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Cross-Linking Evidence for Motional Constraints within Chemoreceptor Trimers of Dimers[†]

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ABSTRACT: Chemotactic behavior in bacteria relies on the sensing ability of large chemoreceptor clusters that are usually located at the cell pole. In *Escherichia coli*, chemoreceptors exhibit higher-order interactions within those clusters based on a trimer-of-dimers organization. This architecture is conserved in a variety of other bacteria and archaea, implying that receptors in many microorganisms form trimer-of-dimer signaling teams. To gain further insight into the assembly and dynamic behavior of receptor trimers of dimers, we used in vivo cross-linking targeted to cysteine residues at various positions that define six different levels along the cytoplasmic signaling domains of the aspartate and serine chemoreceptors, Tar and Tsr, respectively. We found that the cytoplasmic domains of these receptors are close to each other near the trimer contact region at the cytoplasmic tip and lie farther apart as the receptor dimers approach the cytoplasmic membrane. Tar and Tsr reporter sites within the same or closely adjacent levels readily formed mixed cross-links, whereas reporters located different distances from the tip did not. These findings indicate that there are no significant vertical displacements of one dimer with respect to the others within the trimer unit. Attractant stimuli had no discernible effect on the cross-linking efficiency of any of the reporters tested, but a strong osmotic stimulus reproducibly enhanced cross-linking at most of the reporter sites, indicating that individual dimers may move closer together under this condition.

Motile bacteria possess sensory systems that allow them to monitor and track chemical gradients in their environment, a behavior known as chemotaxis. The chemotaxis signal transduction pathway has been very well characterized in *Escherichia coli* (see refs *1* and *2* for recent reviews). A multiprotein complex localized at the cell pole monitors attractant and repellent concentrations and transmits signals to the flagellar motors whenever chemoeffector levels change. Increases in attractant concentrations, for example, trigger signals that promote forward swimming, corresponding to counterclockwise (CCW) rotation of the flagellar motors. Conversely, increases in repellent concentrations promote clockwise (CW) motor rotation, which causes the cell to execute random directional changes, or tumbles.

E. coli contains five homodimeric sensory receptors with different detection specificities that are anchored in the cytoplasmic membrane. Four of the receptor types (Tar, aspartate and maltose; Tsr, serine; Tap, dipeptides and pyrimidines; and Trg, ribose and galactose) contain a periplasmic ligand-binding domain delimited, in each subunit, by a pair of transmembrane segments. The fifth receptor, Aer, lacks a periplasmic domain and has instead a cytoplasmic N-terminal FAD-containing PAS domain that receives redox signals from the electron transport chain. The four canonical receptors, collectively known as methyl-accepting chemotaxis proteins (MCPs),[1] contain a highly conserved cytoplasmic domain that forms a long, four-helix bundle, with membrane-distal hairpin turns in each subunit. Aer, although not methylated, shares this feature. The cytoplasmic tips of the receptors bind the histidine kinase CheA and the coupling protein CheW to form ternary signaling complexes whose phosphorylation activity responds to chemoreceptor control. Chemical stimuli elicit rapid changes in CheA activity to trigger locomotor responses, followed by a return of kinase activity to prestimulus levels. The sensory adaptation process depends on methylation and demethylation of the receptor signaling domains by an MCP-specific methyltransferase (CheR) and methylesterase (CheB).

The transmission of conformational changes between the sensing and signaling domains of chemoreceptor molecules and the mechanisms of kinase control are still poorly understood. A detailed understanding of the architecture of the ternary signaling complex is necessary to explain the extraordinary sensitivity, high gain, integration of signals, and robust adaptation mediated by these relatively simple, yet remarkably efficient, chemoreceptors.

