Mutationally Altered Signal Output in the Nart (NarX-Tar) Hybrid Chemoreceptor

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Signal-transducing proteins that span the cytoplasmic membrane transmit information about the environment to the interior of the cell. In bacteria, these signal transducers include sensor kinases, which typically control gene expression via response regulators, and methyl-accepting chemoreceptor proteins, 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 targeting a highly conserved sequence (the P box) in the periplasmic domain alter chemoreception by Nart and signaling by NarX similarly. In particular, the G51R substitution converts Nart from a repellent receptor into an attractant receptor for nitrate. Our results underscore the conclusion that the fundamental mechanism of transmembrane signaling is conserved between homodimeric sensor kinases and chemoreceptors. They also highlight the plasticity of the coupling between ligand binding and signal output in these systems.

The Tar chemoreceptor of *Escherichia coli* mediates attractant responses to aspartate and maltose (39), the latter via maltose-binding protein (18), and repellent responses to Ni^{2+} and Co^{2+} (45). The other high-abundance chemoreceptor, Tsr, mediates attractant and repellent responses to serine (39) and leucine (45), respectively. Tar and Tsr form homodimers in the presence or absence of ligands (5, 31), and these dimers associate into both homogeneous (19, 21, 43, 46) and mixed (3, 25, 36, 43, 46) trimers of dimers. A number of sensor kinases, including NarX and EnvZ, share predicted membrane topology with these chemoreceptors (10, 13).

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 (23, 24). Tar binds aspartate at the dimer interface near the apexes of the periplasmic domains (8, 27, 28, 31), whereas ligand-bound maltose-binding protein binds asymmetrically at the apex of the Tar homodimer (6, 16, 53). Serine and leucine bind directly to the periplasmic domain of Tsr (23). The cytoplasmic regions of the high-abundance transducers are responsible for transmitting the signal received from the periplasmic region to the CheA kinase (17, 30, 44). 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 (37). The addition of attractants, or the removal of repellents, inhibits the activation of CheA by the cognate receptor, thereby decreasing the rate of phosphoryl group transfer from receptor-associated 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 the review of chemotaxis by Stock and Surette [42].)

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 (22, 48). CheR activity is antagonized by the CheB methylesterase, which, when it is phosphorylated by CheA, removes the methyl groups (41). 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 unstimulated basal 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. The nitrate/nitrite-responsive sensor kinases NarX and NarQ, which phosphorylate the DNA-binding response regulators NarL and NarP, also show much greater sequence conservation in their cytoplasmic output domains than in their periplasmic input domains (26, 33, 34). 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 (52).

Within their periplasmic domains there is a sequence of 18 residues that differs at only three positions between NarX and NarQ (52). This region is called the P box (9), and it is located immediately after (C-terminal to) the first transmembrane helix (TM1). The P box is predicted to play an integral role in ligand binding and signaling through interaction with a region of similar size, called the P′ box, located just before (N-terminal to) TM2 (40). There are two invariant residues (Gly-51 and Met-55) within the P boxes of all known NarX or NarQ homologs (40).

Mutational analyses indicate that Lys-49 is responsible for

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TABLE 1. Phenotypes of P box mutations in NarX[a]

Residue substitution	Associated phenotype
H45E	Nitrate insensitive
A46V	Mimics nitrate occupancy
K49I	Nitrate-nitrite nondifferentiating
G51R	Nitrate insensitive
R54K	Ligand insensitive
M55K	Mimics nitrite occupancy

[a] Phenotypes are described by Williams and Stewart (52).

the nitrate/nitrite-distinguishing capacity of NarX; the residue at the equivalent position in NarQ is Ile-45 (52). Mutations altering other residues within the P box of NarX (Table 1) result in a variety of phenotypes, including mimicking of nitrate or nitrite occupancy and inability to respond to one or both ligands (7, 52).

