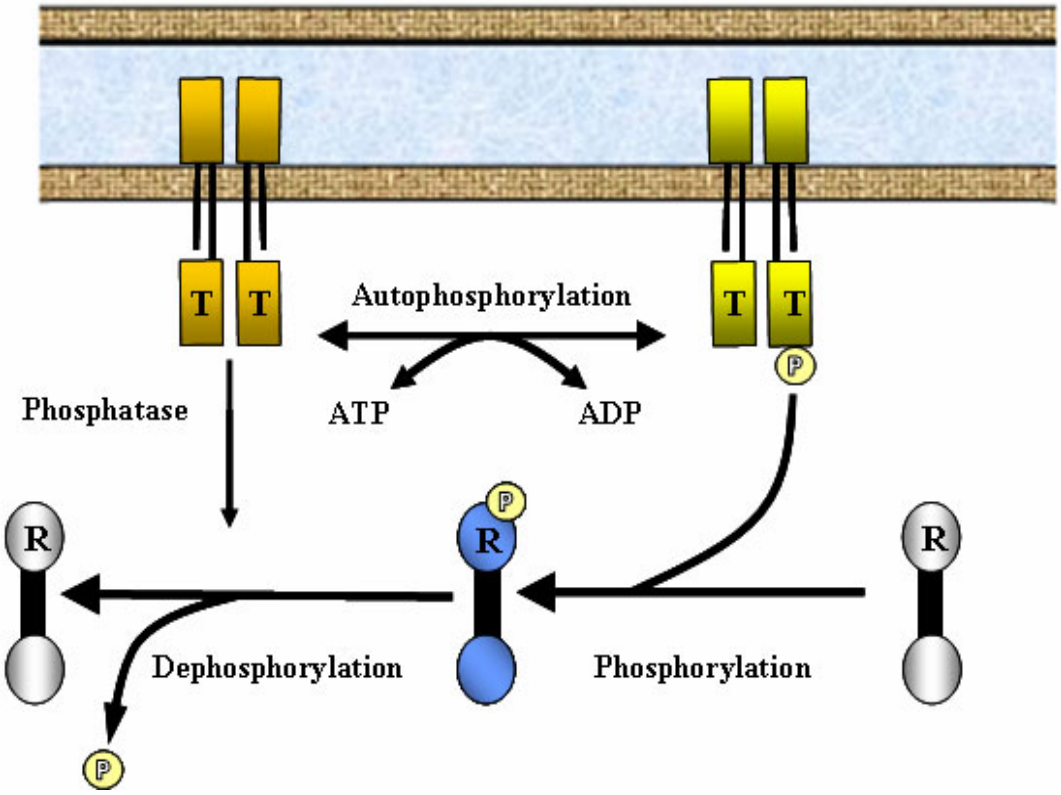

Fig. 4. A hypothetical two-component signaling pathway. The sensor kinase has three biochemical activities. Unstimulated sensor kinases show phosphatase activity towards their cognate phosphorylated response regulator(s). Stimulated sensor kinases autophosphorylate at a conserved His residue (the phosphorylation appears to be intersubunit rather than intrasubunit) and then transfer the phosphoryl groups to their cognate response regulators to activate them. The phosphoryl group is added to a highly conserved Asp residue. The active response regulators then interact with their output DNA targets.

A complex ligand-sensing two-component system

One well-studied two-component pathway provides insight into the paradigm of communication between sensor kinases and response regulators. The nitrate reductase (Nar) system controls expression of genes involved in anaerobic respiration and fermentation in response to extracellular nitrate and nitrite (Darwin and Stewart, 1996; Stewart and Rabin, 1995). During anaerobic growth, both nitrate and nitrite function as efficient respiratory oxidants (Gennis and Stewart, 1996). The Nar regulatory system in *E. coli* comprises two overlapping sets of homologous two-component pathways (Fig. 5), NarX-NarL and NarQ-NarP. NarX and NarQ sense environmental nitrate/nitrite levels and transmit this information through the membrane to control intracellular levels of phosphorylated NarL and NarP (Rabin and Stewart, 1992).

Both NarX and NarQ bind nitrate and nitrite, and ligand binding stimulates autophosphorylation at a conserved His residue. Although both sensors recognize nitrate and nitrite, NarX discriminates between the two to elicit differential responses, whereas NarQ signals similarly in response to either ligand (Williams and Stewart, 1997). Phospho-NarL and phospho-NarP bind to promoters of certain genes whose products are involved in anaerobic respiration (Stewart, 1993). These genes encode two nitrate reductases (*narGHI* and *napA*), two nitrite reductases (*nirBDC* and *nrfABCDEFG*), a nitrite export protein (*narK),* a formate dehydrogenase *(fdnGHI),* a dimethyl sulfoxide/trimethylamine-N-oxide reductase *(dmsABC),* and a fumarate reductase *(frdABCD)*.

NarX and NarQ each possess a phosphatase activity that stimulates the

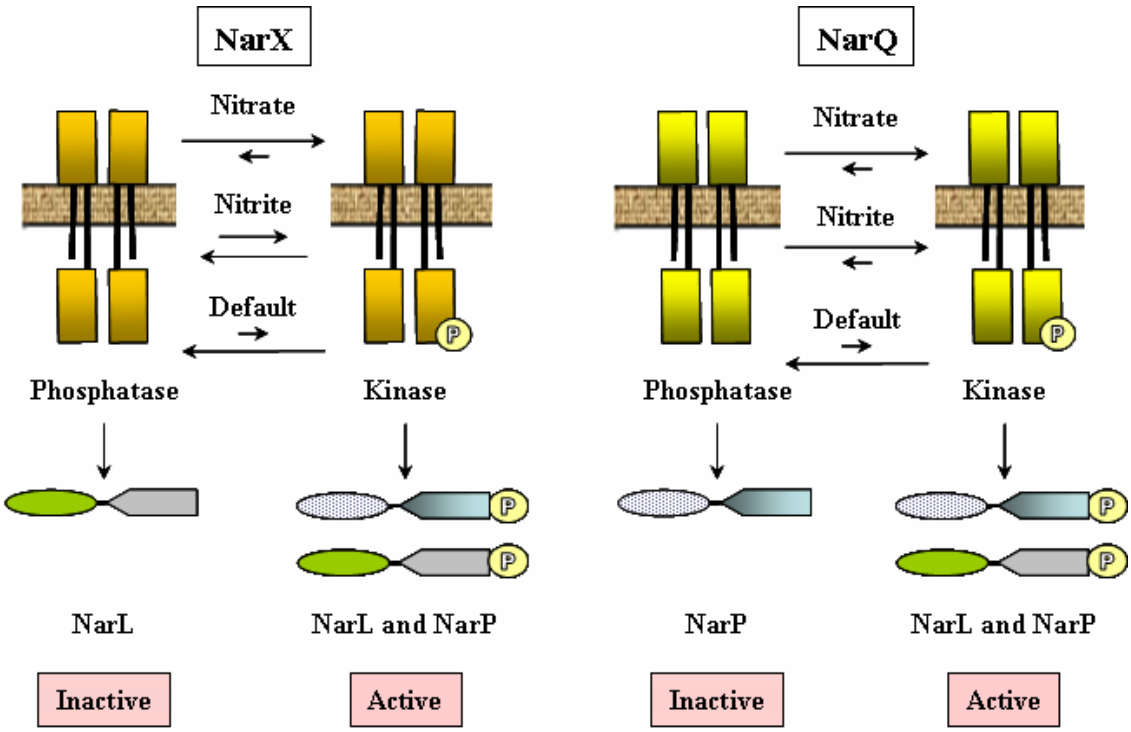

Fig. 5. The NarX-NarL and NarQ-NarP two-component pathways. The NarX/L-NarQ/P system of *E. coli* responds to extracellular nitrate and nitrite. Either NarX or NarQ is capable of phosphorylating either of the response regulators NarL and NarP. In the absence of ligands, NarX and NarQ act primarily as phosphatases. Responses to nitrate mediated by both sensors are similar and favor phosphoylation of NarL and NarP. NarQ phosphorylates both NarL and NarP in the presence of nitrite, whereas nitrite enhances favors the phosphatase activity of NarX towards NarL.

dephosphorylation of phospho-NarL and phospho-NarP (Cavicchioli *et al.,* 1995; Schroeder *et al.*, 1994; Walker and DeMoss, 1993). NarX phosphorylates NarL in the presence of nitrate but dephopshorylates NarL in the presence of nitrite. In contrast, NarQ kinase functions toward both NarL and NarP is stimulated in the presence of nitrate or nitrite (Darwin and Stewart, 1996; Stewart and Rabin, 1995).

NarX and NarQ have similar putative architectures. Both function as homodimers. Each contains an N-terminal periplasmic domain, two hydrophobic transmembrane helices (TM1 and TM2), and a cytoplasmic C-terminal transmitter domain that is connected to the periplasmic domain by TM2. The periplasmic regions are roughly the same size (about 100 residues), although they have very little sequence similarity. An exception is an 18-residue conserved region known as the P-box, which is predicted to play a role in ligand detection and signaling. The cytoplasmic transmitter domain of both Nar sensors contains a conserved His residue that is the target for autophosphorylation and serves as the donor for subsequent phosphoryl transfer to NarL or NarP.

Transmembrane signaling

The chemotaxis system has revealed much of what is known about transmembrane signaling. As a result of interaction with ligand, the periplasmic domain of a chemoreceptor undergoes a conformational change. This change translates into motion of one of the transmembrane helices of one subunit in relation to the other. This asymmetric motion, in turn, is predicted to cause a conformational change within the

cytoplasmic domain that alters receptor-coupled CheA activity (Borkovich and Simon, 1990).

Salmonella Tar undergoes a 1-2 Å displacement between TM2 and TM1 upon binding of aspartate (Chervitz and Falke, 1996; Ottemann and Koshland, 1998). Current models predict that helix α4 of Tar, which connects directly to TM2, moves downward relative to TM1, which is an N-terminal extension of helix α1 (Fig. 3).

Some models have proposed that this slight downward shift of TM2 moves the HAMP domain away from the cytoplasmic membrane, preventing association of an amphipathic sequence (AS1) with the membrane. Although this idea is attractive, Ottemann and Koshland (1997) show that some ligand-induced signaling occurs in the absence of TM1 and TM2 and membranes *in vitro*. This result suggests that the relative movement of the TM helices is not absolutely required for communication between the ligand-binding and signaling domains.

The 'piston' model proposed by Chervitz and Falke (1996) suggests that the movement of TM2 is perpendicular to the membrane and essentially parallel to the helix of TM1. This model combines x-ray crystallographic data (Milburn *et al.* 1991), solution ^{19}F NMR (Danielson *et al.*, 1994), and disulfide-engineering studies (Chervitz *et al.,* 1995; Chervitz and Falke, 1995). Other models suggest that helix α4 may rotate relative to helix α1 (Maruyama *et al.*, 1995; Cochran and Kim, 1996). These models are not mutually exclusive, and α4 helix may move downward while also undergoing tilt and rotation. The exact mechanics of the motion remain elusive, and further experiments are needed to determine the precise conformational changes induced by ligand binding.

Transmembrane receptor similarities

Sensor kinases and chemoreceptors have evolved to recognize different ligands and to communicate with and control diverse signaling pathways and output responses. However, they share several important structural features. As type I membrane receptors, they have a modular construction consisting of an N-terminal sensing domain connected to a C-terminal signaling domain via a HAMP linker. All of these receptors contain two N-terminal transmembrane regions (TM1 and TM2), which flank a periplasmic extension of 100-160 residues. Both types of receptor typically function as homodimers, and dimerization is independent of ligand. Both types of receptors function within their respective systems to modulate a phosphorelay in response to changing environmental conditions, although kinases typically have intrinsic autophosphorylation and phosphatase activities, whereas chemoreceptors control the activity of the CheA kinase (Borkovich and Simon, 1990) and, perhaps, the CheZ phosphatase (Cantwell *et al.*, 2003).

The HAMP region, located just C-terminal to TM2, is involved in transmission of the periplasmic and transmembrane input signal to the cytoplasmic output domain. HAMP regions contain approximately 50 amino acids and consist of two predicted amphipathic α-helices (AS1 and AS2) joined via an unstructured connector (see Fig. 6). HAMP regions are found in all *E. coli* chemoreceptors and in about half of the known sensor kinases. Although their general chemical features are similar, they show little sequence identity. This overall structural similarity allows the HAMP region to be used as a fusion target to create hybrid receptors, although the function of a hybrid protein is

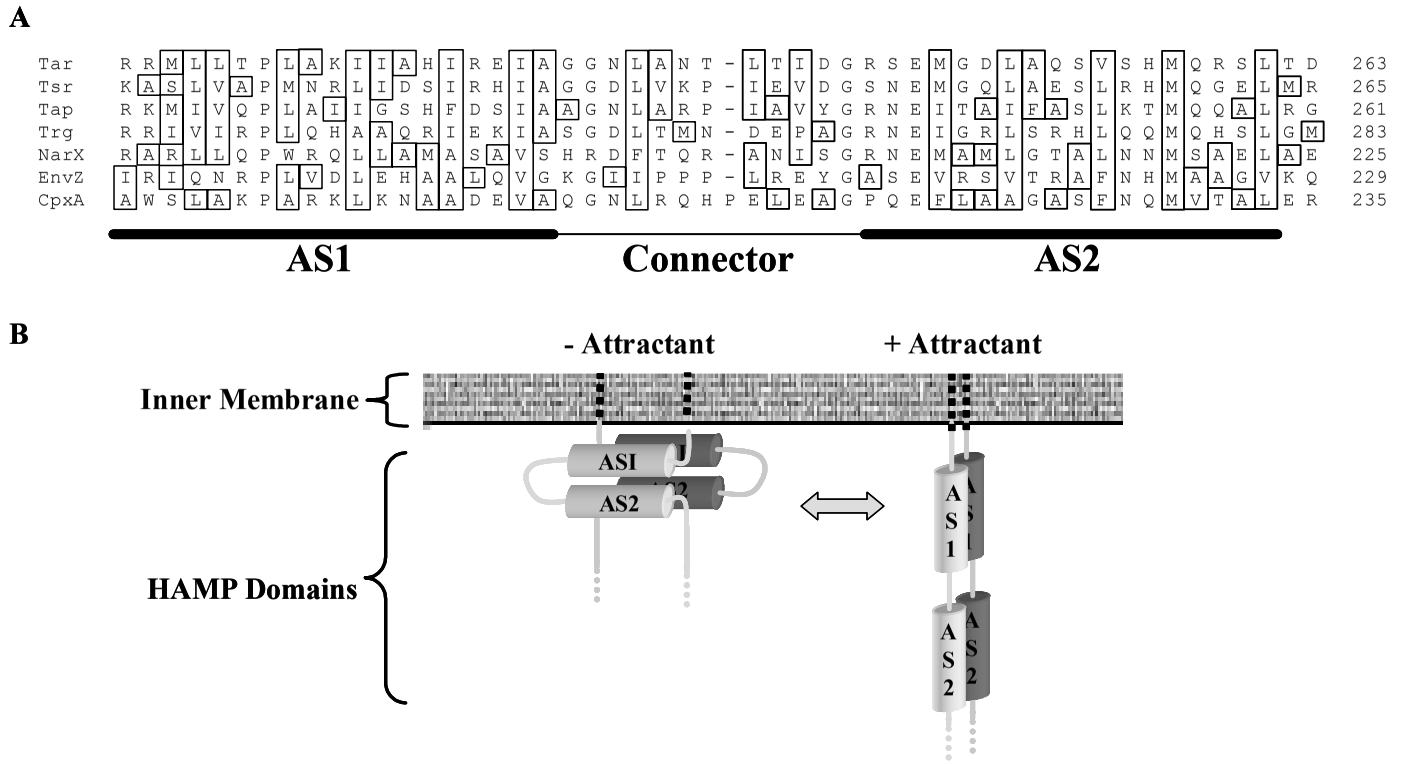

Fig. 6. HAMP linkers from *E. coli.* (A) The approximate limits of the two amphipathic sequences (AS1 and AS2) and the connector are indicated. Hydrophobic residues are boxed in the sequences. (B) HAMP linkers are predicted to adopt a four-helix bundle structure in a dimer receptor (Zhu and Inouye, 2003). The two amphipathic sequences (AS1 and AS2) in the HAMP linker are shown as cylinders. One model for linker function is that upon interaction of an attractant with the periplasmic domain of the receptor, a putative association of AS1 with the membrane is disrupted by movement of TM2, resulting in a conformational rearrangement of AS1 and AS2. This cartoon illustrates one possible, and rather extreme, change in conformation.

based, in part, upon its containing most of the HAMP domain from one of the two components of the chimera (Appleman *et al.,* 2003).