An important clue about the higher-order structure of receptor complexes was provided by a crystal structure of the cytoplasmic domain of *E. coli* Tsr (*3*). In that structure, three dimeric Tsr fragments were arranged in a trimer of dimers, with contacts between dimers near the hairpin tip, the region that binds CheW and CheA (*4*–*6*). Remarkably, the 11 residues promoting interdimer

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[1] Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; MCP, methyl-accepting chemotaxis protein; NMR, nuclear magnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TMEA, tris(2-maleimidoethyl)amine.

contacts in the trimers are highly conserved in all MCPs and identical in the five *E. coli* chemoreceptors, suggesting that receptors of different types might form mixed trimers of dimers in many prokaryotic chemotaxis systems. In vivo cross-linking studies subsequently provided several lines of evidence showing that *E. coli* receptors are indeed organized in trimers of dimers, whose composition depends on the relative abundance of different receptor types in the cell (*7*, *8*). Conformational suppression between receptor molecules with amino acid changes in the trimer contact region also showed that trimer formation and dynamics are important factors for proper in vivo signaling behavior of receptor arrays (*9*). Finally, recent electron cryotomography images of receptor clusters in a variety of bacteria and archaea have revealed hexagonal arrays in which the volume and spacing of vertices are consistent with trimers of dimers (*10*−*12*).

Thus, multiple lines of evidence support the physiological importance of trimers in receptor signaling. However, alternative receptor−receptor geometries have been proposed, albeit for receptors and signaling complexes under non-native in vitro conditions. A cytoplasmic fragment of a *Thermotoga maritima* receptor crystallized in a hedgerow arrangement (*13*). Electron microscopy of a signaling complex assembled with soluble fragments of *E. coli* Tar revealed flattened trimers in a tip-to-tip arrangement (*14*, *15*). Solid state NMR studies of receptor−receptor distances in a similar Tar signaling complex suggested that the receptor dimers might be arranged in a parallel fashion inconsistent with a trimer of dimers (*16*).

In this study, we used a trifunctional thiol cross-linking reagent (TMEA) to test in vivo predictions of the trimer-of-dimers model. In previous work, we employed a cysteine reporter (Tsr-S366C or Tar-S364C) near the receptor's cytoplasmic tip, immediately above the trimer interface region, and found that TMEA trapped ~50% of the receptor subunits in two- and three-subunit cross-linking products, suggesting that TMEA reacts with the axial subunits of receptor dimers organized as trimers (*8*). Here, we examined TMEA cross-linking of cysteine reporters at different positions along the cytoplasmic domain of Tsr and Tar molecules. We also assessed the possibility of movements of individual dimers within the trimer along its central axis by asking whether different reporters, located at different distances from the tip, can mediate inter-receptor cross-linking. Finally, we tested whether the efficiency of cross-linking depends on the signaling state of the receptors. Our results are fully consistent with an in vivo trimer-of-dimers organization for *E. coli* chemoreceptors. The trimers have tight contacts at their tips that preclude significant movements between receptor dimers along the central axis of the trimer. The decreased efficiency of cross-linking between receptor reporter sites that are farther from the tip indicates that the separation between dimers increases with distance.

EXPERIMENTAL PROCEDURES

Bacterial Strains. Strains were derivatives of *E. coli* K12 strain RP437 (*17*) and carried the following genetic markers relevant to this study: UU1581 [*(flhD-flhB)4* Δ(*tsr*)*-7028* Δ(*trg*)*-100*] and UU1613 [*tar-S364C* Δ(*tsr*)*7028* Δ(*trg*)*100* Δ(*tap-cheB*)*-2234* Δ(*cheA-cheW*)*2167 zec::Tn10-980*] (*18*).

Plasmids. The following plasmids were derived from pACYC184 (*19*), which confers chloramphenicol resistance: pRR31 (salicylate-inducible expression vector) (*18*), pCS12 (salicylate-inducible wild-type *tsr*) (*18*), and derivatives of pCS12 encoding Tsr molecules with single-cysteine replacements (ref *18* and this work).

The following plasmids were derived from pBR322 (*20*), which confers ampicillin resistance: pNP1 [isopropyl β-D-thiogalactopyranoside (IPTG)-inducible expression vector] (*21*), pPA818 (IPTG-inducible *tar-6His*) (P. Ames, personal communication), and single-cysteine-containing derivatives (this work). pPA818 encodes a fully functional Tar protein that has a six-histidine tag at its C-terminus.

Site-Directed Mutagenesis. Mutations in *tsr* or *tar-6His* were introduced with the QuikChange site-directed mutagenesis kit (Stratagene), using pCS12 or pPA818, respectively, as the template plasmid. Candidate mutants were verified by sequencing the entire chemoreceptor coding region.