Functional chimeras between chemoreceptors and sensor kinases have been created by fusing genes at an in-frame NdeI restriction site (CATATG [His-Met]) introduced into the sequence encoding AS2 (amphipathic sequence 2) of the cytoplasmic HAMP linker domain (12, 23, 47, 50, 51). 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 (50). 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 nitrate and nitrite in a manner consistent with the phenotypes introduced by the same changes in intact NarX (52). Most strikingly, the G51R substitution, which renders NarX insensitive to nitrate, reverses receptor signaling by converting nitrate into an attractant rather than a repellent.

MATERIALS AND METHODS

Strains and plasmids. Strain RP437 (32) is wild type for motility. Strain MM509 (15) is an eda^+ Δtar-$tap5201$ derivative of strain RP437. *E. coli* VB13 (51) is a thr^+ eda^+ $tsr7021$ trg::Tn10 Δtar-$tap5201$ (38) derivative of strain RP437. Plasmid pMK113 (15) contains the *E. coli* tar gene and the single-stranded origin of phage M13 from plasmid pZ150.

The pNart plasmid (50) encodes the hybrid protein NarX-Tar (Nart). Nart contains N-terminal residues 1 to 218 of NarX fused to C-terminal residues 257 to 553 of Tar. The *narX* sequences for the fusion were obtained by PCR using pLK633 (20) as a template. PCR-generated mutations were created using primers from Invitrogen and were confirmed by DNA sequencing.

Media. Growth media were prepared according to the method of Miller (29). Tryptone broth (TB) was used for growing highly motile cells. It contains 1% (wt/vol) tryptone extract and 0.8% (wt/vol) NaCl. Luria broth (LB), used for standard overnight cultures, contains 1% tryptone extract, 0.5% (wt/vol) yeast extract, and 0.5% NaCl. TB swarm plates contain 0.325% (wt/vol) Difco agar, and LB solid agar has 1.5% (wt/vol) Difco agar. Incubation temperatures were 37°C for LB and 32°C for TB. Ampicillin (final concentration, 50 μg/ml) and tetracycline (final concentration, 5 μg/ml) were added as needed.

Methylation assay. Steady-state and attractant-induced methylation assays were done as described by Weerasuriya et al. (51). Dried samples were resuspended in 200 μl of loading buffer (2% [wt/vol] sodium dodecyl sulfate [SDS], 5% [vol/vol] 2-mercaptoethanol, 8.5% [vol/vol] glycerol, 60 mM Tris [pH 6.8], 0.0004% [wt/vol] bromophenol blue). The extracts were resuspended by incubation at 60°C prior to being loaded onto gels for SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were separated by SDS-PAGE (12% acrylamide) and transferred to nitrocellulose filters. An antibody against the conserved cytoplasmic domain of Tsr (2) that also recognizes Tar was used to probe the immuno-

blots. The cross-reacting primary antibody was visualized with an alkaline phosphatase-conjugated goat anti-rabbit antibody (Bio-Rad). The blot was developed with SigmaFast (Sigma).

Tethered-cell assays. Cells were tethered as described by Weerasuriya et al. (51) with the following modifications. Cells were grown with swirling to an A_{590} of 0.5 at 32°C, flagella were sheared by 10 7-s pulses at the highest power setting of a Waring blender, and chloramphenicol was present in the tethering buffer at 200 μg/ml. The cells were mixed with an anti-filament antibody and tethered to glass coverslips (37). These coverslips were affixed to a flow chamber (4), which was used to introduce chemoeffectors to the tethered cells. Sensitive cells responded to the addition of repellents by exhibiting a brief period of exclusively CW flagellar rotation before adapting and returning to baseline CCW/CW reversing behavior. Removal of repellent by introduction of tethering buffer induced a longer period of exclusively CCW 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 seconds) of the response time, since it takes several seconds for the new medium to reach the cells. Cells that responded for less than 15 s were scored as nonresponders (0 s), 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.

Attractant-in-tube capillary assay. The attractant-in-tube capillary assay was carried out as described by Adler (1), with the following modification. Bacteria were resuspended in chemotaxis buffer at 1×10^5 cells per ml and placed in the pond. The higher cell density was required because of the relatively poor motility (~10% motile cells) of the MM509/pNart strain, which expressed nonphysiologically high levels of receptor and had to be grown in the presence of ampicillin. Then, capillary tubes containing different concentrations of a potential attractant were placed in the pond. In this assay, attractant diffuses out of the capillary to create a gradient of considerable extent in the pond (14). Sensitive cells swim up the gradient and enter the capillary. After 45 min of incubation at 30°C, capillaries were removed, and the contents were suitably diluted and plated onto LB agar. Colonies were counted after overnight incubation. Assays were done in triplicate.