Receptor hybrids

The creation of a functional hybrid protein can provide insight into the signaling properties of each component. As discussed earlier, functional chimeras can be created between chemoreceptors (Krikos *et al.,* 1985; Feng *et al.*, 1997; Weerasuriya *et al.*, 1998). The fusion joint for these hybrids was a conserved *NdeI* restriction site (CATATG) found in *tar* and *tsr* and introduced at the equivalent position in *trg* and *tap*. This sequence represents in-frame His and Met codons and is only six codons before the end of the sequence encoding AS2.

Other hybrid transducers have been created to determine if the transmembrane signaling mechanisms of sensor kinases and chemoreceptors are conserved. These hybrids include the fusion of the input domain of Tar to the signaling domain of the osmosensor kinase EnvZ to create Taz1 (Utsumi *et al*., 1989). EnvZ is a kinase and phosphatase for its cognate response regulator, OmpR, which modulates the transcription of the *ompF* and *ompC* porin genes. Signaling by Taz1 was measured by the β-galactosidase activity expressed from a *ompC-lacZ* fusion. The Taz1 protein was reported to signal in response to aspartate (Utsumi *et al*., 1989) by downregulating the phosphatase activity of the EnvZ signaling domain towards phospho-OmpR. Similarly, a chimera combining the periplasmic, transmembrane and linker domains of the chemoreceptor Trg with the cytoplasmic kinase/phosphatase domain of EnvZ created

Trz1 (Baumgartner *et al.*, 1994). Trz1 mediated a response to sugar-occupied ribose-binding protein to the EnvZ kinase/phosphatase domain by activating *ompC-lacZ* transcription.

Parent proteins of hybrid transducers share predicted structural identity without extensive sequence identity, especially in their ligand-discrimination regions. The varying catalytic regions of sensor kinases and chemoreceptors seem to employ a signaling mechanism that does not involve specific interactions among side chains within these regions. If signaling mechanisms are conserved, it is probable that regions necessary for signaling will exhibit more conservation and that mutations targeting regions required for signaling will have similar effects on both hybrid and parent receptors.

Research goals and rationale

The ability to create stable and functional chimeras by domain swapping provides a powerful tool to examine the mechanism of transmembrane signaling. To overcome obvious shortcomings of previous efforts to study the function of similar chimeras, I utilized the sensitivity and multiple readouts of the chemotaxis system. I fused the periplasmic region of the nitrate/nitrite sensor kinase NarX to the cytoplasmic signaling region of Tar. The NarX-Tar (Nart) hybrid is predicted to detect ligands using the periplasmic ligand-binding region of NarX and to transmit this information to the signaling region of Tar to modulate flagellar rotation. The differential responses NarX exhibits to nitrate and nitrite allow me to determine if this property is retained in the

hybrid protein. The effects of known mutations targeting the predicted NarX ligand-interaction region in Nart can be evaluated and compared to their phenotypes in the intact NarX protein. This work should unequivocally establish the similarities and differences in transmembrane signaling by the two dominant families of bacterial transmembrane sensors.

CHAPTER II

A NARX-TAR CHIMERA MEDIATES REPELLENT CHEMOTAXIS TO NITRATE/NITRITE*

Summary

Membrane receptors communicate between the external world and the cell interior. In bacteria, these receptors include the transmembrane sensor kinases, which control gene expression via their cognate response regulators, and chemoreceptors, which control the direction of flagellar rotation via the CheA kinase and the CheY response regulator. Here, we show that a chimeric protein that joins the ligand-binding, transmembrane and linker domains of the NarX sensor kinase to the signaling and adaptation domains of the Tar chemoreceptor of *Escherichia coli* mediates repellent responses to nitrate and nitrite. Nitrate induces a stronger response than nitrite and is effective at lower concentrations, mirroring the relative sensitivity to these ligands exhibited by NarX itself. We conclude that the NarX−Tar hybrid functions as a bona fide chemoreceptor whose activity can be predicted from its component parts. This observation implies that ligand-dependent activation of a sensor kinase and repellent-initiated activation of receptor-coupled CheA kinase involve a similar transmembrane signal.

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Introduction

The Tar protein of *Escherichia coli* mediates attractant responses to aspartate and maltose (Springer *et al*., 1977) and repellent responses to Ni^{2+} and Co^{2+} ions (Tso and Adler, 1974). It is one of two high-abundance chemoreceptors in *E. coli* (Koman *et al*., 1979). The other is Tsr, which mediates attractant responses to serine and repellent responses to leucine. The x-ray crystallographic structures of the periplasmic domain of Tar (Milburn *et al*., 1991; Bowie *et al*., 1995) and the cytoplasmic domain of Tsr (Kim *et al*., 1999) show that these proteins form homodimers in the presence or absence of ligands. The osmosensing EnvZ kinase of *E. coli* is also a homodimer (Yang and Inouye, 1991), and it has a predicted membrane topology similar to that of the chemoreceptors (Forst *et al*., 1987).

Each subunit of Tar spans the membrane twice and extends an N-terminal ligand recognition loop into the periplasmic space and a C-terminal signaling and adaptation domain into the cytoplasm (Krikos *et al*., 1983). The periplasmic domain is responsible for the interaction with most attractant and repellent ligands, including aspartate, maltose-binding protein (MBP) and Ni^{2+} for Tar and serine and leucine for Tsr (Krikos *et al*., 1985). Aspartate binds at the dimer interface of Tar near the apex of the periplasmic domains (Milburn *et al.,* 1991). Ligand-bound MBP in a closed conformation (Spurlino *et al*., 1991) is predicted to bind in quasi-symmetrical fashion at the apex of the Tar dimer, with the N-terminal domain in contact with one Tar subunit and the C-terminal domain in contact with the second subunit (Zhang *et al*., 1999).

A number of functional chimeric chemoreceptors have been constructed. The fusion joint for these constructs is in the cytoplasmic domain near the C-terminal end of the linker region (Kalman and Gunsalus, 1990; Williams and Stewart, 1999), which connects the second transmembrane helix (TM2) to the signaling and adaptation domains. This position corresponds to a conserved *NdeI* restriction site present in the *tar* and *tsr* genes of *E. coli*. This site spans a CATATG sequence that encodes adjacent His and Met residues. The Tasr and Tsar hybrids, made by reciprocal exchange of the coding regions on either side of the *NdeI* site, have the ligand-sensing properties that correspond to the periplasmic domain of the hybrid (Krikos *et al*., 1985). Subsequent studies have shown that *NdeI* sites introduced at the same relative positions in the *trg* and *tap* genes can be used to generate functional chimeras between the Trg (ribose/galactose) receptor and Tsr (Feng *et al*., 1998) and between the Tap (dipeptide) receptor and Tar (Weerasuriya *et al*., 1998)

To test whether a similar architecture dictates a similar mechanism of transmembrane signaling, chimeras have been constructed between Tar and EnvZ (Utsumi *et al*., 1989) and between Trg and EnvZ (Baumgartner *et al*., 1994). An *NdeI* site was generated in *envZ* at a location judged to be comparable with that of the *NdeI* site in *tar* and was used to join the sensing domain of Tar or Trg to the signaling domain of EnvZ. The level of β-galactosidase expressed from an *ompC-lacZ* fusion gene was used to monitor the activity of the resulting Taz and Trz hybrids. (Transcription of *ompC* requires a high concentration of the phosphorylated form of OmpR, the cognate response regulator for EnvZ.) The addition of aspartate to cells expressing Taz, or of ribose or

galactose to cells expressing Trz, led to a substantial increase in β-galactosidase activity. However, the addition of maltose to cells producing Taz did not boost the expression of *ompC-lacZ* (Utsumi *et al*., 1989).

One problem with using EnvZ to create chemoreceptor-sensor kinase hybrids is that the function of the reciprocal chimera cannot be tested, because EnvZ lacks a known ligand. Deletion analysis indicates that the periplasmic domain of EnvZ is largely dispensable for osmosensing. It can even be exchanged with the periplasmic domain of a non-homologous sensor kinase, PhoR of *Bacillus subtilis*, without impairing osmoregulation (Leonardo and Forst, 1996). Without knowing how the reciprocal hybrids function, it is impossible to evaluate fully how similar the process of transmembrane signaling is in sensor kinases and chemoreceptors.

We thus decided to construct hybrids between the NarX sensor kinase of *E. coli* (Stewart and Berg, 1988; Kalman and Gunsalus, 1990) and Tar. NarX regulates the expression of genes whose products (e.g. nitrate or nitrite reductase) are involved in the utilization of nitrate or nitrite as terminal electron receptors under anaerobic conditions (Rabin and Stewart, 1993). These ligands bind to the periplasmic domain of NarX (Cavicchioli *et al.,* 1996; Williams and Stewart, 1997; Lee *et al.,* 1999) and thereby increase its autophosphorylation activity. The phosphoryl group is transferred to either of two response regulators, NarL or NarP, which act as positive or negative transcription factors for a number of genes (Stewart, 1993).

We report here that a NarX-Tar chimera (Nart) can serve as a repellent chemoreceptor for nitrate and nitrite. The active fusion was made using an *NdeI* site

introduced into *narX* at the same relative position as that introduced into *envZ* to make Taz and the natural *NdeI* site in *tar*. The chemotactic behavior of cells producing the chimeric receptor suggests that ligands stimulate the kinase activity of CheA associated with Nart. We infer that the two responses are elicited by a similar transmembrane signal.

Results

The pAD56 plasmid produces a stable NarX-Tar hybrid protein

Two *narX-tar* fusion genes were constructed using *NdeI* restriction sites introduced at two places in *narX* (Fig. 7). Antibody directed against the conserved cytoplasmic domain of Tsr (Ames and Parkinson, 1994) was used as a probe for the hybrid proteins on immunoblots. Plasmid pAD56, in which codons 1-218 of *narX* are joined to codons 257-553 of *tar* (*narX(218)-tar*), produced a cross-reacting protein (Nart) of the expected size (~55 kDa) in the transducer-deleted (ΔTransducer) strain VB13 (Fig. 8). The fusion joint in this hybrid was at the end of the predicted linker domain (Williams and Stewart, 1999). No cross-reacting protein was seen in extracts from strain VB13 containing plasmid pAD48, in which codons 1-269 of *narX* were joined to codons 257-553 of *tar*. The Nart hybrid protein was found in significantly higher amounts in cells that make Tsr and Trg (Δ*tar-tap* strain MM509) than in cells from strain VB13 (Fig. 8). We presume that the presence of Tsr and/or Trg stabilizes the fusion protein.

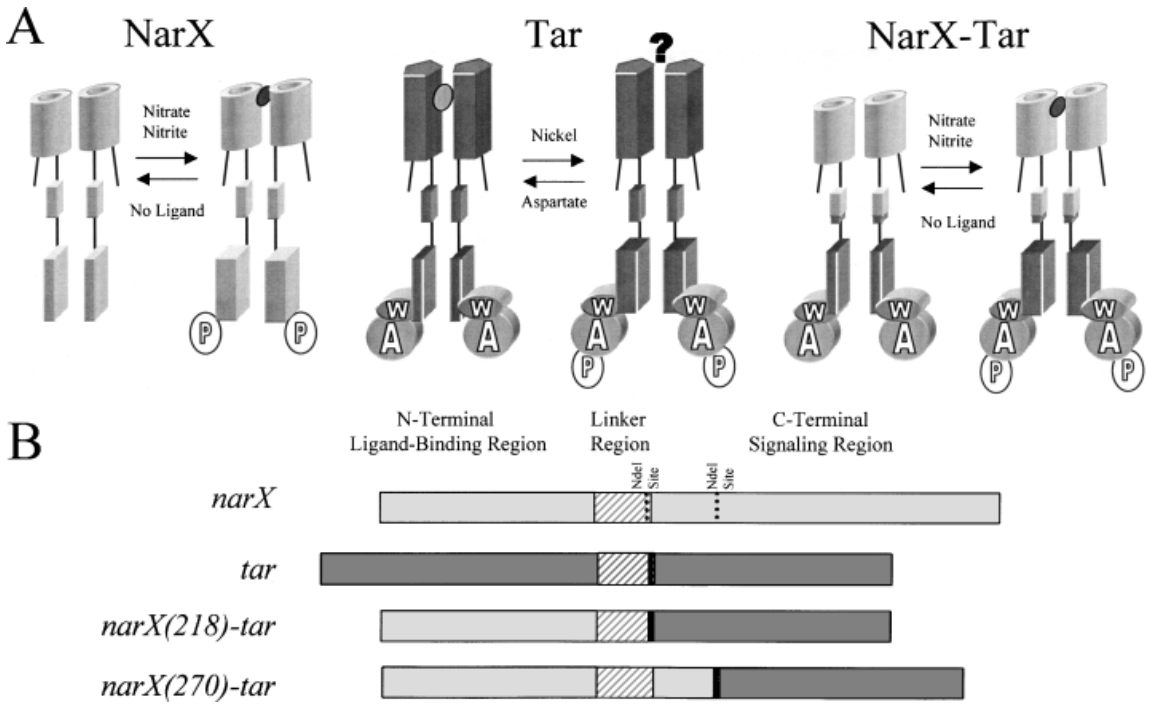

Fig. 7. NarX-Tar chimera function and construction. (**A**) Under anaerobic conditions NarX *coli* mediates a response to nitrate and nitrite (small oval) by increasing its rate of autophosphorylation. The Tar receptor binds the attractant ligand aspartate (large oval) and, in an unknown fashion, the repellent ligand Ni^{2+} (question mark). Attractants inhibit (and repellents enhance) Tar stimulation of CheA autophosphorylation. The NarX-Tar hybrid contains the periplasmic ligand-binding, transmembrane, and linker regions of NarX and the cytoplasmic signaling and adaptation regions of Tar. (**B**) Two *NdeI* sites introduced in the *narX* gene (broken lines) permit fusion of the N-terminal portion of NarX to the C-terminal portion of Tar at a naturally occurring *NdeI* site (bold line). Thin vertical lines show boundaries between transmembrane/periplasmic and linker regions and the linker and signaling domains, respectively, in each gene. Fusions were made after codons 218 and 270 of *narX*.