TMEA Cross-Linking Assay. Cells were grown at 30 °C to midlog phase in tryptone broth (1% tryptone and 0.5% NaCl), harvested by centrifugation, and resuspended at an OD_{600} of 2 in 10 mM potassium phosphate (pH 7.0) and 0.1 mM EDTA. Cell suspensions (0.5 mL) were incubated for 5 min at 30 °C and then treated with 50 μM tris(2-maleimidoethyl)amine (TMEA, Pierce) for 20 s at 30 °C. Reactions were quenched by the addition of 10 mM *N*-ethylmaleimide. Cells were pelleted and then lysed by boiling in 50 μL of sample buffer (*22*). Lysate proteins were analyzed by electrophoresis in sodium dodecyl sulfate−polyacrylamide gels as described previously (*8*) and visualized by immunoblotting with an antiserum directed against the highly conserved portion of the Tsr signaling domain (*4*). In some cases, longer gels with a lower percentage of acrylamide (9%) were used to increase the resolution of mixed cross-linking bands. As secondary antibodies, we used either Cy5-labeled (Amersham) or alkaline phosphatase-conjugated (Sigma) goat anti-rabbit immunoglobulin. Cy5-labeled antibodies were detected with a Storm 840 fluorimager (Amersham); alkaline phosphatase-conjugated antibodies were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (both from Sigma) and converted to grayscale images with a digital scanner. All gel images were analyzed with ImageQuant (Amersham).

For analysis of stimulus effects on cross-linking efficiency, cells were washed twice with phosphate buffer before being resuspended at an OD_{600} of 2 in phosphate buffer. Cell suspensions (0.5 mL) were incubated for 5 min at 30 °C, and then an equal volume of glycerol (repellent stimulus) was added to bring the final concentration to 1 M immediately before (~10 s) the addition of TMEA. For attractant stimulation, 10 mM L-serine or L-aspartate was used. After 20 s at 30 °C, the reaction was quenched with 10 mM *N*-ethylmaleimide, and samples were processed as described above.

In Vivo Accessibility of Thiol Groups. Cells expressing the cysteine-substituted receptors at physiological concentrations were grown to midlog phase, harvested by centrifugation, and resuspended at an OD_{600} of 2 in 10 mM potassium phosphate (pH 7.0) and 0.1 mM EDTA. Cell suspensions (0.5 mL) were or were not treated with 5 mM *N*-ethylmaleimide for 15 min at room temperature. Reactions were quenched by the addition of 20 mM dithiothreitol for 15 min at room temperature. Cells were pelleted, washed twice with potassium phosphate buffer to remove excess dithiothreitol, and then lysed by being boiled in 50 μL of sample buffer (*22*). Unblocked thiol groups were then labeled by treatment with 5 mM maleimide-polyethylene glycol (5 kDa) (Boehringer Mannheim) for 15 min at room temperature. Total protein extracts were analyzed by electrophoresis in sodium dodecyl sulfate−polyacrylamide gels as described previously (*8*) and visualized by immunoblotting