Repellent-in-pond capillary assay. Cells were prepared for the repellent-in-pond assay as for a standard capillary assay and were placed in a pond containing buffer and various concentrations of a potential repellent. Capillaries filled with buffer were then placed in the pond. As repellent diffuses into the capillary, a very local gradient of decreasing repellent concentration toward the capillary mouth is established. Sensitive cells swim down this gradient and enter the capillary. After 1 h, the cells within the capillary were diluted and plated. The number of cells within the capillary was compared to the baseline number of cells that swam from a buffer-repellent mixture to a capillary filled with buffer and repellent at the same concentration as that in the pond. Assays were done in triplicate.

RESULTS

Behavior of unstimulated tethered cells. Tethered cells of strain MM509, which has the chemoreceptor gene configuration [(Δtar-tap)5201] tsr^+ trg^+ aer^+, exhibited the same rotational bias (60 to 80% CCW flagellar rotation) as cells of the wild-type strain RP437 (tar^+ tap^+ tsr^+ trg^+ aer^+). However, MM509 cells expressing Nart were strongly CW biased (33% CCW flagellar rotation). Cells expressing Nart proteins bearing the H45E, K49I, or R54K substitution in the P box showed the same rotational bias (30 to 33% CCW), whereas cells expressing Nart proteins bearing the A46V, G51R, or M55K substitution had an increased CW bias (20 to 25% CCW).

Responses of tethered cells to addition of ligands. MM509 cells expressing Nart exhibited brief periods (less than 1 min) of exclusively CW rotation after the addition of nitrate or nitrite (Table 2), a typical response to the addition of repellent. The responses of MM509 cells expressing Nart proteins with the P box substitutions described by Williams and Stewart (52) are also shown in the top half of Table 2. Cells expressing the H45E (nitrite-insensitive) or K49I (nitrate-nitrite nondifferentiating) Nart exhibited a lower threshold concentration for the

TABLE 2. Responses of tethered cells to addition and removal of ligand

Protein	Time of CW-only rotation (s) until first CW-to-CCW reversal after addition or removal of ligand at the following concn (M)[a]:											
	Nitrate						Nitrite					
	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
Addition of ligand												
Wild type	0	0	0	0	24 ± 1	35 ± 8	0	0	0	0	26 ± 6	53 ± 8
H45E	0	0	0	20 ± 3	21 ± 6	31 ± 9	0	0	0	0	0	21 ± 3
A46V	0	0	0	0	0	0	0	0	0	0	23 ± 4	26 ± 4
K49I	0	0	19 ± 31	20 ± 21	22 ± 3	29 ± 3	0	0	0	0	0	80 ± 10
G51R[b]	**0**	**0**	**0**	**19 ± 4**	**102 ± 17**	**144 ± 15**	**0**	**0**	**0**	**0**	**40 ± 7**	**31 ± 14**
R54K	0	0	0	0	0	26 ± 4	0	0	0	0	0	27 ± 4
M55K	0	0	0	0	0	20 ± 3	0	0	0	0	0	0
Removal of ligand												
Wild type	66 ± 17	126 ± 16	315 ± 27	753 ± 58	790 ± 52	389 ± 32	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	0	0	0	0	0	25 ± 6
A46V	0	0	0	0	37 ± 6	49 ± 8	0	0	0	19 ± 2	77 ± 10	118 ± 22
K49I	89 ± 11	208 ± 28	325 ± 31	356 ± 25	288 ± 27	167 ± 16	0	0	0	46 ± 10	151 ± 14	172 ± 16
G51R[c]	**0**	**0**	**0**	**0**	**0**	**29 ± 4**	**0**	**0**	**0**	**0**	**0**	**0**
R54K	0	0	0	35 ± 5	39 ± 4	132 ± 8	0	0	0	0	32 ± 4	146 ± 11
M55K	0	0	0	45 ± 8	150 ± 12	342 ± 23	0	0	0	0	0	26 ± 6

