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Residues at the Cytoplasmic End of Transmembrane Helix 2 Determine the Signal Output of the Tar_{Ec} Chemoreceptor

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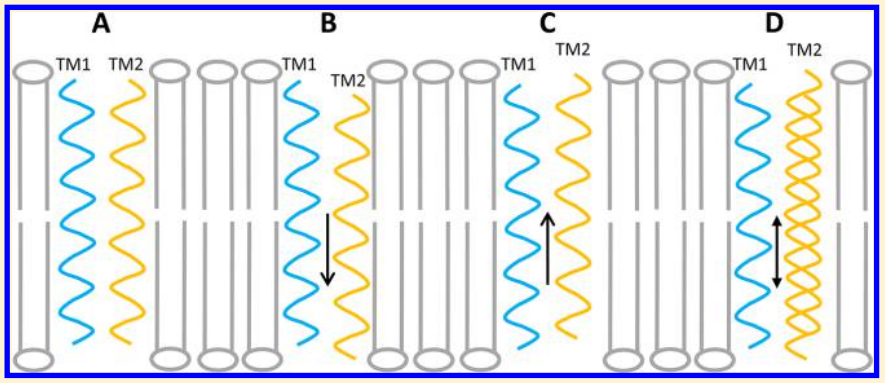
Supporting Information



ABSTRACT: Baseline signal output and communication between the periplasmic and cytoplasmic domains of the *Escherichia coli* aspartate chemoreceptor Tar_{Ec} are both strongly influenced by residues at the C-terminus of transmembrane helix 2 (TM2). In particular, the cytoplasmic aromatic anchor, composed of residues Trp-209 and Tyr-210 in wild-type Tar_{Ec}, is important for determining the CheA kinase-stimulating activity of the receptor and its ability to respond to chemoeffector-induced stimuli. Here, we have studied the effect on Tar_{Ec} function of the six-residue sequence at positions 207−212. Moving various combinations of aromatic residues among these positions generates substantial changes in receptor activity. Trp has the largest effect on function, both in maintaining normal activity and in altering activity when it is moved. Tyr has a weaker effect, and Phe has the weakest; however, all three aromatic residues can alter signal output when they are placed in novel positions. We also find that Gly-211 plays an important role in receptor function, perhaps because of the flexibility it introduces into the TM2−HAMP domain connector. The conservation of this Gly residue in the high-abundance chemoreceptors of *E. coli* and *Salmonella enterica* suggests that it may be important for the nuanced, bidirectional transmembrane signaling that occurs in these proteins.

An *Escherichia coli* cell moves through a chemically homogeneous environment in a three-dimensional random walk consisting of a series of smooth-swimming "runs" interspersed with brief "tumbles" that randomly reorient the bacterium. When flagella rotate counterclockwise (CCW), the flagellar filaments coalesce into a bundle at one end of the cell and propel it in a relatively linear manner.[1,2] When one or more flagella switch from CCW to clockwise (CW) rotation, this bundle is disrupted, resulting in a random reorientation of the bacterium.[2]

Several transmembrane chemoreceptors, known as methyl-accepting chemotaxis proteins (MCPs), mediate behavioral responses to specific sets of compounds. These MCPs, along with the aerotaxis receptor (Aer_{Ec}), are members of a common chemotaxis circuit that couples detection of environmental compounds to modulation of the rotational bias of the flagellar motors. By decreasing the probability of CCW rotation, a cell can extend the duration of runs in a favorable direction, either up an attractant gradient or down a repellent gradient.[3,4]

The aspartate chemoreceptor of *E. coli* (Tar_{Ec}) detects the presence of attractants both directly (aspartate) and indirectly (maltose). Aspartate binds at one of two rotationally symmetric binding sites in the periplasmic domain of the Tar_{Ec} homodimer. Maltose, in contrast, first associates with the periplasmic maltose-binding protein (MBP), causing MBP to adopt a conformation that facilitates interaction with Tar_{Ec}.[5,6] Maltose-bound MBP binds to the apex of the Tar periplasmic four-helix bundle.[7,8] Tar_{Ec} also mediates repellent taxis to Ni^{2+} and Co^{2+},[9,10] which bind directly to its periplasmic domain, as shown by isothermal titration calorimetry (ITC) (I. Kawagishi, personal communication).

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Attractant binding also induces covalent modification of the cognate MCP.[11] Methyl groups are added by a methyltransferase, CheR, and removed by a methylesterase, CheB.[12,13] An increased level of methylation of eight specific glutamyl residues within the receptor homodimer stimulates the activity of the CheA kinase, whereas a decreased level of methylation lowers the level of CheA stimulation.[14]

E. coli MCP subunits contain two transmembrane helices. Transmembrane helix 1 (TM1) is an N-terminal extension of helix 1 of the periplasmic domain, whereas transmembrane helix 2 (TM2) is a C-terminal extension of helix 4 of the periplasmic domain. TM2 communicates conformational changes induced by ligand binding to the cytoplasmic HAMP domain (originally identified within histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and certain phosphatases).[15] The mechanism of signal transduction from the periplasmic domain to the HAMP domain is not fully understood, and several hypotheses have been advanced to explain the process.[16−18] Whatever the signaling mechanism may be, a preponderance of evidence suggests that a small (∼1−3 Å) inward displacement of α helix 4 and TM2 roughly perpendicular to the plane of the membrane occurs upon binding of aspartate.[19−25]

Substitutions of the cytoplasmic aromatic tandem (Trp-209/Tyr-210) within TM2 of Tar_{Ec} with other aromatic or alanine residues result in a steady-state signal output consistent with an aspartate-bound conformation.[26] Repositioning the Trp-209/Tyr-210 tandem up to several residues from its original position incrementally modulates Tar_{Ec} signal output.[25]

Here, we have extended that study by examining in detail how the amino acid sequence of residues 207−212 affects the signaling properties of Tar_{Ec} and its ability to mediate aspartate chemotaxis. The results identify the distributions of aromatic residues within this region that support normal receptor function and highlight the importance of residue Gly-211 in overall receptor performance. These data explore the multiplicity of factors that affect the function of the aromatic anchor and perhaps help explain how aromatic anchors may function in different transmembrane signaling proteins.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Strains HCB436 [Δ*tsr7021* Δ*trg100* Δ(*tar-cheB*)*2234*],[27] RP3098 [Δ(*flhD-flhB*)*4*],[28] and VB13 (Δ*tsr7021* Δ*tar-tap5201 trg*::Tn*10*)[29] are derived from *E. coli* K-12 strain RP437.[30] Strain VB13 is deleted for all chemoreceptor genes other than *aer*, as is strain HCB436, which is also deleted for *cheRB*. We therefore designate these strains as ΔT, for deleted for transducers. All in vivo assays of receptor activity were conducted with these two strains. Strain RP3098 contains a deletion of the master regulator *flhDC* and therefore fails to produce any flagellar or chemotaxis proteins from their native promoters.