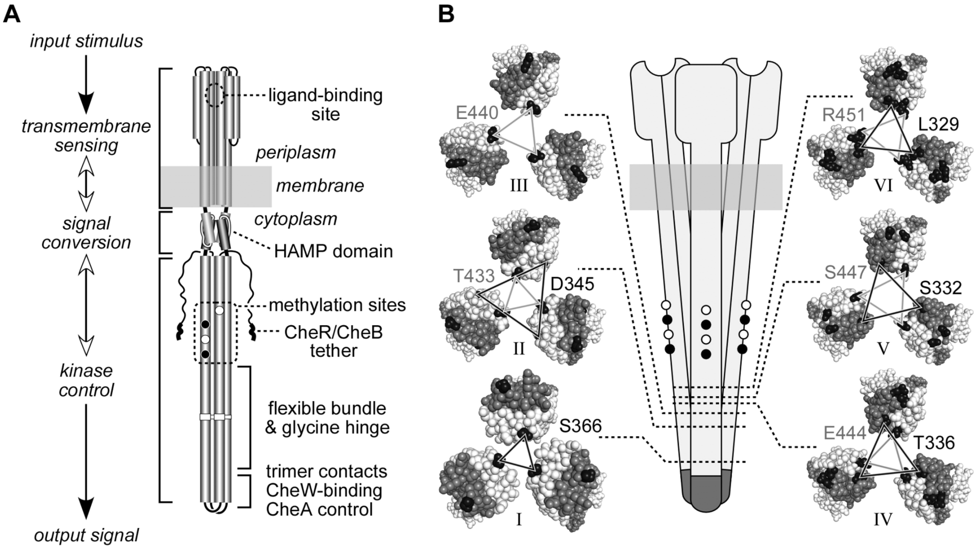

FIGURE 1: Structural features of receptor molecules and trimers of dimers. (A) Functional architecture of Tsr and Tar homodimers. Cylindrical segments represent α-helices. Black circles represent methyl-accepting residues that are translated as glutamines and then deamidated to glutamate by CheB. The CheR-CheB tether represents the C-terminal region to which the methylation enzymes bind. (B) Schematic representation of a trimer of dimers (center) and cross sections corresponding to the six levels, indicated by dotted lines and Roman numbers, defined in Table 1. Cross section images were made with Pymol, viewing the trimer of dimers from the cytoplasm toward the membrane. Residues between the cytoplasmic tip and the corresponding level are hidden. Axial subunits (subunits that face the interior of the trimer at level I) are colored light gray; peripheral subunits are colored dark gray. Representative residues from each level that were substituted with cysteine residues are colored black. Lines connecting the cysteine-substituted residues are colored black when the residues reside in the N-helix of the subunit (helix running from the membrane to the cytoplasmic tip) and gray when the residues reside in the C-helix of the subunit (helix running from the cytoplasmic tip to the C-terminus). Residue identification uses a matching shading scheme and Tsr residue numbers, even though some replacements were made in Tar (see the text and Table 1).

Table 1: Cross-Linking Efficiencies of Reporters Located along the Cytoplasmic Domain[a]

level	distance from the tip (Å)[b]	position[c]	receptor	% three-subunit product[d]	Cα−Cα (Å)[e]	subunit[f]	helix[f]
I	37	S366	Tsr/Tar	100	12.86	axial	N
II	60.5	V430 (T)	Tar	11 ± 7	19.33	axial	C
	63	T433	Tsr	23 ± 5	16.86	axial	C
	67	E436	Tar	14 ± 3	20.65	axial	C
	69	D345	Tar	0	32.09	axial	N
III	74	E440	Tsr	19 ± 4	20.83	axial	C
IV	81	T336	Tsr	1 ± 1	27.27	peripheral	N
	81	E444 (N)	Tar	4 ± 3	22.17	axial	C
V	87	S447 (N)	Tsr	12 ± 3	25.28	axial	C
	87	S332	Tar	5 ± 3	28.21	peripheral	N
VI	92	L329	Tar	1 ± 0.5	26.67	peripheral	N
	93	R451	Tsr	1 ± 1	28.68	axial	C
	96	Q325	Tar	3 ± 1	26.81	peripheral	N

[a]Strain UU1581 cells expressing cysteine-substituted Tsr or Tar receptors at physiological levels were treated with TMEA, and the cross-linking products were analyzed by Western blotting with an anti-Tsr antibody that reacts with both receptors. [b]Distance measured from the α-carbon of the corresponding residue to the α-carbon of G390, located at the cytoplasmic tip. [c]Residue numbering corresponds to that of Tsr. When a cysteine replacement was made in Tar at the equivalent position, the actual residue that was replaced is indicated in parentheses (if different from the corresponding Tsr residue). [d]The fraction of three-subunit cross-linking products was calculated relative to that obtained with the S366C reporter. [e]Distance measured between α-carbons of the corresponding residues in subunits that face the trimer axis. [f]Subunits and helices defined as in Figure 1.

with an antiserum directed against the highly conserved portion of the Tsr signaling domain (*4*). Gel images were analyzed with ImageQuant (Amersham), and the accessibility of thiol groups was calculated as follows: the percentage of total MCP with shifted mobility in the NEM-treated samples was expressed as a fraction of that percentage in the nontreated cells. This fraction represented the residues that were not accessible for blockage by the NEM treatment. Accessibility could then be expressed as 1 − (fraction of inaccessible residues). Accessibility values for residues chosen as controls and calculated from three independent experiments were 0.85 ± 0.08 (S366C, exposed) and 0.35 ± 0.17 (T441C, hidden).