[a] Bold type indicates the responses of cells containing G51R NarX, which were opposite those of cells of all other responding strains.
[b] Unlike cells of all other strains, G51R cells rotated exclusively CCW after addition of ligand and underwent a CCW-to-CW reversal at the time indicated.
[c] Unlike cells of all other strains, G51R cells rotated exclusively CW after removal of ligand and underwent a CW-to-CCW reversal at the time indicated.

nitrate response and a higher threshold concentration for the nitrite response. Cells expressing A46V (nitrate occupancy-mimicking) Nart responded much like the wild-type cells to nitrite but did not respond to nitrate. Cells expressing R54K (ligand-insensitive) Nart responded only at 100 mM nitrate and nitrite, and cells expressing M55K (nitrite occupancy-mimicking) Nart responded only at 100 mM nitrate and not at all to nitrite.

The behavior of cells expressing G51R (nitrate-insensitive) Nart was totally unexpected. They responded to high concentrations of either ligand (≥1 mM nitrate and ≥10 mM nitrite) with exclusively CCW flagellar rotation, which lasted more than 2 min with 100 mM nitrate. Thus, the G51R Nart receptor senses both nitrate and nitrite as attractants when they are added at millimolar concentrations.

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 proteins are shown in the bottom half of Table 2. In this situation, which resembles the physiologically relevant response to repellents, cells expressing wild-type Nart rotated their flagella exclusively CCW for extended periods. Responses, albeit brief, were seen even after removal of 1 μM nitrate or nitrite. Qualitatively similar results were obtained with cells expressing the H45E and K49I versions of Nart, although extended CCW flagellar rotation was evoked only after removal of 1 mM or higher concentrations of nitrite, and the length of responses at equivalent concentrations was usually shorter. Cells producing the R54K and M55K mutant receptors gave shorter responses than cells expressing wild-type Nart, and they responded only after removal of higher concentrations of nitrate and nitrite. The R54K protein seemed relatively more debilitated as a nitrate sensor, and the M55K protein was relatively more defective as a nitrite sensor. The A46V protein was unique in that cells expressing it responded longer to nitrite removal than to nitrate removal and responded at a lower concentration of nitrite than of nitrate.

Cells expressing G51R Nart rotated exclusively CW for about 30 s after removal of 100 mM nitrate and did not respond to removal of any concentration of nitrite. As is typical for attractants, the CCW response to the addition of nitrate and nitrite was significantly longer than the CW response to their removal. Thus, G51R Nart behaves in all respects like a typical attractant receptor for nitrate, and perhaps nitrite, so that this residue replacement reverses the sign of the chemotactic response. It should be noted that the affinity for both substrates seems low and that the attractant response is not particularly robust, especially given that the proteins were overproduced. However, there is no question that it is significant.