Plasmid pRD200,[24] derived from pMK113,[8] was used to express wild-type or mutant *tar* genes constitutively for all in vivo experiments. An in-frame coding sequence for a seven-residue linker (GGSSAAG)[31] and a C-terminal V5 epitope tag (GKPIPNPLLGLDST)[32] were added to the 3′ end of *tar*. Plasmid pRD300[24] is a derivative of pBAD18[33] that expresses *tar* with the same C-terminal linker and V5 epitope tag from the *E. coli araBAD* promoter upon induction with L-arabinose. Strain RP3098 was used with pRD300 as previously described[24] to produce standards for the in vivo methylation assay. Mutations in *tar* were introduced via site-directed mutagenesis (Stratagene).

Observation of Tethered Cells. HCB436 and VB13 cells containing plasmid pRD200 were grown overnight at 30 °C in tryptone broth[34] supplemented with 100 μg/mL ampicillin. Overnight cultures were then back-diluted (1:100) in tryptone broth and grown at 30 °C with agitation until the OD_{600} reached ∼0.6. At this time, a 10 mL aliquot of cells was pelleted, and the cells were resuspended in 10 mL of tethering buffer [10 mM potassium phosphate (pH 7.0), 100 mM NaCl, 10 μM EDTA, 20 μM L-methionine, 20 mM sodium DL-lactate, and 200 μg/mL chloramphenicol]. Flagella were sheared in a 50 mL stainless steel cup of a Waring blender during eight repetitions with 7 s intervals of shearing at high speed interspersed with 13 s pauses to prevent overheating.[35] Cells were collected by centrifugation, washed three times in tethering buffer, and mixed with an equal volume of a 1:200 dilution of antiflagellar filament antibody. A 40 μL aliquot of this cell/antibody mixture was added to the center of a glass coverslip. Coverslips were then incubated in a humidity chamber for 30 min at 30 °C and affixed to a flow chamber;[36] nontethered cells were removed when the chamber was flushed with chemotaxis buffer.

Tethered cells were observed under reverse phase contrast at 1000× magnification, using an Olympus BH-2 microscope. Rotating cells were digitally recorded, and at least 50 (usually 100) cells expressing each receptor variant were monitored visually during a 20 s playback. Cells were assigned to one of five rotational categories as previously described:[26] exclusively CCW, mostly CCW with occasional reversals, reversing frequently with no clear bias, mostly CW with occasional reversals, and exclusively CW. The reversal frequency was determined by tallying the number of reversals for each cell during video playback. The percentage of CW rotation was determined using the method of Ames et al.[37] Briefly, cells were weighted by their rotational category: exclusively CCW, 0.0; mostly CCW (cw/CCW), 0.25; reversing frequently (cw/ccw), 0.5; mostly CW (CW/CCW), 0.75; >75% CW (CW/ccw and CW), 1.0. None of our mutant receptors produced CW/ccw or CW output.

Determination of the Methylation State of Receptors in Vivo. HCB436 or VB13 cells containing plasmid pRD200 were grown overnight at 30 °C in tryptone broth supplemented with 100 μg/mL ampicillin. Overnight cultures were then back-diluted (1:100) in tryptone broth without ampicillin and grown at 30 °C until the OD_{600} reached ∼0.6. Cells were harvested by centrifugation, washed three times with 10 mL of 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, and resuspended in 5 mL of 10 mM potassium phosphate (pH 7.0) containing 0.1 mM EDTA, 10 mM sodium DL-lactate, and 200 μg/mL chloramphenicol. Aliquots (1 mL) were transferred to 10 mL scintillation vials and incubated for 10 min at 32 °C, with agitation. Cells were then incubated for an additional 30 min after the addition of L-methionine to a final concentration of 100 μM. A 100 μL aliquot of 100 mM L-aspartate or 10 mM $NiSO_4$ solutions, or 100 μL of buffer as a negative control, was added at this time, and the cells were incubated for an additional 20 min. Reactions were terminated by addition of 100 μL of ice-cold 100% TCA, and the samples were then incubated on ice for 15 min. Denatured proteins were pelleted, washed with 1% TCA and acetone, and resuspended in 100 mL of 2× SDS loading buffer. A 15 μL aliquot of each sample was subjected to sodium dodecyl