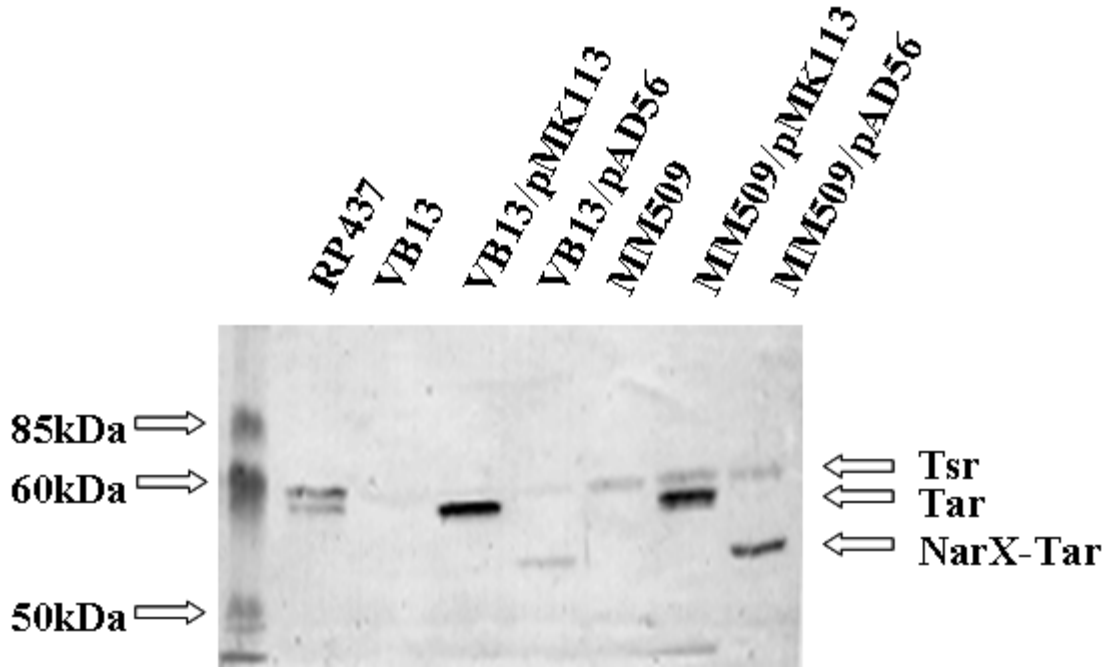

Fig. 8. Levels of NarX, Tar, and NarX-Tar in strains VB13 (ΔTransducer) and MM509 (Δ*tar-tap*). Immunoblots of proteins encoded by plasmid-borne *tar* (pMK113) or *narX-tar* (plasmid AD56) genes were determined in immunoblots developed with antibody against a highly conserved region from the cytoplasmic domain of the Tsr (serine) chemoreceptor. Proteins in membrane extracts from equal numbers of cells were loaded in each lane and separated by SDS-PAGE. An extract from the parental RP437 wild-type strain was included to indicate the normal levels of Tsr and Tar produced from the chromosomal genes. A faint band of unknown provenance appears in most lanes between the positions occupied by Tsr and Tar. The NarX-Tar hybrid migrates to a position corresponding to an apparent molecular mass of about 55 kD. Molecular weight markers are shown in the leftmost lane.

Several other reproducible features of the immunoblots should be mentioned. First, cells of the wild-type strain RP437 contained about twice as much Tsr as cells of strain MM509, although these strains are isogenic except for the *tar-tap* deletion in MM509. The presence of plasmid-encoded Tar or Nart in strains MM509/pMK113 and MM509/pAD56 did not restore the level of Tsr seen in strain RP437. Secondly, although the level of Nart encoded by plasmid pAD56 was much reduced in strain VB13 relative to strain MM509, the level of plasmid-encoded Tar was approximately the same in strains VB13/pMK113 and MM509/pMK113. We presume that interactions among the receptors and their attendant Che proteins with the receptor patch (Maddock and Shapiro, 1993; Shimizu *et al.*, 2000) can lead to their differential stabilization. Finally, a faint band corresponding to a protein of the same size as Tsr was consistently seen in strain VB13, which lacks Tsr, Tar, Tap and Trg (compare the VB13, VB13/pMK113 and VB13/ pAD56 lanes in Fig. 8). As the only other chemotactic signal transducer identified in *E. coli*, the oxygen receptor Aer, runs with an apparent molecular weight of ~55 kDa (Bibikov *et al.,* 1997), this phantom band is unlikely to be Aer, and its provenance remains a mystery.

Behavior of strain VB13/pAD56 on swarm plates

Plasmid pAD56 allowed strain VB13 to form a spreading colony in TB semi-solid agar, whereas plasmid pAD48 did not. VB13 colonies do not spread because the absence of chemoreceptors renders them exclusively smooth swimming. (The formation of a spreading colony in semi-solid agar requires that cells are able to run and tumble; Wolfe

and Berg, 1989.) Thus, Nart must stimulate the activity of CheA kinase to produce enough CheY-P to induce tumbling, which corresponds to clockwise (CW) flagellar rotation.

The spreading colonies did not form chemotactic rings in semi-solid TB agar containing various concentrations of nitrate or nitrite. This result indicates that cells producing Nart as their sole transducer do not respond to any of the normal attractants for *E. coli* that are present in TB (e.g. amino acids). However, it does not indicate that these cells do not respond to nitrate or nitrite. The formation of chemotactic rings requires not only that a compound is an attractant but also that its metabolism creates a relatively steep gradient whose concentration increases away from the point of inoculation.

Behavior of unstimulated tethered cells

The flagella of tethered cells of strain VB13/pAD48 were counterclockwise (CCW) biased (~90% CCW flagellar rotation), although they did reverse, unlike the flagella on tethered cells of strain VB13, which rotated almost exclusively CCW (Table 1). The relatively low level of Nart present in these cells could explain the CCW bias. Tethered MM509 cells (~70% CCW flagellar rotation) behaved essentially like wild-type cells (data not shown), whereas the flagella of MM509/pAD56 cells were substantially more CW biased (only 20% CCW flagellar rotation). These results support the notion that Nart facilitates random spreading of VB13 cells in semi-solid TB agar by increasing their tumbling frequency.

Table 1. Rotational biases of tethered cells.

Strain	Percentage time in CCW rotation[a]
VB13	99.5 ± 0.5
VB13/pAD56	91 ± 1
MM509	71 ± 5
MM509/pAD56	20 ± 2

a. The values shown are the means of the percentage time spent rotating CCW out of 60 s, ± the standard error. Data from 20 cells were averaged for each strain.

Responses of tethered cells to the addition of repellents

The adaptation times for tethered cells exposed to repellents are compiled in Table 2. RP437 (wild-type) cells gave brief (~30 s) CW responses to the addition of 10^{-3} M leucine (sensed by Tsr) or Ni^{2+} (sensed by Tar). The adaptation times decreased at lower concentrations. As expected, MM509 cells (Δ*tar-tap*) did not respond to the addition of 10^{-3} M Ni^{2+}. However, they also did not respond to the addition of 10^{-3} M leucine. (Note that responses of <15s would not be recorded.) The introduction of pMK113 into MM509 restored a remarkably long CW response (480s) to Ni^{2+}. This effect was even more pronounced with VB13/pMK113 cells, which had a CW response of 740s after the addition of 10^{-3} M Ni^{2+}. However, MM509 cells containing pMK113 did not show a significant CW response to the addition of leucine. As expected, the pAD56 plasmid had no measurable effect on the responses to leucine or Ni^{2+} addition in either MM509 or VB13.

Neither nitrate nor nitrite evoked a detectable response when added to tethered cells of strains RP437 (wild type), VB13 or MM509 that lacked pAD56. In contrast, when tethered VB13 cells containing pAD56 were exposed to nitrate or nitrite, both induced a period of exclusively CW flagellar rotation (Table 3). The threshold concentrations required for this response with VB13/pAD56 cells were 10^{-5} M for nitrate and 10^{-3} M for nitrite. The longest mean adaptation times (110s for nitrate and 37s for nitrite) were seen at 10^{-2} M. The responses became shorter at 10^{-1} M for both ions, perhaps because, at these high concentrations, the health of the cells was compromised. Tethered MM509/pAD56 cells also responded to the addition of nitrate and nitrate by

Table 2. Responses of tethered cells to the addition of repellents.

	Time (s) of CW-only rotation until the first CW to CCW reversal after repellent added at the indicated concentration (M)								
Leucine added	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**Nickel added**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**
Strains					Strains				
RP437	28 ± 1	10 ± 0.8	6 ± 0.8	5 ± 0.4	RP437	26 ± 2	19 ± 1	5 ± 0.4	3 ± 0.3
MM509	0	0	0	0	MM509	ND	ND	ND	ND
MM509/pMK113	5 ± 0.4	2 ± 0.3	0	0	MM509/pMK113	480 ± 19	350 ± 25	25 ± 2	0
MM509/pAD56	0	0	0	0	MM509/pAD56	0	ND	ND	ND
VB13	0	0	0	0	VB13	0	0	0	0
VB13/pMK113	0	ND	ND	ND	VB13/pMK113	740 ± 28	610 ± 26	2 ± 0.4	0
VB13/pAD56	ND	ND	ND	ND	VB13/pAD56	ND	ND	ND	ND
Nitrate added	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**Nitrite added**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**
Strains					Strains				
RP437	0	ND	ND	ND	RP437	0	ND	ND	ND
MM509	ND	ND	ND	ND	MM509	ND	ND	ND	ND
MM509/pMK113	ND	ND	ND	ND	MM509/pMK113	ND	ND	ND	ND
MM509/pAD56	0	ND	ND	ND	MM509/pAD56	0	ND	ND	ND
VB13	0	0	0	0	VB13	0	0	0	0
VB13/pMK113	0	ND	ND	ND	VB13/pMK113	0	ND	ND	ND
VB13/pAD56	84 ± 7	11 ± 1	6 ± 1	0	VB13/pAD56	10 ± 0.6	11 ± 1	6 ± 1	0

ND, not determined.

turning their flagella only CW. The longest mean adaptation times (90s for nitrate and 48s for nitrite) were also observed at 10^{-2} M (data not shown). The threshold concentration for the response to nitrite was again 10^{-3} M, but nitrate did not elicit a response at concentrations below 10^{-2} M. We currently have no good explanation for this somewhat surprising result.

The addition of leucine produced CW flagellar rotation in MM509 and MM509/pAD56 cells, but not in VB13 or VB13/pAD56 cells, as expected. The longest response was seen after the addition of 10^{-1} M leucine, the highest concentration tested. It was of the same duration (37 and 38s respectively) in both strains (data not shown). However, the threshold concentrations needed to generate a measurable response were very different: $<10^{-6}$ M with MM509 and 10^{-3} M with MM509/pAD56.

Responses of tethered cells to the removal of nitrate and nitrite

The physiologically relevant response of *E. coli* cells to repellents is to decrease their tumble frequency as the concentration of a repellent drops (Berg and Tedesco, 1975). Therefore, we looked at the behavior of tethered cells upon removal of nitrate and nitrite. The response was dramatic. VB13/pAD56 cells responded with extended periods of CCW rotation with thresholds of 10^{-5} and 10^{-3} M for nitrate and nitrite respectively. The corresponding peak adaptation times, both at 10^{-2} M, were 1100 and 420 s. Adaptation times dropped off somewhat at 10^{-1} M but, in this case, an alternative explanation to cell damage at high anion concentration is that the 15s flow did not remove all the nitrate or nitrite.

Table 3. Responses of tethered cells to the addition of attractants or removal of repellents.

	Time (s) of CCW-only rotation until the first CCW to CW reversal after addition of attractant at the indicated concentration (M) or replacement of repellent present at the indicated concentration with unadulterated buffer								
Serine added	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**Leucine removed**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**
Strains					**Strains**				
RP437	470 ± 18	230 ± 3	100 ± 2	0	RP437	64 ± 2	62 ± 2	50 ± 1	43 ± 1
MM509	690 ± 8	340 ± 4	60 ± 2	1 ± 0.3	MM509	31 ± 1	13 ± 1	10 ± 1	9 ± 0.6
MM509/pMK113	180 ± 5	41 ± 3	30 ± 2	14 ± 2	MM509/pMK113	120 ± 3	50 ± 3	24 ± 2	6 ± 1
MM509/pAD56	420 ± 8	22 ± 2	ND	ND	MM509/pAD56	3 ± 0.5	ND	ND	ND
VB13/pMK113	0	ND	ND	ND	VB13/pMK113	0	ND	ND	ND
VB13/pAD56	0	ND	ND	ND	VB13/pAD56	0	ND	ND	ND
Aspartate added	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	Nickel removed	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**
Strains					**Strains**				
RP437	340 ± 6	230 ± 6	100 ± 4	6 ± 0.8	RP437	140 ± 3	130 ± 2	53 ± 1	44 ± 1
MM509	0	ND	ND	ND	MM509	0	ND	ND	ND
MM509/pMK113	1130 ± 32	1030 ± 24	690 ± 20	540 ± 10	MM509/pMK113	650 ± 18	600 ± 18	120 ± 9	39 ± 3
MM509/pAD56	0	ND	ND	ND	MM509/pAD56	0	ND	ND	ND
VB13/pMK113	930 ± 21	710 ± 10	400 ± 12	105 ± 7	VB13/pMK113	680 ± 11	600 ± 8	65 ± 6	38 ± 2
VB13/pAD56	0	ND	ND	ND	VB13/pAD56	0	ND	ND	ND
Nitrate removed	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	Nitrite removed	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**
Strains					**Strains**				
RP437	0	ND	ND	ND	RP437	0	ND	ND	ND
MM509	0	ND	ND	ND	MM509	0	ND	ND	ND
MM509/pMK113	0	ND	ND	ND	MM509/pMK113	0	ND	ND	ND
MM509/pAD56	740 ± 13	300 ± 6	110 ± 4	52 ± 4	MM509/pAD56	0	ND	ND	ND
VB13/pMK113	0	ND	ND	ND	VB13/pMK113	0	ND	ND	ND
VB13/pAD56	990 ± 15	880 ± 25	120 ± 6	ND	VB13/pAD56	110 ± 4	ND	ND	ND

ND, not determined.