Responses in capillary assays. Cells were tested for attractant and repellent taxis using the standard capillary assay (1) and a repellent-in-pond variant of that assay (45). In the standard assay, cells in chemotaxis buffer swim toward and then into a capillary that is a diffusive source of attractant. If the attractant concentration is high enough, the chemoreceptors of a cell become saturated while it is still in the pond, and fewer cells accumulate in the capillary. Thus, peak accumulations of cells can be seen with intermediate concentrations of attractant. In the repellent-in-pond variant, cells are suspended in chemotaxis buffer containing different concentrations of a repellent and can move 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 will be far higher than the accumulation in a capillary containing buffer in the repellent-in-pond assay. Again, saturation of receptors at higher concentrations of repellent prevent them from sensing a gradient, and peak accumulations of cells may occur at intermediate concentrations of repellent.
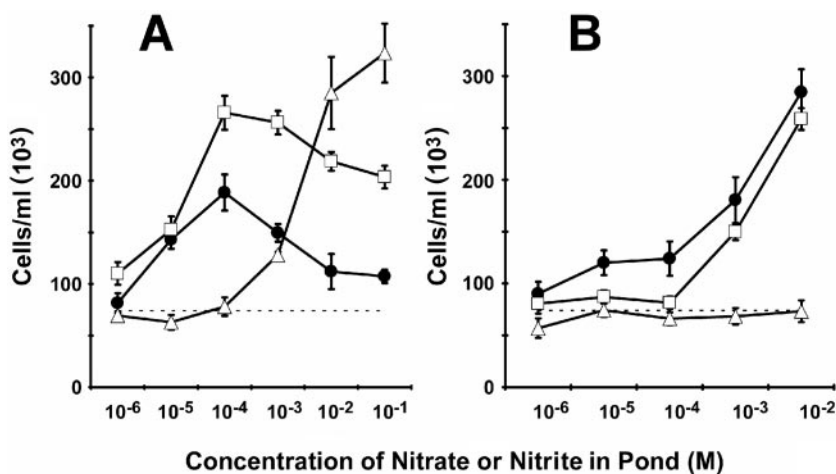
FIG. 1. Repellent-in-pond capillary assay with nitrate and nitrite. The accumulation of MM509 cells containing plasmid pAD56 expressing Nart (closed circles), H45E Nart (open triangles), or K49I Nart (open squares) in buffer-containing capillaries was recorded. The pond contained nitrate (A) or nitrite (B) at the indicated concentrations. The accumulation of cells when only buffer was present in the pond was 8×10^4 (dashed line). Assays were done in triplicate.

Figure 1 presents the data for MM509 cells expressing the wild-type, H45E, and K49I Nart proteins. Cells expressing wild-type Nart gave peak responses in the repellent-in-pond assay when nitrate and nitrite were present at 0.1 mM and ≥ 10 mM, respectively. K49I Nart supported a somewhat stronger response with nitrate than did wild-type Nart and a somewhat weaker response to nitrite. With H45E Nart, a robust repellent response to nitrate was seen, but only at concentrations of 1 mM or above. Cells containing H45E Nart did not respond at concentrations up to 10 mM nitrite. Higher concentrations could not be tested, because high levels of nitrite are toxic 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. 2. An accumulation to 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 nitrate concentrations of ≤ 1 mM. No significant accumulation was seen with nitrite up to 10 mM. Cells expressing the other Nart proteins, including the wild type, gave no accumulation above background at any concentration of nitrate or nitrite. These results confirm that G51R Nart is an attractant chemoreceptor for nitrate.

Methylation pattern of Nart mutant proteins. The basal and ligand-adapted methylation patterns of wild-type or mutant Nart proteins expressed from plasmid pAD56 were tested in the transducer-deleted (ΔT) strain VB13. Nart can potentially exist in any of five possible states of covalent modification, with 0, 1, 2, 3, or all 4 glutamyl residues being methyl esters. These different forms can be distinguished on immunoblots because each methyl group neutralizes one negative charge, allowing more SDS to bind and thus increasing the migration rate of the modified protein. Under our standard conditions for immunoblotting, we did not achieve clear resolution of the bands corresponding to individual methylation states. One contributing factor could be that each level of methylation is potentially represented by multiple proteins, and thus by multiple bands. For example, a singly methylated protein could be modified at the first, second, third, or fourth glutamyl residue, and each of these forms might migrate slightly differently.

The H45E, K49I, and R54K proteins showed a banding pattern similar to that of wild-type Nart (Fig. 3). The R54K
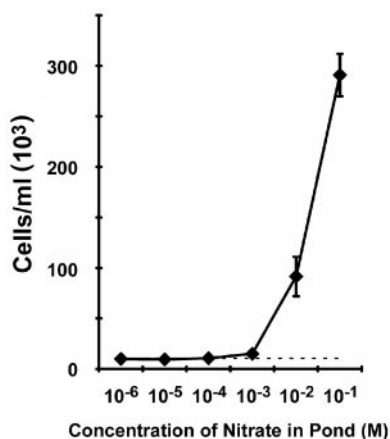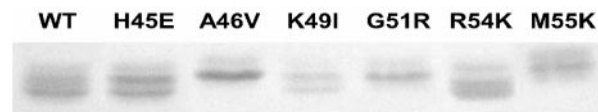

FIG. 2. Attractant-in-capillary assay with nitrate. The accumulation of MM509 cells containing plasmid pAD56 expressing G51R Nart in capillaries containing nitrate at the indicated concentrations was recorded. The accumulation seen when only buffer was present in the capillary was 1×10^4 cells (dashed line). Capillary assays were done in triplicate.