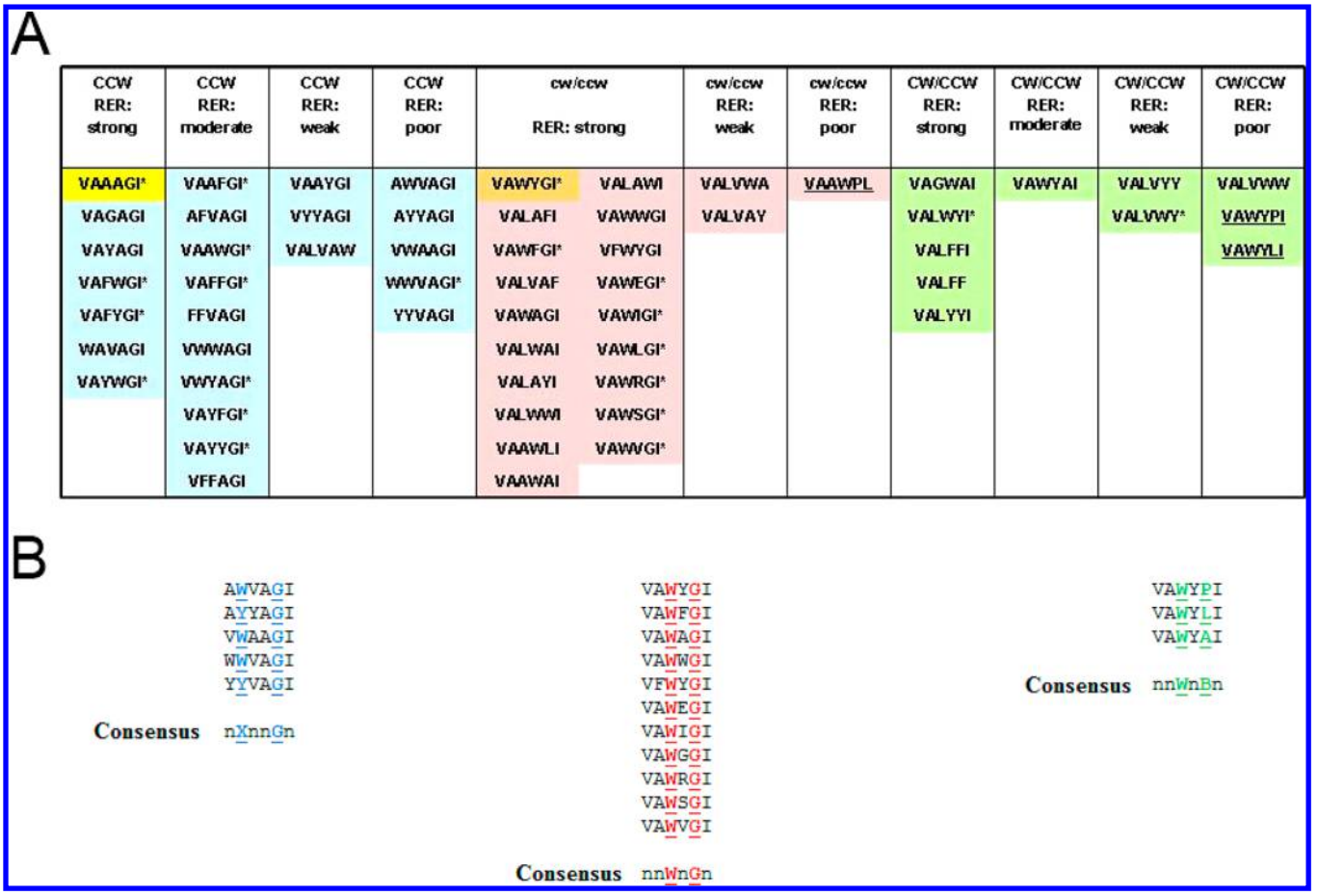

Figure 1. Library of TM2 aromatic anchor mutants. (A) Amino acid sequence of the six residues potentially altered at the C-terminal region of TM2 and the start of the connector region between TM2 and the HAMP domain. Mutants that have been described previously are noted with an asterisk. The receptor with tandem Ala residues (AA) is highlighted in yellow, and the wild-type receptor is highlighted in orange. Mutant receptors with similar rotational biases in Δ*cheRB* cells (Figure 2) are highlighted with the same color: CCW, ≤2% CW rotation; cw/ccw, 25−50% CW rotation; CW/CCW, 50−75% CW rotation. The following shorthand designations are used to group RER values: poor, 0−25% of the wild-type RER value; weak, 25−50% of the wild-type RER value; moderate, 50−75% of the wild-type RER value; strong, ≥ 75% of the wild-type RER value. The sequences for the three receptors that did not change their pattern of covalent modification in response to addition of the attractant aspartate or the repellent Ni^{2+} (Figure 4) are underlined. (B) Consensus sequences at residues 207−212 for the aromatic anchor in receptors with different signaling properties. X indicates either of the amphipathic aromatic residues (W and Y). B indicates (any?) residue other than G. n indicates (probably) many different residues. The sequence colored blue (nXnnGn) leads to the most extreme CCW bias in baseline signaling and poor aspartate taxis in semisold agar. The sequence colored red (nnWnGn) leads to near normal baseline signaling and good aspartate taxis. The sequence colored green (nnWnBn) leads to normal or CW baseline signaling biases but little or no ability to support aspartate taxis or to undergo normal covalent modification (see the text).

sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and immunoblotting with antibodies raised against the V5 epitope that were conjugated to alkaline phosphatase (Invitrogen). Standards were run as a mixture of Tar proteins containing equal proportions of V5-tagged versions of the EEEE, QEQE, and QQQQ forms of Tar_{Ec} produced from RP3098 cells containing pRD300 and induced with arabinose. The Gln residues affect protein migration like methylated Glu residues, such that the standards migrate like the unmethylated, doubly methylated, and quadrupally methylated subunits of the receptor, respectively.

Chemotactic Swim-Plate Assays. Semisolid motility agar contained 3.25 g/L Difco BactoAgar (Difco) in motility medium [10 mM potassium phosphate (pH 7.0), 1 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 1 mM $MgCl_2$, 1 mM glycerol, and 90 mM NaCl] and was supplemented with 20 μg/mL L-threonine, L-histidine, L-methionine, and L-leucine, and 1 μg/mL thiamine. Aspartate and maltose were added to final concentrations of 100 μM. Plates were inoculated with toothpicks from isolated colonies of strain VB13 expressing one of the various Tar receptors from pRD200. Semisolid agar motility plates were incubated at 30 °C. Once chemotaxis rings became visible (typically 8 h for aspartate and 12 h for maltose plates), their diameter was measured every 4 h to determine a ring expansion rate (RER value). Cells expressing different receptors were grouped into categories on the basis of their RER values: 0−25% of the wild-type value, poor; 25−50% of the wild-type value, weak; 50−75% of the wild-type value, moderate; >75% of the wild-type value, strong.