MM509 cells responded for a longer time (690s) than RP437 cells to the addition of 10^{-3} M serine. Thus, a reduced level of Tsr, the absence of Tar, possible polarity of the *Δtar-tap* deletion on the expression of the downstream *cheRBYZ* genes, or some combination thereof, lowered the sensitivity, or accelerated the adaptation, to a step increase in the repellent leucine. These same factors may have heightened the sensitivity, or slowed the adaptation, to a step increase in the attractant serine.

MM509/pAD56 cells behaved in a similar way (Table 2), but they exhibited a lower threshold (both nitrate and nitrite elicited significant CCW responses at 10^{-6} M, the lowest concentration tested), and the peak adaptation times, although still at 10^{-2} M for both compounds, were slightly shorter than with VB13/pAD56 (790s versus 1100s for nitrate and 380s versus 420s for nitrite). In MM509 cells with or without pAD56, removal of leucine also evoked a CCW response, but it was much briefer (adaptation times after dilution from 10^{-2} M of 66 and 79s in the absence and presence of pAD56). Although the plasmid did not significantly affect the maximum adaptation time to leucine, it did shift the threshold significantly, from $<10^{-6}$ M in MM509 cells without the plasmid to 10^{-3} M in MM509/pAD56. Here, one could argue that the strong CW bias of MM509/pAD56 cells (Table 1) reduced the adaptation time upon removal of leucine.

Repellent-in-pond capillary assays

The repellent-in pond assay (Tso and Adler, 1974) provides a direct method of demonstrating negative chemotaxis. This assay is a variant of the standard chemotaxis assay, in which cells enter a capillary as they move up a diffusion gradient of an

attractant (Adler, 1973). The operating principle is that cells suspended in buffer containing a repellent will flee into capillary tubes filled with repellent-free buffer. With strain MM509, when 10^{-2} M leucine or acetate (both sensed by Tsr) was present in the pond, cells accumulated in the capillaries to four- or sixfold higher levels, respectively, than in the buffer controls (Fig. 9A). Neither nitrate nor nitrite in the pond led to increased accumulation of cells in the capillaries.

When strain MM509/pNarX-Tar was tested, cells accumulated to densities three- and fivefold higher than the buffer control when 10^{-4} M nitrate or 10^{-3} M nitrite (the concentrations that gave the peak responses) was present in the pond (Fig. 9B). Thus, nitrate and nitrate both act as repellents when Nart is expressed in strain MM509 and are effective at lower concentrations than leucine or acetate. As in the tethered cell assay, the presence of Nart desensitizes strain MM509 to leucine (and also to acetate). VB13/pAD56 cells responded to nitrite essentially the same as the MM509/pAD56 strain (Fig. 9C), although their response to nitrate was weaker. As Tsr is absent in strain VB13, neither leucine nor acetate caused cells to accumulate in the capillaries.

Discussion

Nitrate and nitrite both stimulate autophosphorylation of NarX. This response is consistent with the induction of a transient period of CW flagellar rotation (tumbling) when these ions bind to the NarX(218)-Tar chimera (Nart), as CW rotation requires higher levels of CheY-P produced by increased CheA kinase activity. The responses to the addition and removal of nitrate and nitrite by MM509*(Δtar-tap)*/pAD56 and

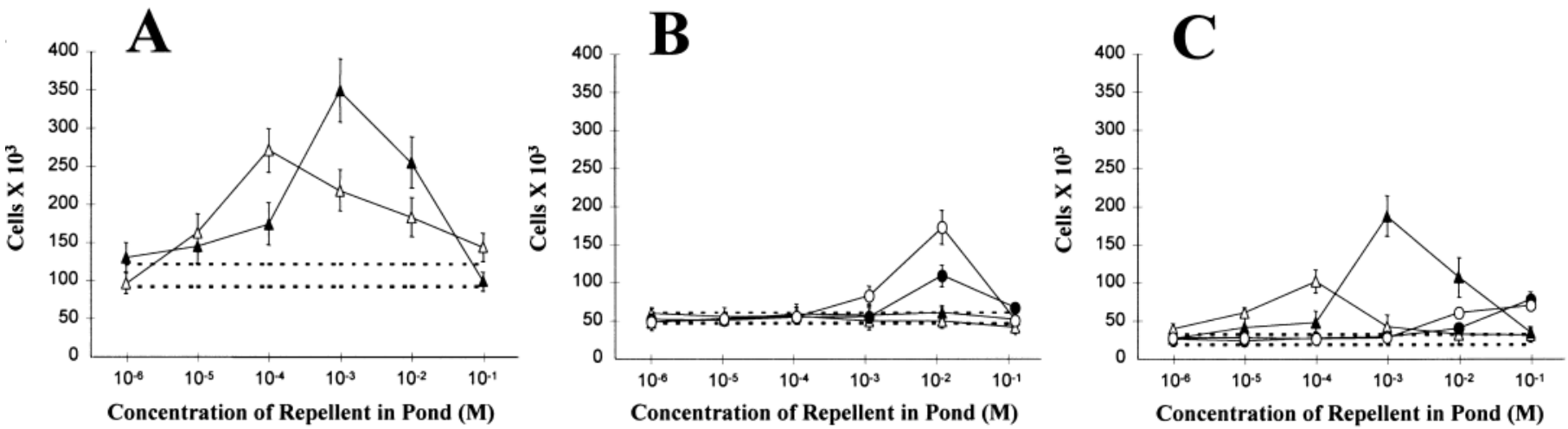

Fig. 9. Repellent-in pond capillary assays. The accumulation of strain MM509 harboring (**A**) pMK113 (*tar*) or (**B**) pAD56 (*narX-tar*) in buffer-containing capillaries out of suspensions of cells in buffer containing the indicated concentrations of the repellents acetate (open circles), leucine (closed circles), nitrate (open triangles), and nitrite (closed triangles) were compared. The background accumulation of cells when only buffer was present in the pond and capillary or when repellent was present at the same concentration in the pond and capillary was 2-4 X 10^4. Capillary assays were done in triplicate according to the method of Adler (1973) as modified for repellent chemotaxis (Tso and Adler, 1974).

VB13(ΔTransducer)/pAD56 cells resemble those seen after the addition and removal of Ni^{2+} from MM509/pMK113(*tar*$^{+}$) and VB13/pMK113 cells. This similarity suggests that nitrate and nitrite elicit bona fide repellent responses. Furthermore, the ability of pAD56 to support CW flagellar rotation in strain VB13 demonstrates that the hybrid receptor must be able to interact with CheW and CheA in a productive manner. The avoidance of nitrate and nitrite in the repellent-in-pond capillary assay by MM509 and VB13 cells expressing Nart reinforces the conclusion that the NarX-Tar fusion protein mediates normal sensing, signaling and adaptation.

Nitrate typically elicited responses at lower concentrations than nitrite, and the peak responses induced by nitrate were longer than those seen with nitrite. Thus, nitrate appears to have a higher affinity for Nart than nitrite does, and it evokes a more robust response. This same relative efficacy of nitrate and nitrite has been observed with NarX itself (Williams and Stewart, 1997; Lee *et al*., 1999; Wang *et al*., 1999), bolstering our confidence that NarX and Nart bind ligands and carry out transmembrane signaling in a similar fashion.

The NarX(218)-Tar hybrid joins residues 1-218 of NarX to residues 257-553 of Tar. The fusion joint is at the end of the linker region, which contains the HAMP domain. The HAMP domain is highly conserved in a large family of homodimeric transmembrane receptors that couple ligand binding to conformational changes which alter the signal produced by the receptor (Aravind and Ponting, 1999). The NarX(270)-Tar hybrid fuses residues 1-270 of NarX, which still precede the conserved sensor kinase signaling region of NarX, to residues 257-553 of Tar. The translation product was not

detected on immunoblots, presumably because it is unstable. This second hybrid was constructed to test whether a second region of extended amphipathic helical structure, similar to the one preceding the fusion joint in Nart, could also be coupled to Tar to generate a functional chemoreceptor. The instability of the protein limits the inferences that can be drawn, but fusions clearly cannot be made at any arbitrary point and still generate a completely active chimeric protein.

The Taz (Utsumi *et al*., 1989) and Trz (Baumgartner *et al.,* 1994) proteins couple the ligand-binding domain of Tar or Trg to the kinase domain of EnvZ. The fusion joints in these proteins are in the same relative position with respect to the linker regions and HAMP domains as in Nart. Both proteins mediate enhanced transcription of *ompC*, which requires increased levels of phospho-transfer to OmpR from the signaling domain of EnvZ, in response to their respective ligands.

Taz clearly changes its signaling behavior in response to aspartate, and Trz alters its signaling behavior in response to ribose and galactose. However, several limitations in the results reported for these two proteins prevent those authors from making an airtight case for a common mechanism of transmembrane signaling by chemoreceptors and sensor kinases. (i) As attractants inhibit the activity of CheA kinase, one might expect that attractants should decrease levels of Taz or Trz autophosphorylation, and therefore decrease levels of OmpR-P. The opposite effect is observed. (Aspartate is proposed to increase OmpR-P levels by inhibiting a phosphatase activity of EnvZ, but a similar effect on CheA has not been observed with chemoreceptors.) (ii) Although the ligand-occupied ribose and galactose/glucose-binding proteins elicit an increase in

OmpC expression, ligand-occupied maltose-binding protein does not have this effect. The reason for this apparent discrepancy has not been explained. (iii) The role of Taz and Trz on the expression of the *ompF* gene, which should go down when OmpR-P concentrations rise to a level that induces *ompC* expression, has not been reported. (iv) Finally, EnvZ has no known ligand, so the function of reciprocal constructs fusing the EnvZ sensing domain to a chemoreceptor cytoplasmic signaling domain cannot be addressed.

We are in the process of testing a reciprocal Tar-NarX hybrid (Tarx), which fuses residues 1-256 of Tar to residues 219-598 of NarX, for its ability to phosphorylate the NarL (or NarQ) response regulator and to modify this activity in response to the Tar attractant ligands aspartate and maltose and the Tar repellent ligands Ni^{2+} and Co^{2+}. We predict that cells expressing Tarx will respond to aspartate, and perhaps maltose, by decreasing the expression of genes whose transcription requires NarL-P and increasing the expression of genes whose transcription is repressed by NarL-P. Conversely, Ni^{2+} and Co^{2+} should induce the transcription of the first set of genes and repress the expression of the second. It has already been proposed, based on mutational analysis, that NarX and Tsr share a transmembrane signaling function (Collins *et al.,* 1992). Demonstration that repellents and attractants sensed by Tar regulate the activity of Tarx in the expected manner will solidify our conclusion that transmembrane signaling operates by fundamentally the same mechanism for bacterial chemoreceptors and sensor kinases.

VB13/pAD56 cells, which overexpress Nart, gave a longer than normal repellent (CW) response (84 s) to the addition of 10^{-3} M nitrate. We presume that CheB methylesterase becomes limiting under these conditions, as adaptation to repellents involves demethylation of the receptors. This effect was considerably exaggerated when 10^{-3} M Ni^{2+} was added to VB13/pMK113 cells that overexpress Tar, in which the adaptation time was 740 s. This difference could result from the higher level of expression of Tar compared with Nart (Fig. 8). When MM509 cells containing the same two plasmids were tested, however, there was no detectable CW response to adding 10^{-3} M nitrate, and the CW response to 10^{-3} M Ni^{2+} decreased to 480 s. Thus, the presence of Tsr apparently accelerates adaptation to non-cognate repellents under these conditions. When repellents were removed (Table 3), very long CCW responses were seen with both nitrate and Ni^{2+}, but here the differences between the VB13 and MM509 strains were much less pronounced. In this case, the extended adaptation times could result from a shortage of CheR methyltransferase, which sets the rate of adaptive methylation after the addition of attractant.

The effects of the expression level of Tar and Nart on responses mediated by the chromosomally encoded Tsr receptor, although not the focus of this work, beg comment. The absence of Tar in Δ*tar-tap* strain MM509 lengthened the period of exclusively CCW rotation (the adaptation time) after the addition of a saturating (10^{-3} M) concentration of serine (Table 3), and overproduction of Tar in MM509/pMK113(*tar*$^{+}$) decreased the adaptation time. However, the absence of Tar decreased the adaptation time after the addition of 10^{-5} M serine, and overproduction of Tar results in relatively longer

adaptation times at low serine concentrations. In contrast, overproduction of Nart in strain MM509/pAD56(*NarX218-Tar*) decreased the adaptation time after the addition of serine (420 and 22s at 10^{-3} and 10^{-4} M, respectively, compared with 470 and 220s for RP437 wild-type cells).

A totally different pattern was seen with the repellent leucine, which induces CW rotation upon addition and CCW rotation upon removal. The longest adaptation times to leucine addition (28s at 10^{-3} M) were seen with strain RP437, and strain MM509, with or without the pMK113 or pAD56 plasmids, exhibited essentially no response to leucine addition (Table 2). The longest adaptation times after the removal of high concentrations of 10^{-3} M leucine were seen with MM509/pMK113 cells, and the adaptation times became progressively shorter in strains RP437 and MM509 (Table 3). Strain MM509/pAD56 did not respond at all. However, the relative reduction in adaptation times as the initial leucine concentration decreased was much more abrupt in strain MM509/pMK113 than in either strain RP437 or strain MM509.

These results are complicated enough to defy a simple explanation, at least by us. It seems likely that some combination of effects contributes to these phenomena: (i) titration of limiting Che proteins (the CheR methyltransferase and CheB methylesterase, whose competing activities regulate chemotactic adaptation, are good candidates); (ii) the effect of the level of receptor methylation in the receptor-CheW-CheA tertiary complex on ligand affinity; and (iii) higher order associations of different receptors and Che proteins within the receptor patch (Maddock and Shapiro, 1993; Shimizu *et al.*, 2000; Sourjik and Berg, 2000; 2002). These data reinforce the conclusion of Barak and

Eisenbach (2001) that the response to attractant addition is mechanistically different from the response to repellent removal. However, much more thought and more experimentation will be required to untangle this particular Gordian knot.

Returning to the main theme after this digression, the ability of the Nart hybrid to function like a canonical homodimeric chemoreceptor may herald an opportunity to design novel receptors and sensor kinases by a mix-and-match approach. The chemical recognition domains (including those of the cognate periplasmic-binding proteins) of such designer receptors can potentially be modified to recognize novel ligands (Hellinga and Marvin, 1998). These engineered signal transduction systems can, in principle, be coupled to a wide variety of outputs and may prove to be of considerable utility.

Experimental procedures

Media

Routine media were prepared according to the method of Miller (1972). Tryptone broth (TB) is 1% (w/v) tryptone extract and 0.8% (w/v) NaCl. Luria broth (LB) contains 1% tryptone extract, 0.5% (w/v) yeast extract, 0.5% NaCl. LB solid agar contains 1.5% (w/v) Difco agar, and TB swarm plates contain 0.325% (w/v) Difco agar. Liquid cultures and agar plates were incubated at 37°C for LB or 32°C for TB. Media were supplemented with ampicillin (Amp, 50μg ml^{-1}) and tetracycline (Tet, 5μg ml^{-1}) as needed.