FIG. 3. In vivo basal methylation patterns of mutant Nart proteins. Proteins were expressed in the ΔT strain VB13. The greater the number of methyl groups (from zero to 4) per receptor, the faster the migration of the band. The primary antibody was raised against the conserved cytoplasmic domain of Tsr (2) and reacts well with the cytoplasmic domain of Tar. WT, wild type.
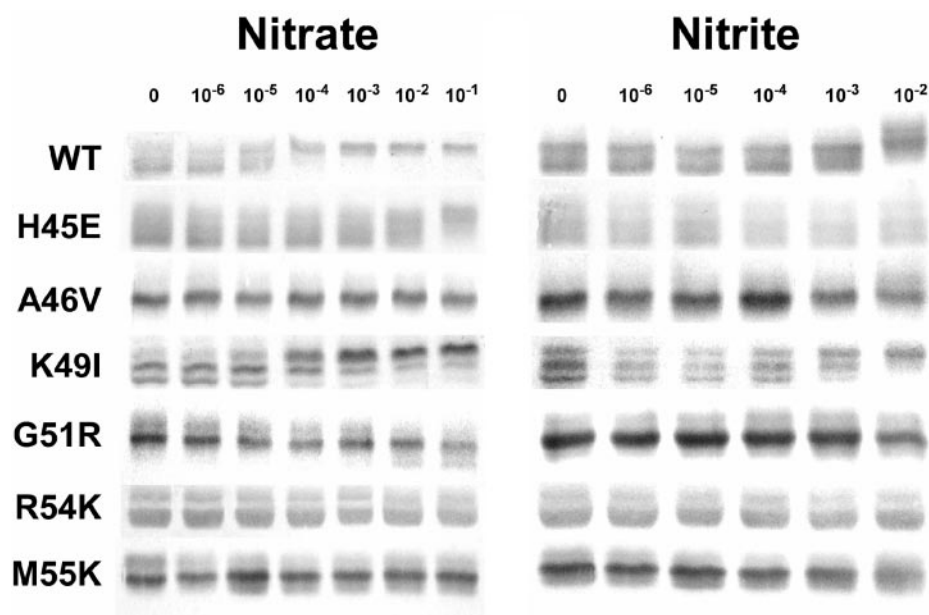
FIG. 4. In vivo methylation patterns of mutant Nart proteins after complete adaptation to nitrate or nitrite. Increasing concentrations of nitrate or nitrite were added to VB13 cells expressing either wild-type Nart (WT) or one of the mutant Nart proteins. Cells were incubated with the ligand for 20 min before the methylation states of the proteins were determined.

substitution is charge neutral, and the H45E and K49I substitutions introduce one negative or eliminate one positive charge, respectively, changes that should, if anything, slow migration during SDS-PAGE. Thus, these three proteins must be at least as highly methylated as wild-type Nart. An upward shift in the band pattern was seen with the A46V, G51R, and M55K Nart proteins. The A46V substitution is charge neutral, and the G51R and M55K substitutions introduce positive charges, a change that should, if anything, speed migration during SDS-PAGE. Thus, these three proteins are clearly less methylated than wild-type Nart. The overall methylation patterns of the proteins were similar whether they were expressed in strain VB13 or in the Δtar-tap tsr⁺ strain MM509, although the differences in band pattern were not as pronounced in strain MM509, 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 nitrate and nitrite (Fig. 4). The cells were harvested 20 min after the addition of ligand, by which time adaptation should be complete (see Table 2). Wild-type Nart showed the expected decrease in methylation level with both nitrate and nitrite, as did K49I Nart. The shifts were first seen at 0.1 mM nitrate and 10 mM nitrite. H45E Nart exhibited no change in methylation up to 10 mM nitrite and a significant decrease only with 100 mM nitrate. The A46V, R54K, and M55K proteins did not change their methylation levels at any concentration of nitrate or nitrite. G51R Nart was an outlier. It did not change its methylation pattern significantly with any concentration of nitrite, but it showed a distinct shift toward more highly methylated, faster-migrating forms after addition of 100 mM nitrate, as is expected after adaptation to addition of a relatively weak attractant.