RESULTS

Generation of Tar_{Ec} Variants with Altered TM2 C-Termini. The replacement of Trp-Tyr (VAWYGI wild-type receptor) at positions 209 and 210 with Ala-Ala (VAAAGI receptor) in Tar_{Ec} results in a CCW-locked signal output when the mutant receptor is expressed in ΔT Δ*cheRB* strain

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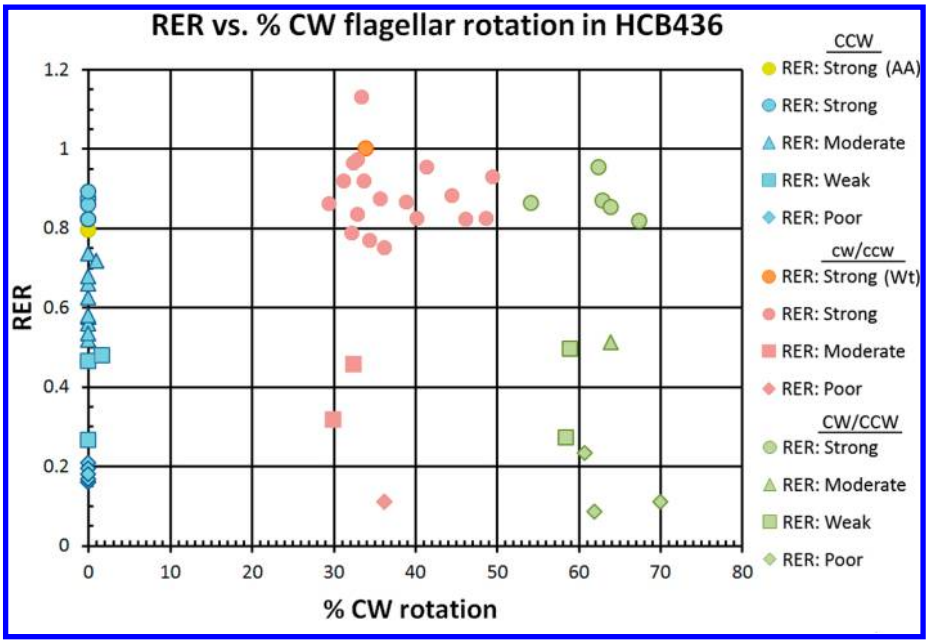

Figure 2. RER values vs percent CW flagellar rotation of HCB436 (Δ*cheRB* cells) cells expressing Tar_{Ec} or its mutant variants. RER values were determined for VB13 (ΔT $cheR^+B^+$) cells expressing the V5-tagged wild-type or mutant Tar_{Ec} variants expressed from pRD200.[24] The RER value for each mutant was normalized to the wild-type RER value for each individual assay. Flagellar rotation was analyzed from tethered HCB436 (ΔT Δ*cheRB*) cells expressing Tar_{Ec} variants from pRD200.[24] Cells were tethered, observed for 20 s, and assigned to one of five categories based on their rotational bias. The percent CW flagellar rotation was calculated as described by Ames et al.[37] The color code for the symbols is the same as in Figure 1: (circles) RER values of ≥75% (strong aspartate taxis), (triangles) RER values of ≥50% (moderate aspartate taxis), (squares) RER values of ≥25% (weak aspartate taxis), and (diamonds) RER values of ≤25% (poor aspartate taxis).

HCB436,[26] which lacks all four MCPs. However, the plasmid-expressed VAAAGI receptor can support a chemotaxis ring expansion rate (RER) of ΔT $cheR^+B^+$ strain VB13 in semisolid aspartate minimal agar at ∼80% of the wild-type RER value (strong aspartate taxis) obtained with cells expressing the wild-type receptor (Figure 1). The VAAAGI receptor can support chemotaxis because, in the $cheR^+B^+$ strain, adaptive methylation restores enough CW signal output to allow the run−tumble behavior required to conduct a biased random walk.[26]

Because an Ala-Ala tandem has minimal positioning character with respect to the membrane,[38] we considered that the Tar_{Ec} VAAAGI receptor provides the default behavior observed in the absence of a cytoplasmic aromatic anchor in TM2. The properties of the Tar_{Ec} VAAAGI receptor thus serve as a standard against which the properties of other mutant Tar_{Ec} receptors can be evaluated.

Our strategy was to create a library of mutants containing different combinations of single and tandem-double aromatic residues at positions 207−212 of Tar_{Ec}. Including the wild-type Tar_{Ec} receptor, 59 receptor variants were analyzed. A majority of the receptors were constructed for this study, but some previously published mutants were analyzed as well and are designated as such with asterisks in Figure 1. Each of the receptors is identified by its sequence at residues 207−212. All of the Tar_{Ec} constructs carried a V5 epitope tag at their C-termini, but this modification has little effect on the output of the receptor.[26]

Plasmids expressing Tar_{Ec} variants were expressed in VB13 and HCB436 cells. The receptors were modestly overexpressed, and all of them were present at approximately the same concentration. In aspartate semisolid agar, the RER (ring expansion rate) value of a colony of strain VB13 expressing plasmid-encoded, V5-tagged wild-type Tar_{Ec} was similar to that of wild-type strain RP437.[26] We thus set the RER value of strain VB13 expressing wild-type (WY) Tar_{Ec} at 100% and expressed the RER values for all of the other receptors as a percentage of the wild-type RER value. To simplify the presentation, the RER values were used to classify the chemotaxis performance into four categories: strong, moderate, weak, and poor (Figure 1).

The data obtained with the different receptors in different assays are summarized in Figures 2−4. Histograms showing the actual distribution of rotational biases for each receptor are given in Figures S1−S3 of the Supporting Information. The combination of RER values and the percentage CW rotation of the flagellar motors of tethered cells allow mutants with similar receptor outputs to be grouped on the basis of these parameters. Four distinct clusters of mutants were identified. In general, mutants within each cluster had a similar residue composition (Figure 1).

Functional Biases of Receptor Variants in HCB436 Cells (Δ*cheRB*). Because strain HCB436 lacks both methyltransferase and methylesterase/deamidase activities, Tar_{Ec} produced in this strain remains in the QEQE modification state in which it was originally translated. This receptor behaves much like a half-methylated E^mEE^mE receptor because the negative charges of two glutamyl residues are neutralized in each case.