Strains and plasmids

Escherichia coli strain RP437 is wild type for motility (Parkinson and Houts, 1982). Strain MM509 is an *eda*$^+$ Δ*tar-tap5201* derivative of strain RP437 (Gardina *et al*., 1992). Strain VB13 is a *thr*$^+$ *eda*$^+$ *tsr7021 trg::Tn10 tar-tap5201* derivative of RP437. Plasmid pMK113 contains the *E. coli tar* gene and the single-stranded origin of phage M13 from plasmid pZ150 (Gardina *et al.,* 1992).

We constructed plasmids encoding two hybrid proteins, NarX(218)-Tar and NarX(270)-Tar, each of which contains a different number of N-terminal residues of NarX fused to the C-terminal cytoplasmic adaptation and signaling regions of Tar. NarX(218)-Tar, referred to as Nart, contains residues 1-218 of NarX fused to residues 257-553 of Tar. NarX(270)-Tar contains residues 1-270 of NarX fused to residues 257-553 of Tar. The *narX* sequences for both fusions were obtained by polymerase chain reaction (PCR) using pLK633 (Kalman and Gunsalus, 1990) as a template.

To construct the NarX(218)-Tar gene, codon 218 (Asn) of *narX* was converted to a CAT (His) codon by site-directed mutagenesis. This mutation created an *NdeI* site (CATATG) 44 codons after the DNA sequence encoding the second transmembrane helix of NarX. A 0.7 kbp PCR fragment of pLK633 was obtained that contained codons 1218 of *narX* flanked on the 3' end by the introduced *NdeI* site and on the 5' end by a region corresponding to the sequence upstream of the *tar* start codon up to a *BamHI* site in pMK113 (Gardina et al., 1992), which is downstream of the native *tar* promoter. This PCR product was cut with *BamHI* and *NdeI* and ligated into a 5.3 kbp *BamHI-NdeI* fragment from pMK113 generated by digestion with *BamHI* and partial digestion with

NdeI. (There is a second *NdeI* site in pMK113 downstream of *tar*.) This ligation yielded plasmid pAD56, which should transcribe NarX(218)-Tar from the modified *tar* promoter of pMK113.

NarX(270)-Tar was constructed using a similar strategy. The only difference was that the *NdeI* site was created at codons 270 (Pro) and 271 (Val), which were converted to the CAT (His) and ATG (Met) codons by site-directed mutagenesis. A 0.8 kbp fragment from pLK633 was obtained that contained codons 1270 of *narX*, again flanked on the 3' end by the introduced *NdeI* site and on the 5' end by the same sequence upstream of the tar start codon up to the *BamHI* site. This PCR product was ligated into the 5.3 kbp *BamHI-NdeI* fragment of pMK113 to yield plasmid pAD48, which should also transcribe NarX(270)-Tar from the modified *tar* promoter.

Immunoblotting

Cultures were grown in TB with swirling in 125 ml Erlenmeyer flasks. Overnight cultures were diluted 1:100 (v/v) into a 10 ml volume of TB. Cells were harvested at an A590 of 0.8. An equal number of cells from the exponential phase culture was pelleted and washed once with TE buffer (10 nM Tris, pH 7.5, 0.1 mm EDTA), and the cells were resuspended in 50 μl of loading buffer [2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 8.5% (v/v) glycerol, 60 mm Tris, pH 6.8, 0.0004% (w/v) bromophenol blue]. Freeze-thaw extracts were prepared from these resuspended cells by three alternating cycles of 5 min of boiling and 5 min on ice. Proteins were separated by 12% acrylamide SDS-PAGE and transferred to nitrocellulose. Antibody against the conserved

cytoplasmic domain of Tsr (Ames and Parkinson, 1994) was used to probe the immunoblots, and cross-reacting proteins were visualized with alkaline phosphatase-conjugated goat anti-rabbit antibody (Bio-Rad). The blot was developed using SigmaFast (Sigma).

Swarm plate assays

The motility of each strain was assessed by inoculating colonies onto TB swarm plates. Plates were incubated at 32°C for 6-8 h, and the swarm diameters were measured and recorded.

Tethered cell assays

The flagellar filaments of highly motile cells were sheared to short stubs, and the cells were mixed with antifilament antibody and tethered to glass coverslips (Silverman and Simon, 1974). These coverslips were affixed to a flow chamber (Berg and Block, 1984), which was used to introduce chemoeffectors to the tethered cells. Adaptation times are defined as the time a cell undergoes unidirectional rotation before the first reversal to the opposite direction. Sensitive cells responded to repellents with a brief period of exclusively clockwise (CW) flagellar rotation before adapting and returning to baseline reversing behavior. Removal of repellent after the introduction of buffer induced a much longer period of exclusively counterclockwise (CCW) flagellar rotation in sensitive cells, again followed by a return to reversing behavior. The adaptation time was

measured from the instant that flow was initiated, leading to a systematic overestimation (by a few seconds) of the adaptation time, as several seconds are required for the new medium to reach the cells. Cells that responded for <15s were scored as non-responders (0s) because the direction of rotation cannot be scored reliably during the flow. The responses of at least 20 cells were averaged for each measurement. All experiments with tethered cells were carried out at room temperature (22-24°C).

Repellent-in-pond capillary assay

Cells were placed in a pond containing repellents at various concentrations. A capillary filled with buffer was inserted into each pond. After 1 h of incubation at 30°C, the capillary contents were blown out, serially diluted and plated on LB agar. The number of cells within the capillary was calculated from colony counts. Capillary assays were carried out in triplicate according to the method of Adler (1973) as modified for repellent chemotaxis (Tso and Adler, 1974).

CHAPTER III

MUTATIONALLY REVERSED SIGNAL OUTPUT IN THE NART (NARX-TAR) HYBRID CHEMORECEPTOR

Summary

Proteins that span the cytoplasmic membrane transmit information from outside the cell to the cell interior. In bacteria, these signal transducers include sensor kinases, which typically control gene expression via response regulators, and methyl-accepting chemoreceptor proteins (MCPs), which control flagellar rotation via the CheA kinase and CheY response regulator. We previously reported that a chimeric protein (Nart) that joins the ligand-binding, transmembrane, and linker regions of the NarX sensor kinase to the signaling and adaptation domains of the Tar chemoreceptor elicits a repellent response to nitrate and nitrite. As with NarX, nitrate evokes a stronger response than nitrite. Here, we show that mutations in a highly conserved sequence (the P-box) in the periplasmic domain of the hybrid receptor alter chemotactic signaling in a manner consistent with their effects in the intact NarX protein. The most dramatic phenotype is associated with the G51R substitution, which confers a reversed-signal phenotype that converts nitrate into an attractant. Our results provide further evidence for conservation of the mechanism of transmembrane signaling between homodimeric sensor kinases and chemoreceptors and highlight the plasticity of the coupling between ligand binding and signal output in these systems.

Introduction

The *Escherichia coli* Tar chemoreceptor mediates attractant responses to aspartate and maltose (Springer *et al.*, 1977), the latter via maltose-binding protein (Hazelbauer, 1975), and repellent responses to Ni^{2+} and Co^{2+} (Tso and Adler, 1974). The other high-abundance chemoreceptor, Tsr, mediates attractant and repellent responses to serine and leucine, respectively. Tar and Tsr form homodimers in the presence or absence of ligands (Milburn *el al.*, 1991; Bowie *et al.*, 1995), and much evidence indicates that these dimers can associate into both homogeneous and mixed trimers of dimers (Kim *et al.*, 1999; Ames *et al.*, 2002; Studdert and Parkinson, 2004; Umemura *et al.,* 1998; Homma *et al.,* 2004). A number of sensor kinases, including NarX and EnvZ, share predicted membrane topology with these chemoreceptors (Forst *et al.*, 1987; Collins *et al.*, 1992).

The Tar and Tsr proteins contain two membrane-spanning regions that connect an N-terminal, periplasmic ligand-recognition domain to a C-terminal, cytoplasmic signaling and adaptation domain (Krikos *et al.*, 1983; Krikos *et al.*, 1985). Tar binds aspartate at the dimer interface near the apex of the periplasmic domains (Mesibov and Adler, 1972; Milburn *et al.*, 1991; Milligan and Koshland, 1993), whereas ligand-bound MBP binds asymmetrically at the apex of the Tar homodimer (Hazelbauer, 1975; Brass and Manson, 1984; Gardina *et al.*, 1997; Zhang *et al.*, 1999). Serine and leucine are predicted to bind to the periplasmic domain of Tsr (Krikos *et al.*, 1985). The cytoplasmic regions of the high-abundance transducers are responsible for transmitting the signal received from the periplasmic region to the CheA kinase (Milligan and Koshland, 1991;

Tatsuno *et al.*, 1996; Gardina and Manson, 1996). When the chemical environment is homogeneous, CheA activity is at its baseline level, the flagellar motors alternate between counterclockwise (CCW) and clockwise (CW) rotation, and the cell exhibits normal run-tumble motility. The addition of attractants or removal of repellents inhibits the activation of CheA by the receptor, thereby decreasing the rate of phosphoryl-group transfer from CheA to the response regulator CheY. Lowering the cytoplasmic level of phosphorylated CheY (CheY-P) suppresses tumbling and results in the cells running for a longer time in the direction of increasing attractant (or decreasing repellent). See Stock & Surette (1996) for a review of chemotaxis.

The cytoplasmic domain of each subunit of Tar or Tsr contains four conserved glutamyl residues, two of which are originally translated as glutaminyl residues. Each of these glutamyl residues is a target for methylation by CheR methyltransferase (Kleene *et al.*, 1977; Van der Werf and Koshland, 1977). CheR activity is antagonized by the CheB methylesterase, which, when it is phosphorylated by CheA, removes the methyl groups (Stock and Koshland, 1978). The interplay of CheR and CheB activities provides cells with a short-term memory that is essential for detecting temporal changes in chemoeffector concentrations. Adaptation to an attractant leads to a net increase in methylation above the baseline, unstimulated level, and adaptation to a repellent leads to net demethylation.

Tar and Tsr share 70% amino acid identity in their cytoplasmic regions but only 33% identity within the periplasmic regions where ligand discrimination occurs. A similar situation is found with the sensor kinases NarX and NarQ, which detect

environmental nitrate and nitrite and evoke responses by phosphorylating the DNA-binding response regulators NarL and NarP (Rabin and Stewart, 1992). Although either NarX or NarQ is capable of increasing autophosphorylation in response to nitrate and nitrite, only NarX is capable of distinguishing between the two ligands; it elicits a stronger response to nitrate than to nitrite (Williams and Stewart, 1997).

The amino acid sequences of the periplasmic regions of NarX and NarQ are quite dissimilar except in a highly conserved region called the P-box (Fig. 10; Chiang *et al.,* 1992; Rabin and Stewart, 1992). This region is located at the C-terminus of the first transmembrane helix (TM1). It comprises 18 residues that vary at only three positions between NarX and NarQ (Williams and Stewart, 1997). This region is predicted to play an integral role in the detection and signaling of NarX ligands by interaction with another region of similar size, called the P'-box, which is located at the N-terminal end of TM2 (Stewart, 2003). Among known NarX or NarQ homologs, two invariant residues (Gly-51 and Met-55) exist within the P-box (Stewart, 2003).

Mutational analyses indicate that Lys-49 in NarX provides the nitrate/nitrite distinguishing capacity; the residue at the equivalent position in NarQ is Ile-45 (Williams and Stewart, 1997). Mutations altering other residues within the P-box of NarX (Table 4) result in a variety of phenotypes, including mimicked occupancy by nitrate or nitrite or inability to respond to one or both of the ligands (Cavicchioli *et al.,* 1996; Williams and Stewart, 1997).

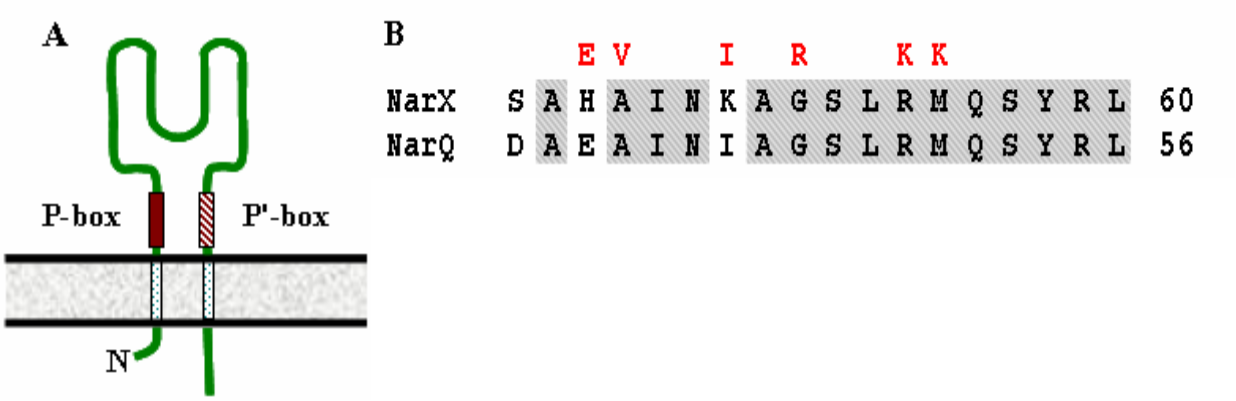

Fig. 10. The NarX periplasmic region and P-box sequences. (**A**) The linear, monomeric NarX periplasmic and transmembrane regions are depicted, in highly schematic form. The P-box (shaded rectangle; Rabin and Stewart, 1992) and P'-box (hatched rectangle; Stewart, 2003) are shown just outside the cytoplasmic membrane. (**B**) P-box sequences from *E. coli* NarX and NarQ sensors shown with identical residues shaded. P-box mutations discussed in the text are shown above the sequence. Numbers indicate the terminal residue in each sequence.

Table 4. Phenotypes of P-box mutations in NarX[a].

Amino Acid	Phenotype
H45E	Nitrite insensitive
A46V	Mimicked nitrate occupancy
K49I	Nitrate-nitrite non-differentiating
G51R	Nitrate insensitive
R54K	Ligand insensitive
M55K	Mimicked nitrite occupancy

a. Phenotypes described by Williams and Stewart (1997) in Table 4

Functional chimeras between chemoreceptors and sensor kinases have been created by fusing genes at an *NdeI* restriction site (CATATG) present near the end of the cytoplasmic HAMP linker domain (Krikos *et al.*1985; Feng *et al.*, 1998; Weerasuriya *et al.*, 1998; Utsumi *et al*., 1989). One of these chimeras, which we call Nart, fuses the nitrate/nitrite detecting periplasmic domain of NarX to the cytoplasmic signaling and adaptation domains of Tar (Ward *et al.*, 2002). Nart acts like a high-abundance transducer when it is expressed at appropriate levels, and it endows cells with the ability to respond to nitrate, and to a lesser extent nitrite, as repellents. Here, we show that mutations affecting the P-box of Nart affect both basal levels of methylation and the response to one or both NarX ligands in ways consistent with the phenotypes introduced by the same changes in intact NarX (Williams and Stewart, 1997). Most strikingly, the G51R substitution, which renders NarX insensitive to nitrate, converts nitrate from a repellent into an attractant when it interacts with G51R Nart.