DISCUSSION

The NarX sensor kinase increases its autophosphorylation activity in the presence of nitrate or nitrite (26, 35, 49). NarX ligands may also alter the phosphatase activity of NarX toward the phosphorylated forms of its cognate response regulators, NarL and NarP (35, 49). 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 (50), 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 an output of increased or decreased CheA activity.

We use the designation "wild-type Nart" for 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 (50). Evidence to support this conclusion comes from observations of tethered cells (Table 2), the behavior of cells in repellent-in-pond capillary assays (Fig. 1), and experiments that record adaptive methylation after the addition of nitrate and nitrite (Fig. 4). 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 1).

The phenotypes associated with residue substitutions in the P box region of NarX (52), which is thought to be responsible for ligand detection, were consistent between the intact sensor kinase and Nart. This correlation could be seen with the responses, or lack of response, of cells in the tethered-cell and
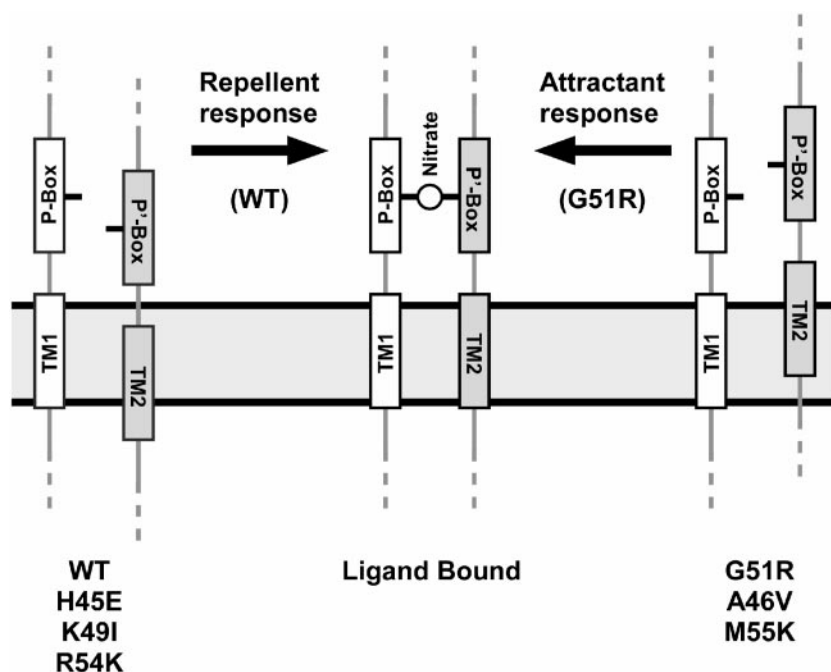
FIG. 5. Model for transmembrane signaling by Nart. Three potential signaling states are shown: on the right, a highly methylated "off" state; on the left, a lightly methylated "on" state; and in the center, a ligand-bound state with an intermediate level of methylation. By analogy with what is known about transmembrane signaling in Tar (7, 10), the P′ box in the wild-type (WT), H45E, K49I, and R54K proteins is displaced toward the membrane relative to the P box, which pushes TM2 further toward the cytoplasm. In the A46V, G51R, and M55K proteins, the P′ box is displaced away from the membrane relative to the P box, which pulls TM2 further out off the cytoplasm. When nitrate (or nitrite) binds, the P′ box is in an intermediate position. With the wild-type protein, ligand binding causes the P′ box and TM2 to move upward, which represents activation of signaling (i.e., a repellent response) relative to the ligand-free state. With the G51R protein, ligand binding moves the P′ box and TM2 toward the cytoplasm, which represents inactivation of signaling (i.e., an attractant response) relative to the ligand-free state. To simplify, only three states are shown, corresponding to ligand-free wild-type, H45E, K491, and R54K proteins (left), ligand-free A46V, G51R, and M55K proteins (right), and ligand-bound wild-type and G51R proteins (middle).

capillary assays for chemotaxis (compare Table 1 with Table 2 and Fig. 1). However, it is best quantified with adaptive methylation.