Twenty-six of the 59 receptors (highlighted in blue, except for the VAAAGI receptor highlighted in yellow, in Figure 1) were essentially CCW-locked (≤2% CW rotation, defined as CCW) when tethered HCB436 cells were observed (Figure 2). All of the 33 remaining receptors supported CW rotation. A majority of these were in the wild-type (cw/ccw) category (highlighted in light red in Figure 1). The wild-type receptor (highlighted in orange) supported 34% CW rotation. Thus,

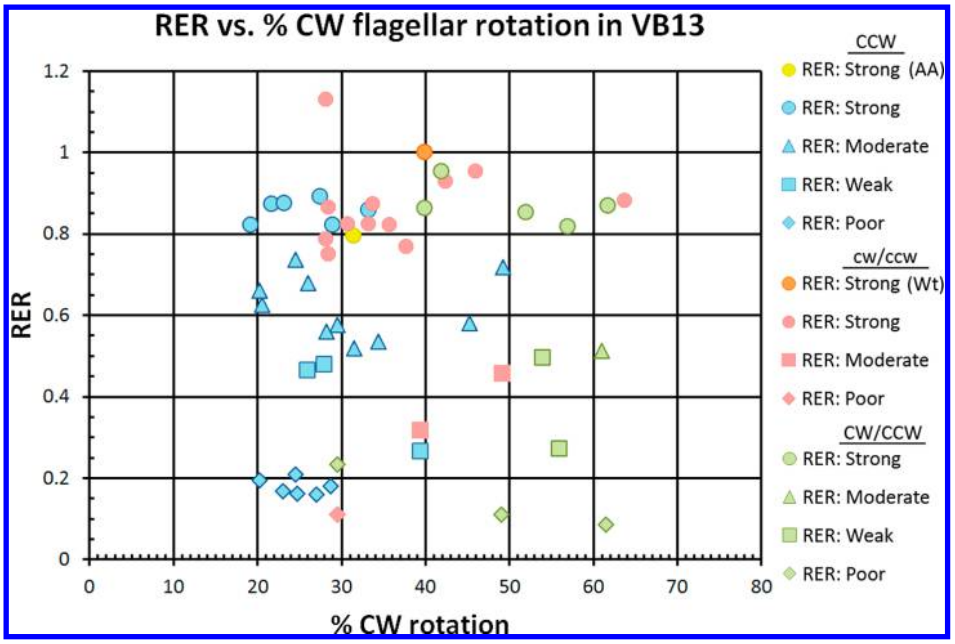

Figure 3. RER values vs percent CW flagellar rotation of VB13 (*cheR*$^+$*B*$^+$) cells expressing wild-type or mutant variants of Tar_{Ec}. RER data were collected from VB13 (ΔT *cheR*$^+$*B*$^+$) cells expressing wild-type or mutant Tar_{Ec} variants from pRD200.[24] The RER value for each mutant was normalized to the wild-type average for each individual assay. Flagellar rotation was analyzed from tethered VB13 (ΔT *cheR*$^+$*B*$^+$) cells expressing Tar_{Ec} variants from pRD200.[24] Data are presented as in Figure 2.

there was a large gap between the signal output of "CCW" receptors and the receptors that could support CW rotation. Eleven receptors (highlighted in green in Figure 1) were designated as CW/CCW because they supported >50% CW rotation.

Functional Biases of Receptor Variants in VB13 Cells (*cheR*$^+$*B*$^+$). In strain VB13, the inherent signaling bias of mutant Tar_{Ec} receptors can be offset by increasing or decreasing the level of covalent modification through the combined activities of CheR and CheB. The effectiveness of this mechanism is evidenced by the fact that all 53 receptors tested in strain VB13 supported ≥20% CW rotation (Figure 3). (The rotational biases supported by six receptors, VAWEGI, VAWIGI, VAWLGI, VAWRGI, VAWSGI, and VAWVGI,- were not determined in the *cheR*$^+$*B*$^+$ strain, but they supported wild-type rotational biases in the Δ*cheRB* strain.) The wild-type receptor expressed in this strain supported 40% CW rotation, a slightly higher value than in strain HCB436. The highest level of CW rotation observed was 64%, and the lowest was 19%. Thus, the range of CW rotation in cells with a functional adaptation system was narrower than the range in cells lacking the adaptation machinery.

All receptors that produced CCW-locked behavior in strain HCB436 supported 20−50% percent CW rotation when adaptive methylation could occur. In contrast, there was only a 10% decrease in the CW signal output of the receptors that were most CW-biased in strain HCB436 when they were expressed in strain VB13. The distribution of CW percentages decreased over the ensemble of receptors from 0−68% in strain HCB436 to 19−64% in strain VB13 (Figures 2 and 3).

Ability of the Mutant Receptors To Support Aspartate Chemotaxis. The chemotaxis ring formed by strain VB13 expressing wild-type Tar_{Ec} expands in minimal aspartate semisolid agar at a rate of 1.7−2.1 mm/h at 30 °C (Figures S4−S6 of the Supporting Information). The variation is presumably caused by slight differences in agar concentration or incubation temperature. Thus, for each set of measurements, the RER value was set relative to an RER of 100% for cells expressing wild-type Tar in the same experiment. The colony formed by the negative control, VB13 cells containing the empty vector, expanded at a rate of only 0.1 mm/h (~5% of the wild-type rate) under these conditions.

A number of the receptors supported aspartate taxis nearly as well or, in one case, better than the wild type. The highest RER value (113%) was seen with the WF receptor.[26] Overall, 31 of the 59 receptors supported strong aspartate taxis, and 42 of 59 (71%) supported at least moderate aspartate taxis. Of the 26 CCW receptors, seven (27%) supported strong aspartate taxis and 17 (65%) supported at least moderate aspartate taxis. These data demonstrate the effectiveness of adaptive methylation. They may also testify to the forgiving nature of the semisolid agar assay, in which the chemotaxis ring is localized within a region in which there is a sharp concentration gradient of aspartate at the edge of the expanding colony.

At the other end of the distribution, 17 of 59 receptors (29%) supported at best weak aspartate taxis, and 10 (17%) supported poor aspartate taxis. Of the 17, nine had CCW-locked signal output, five supported cw/ccw rotation, and three were CCW-biased (CW/CCW). Of the bottom 10, six had CCW-locked output, three supported cw/ccw rotation, and one had CW-biased (CW/CCW) signal output.