RESULTS

TMEA Cross-Linking Sites along the Cytoplasmic Domains of Tar and Tsr. To assess the native organization of *E. coli* MCP trimers of dimers by cysteine-directed TMEA cross-linking, we constructed single-cysteine replacements at various surface residues along the cytoplasmic domain of the Tar and Tsr chemoreceptors. The locations of representative reporter sites are shown in Figure 1B; the complete list of cysteine replacements is given in Table 1. For the sake of simplicity, all residue positions are designated with Tsr numbering (Tar number = Tsr number − 2), using italic designations for replacements made in Tar.

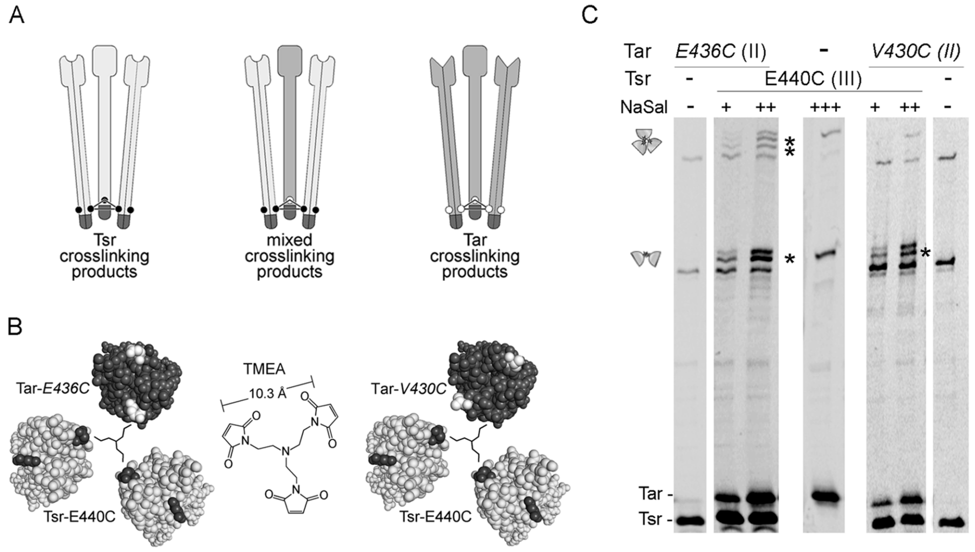

FIGURE 2: Inter-receptor cross-linking tests with proximal reporter sites. (A) Schematic representation of a cross-linking experiment in which two different receptors bear cysteine reporters at the same or closely adjacent levels. Individual dimers represent Tsr (light gray) or Tar (dark gray) molecules; small circles represent cysteine replacements. The three dimers form trimers through interactions between their tips (dark region). Upon TMEA treatment, cells expressing both receptor types can form pure three-subunit cross-linking products (Tsr-only, left; Tar-only, right) and may also form mixed cross-linking products (center), if the reporter sites are sufficiently close to one another. (B) Cross sections of mixed trimers bearing reporters at adjacent levels: left, Tar *436C* (level II) and Tsr 440C (level III); right, Tar *430C* (level II) and Tsr 440C (level III). Cross section images were made with Pymol, viewing the trimer of dimers from the cytoplasm toward the membrane. Residues between the cytoplasmic tip and the corresponding level are hidden. Tsr subunits are colored light gray, with the cysteine reporters colored black. Tar subunits are colored dark gray, with the cysteine reporters colored white. A schematic representation of the TMEA cross-linking reagent (center) is shown to scale in the center of each trimer. (C) UU1581 cells carrying pCS12 Tsr-E440C and pPA818 Tar-6His-*E436C* or pPA818 Tar-6His-*V430C* were grown to midlog phase and treated with TMEA. Tar expression was induced at 10 μM IPTG and Tsr expression at 0.15 (+), 0.30 (++), or 0.60 μM sodium salicylate (+++). Cells expressing each receptor individually were also included to show the mobility of the pure cross-linking products. Samples were analyzed by SDS−PAGE in long, 9% acrylamide gels and visualized by immunoblotting with an anti-Tsr antibody that reacts with both receptors. Cartoons on the left indicate the region of the gel in which two- and three-subunit cross-linking products are found. Mixed cross-linking products are denoted with asterisks.