The addition of either nitrate or nitrite led to demethylation of wild-type Nart (Fig. 4), as expected for a repellent. The H45E (nitrite-insensitive), K49I (nitrate-nitrite nondifferentiating), and R54K (ligand-insensitive) Nart proteins showed basal levels of methylation much like those of wild-type Nart. However, the changes in methylation seen after addition of ligands were quite different. Cells expressing H45E Nart required 1,000 times more nitrate to elicit any detectable demethylation than cells expressing wild-type Nart, and H45E Nart showed no demethylation at the highest concentration of nitrite tested (10 mM). The effect of this replacement is most parsimoniously interpreted as decreased affinity for both ligands, since NarX and, at least in the methylation assay, Nart (Fig. 4) have a 1,000-fold lower affinity for nitrite than for nitrite. The result of an equal decrease in the affinities for both ligands would be a "nitrite-blind" receptor. R54K Nart indeed appears to be blind to both ligands, since no change in methylation level was seen after addition of any amount of nitrate or nitrite. The changes in methylation of K49I Nart are similar to those of wild-type Nart. However, the methylation patterns of K49I Nart after adaptation to 100 mM nitrate and 10 mM nitrite are quite similar, whereas with wild-type Nart, 100 mM nitrate leads to considerably more demethylation than 10

mM nitrite. This behavior is in keeping with the nitrate-nitrite nondifferentiating phenotype associated with the mutation that created K49I NarX.

The A46V (nitrate occupancy-mimicking), G51R (nitrate-insensitive), and M55K (nitrite occupancy-mimicking) Nart proteins were all considerably less methylated than the wild-type protein in the absence of ligands. These three proteins also confer an increased CW bias to tethered cells. 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 (52). Neither A46V nor M55K Nart showed any change in methylation after addition of nitrate or nitrite, a result consistent with their ligand occupancy-mimicking phenotypes.

The most surprising effect was seen with G51R. Although G51R Nart did not change its methylation pattern upon addition of nitrite, its level of methylation increased modestly after the addition of 10 mM or 100 mM nitrate. It is noteworthy that nitrate causes a twofold reduction in G51R NarX activity (52), which suggests that the G51R change also reverses the sign of the nitrate response mediated by intact NarX. Although 10 mM nitrite did not increase methylation of G51R Nart, high concentrations of nitrite (10 mM and 100 mM) produced a brief period of exclusively CCW flagellar rotation in tethered cells expressing this protein (Table 2).

G51R Nart differs from A46V Nart and N55K Nart in that it still binds ligand, although with decreased affinity compared to wild-type Nart. The difference in the sign of the ligand response—negative (repellent) for NarX and positive (attractant) for G51R Nart—may be determined by the basal signaling state before ligand is 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. A diagram to illustrate our model is shown in Fig. 5. The model is clearly wrong in detail, since it shows only three conformations, and hence three signaling states: an "off" state assumed by wild-type, H45E, K49I, and R54K Nart in the absence of ligand, an "on" state assumed by A46V, G51R, and M55K Nart in the absence of ligand, and an "intermediate" signaling state adopted by wild-type and G51R Nart proteins when they are bound to ligand. Clearly, the different phenotypes observed with the different mutant proteins defy such a simplistic analysis. We also do not mean to imply that nitrate-bound wild-type Nart and nitrate-bound G51R Nart are in identical conformations. The sole purpose of the drawing is to show that the different mutant proteins start in different conformational/signaling states and that, consequently, their activities can be pushed in different directions by the same ligand.

Draheim et al. (11) 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. The 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 hybrid proteins that fuse 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.

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