A striking feature of these data is that there is little correlation between the baseline signal output in the Δ*cheRB* strain and the ability to support aspartate chemotaxis in the *cheR*$^+$*B*$^+$ strain (Figure 2). The percentage CW rotation seen in the *cheR*$^+$*B*$^+$ strain also shows no obvious correlation with the ability to support aspartate chemotaxis (Figure 3). The overall conclusion is that baseline CW signal output, a measure of the baseline intracellular CheY-P concentration, is a poor predictor of the ability to support aspartate chemotaxis.

Adaptive Methylation Determines Whether a Receptor Can Support Aspartate Chemotaxis. If receptor

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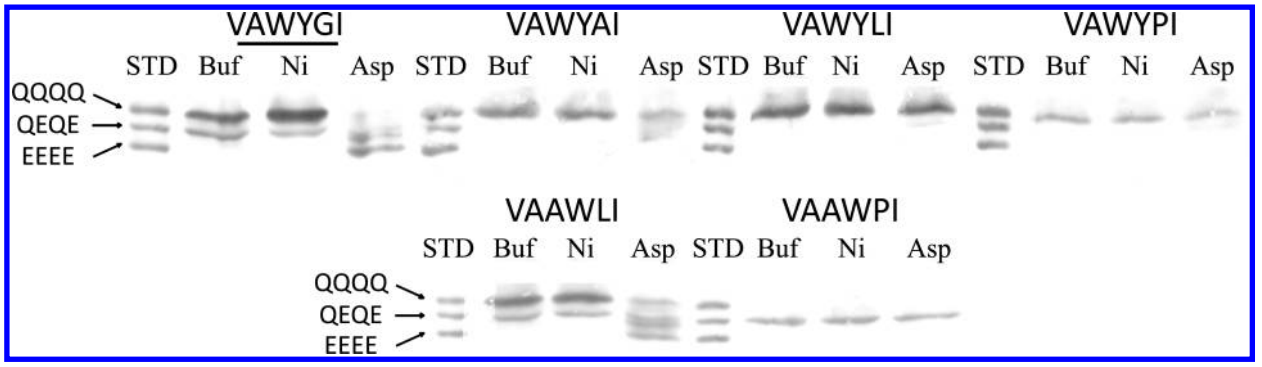

Figure 4. Steady-state patterns of covalent modification of Tar_{Ec} receptor variants in VB13 ($cheR^+B^+$) cells. Total protein from cells expressing one of the Tar_{Ec} variants from pRD200 was analyzed. Cells were exposed to buffer only, 10 mM $NiSO_4$, or 100 mM aspartate (Asp). A mixture of equal amounts of protein representing the unmodified (EEEE), partly modified (QEQE), and fully modified (QQQQ) forms of Tar_{Ec} (STD) was run as a standard for each receptor variant. The rate of migration during SDS–PAGE is related to the extent of modification, with the more highly modified forms migrating faster. The wild type is underlined.

baseline signal output is not a good measure of receptor function in chemotaxis, what is? Presumably, it is the ability of a receptor to communicate ligand-induced conformational changes across the membrane. Although transmembrane signaling can, in principle, be assayed directly with in vitro receptor-linked assays of CheA-kinase activity,[24] it is more conveniently assessed by determining whether a receptor is capable of changing its level of adaptive methylation in response to chemoeffectors.

The VAAWPI receptor, which produced essentially wild-type levels of CW rotation in both Δ*cheRB* and $cheR^+B^+$ cells (Figures 2 and 3), and the VAWYLI and VAWYPI mutant proteins, which had CW-biased signal outputs, supported only poor aspartate taxis. All three of these receptors migrate as a single band when produced in $cheR^+B^+$ cells, both in the absence of chemoeffectors and after the addition of 100 mM aspartate or 10 mM $NiSO_4$ (Figure 4). The VAAWPI receptor migrated at the position of the QEQE modification state of wild-type Tar_{Ec} (Figure S7 of the Supporting Information). The VAWYLI and VAWYPI receptors migrated at the position of the EEEE modification state of Tar_{Ec} in the presence and absence of ligand stimuli (Figure 4). The VAWYAI receptor, which supported weak to moderate aspartate taxis, showed a pattern similar to that of the VAWYLI and VAWYPI receptors, except that it increased its level of covalent modification slightly in response to aspartate (Figure 4). When expressed in HCB436 cells, all four of these receptors migrated at the position of QEQE wild-type Tar_{Ec} (Figure S7 of the Supporting Information), indicating that the slower migration of the VAWYI, VAWYLI, and VAWYPI receptors when expressed in VB13 cells is caused by CheB-mediated deamidation and/or demethylation rather than an intrinsic change in mobility during SDS–PAGE.

The VALVWW receptor, which supported CW/CCW rotation in HCB436 cells, produced cw/ccw rotation in strain VB13. This receptor was undermethylated in its baseline state in strain VB13 and showed a relatively small increase in its level of methylation after the addition of aspartate (Figure S8 of the Supporting Information). The AWVAGI, VWAAGI, WWVAGI, WYVAGI, and YYVAGI receptors, all of which have CCW-locked signal output in strain HCB436, were heavily overmethylated in their baseline state in strain VB13 (Figures S8 and S9 of the Supporting Information), which explains their ability to support CW rotation in that strain. However, their high baseline level of methylation allows them only slight increases in the level of methylation after the addition of aspartate. Thus, they have a limited dynamic range in their attractant responses and show impaired performance in aspartate taxis.

DISCUSSION

The dynamic bundle model of signaling by the HAMP domain[39,40] proposes that the stability of the HAMP domain is regulated by its attachment to TM2 via the control cable.[17,40] Previously published experimental data[23−25] and recent computer simulations[41,42] suggest that the aromatic anchor at the cytoplasmic end of TM2 is essential for the maintenance of normal signal output from Tar_{Ec}. The work presented here builds upon previous results that demonstrate the importance of the residue composition and position of the aromatic anchor of Tar_{Ec}.[26]

We examined, in aggregate, 59 receptors with different amino acid sequences at residues 207–212 of Tar_{Ec}. Some of these receptors have been described previously;[24−26] the remainder were constructed for this study. To make sense of the large amounts of data that were generated, it is useful to consider the different phenotypes that were observed and then identify some generalizations about the properties of the TM2–HAMP connection.