Results

Behavior of unstimulated tethered cells

The flagella of tethered cells of strain MM509 (Δ*tar-tap*) exhibit a normal wild-type CCW rotational bias (~70% CCW flagellar rotation), whereas cells expressing Nart are more strongly CW biased (33% CCW flagellar rotation). The residue substitutions H45E, K49I and R54K do not alter the rotational bias (30-33% CCW). In contrast,

introduction of the A46V, G51R or M55K substitution into Nart increases the CW bias even further (20-25% CCW).

Responses of tethered cells to addition of ligands

MM509 cells containing Nart exhibit brief periods of exclusively CW rotation after the addition of nitrate or nitrite (Table 5). MM509 cells expressing Nart proteins with the substitutions in the P-box described by Williams and Stewart (1997) behave similarly with the following differences: the H45E and K49I mutants respond at lower concentrations of nitrate, the A46V mutant does not respond to nitrate, the M55K mutant does not respond to nitrite, and the G51R mutant responds to both ligands with extended (up to several minutes for nitrate) intervals of exclusively CCW flagellar rotation. This remarkable result suggests that the G51R Nart receptor senses both nitrate and nitrite as attractants.

Responses of tethered cells to removal of ligands

Adaptation times upon removal of nitrate and nitrite from MM509 cells expressing the various wild-type and mutant Nart protein are also shown in Table 5. In this situation, which resembles the physiologically response to removal of repellents, cells expressing wild-type Nart rotated their flagella exclusively CCW for extended periods. Responses, albeit brief, were seen even after removal of only 1 μM nitrate or nitrite. Qualitatively similar results were obtained with cells expressing the H45E and K49I versions of Nart,

Table 5. Responses of tethered cells to the addition and removal of ligand.

	Time of CW-only rotation[a] (sec) until the first CW to CCW reversal after ligand added at the indicated concentration (M)												
Nitrate added	**10^{-6}**	**10^{-5}**	**10^{-4}**	**10^{-3}**	**10^{-2}**	**10^{-1}**	**Nitrite added**	**10^{-6}**	**10^{-5}**	**10^{-4}**	**10^{-3}**	**10^{-2}**	**10^{-1}**
Mutation							Mutation						
Wild-type	0	0	0	0	24 ± 7	35 ± 8	Wild-type	0	0	0	0	26 ± 6	53 ± 8
H45E	0	0	0	20 ± 3	21 ± 6	31 ± 9	H45E	0	0	0	0	0	21 ± 3
A46V	0	0	0	0	0	0	A46V	0	0	0	0	23 ± 4	26 ± 4
K49I	0	0	19 ± 3	20 ± 2	22 ± 3	29 ± 3	K49I	0	0	0	0	0	80 ± 10
G51R [a]	***0***	***0***	***0***	***19 ± 4***	***102 ± 17***	***144 ± 15***	***G51R***	***0***	***0***	***0***	***0***	***40 ± 7***	***62 ± 14***
R54K	0	0	0	0	0	26 ± 4	R54K	0	0	0	0	0	27 ± 4
M55K	0	0	0	0	0	20 ± 3	M55K	0	0	0	0	0	0

	Time of CCW-only rotation[b] (sec) until the first CCW to CW reversal after ligand added at the indicated concentration (M)												
Nitrate removed	**10^{-6}**	**10^{-5}**	**10^{-4}**	**10^{-3}**	**10^{-2}**	**10^{-1}**	**Nitrite removed**	**10^{-6}**	**10^{-5}**	**10^{-4}**	**10^{-3}**	**10^{-2}**	**10^{-1}**
Mutation							Mutation						
Wild-type	66 ± 17	126 ± 18	315 ± 27	753 ± 58	790 ± 52	389 ± 32	Wild-type	29 ± 5	55 ± 6	69 ± 13	190 ± 13	380 ± 44	200 ± 34
H45E	26 ± 7	27 ± 6	38 ± 8	54 ± 13	182 ± 38	443 ± 69	H45E	0	0	0	0	0	25 ± 6
A46V	0	0	0	0	37 ± 6	49 ± 8	A46V	0	0	0	19 ± 2	77 ± 10	118 ± 22
K49I	89 ± 11	208 ± 28	325 ± 31	356 ± 25	288 ± 27	167 ± 16	K49I	0	0	0	46 ± 10	151 ± 14	172 ± 16
G51R [b]	***0***	***0***	***0***	***0***	***0***	***29 ± 4***	***G51R***	***0***	***0***	***0***	***0***	***0***	***0***
R54K	0	0	0	35 ± 5	39 ± 4	132 ± 8	R54K	0	0	0	0	32 ± 4	146 ± 11
M55K	0	0	0	45 ± 8	150 ± 12	342 ± 23	M55K	0	0	0	0	0	26 ± 6

a. Cells rotated exclusively CCW after ligand addition and underwent a CCW to CW reversal; shown bold and italicized

b. Cells rotated exclusively CW after ligand removal and underwent a CW to CCW reversal; shown bold and italicized

although extended CCW flagellar rotation was evoked only after removal of 1 mM or higher concentrations of nitrite. Cells producing the R54K and M55K mutant receptors simply gave shorter responses and only responded after removal of higher concentrations of nitrate and nitrite. The response mediated by the A46V protein was unique in that removal of nitrite produced a response that was stronger and appeared at a lower concentration for nitrite than for nitrate.

G51R Nart again behaved like an attractant receptor for nitrate, supporting about 30 sec of CW rotation after the removal of 100 mM nitrate. As is typical for responses to the removal of chemoattractants, the CCW response to the addition of nitrate and nitrite was much stronger than the CW response to their removal. Thus, it appears that a single residue substitution totally reverses the sign of the chemotactic response mediated by Nart.

Responses in capillary assays

Cells were tested for both negative and positive chemotaxis using the standard capillary assay and a repellent-in-pond variant. (Adler, 1973; Tso and Adler, 1974). In the standard assay, cells swimming in chemotaxis buffer swim towards and then into a capillary tube filled with buffer containing different concentrations of attractant. In the repellent-in-pond variant, cells are suspended in chemotaxis buffer containing different concentrations of a repellent and can flee into a capillary containing only buffer. The geometry of the assay dictates that, for a response of a given magnitude, the accumulation of cells in the capillary in response to an attractant in the standard will be

far higher than the accumulation in the capillary containing buffer in the repellent-in-pond assay.

Our previous work showed that MM509 cells expressing a wild-type Nart give peak responses in the repellent-in-pond assay when nitrate or nitrite are present at 0.1 mM and 1 mM, respectively. Fig. 11 presents the data for cells expressing the H45E and K49I Nart proteins, with the data for cells expressing wild-type Nart shown for comparison. HK49I Nart gave a stronger response with nitrate and a weaker response with nitrite, but was otherwise quite similar to wild-type Nart in this assay. With H45E Nart, a robust nitrate response was seen, but only at concentrations of 1 mM or above. In accord with its moniker of "nitrite insensitive," cells containing H45E Nart gave no response to nitrite up to 10 mM. Higher concentrations could not be tested because of the toxicity of high levels of nitrite to *E. coli*. The A46V, R54K, and M55K mutants produced negligible responses to either compound.

The response of cells expressing G51R Nart in the standard capillary assay is shown in Fig. 12. An accumulation about 30-fold above the buffer level was seen with 100 mM nitrate in the capillary. Nitrate at 10 mM also elicited a significant response, but little or no accumulation was seen with capillary nitrate concentrations at or below 1 mM. No significant accumulation was seen with nitrite up to the highest concentration (10 mM) that could be tested. These results confirm that G51R Nart is an attractant chemoreceptor for nitrate.

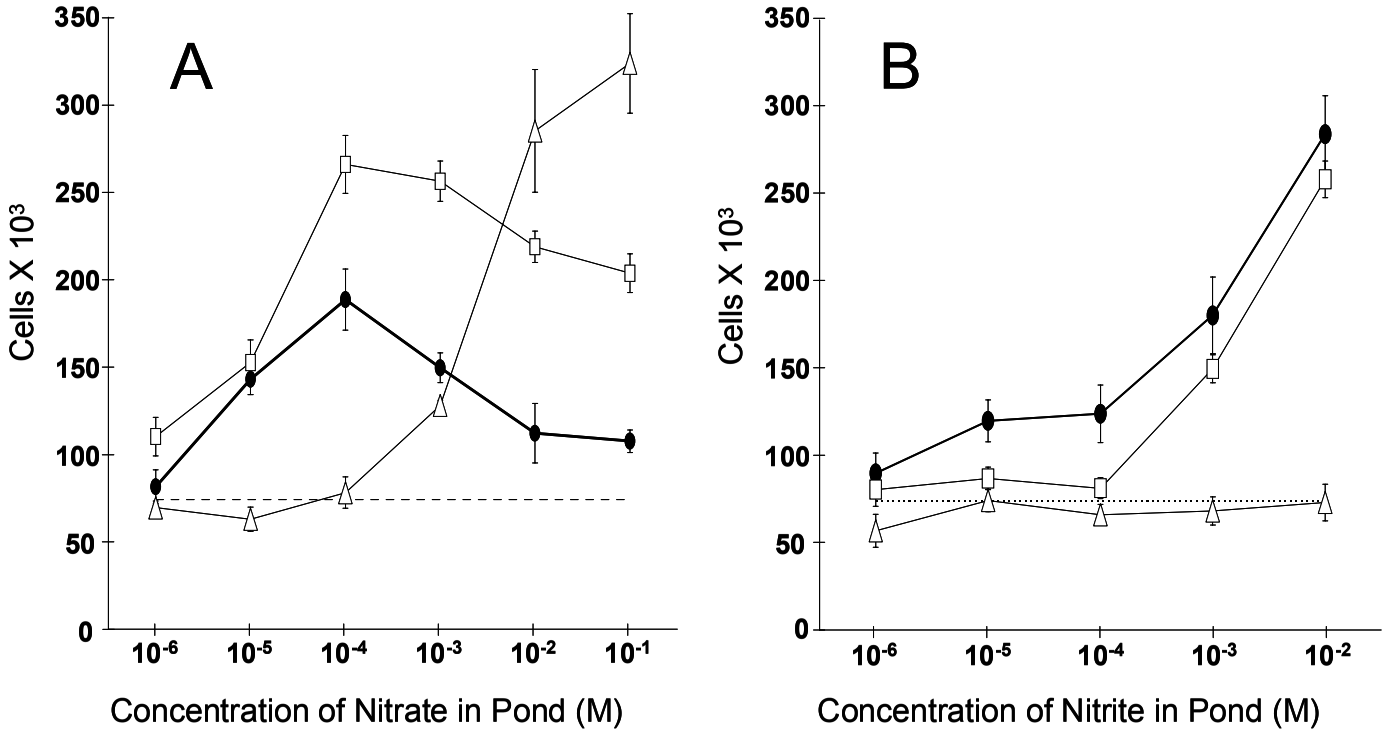

Fig. 11. Repellent-in pond capillary assay with Nart mutant proteins. The accumulation of MM509 cells containing plasmid pAD56 expressing Nart (closed circles), H45E Nart (open triangles), or K49I Nart (open squares) in buffer-containing capillaries was recorded. The pond contained nitrate (**A**) or nitrite (**B**) at the indicated concentrations. The background accumulation of cells when only buffer was present in the pond 8 X 10^4 (dotted line). Capillary assays were done in triplicate according to the method of Adler (1973) as modified for repellent chemotaxis (Tso and Adler, 1974).

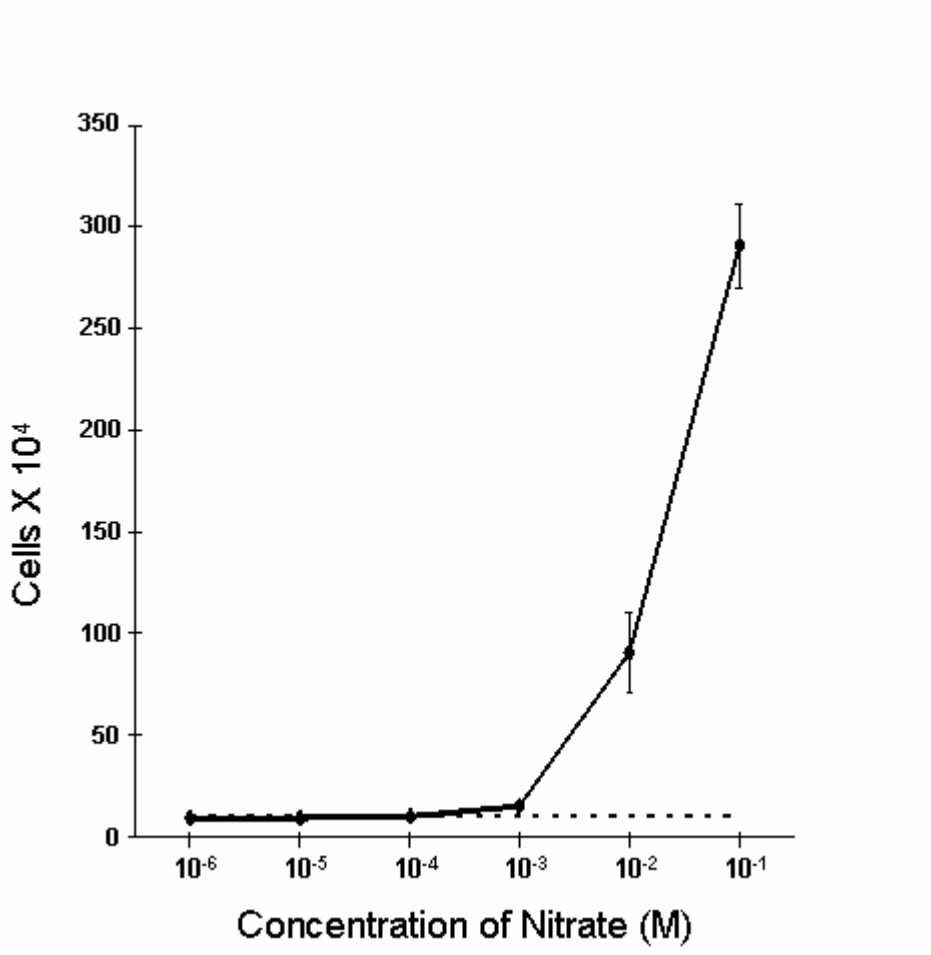

Fig. 12. Capillary assay with the Nart G51R mutant. The accumulation of MM509 cells containing plasmid pAD56 expressing G51R Nart in capillaries containing nitrate at the indicated concentrations was recorded. The accumulation seen when only buffer was present in the capillary was 1 X 10^5 (dotted line). Capillary assays were done in triplicate according to the method of Adler (1973).