CCW-Locked Receptors. Twenty-six of the mutant receptors had a CCW-locked signal output in strain HCB436 cells [Δ*cheRB* (Figures 1 and 2 and Figures S1–S3 of the Supporting Information)]. All of these receptors were overmethylated in VB13 cells ($cheR^+B^+$) relative to the wild-type receptor, although the extent of overmethylation varied (Figure 4 and Figures S8 and S9 of the Supporting Information). This increased level of methylation allowed all of the mutant receptors to produce a significant CW signal output in strain VB13 (Figure 3 and Figures S10–S12 of the Supporting Information), but the different receptors varied enormously in their ability to support aspartate taxis. The five CCW-locked receptors supporting poor aspartate taxis all had either Trp or Tyr, the two amphipathic aromatic amino acids, at residue 208 (Figure 1). The residue at position 207 seemed to have little effect, as cells expressing the WAVAGI receptor supported good aspartate taxis. The VAAAGI receptor, which has no aromatic residue in the range of positions 207–212, also supported good aspartate taxis.

It is striking that, when Gly is present at position 211, having any residue other than Trp at position 209 leads to a CCW-

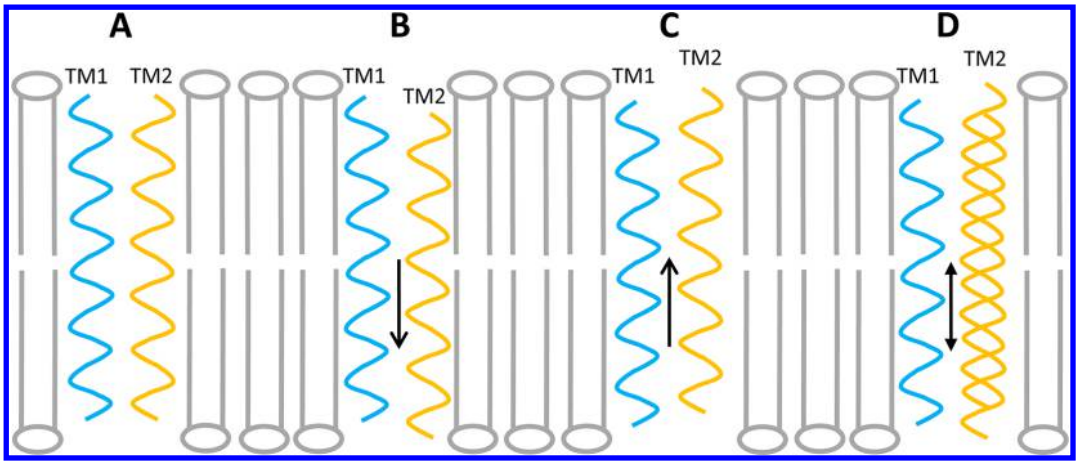

Figure 5. Model for the different roles of TM2 in transmembrane signaling. (A) In the simplest case, a static TM2 holds the cytoplasmic HAMP domain (C-terminal to TM2) in a particular position with respect to the membrane. (B) In functioning as an on-to-off binary switch, TM2 propagates in inward pistonlike movement to the HAMP domain. (C) In functioning as an off-to-on binary switch, TM2 propagates an outward pistonlike movement to the HAMP domain. (D) In a high-abundance *E. coli* chemoreceptor, TM2 has to communicate signals induced by binding of an attractant and a repellent to the periplasmic domain through the membrane to modulate the conformation of a dynamic HAMP domain. It also has to communicate signals from the cytoplasmic adaptation domain through the membrane to control the binding affinity of the periplasmic domain for its chemoeffector ligands in response to the methylation level. We propose that the ability to support this multifaceted response requires particular sequences in the aromatic anchor, one of which is the nnWnGn consensus over residues 207−212 colored red in Figure 1B.

locked signal output in Δ*cheRB* cells (Figure 1). An intact adaptation system in strain VB13 can restore CW signaling capacity to all of these receptors (Figure 3 and Figures S1−S3 and S10−S12 of the Supporting Information) and reasonably good aspartate taxis with many of them (Figures S4−S6 of the Supporting Information). The only CCW-locked receptor without Gly-211 is VALVAW, which has Ala at position 211 and Trp at position 212. Trp-212 may be displaced so far that it no longer interacts with the hydrophobic−hydrophilic membrane interface and therefore cannot pull TM2 outward (i.e., into the membrane) to promote CW signaling. Trp-212 must still have some influence in orienting TM2 in an attractant mimicking position, however, because the VALVAF and VALVAY receptors both support normal levels of CW rotation in HCB436 cells.

CW-Biased Receptors. The CW-biased receptors represent a mixed bag, with respect to both the sequence over residues 207−212 and their ability to support aspartate taxis. The VAWYLI and VAWYPI receptors were both poor in supporting aspartate taxis (Figure 1). Both of these receptors shared the property of being in the EEEE modification state in *cheR*$^+$*B*$^+$ cells and were unable to conduct adaptive covalent modification toward either attractant or repellent (Figure 4). As would be expected, because of their inability to change from the EEEE modification state, they also had almost identical baseline signal outputs in the *cheR*$^+$*B*$^+$ and Δ*cheRB* strains (Figures 2 and 3). The VAWYAI receptor was weak to moderate in supporting aspartate taxis, but it was also undermethylated in *cheR*$^+$*B*$^+$ cells and showed only a slight increase in its level of methylation when exposed to aspartate (Figure 4).

The CW-biased VALVWW receptor was also poor in supporting aspartate taxis, but it showed a very different response to the adaptation system. Demethylation and/or deamidation compensated for its intrinsically CW-biased signal output in Δ*cheRB* cells (Figure S7 of the Supporting Information). Thus, it was an inability to increase its level of methylation sufficiently that limited the dynamic range in aspartate taxis. The remaining receptors with CW-biased signal output ranged from weak (the VALVYY receptor) to strong (the VALWYI receptor) in supporting aspartate taxis.