Methylation pattern of Nart mutant proteins

The basal and ligand-adapted methylation patterns of either wild-type or mutant Nart proteins from plasmid pAD56 were tested in the transducer-deleted (ΔTransducer) strain VB13. Antiserum that targets the cytoplasmic region of Tar was used to visualize Nart during immunoblotting. The H45E, K49I and R54K proteins had basal methylation levels similar to that of wild-type Nart (Fig. 13). Decreased basal levels of methylation, defined by an upward shift in the band pattern, were seen with the A46V, G51R and M55K Nart proteins. Methylation patterns of all of the mutant proteins expressed in the Δ*tar-tap tsr*$^{+}$ strain MM509 showed similar changes, although the differences were not as pronounced, possibly due to sequestering of methyl groups by Tsr (data not shown).

Changes in methylation patterns of the same set of Nart proteins produced in strain VB13 were examined after adaptation to the addition of nitrate and nitrite (Fig. 14). The samples were collected 20 min after the addition of ligand, by which time adaptation should be complete (see Table 5). The wild-type protein showed the expected repellent response of decreased methylation with both nitrate and nitrite, as did the K49I protein. The shifts were first seen at 0.1 mM nitrate and 10 mM nitrite. H45E Nart exhibited no change with nitrite up to 10 mM and a significant decrease only with the highest concentration of nitrate tested (100 mM). The A46V, R54K and M55K proteins showed no change in methylation pattern at any concentration of either nitrate or nitrite.

Once more, G51R Nart behaved as an outlier. Although it did not change its methylation pattern with nitrite at any concentration tested, it showed a small but distinct

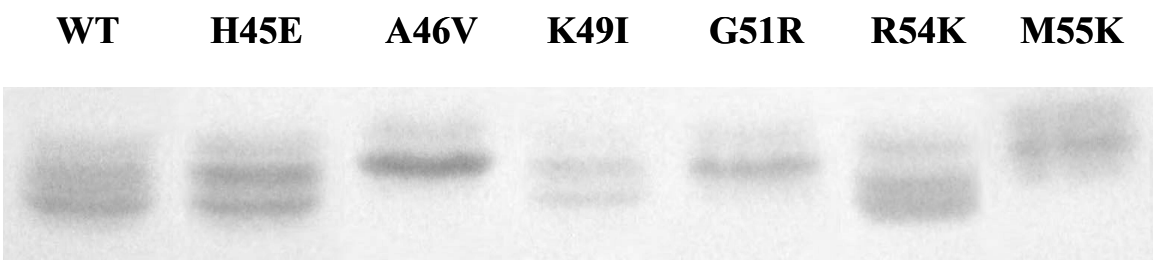

Fig. 13. *In vivo* basal methylation patterns of mutant Nart proteins. The greater the number of methyl groups per receptor (0-4), the faster the migration of the band.

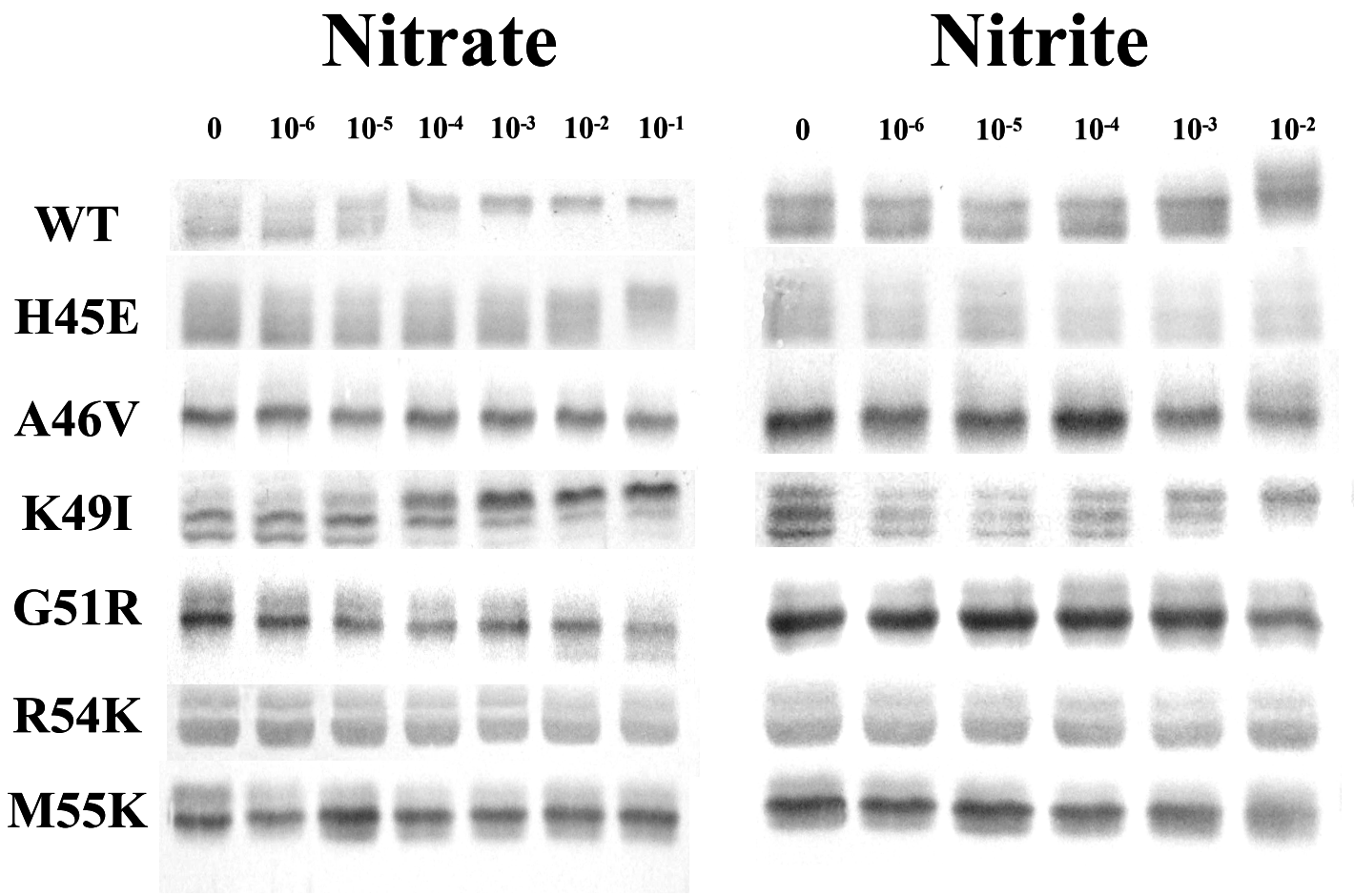

Fig. 14. *In vivo* methylation patterns of mutant Nart proteins after complete adaptation to nitrate or nitrite. Increasing concentrations of nitrate or nitrite were added to VB13 cells expressing either wild-type Nart or one of the mutant Nart proteins. Cells were incubated with ligand for for 20 min before addition of 10% TCA to stop the reaction.

shift toward more highly methylated, faster migrating forms after addition of 100 mM nitrate. Increased methylation is the canonical response seen after adaptation to an attractant.

Discussion

In response to either nitrate or nitrite, the sensor kinase NarX increases its autophosphorylation activity. In addition, NarX ligands may alter the phosphatase activity of NarX toward the phosphorylated forms of its cognate response regulators, NarL and NarP. In the chemoreceptor Tar, ligands that activate the CheA kinase elicit a repellent response and enhance CW flagellar rotation, thereby making cells more likely to change their swimming direction by tumbling. In the Nart hybrid, the cytoplasmic region of NarX responsible for the autophosphorylation, phosphotransfer and phosphatase activities of the protein is replaced with the cytoplasmic domain of the Tar chemoreceptor. With this chimeric protein, the input of NarX ligand binding can be observed as the more simple output of either increased or decreased CheA activity.

We define wild-type Nart as the chimera containing the normal periplasmic, transmembrane, and HAMP linker domains of NarX. This hybrid receptor mediates repellent responses to nitrate and, with lower sensitivity and signal strength, nitrite (Ward *et al*., 2002). Evidence to support this conclusion comes from observations of tethered cells (Table 5), the behavior of cells in repellent-in-pond capillary assays (Fig. 11), and experiments that record adaptive methylation after the addition of nitrate and nitrite (Fig. 14). To test whether the signaling mechanism is truly conserved between the

NarX sensor kinase and chemoreceptors, we studied the effects of previously identified mutations that affect the activity of NarX in a known manner (Table 4).

The phenotypes associated with the mutationally introduced residue substitutions in the P-box region of NarX (Williams and Stewart, 1997), which is thought to be responsible for ligand detection, were consistent between the intact sensor kinase and Nart. This correlation can be seen with the responses, or non-responses, of cells in the tethered-cell and capillary assays for chemotaxis (Table 6; compare Table 4 with Table 5 and Fig. 11). However, it is more clearly seen quantitatively in the patterns of adaptive methylation.

The addition of either nitrate or nitrite to wild-type Nart leads to a net demethylation of the receptor (Fig. 14), as expected for a repellent. The H45E (nitrite-insensitive), K49I (nitrate-nitrite non-differentiating), and R45K (ligand-insensitive) Nart proteins have basal levels of methylation like those of the wild type. However, their response to ligands is quite different. The H45E protein requires 1000 times more nitrate to elicit any detectable demethylation, and it shows no decrease in methylation at the highest concentration of nitrite tested (10 mM). Thus, the effect of this replacement is most parsimoniously interpreted as being to decrease affinity for both ligands. Because Nart apparently has a 1000-fold lower affinity for nitrite than for nitrite, at least with respect to induction of demethylation, the end result is a "nitrite-blind" receptor. The R54K protein appears, indeed, to be blind to ligands, since no change in methylation level is seen after addition of any amount of nitrate or nitrite. The K49I substitution defies such easy explanation, since the changes in methylation seem by and large to

Table 6. Comparison of P-box mutation phenotypes in NarX and Nart.

Phenotype in NarX	**Amino Acid**	**Phenotype in Nart**
Wild-type	-	High basal methylation Response to both nitrate and nitrite
Nitrite insensitive	H45E	High basal methylation No response to nitrite
Mimicked nitrate occupancy	A46V	Low basal methylation Response to nitrite at high concentrations
Nitrate-nitrite non-differentiating	K49I	High basal methylation Response to both nitrate and nitrite
Nitrate insensitive	G51R	Low basal methylation Reversed signaling to nitrate and nitrite
Ligand insensitive	R54K	High basal methylation Response at high nitrate/nitrite concentration
Mimicked nitrite occupancy	M55K	Low basal methylation Response to nitrate at high concentrations

parallel those of wild-type Nart. However, demethylation never reaches the same extent with the K49I protein as it does with the wild type.

The A46V (mimicked nitrate occupancy), G51R (nitrate insensitive) and M55K (mimicked nitrite occupancy) Nart proteins are all considerably less methylated than the wild-type protein in the absence of ligands. In the context of chemotaxis, this suggests that they are more biased toward the "on" (CheA kinase stimulating) state than is the wild-type protein. The same may hold true for their basal signaling state in intact NarX (see Williams and Stewart, 1997, Table 4). Neither A46V nor M55K Nart shows any change in methylation pattern after the addition of nitrate or nitrite, a result consistent with their mimicked ligand-occupancy phenotypes.

The most surprising effect is the one seen with G51R. Although it shows no change in methylation pattern upon addition of nitrite, it increases its level of methylation after the addition of 10mM or 100 mM nitrate. Although increased methylation is not seen after addition of 10 mM nitrite, high concentrations of nitrite (10 mM and 100 mM) do produce a relatively brief period of exclusive CCW flagellar rotation in tethered cells expressing G51R Nart. It is noteworthy that nitrate causes a two-fold reduction in the NarL output activity measured by Williams and Stewart (1997, Table 4), which suggests that this mutation also reverses the sign of the response to nitrate in the context of intact NarX

Our interpretation of the influence of the G51R substitution is that, like the A46V and M55K replacements, it shifts baseline signaling more toward the kinase-stimulating state. Unlike the A46V and M55K proteins, however, G51R Nart still binds nitrate with

reasonable affinity. We propose that the final conformation of the ligand-associated periplasmic domain may be the same for wild-type NarX and G51R NarX (Fig.15). The difference in the sign of the response is imposed by what the signaling state was before the ligand was added. In the case of the wild-type protein, the change is toward a more-active signaling state. In the case of the G51R protein, the change is toward a less-active signaling state. Fig. 15 also indicates which signaling states we speculate are adopted by the H45E, A46V, K49I, R54K and M55K mutant proteins.

The unique advantage of monitoring the signaling state of a receptor in a chimera with a chemoreceptor is that the methylation readout is a very direct measure of the conformation of the ligand-binding domain. Draheim *et al*. (2005) demonstrated that small shifts in the position of TM2 of Tar induced by moving residue Trp-209 near the C-terminal end of TM in single residue increments caused profound changes in CheA kinase activity in the *in vitro* receptor-coupled assay. These same changes also produced major shifts in the basal methylation level of the Tar receptor *in vivo*, although most of the mutant proteins still supported nearly normal aspartate and maltose taxis. These results indicate that inferences made from output activities such as chemotaxis or gene transcription may be misleading, since they do not directly monitor the conformation of the receptor. We propose that studies using hybrid proteins that fuse other sensor kinases to chemoreceptors to make Nart-like chimeras might reveal much about the properties of those kinases, including their basal and ligand-induced signaling states and the effects of mutations on those signaling states.

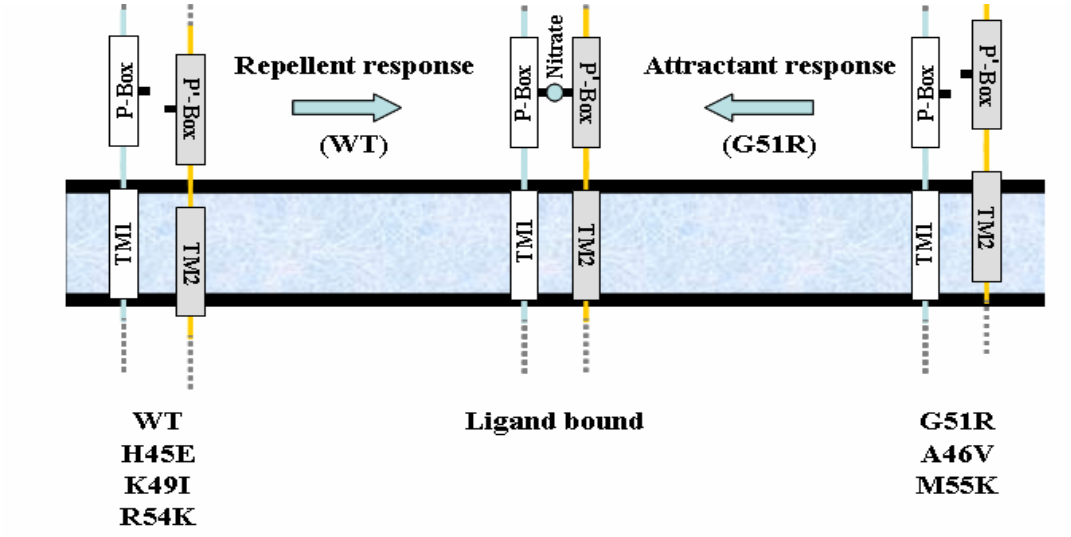

Fig.15. Model for transmembrane signaling by Nart. It is not known whether nitrate or nitrite binds within a NarX monomer or at the subunit interface of the presumed NarX homodimer, as is the case with aspartate binding at the subunit interface of the periplasmic domain of Tar. For simplicity, we have chosen to show nitrate binding to the P box region of only one subunit. Three potential signaling states of Nart are shown. They differ in the relative positions of the P-box relative to the P'-box. The wild-type, H45E, K49I and R54K proteins are proposed to be in an "off" signaling state. By analogy to what is know about transmembrane signaling by Tar (Chervitz and Falke, 1996; Draheim *et al.*, 2005), we propose that the P'-box is displaced toward the membrane relative to the P-box, which pushes TM2 further toward the cytoplasm. The A46V, G51R and M55K proteins are proposed to be in an "on" signaling state with the P'-box displaced away from the membrane relative to the P-box, which pulls TM2 further away from the cytoplasm. We suggest that when nitrate is bound that the P-box and the P'-box are brought into register and that nitrate stabilizes an intermediate signaling conformation. In the case of the wild-type protein, the ligand-bound state represents a movement of the P'-box and TM2 away from the cytoplasm, which would represent an activation of signaling (*i.e.*, a repellent response) relative to the basal signaling state of this receptor. In the case of the G51R protein, the ligand-bound state represents a movement of the P'-box and TM2 toward the cytoplasm, which would represent an inactivation of signaling (*i.e.*, an attractant response) relative to the basal signaling state of this mutant receptor.