A Receptor That Supports a Normal (cw/ccw) Baseline Output but Poor Aspartate Taxis. Most of the receptors that supported cw/ccw rotation in HCB436 cells also supported strong aspartate taxis. Exceptions were the VALVWA and VALVAY receptors, which supported weak aspartate taxis, and, most notably, the VAAWPI receptor, which was very poor in supporting aspartate taxis (Figures 1−3). The VAAWPI receptor seemed to be stuck in the QEQE state under all conditions. Perhaps some distortion of the TM2−HAMP connection in these receptors transfers to the adaptation domain such that the receptor becomes a poor substrate for both the CheR methyltransferase and the CheB deamidase/methylesterase.

What Determines Whether a TM2−HAMP Connector Sequence Supports Good Aspartate Chemotaxis? Of the 59 variant Tar_{Ec} receptors tested, 33 supported good aspartate taxis (Figures 1−3). Of these, seven produced CCW-locked signal output, five produced CW-biased (CW/CCW) signal output in the Δ*cheRB* strain, and 19 have a wild-type signal output. If we define "wild-type" behavior to be cells with cw/ccw rotation and the ability to support strong aspartate taxis, 18 mutant receptors fall into this category. Of the 18, 10 have Trp as residue 209 and Gly as residue 211. The residue at position 210 seems to make relatively little difference if it is flanked by Trp and Gly (Figure 1). The eight remaining "wild-type" receptors all lack Gly-211, having it replaced with an Ala in the VALVAF, VALWAI, and VAAWAI receptors, with Leu in the VAAWLI receptor, and with an aromatic residue in the VALAFI, VALAWI, and VALAYI receptors.

Importance of Position 211. The importance of Trp-209 was established in previous studies.[24−26] The role of Gly-211 in receptor function in Tar_{Ec} was not anticipated. A recent study[17] demonstrated that the equivalent Gly-213 residue in *E. coli* Tsr (Tsr_{Ec}) could be replaced with any amino acid other than Tyr without having a significant effect on serine chemotaxis. Even the Tyr replacement still allowed some level of serine taxis. Here, we found that the replacement of Gly-211 with Ala and

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to a greater extent with Leu and Pro in the VAWYAI, VAWYLI, and VAWYPI receptors, respectively, seriously disrupted aspartate taxis and led to a CW signaling bias. The Tsr sequence over the equivalent regions is AVWFGI. Whether the difference in the apparent importance of the Gly residue is a consequence of the AVWF sequence preceding the Gly residue in Tsr_{Ec} as opposed to the VAWY sequence in Tar_{Ec} is unclear. It should also be noted that the sequence at roughly the equivalent position at the end of TM2 is ALWWTR in the dipeptide receptor Tap_{Ec} and ITFMVL in the ribose/galactose receptor Trg_{Ec}. Thus, even among *E. coli* MCPs, the Gly residue is not crucial in every sequence context.

Some clue about the function of Gly-211 may come from the observation that all of the chemoreceptors in *Salmonella enterica* that contain the $NWE^T/_SF$ CheR-binding pentapeptide[43] at their extreme C-termini (Tar_{Se}, Tsr_{Se}, and Tcp_{Se}) also have the conserved Gly residue. The other three MCPs of *S. enterica*, Trg_{Se}, $McpB_{Se}$, and $McpC_{Se}$,[44] do not have this Gly residue, nor do the Aer receptors of either *E. coli* or *S. enterica*. The driving evolutionary selection may be the requirement to generate graded signals in response to ligands with different free energies of binding: aspartate and ligand-bound maltose-binding protein for Tar,[8] serine and AI-2-bound LsrB protein[45] in Tsr, and citrate and phenol for Tcp.[46] Alternatively, it may be the requirement for feedback signaling to the periplasmic domain in response to the methylation state of the $NWE^T/_SF$ (majority) receptors that represents the crucial difference.

One proposed role of conserved Gly residues in chemoreceptors has been to serve as a conformational hinge between the adaptation and signal output domains in the cytoplasmic four-helix bundle of the receptor dimer.[47] The conserved Gly residue in the control cable may serve a similar function in connecting TM2 to the HAMP domain.

Implications for Transmembrane Signaling by Membrane-Spanning Receptors of Two-Component Systems. The functions of TM2 in different transmembrane sensors vary a great deal, as summarized in Figure 5. In the simplest case, there is no transmembrane signal. In Aer, for example, there is no significant periplasmic domain, and redox sensing is accomplished by the direct interaction between the FAD-containing PAS domain and the HAMP domain.[48] In such proteins, the role of TM2 may be simply to hold the HAMP domain in a conformation that is receptive to direct physical input. In other sensor kinases, ligand binding activates a binary switch, with the ligand-free receptor being in the kinase-on or kinase-off state and changing its signal output in response to ligand binding. Here, the role of TM2 is 2-fold. It must hold the HAMP domain in the proper conformation to execute its baseline signal output, and it must transmit a conformational change across the membrane in response to ligand binding. That this change is analogous to what occurs in chemoreceptors is shown by the ability of the NarX-Tar chimeric protein Nart to mediate chemotaxis to nitrate and nitrite.[49,50] It seems unlikely that a continuous helical connection between TM2 and the HAMP domain is required based on the observation that replacing the four residues preceding the conserved Pro of the HAMP domain with four Gly residues, typically helix breakers, does not seriously disrupt Tar function.[51]

MCPs, with their bidirectional flow of information across the membrane through shifts in the position of TM2 with respect to the phospholipid bilayer, represent a special case. Ligand occupancy affects both kinase-stimulating activity and the respective rates of methylation and/or demethylation of the adaptation domain, and the modification state of the adaptation domain affects the ligand affinity of the periplasmic domain.[52] The TM2 regions that must conduct such manifold signaling tasks, especially in the high-abundance, $NWE^T/_SF$-containing MCPs, may need to be finely tuned to communicate subtle conformational changes. The lower-abundance Tap and Trg chemoreceptors lack the Gly residue, but it is not known whether covalent modification alters their affinity for their binding protein ligands. The fact that only the high-abundance chemoreceptors among *E. coli* transmembrane two-component sensor kinases have Gly at the end of TM2[24] suggests that the conserved Gly residue represents an evolutionary response to the multiple constraints of two-way signaling across the cell membrane.

■ ASSOCIATED CONTENT

Supporting Information

Additional results and controls relating to swarm plates, the methylation state of receptors, and the bias of cells containing wild-type and/or mutant receptors. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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