Experimental procedures

Media

Routine media were prepared according to Miller (1972). Tryptone broth (TB) is 1% (w/v) tryptone extract and 0.8% (w/v) NaCl. Luria broth (LB) contains 1% tryptone extract, 0.5% (w/v) yeast extract, 0.5% NaCl. LB solid agar contains 1.5% (w/v) Difco agar and TB swarm plates contain 0.325% (w/v) Difco agar. Liquid cultures and agar plates were incubated at 37^{o}C for LB or 32^{o}C for TB. Media were supplemented with ampicillin (Amp, 50μg/ml) and tetracycline (Tet, 5μg/ml) as needed.

Strains and plasmids

Strain RP437 is wild type for motility (Parkinson and Houts, 1982). Strain MM509 is an *eda*$^{+}$ Δ*tar-tap*5201 derivative of strain RP437 (Gardina *et al*., 1992). *E. coli* VB13 is a *thr*$^{+}$ *eda*$^{+}$ *tsr*7021 *trg*::Tn10 Δ*tar-tap*5201 derivative of strain RP437 (Parkinson, 1978). Plasmid pMK113 contains the *E. coli tar* gene and the single-stranded origin of phage M13 from plasmid pZ150 (Gardina *et al*., 1992).

We constructed plasmids encoding the hybrid protein, NarX-Tar (Nart) (Ward *et al.,* 2001), and mutant derivatives of NarX-Tar. The NarX-Tar plasmid contains residues 1 to 218 N-terminal residues of NarX fused to the C-terminal cytoplasmic adaptation and signaling regions of Tar. Nart contains residues 1 to 218 of NarX fused to residues 257 to 553 of Tar. The *narX* sequences for both fusions were obtained by PCR using

pLK633 (Kalman & Gunsalus, 1989) as a template. PCR-generated mutations were created using primers from Invitrogen and confirmed by DNA sequencing.

Methylation assay

Our methods were based on methods by Weerasuriya *et al.* (1998). Cultures were grown in TB with swirling in 125ml Erlenmeyer flasks. Overnight cultures were diluted 1:100 (v/v) into 10 ml of the Erlenmeyer flasks. Cells were harvested at an A590 of 0.8. An equal number of cells from the exponential-phase culture was pelleted and washed three times with methylation buffer (10 nM Tris (pH 7.5), 0.1 mM EDTA). Aliquots were incubated at 30^0C for 40 minutes followed by the addition of ligand and subsequent incubation for 20 minutes. Reactions were stopped with 10% TCA. Cells were kept on ice for 15 minutes and pelleted. The pellets were washed in 1% TCA, pelleted, and finally washed in 100% acetone, and pelleted. Dry samples were resuspended in 200 μl of loading buffer (2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 8.5% (v/v) glycerol, 60mM Tris (pH 6.8), 0.0004% (w/v) bromophenol blue). The extracts were resuspended by incubation at 60^0C prior to loading on gels for SDS-PAGE. Proteins were separated by 12% acrylamide SDS-PAGE and transferred to nitrocellulose. Anti-Tsr antibody, which also binds Tar, was used to probe the immunoblots, and the cross-reacting protein was visualized with alkaline phosphatase-conjugated, goat anti-rabbit antibody (BioRad). The blot was developed using SigmaFast (Sigma).

Tethered cell assays

The flagellar filaments of highly motile cells were sheared to short stubs, and the cells were mixed with anti-filament antibody and tethered to glass cover slips (Silverman and Simon, 1974). These cover slips were affixed to a flow chamber (Berg and Block, 1984), which was used to introduce repellents to the tethered cells. Sensitive cells responded to the addition of repellents by exhibiting a brief period of exclusively clockwise flagellar rotation before adapting and returning to baseline reversing behavior. Removal of repellent by introduction of unadulterated buffer induced a much longer period of exclusively counterclockwise flagellar rotation in sensitive cells, again followed by a return to reversing behavior. The response time was measured from the instant that flow was initiated, leading to a systematic overestimation (by a few sec) of the response time, since it requires several seconds for the new medium to reach the cells. Cells that responded for less than 15 sec were scored as non-responders (0 sec) because the direction of rotation cannot be scored reliably while flow is occurring. The responses of at least 20 cells were averaged for each measurement.

Repellent-in-pond capillary assay

Cells are placed in a pond of repellent and capillaries filled with buffer are inserted into the pond, establishing a gradient of decreasing repellent concentration. After one hour, the cells within the capillary were diluted and plated. The number of cells within the capillary was compared to a baseline number of cells that swam from a buffer-repellent

mixture to a capillary filled with buffer-repellent. Capillary assays were done in triplicate according to the method of Adler (1973) as modified for repellent chemotaxis (Tso and Adler, 1074).

CHAPTER IV

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Most cell-surface receptors do transport nothing other than conformationally coded information across the membrane. In the case of chemoreceptors and membrane-bound sensor kinases, a ligand-induced movement of a transmembrane helix is predicted to transmit information about ligand binding from an extracellular domain (in Gram-negative bacteria, a periplasmic domain) to the cytoplasmic signaling domain. Studies of transmembrane signaling have focused on chemoreceptors because of their well-characterized ligand interactions and robust outputs. The mechanism of transmembrane signal transduction by sensor kinases, however, has not been as extensively studied. If the similar architecture of sensor kinases and chemoreceptors dictates similar function, then hybrids between the two types of receptors should be functional. By studying the Nart sensor kinase-chemoreceptor hybrid, I have extended our knowledge of transmembrane signaling and found it to be similar between chemoreceptors and sensor kinases, at least in this example.

In Chapter II, I show that the expressed Nart protein is the size predicted for a fusion of the NarX periplasmic, TM, and HAMP linker regions to the Tar cytoplasmic region. The location of the fusion joint is important. Fusion of residues 1-218 of NarX with Tar residues 257-553, which places the junction near the end of the HAMP region, produced a functional hybrid, NarX(218)-Tar. An attempt to fuse residues 257-553 of

Tar to residues 1-270 of NarX, which comprise the entire HAMP domain and the following amphipathic helical region, did not produce a detectable translational product. Our results suggest that, although the domains are largely modular in nature, fusions at arbitrary points do not typically produce functional chimeras. These data are supported by the work of Appleman *et al.* (2003), who showed that HAMP regions, perhaps because of their predicted functional conservation, often can serve as fusion joints for functional hybrid sensor kinases.

Ligand interaction with NarX activates autophosphorylation of the signaling domain. In the chemotaxis system, repellent binding activates autophosphorylation of the CheA kinase. With the Nart hybrid, the NarX ligands nitrate and nitrite stimulate the CheA kinase to evoke a repellent chemotactic response. Thus, the 'sign' of the signal has been conserved. Furthermore, the relative sensitivity of Nart to these two ligands is maintained. Nitrate elicits a repellent response through Nart at lower concentrations than does nitrite, and the nitrate response is more robust. In NarX, nitrate also elicits a stronger response at lower concentrations that does nitrite (Williams and Stewart, 1997).

In Chapter III, I show the effects of mutations targeting the presumed ligand-binding P-box region of NarX in the context of the Nart chimera. Some of the mutations affect basal levels of methylation in the absence of ligand binding. These mutations have comparable effects on the basal signaling state of NarX (Williams and Stewart, 1997), indicating that the signaling mechanism is conserved between NarX and the hybrid. Most interestingly, a previously characterized mutation in NarX (G51R) resulted in a reversed signaling phenotype in Nart such that nitrate was sensed as an attractant rather

than as a repellent. In NarX, this same substitution was considered to have caused insensitivity to nitrate. I show that a residue substitution in the periplasmic domain reverses signal output upon exposure to nitrate.

The logical continuation of this work would be to construct a reciprocal hybrid containing the Tar periplasmic region connected to the NarX signaling domain. This protein could be tested in the Nar pathway to determine how Tar ligands affect the transcription of target genes for NarL and NarP. Because Tar has both attractant and repellent ligands, both activation and repression of the system could be studied. In this analysis, special attention should be paid to signaling in response to maltose, since TazI, the previously discussed Tar-EnvZ hybrid, signals in response to aspartate but not maltose (Utsumi *et al.*, 1989). Aspartate binds to Tar with higher affinity than maltose-bound MBP (Mowbray and Koshland, 1987; Manson *et al.*, 1985). Perhaps, the cytoplasmic region of EnvZ in Taz forces the Tar periplasmic domain into a conformation that has lower affinity for both ligands, an effect which essentially eliminates association with MBP.

The creation of a hybrid between the periplasmic domain of a sensor kinase with known stimulatory and inhibitory ligands with the Tar signaling domain might also provide valuable data. This construct should allow for both activation and inhibition of the hybrid chemoreceptor. Although other bacteria contain such sensor kinases, such as SasS of *Myxococcus xanthus* (Yang and Kaplan, 1997), none have been discovered in *E. coli* as yet.

The creation of hybrid sensors between proteins from different species has already begun to yield information. For example, Tar has been joined to the human insulin receptor (Moe *et al.*, 1989; Biemann *et al.*, 1996). The insulin receptor signals upon the formation of dimers, a process facilitated by insulin. The Tar-insulin receptor hybrid signals in response to aspartate, also as a result of increased dimerization. In this hybrid, the Tar periplasmic domain apparently facilitates dimerization of the receptor in the presence of aspartate. More recently, the NarX ligand-recognition region has been fused to the signaling domain of the *M. xanthus* sensor kinase DifA to yield a functional hybrid that supports fruiting body development in *M. xanthus* in a nitrate-dependent fashion (Zhao *et al.*, 2005, submitted). This research shows that the signal conservation is widespread.

Another area of recent interest is the role of HAMP domains in signal transduction. This region has been the site of fusion for all successful sensor kinase–sensor kinase and sensor kinase–chemoreceptor hybrids. HAMP regions are found in all chemoreceptors and in about half of the known sensor kinases, both those with and without known ligands. HAMP regions also link functional domains in some soluble proteins (Aravind and Ponting, 1999). Mutations affecting each of the two amphipathic helices of HAMP domains produce marked and predictable phenotypes (Appleman *et al.*, 2003). Furthermore, construction of a chemoreceptor lacking the TM helices showed that interaction of the HAMP domain with the membrane is apparently not required for signaling (Ottemann and Koshland, 1997). The HAMP region may facilitate the formation of higher-order structures, provide signal amplification, or promote intradimer

or interdimer crosstalk. Construction of a hybrid protein using sensors with altered HAMP regions may reveal more about how these linkers function in communication between protein domains.

The effects of P-box mutations in NarX and Nart reveal potentially valuable information about the properties of hybrid receptors. Residue substitutions in the P-box can affect either the kinase or phosphatase activities of the sensor kinase. Nart eliminates the complication of the phosphatase activity and focuses only on the control of the CheA kinase. Furthermore, *narX* mutations that create small signaling differences within the confines of the two-component pathway can exert profound effects in Nart due to the amplification properties of the chemotactic system. This advantage is highlighted by the properties of the G51R substitution, which causes a signal reversal in Nart. Previous research characterizing G51R largely ignored this mutation and simply classified it as 'nitrate insensitive,' since the addition of nitrate merely decreased *narG* expression to basal levels (Williams and Stewart, 1997). In the Nart hybrid, however, it became obvious that G51R resets the basal signaling state of the receptor, resulting in a clear inhibition of kinase activity upon nitrate addition. It is likely that a previously unnoticed mechanism operates within NarX itself.

Combining domains of receptors to create new chimeras may initially appear to be 'Frankenscience.' Our study of signaling mechanisms using hybrid constructs, however, may herald exciting new advances in this field. Receptors that possess kinase and phosphatase activities that are unable to function independently of other receptors or that are targets for activation by other receptors can perhaps have their individual

components placed into a context in which they can be examined rigorously. Thus, properties that remain cryptic or obscure in the original system may be revealed, and complex interactions within a receptor can be elucidated. Finally, since most individual domains retain their signaling properties within a hybrid, it seems likely that a variety of novel and useful sensors can be engineered.

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APPENDIX A

STATEMENT OF CONTRIBUTION TO WORK

-----Original Message-----
From: Scott Ward [mailto:sward@mail.bio.tamu.edu]
Sent: Monday, March 28, 2005 1:36 PM
To: robg@microbio.ucla.edu
Cc: Michael Manson
Subject: Dissertation Statement

Dr. Gunsalus -

I worked with your lab in a collaborative effort in the creation of the NarX-Tar chimera, which we published in Molecular Microbiology (2002). I am about to finish my dissertation and one of my committee members is asking that with any collaborative works, we acquire a statement from our collaborators regarding their contribution to the work.

With this collaboration, Asuncion Delgado created the two hybrid transducers, NarX(218)-Tar and NarX(270)-Tar. I was responsible for the experimentation portion by conducting immunoblots, capillary assays, and tethered cell experiments.

At your convenience, if you could e-mail me stating your labs contribution to the work, I would be very appreciative.

Thank you,

Scott Ward
sward@mail.bio.tamu.edu

>>> "Gunsalus, Rob" <robg@microbio.ucla.edu> 03/28/05 3:55 PM >>>

Dear Scott,

Your description matches exactly what I recall what the case! I hope the thesis writing and other tasks go smoothly.

Best, Rob

Please give my regards to Mike

APPENDIX B

PERMISSION TO REPRINT MANUSCRIPT

-----Original Message-----
From: Scott Ward [mailto:sward@mail.bio.tamu.edu]
Posted At: 28 March 2005 22:20
Posted To: March 29 - Apr 1
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Good luck!

Best wishes,

VITA

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