Near the receptor's cytoplasmic tip one subunit of each dimer, which we designate the axial subunit, resides at the trimer interface; the other, peripheral subunit lies at the outside of the trimer (see cross sections for levels I and II in Figure 1B). Because of supercoiling of the four-helix cytoplasmic bundle, both axial and peripheral subunits face the trimer axis at greater distances from the tip (see cross sections for levels III to VI in Figure 1B). We created cysteine replacements at residues that face the central axis of the trimer in the Tsr crystal structure (*3*), either in the axial or in the peripheral subunit, depending on their distance from the cytoplasmic tip. We also included one residue, *D345*, whose off-axis location near the trimer interface should preclude efficient cross-linking with its counterparts in the other dimers, unless the reporter sites are able to rotate into more proximal positions within the trimer. Reporter residues were located in both the N-helix, running from the HAMP domain to the tip (residues connected by dark gray lines in Figure 1B), and the C-helix, running from the tip to the receptor's C-terminus (residues connected by light gray lines in Figure 1B). Altogether, the chosen reporter sites defined six levels, based on cross section planes through the helix bundle (Figure 1B). Reporters at each level exhibited similar distances from the cytoplasmic tip, measured from the α-carbon of the reporter residue to the α-carbon of residue G390 at the hairpin turn (Table 1).

All cysteine-carrying receptors analyzed in this study supported full chemotaxis, as assessed in soft agar assays (data not shown). Thus, the overall structure of the marked receptors, including their trimer organization, should closely resemble that of their wild-type counterparts.

TMEA Cross-Linking Efficiency of Cysteine Reporters from Level I to VI. The TMEA reagent has three thiol-reactive maleimide groups spaced 10.3 Å apart in a trigonal symmetry (see Figure 2B). TMEA readily permeates into cells and mediates efficient cross-linking of chemoreceptors bearing a cysteine reporter at position 366, generating roughly equal amounts of two- and three-subunit products that together represent ~50% of the receptor subunits (*8*, *18*, *23*) (see Figure 4). These properties are consistent with the premise that TMEA traps the axial subunits of receptor dimers organized in trimers of dimers.

On the basis of the Tsr crystal structure, trimers of dimers should pack closely at the receptor tips but then splay apart as the individual dimers approach the cytoplasmic membrane (see Figure 1). To test this prediction, we measured TMEA cross-linking efficiencies between cysteine reporters at different distances from the receptor tip. We expressed the marked receptors at physiological levels in cells lacking other receptors and chemotaxis proteins. Previous studies have shown that the CheA and CheW components of ternary signaling complexes have no influence on the pattern or extent of TMEA-mediated receptor cross-linking under these conditions (*18*). Following TMEA treatment, cells were lysed and whole cell extracts were subjected to gel electrophoresis and immunoblotting using an antibody that recognizes the cytoplasmic domains of Tsr and Tar. To compare the cross-linking efficiencies of different reporter sites, we used receptors with the 366C reporter as internal controls in every experiment. In this way, the amount of three-subunit cross-linking products obtained with any particular reporter could be expressed relative to the level of cross-linking products obtained with the 366C reporter.

To test the possibility that variations in cross-linking efficiencies merely reflect differences in the accessibility of the incorporated thiol groups, we measured the in vivo accessibility of every reporter site as described in Experimental Procedures. Our assay was based on NEM-mediated blockage of thiol groups in intact cells, followed by denaturation and labeling of any unblocked groups by maleimide-polyethylene glycol treatment. All the reporters analyzed in this work showed an accessibility of >0.7 in this assay (data not shown). Accordingly, we conclude that differences in cross-linking efficiency mainly reflect differences in reporter site geometry rather than intrinsic reactivity.

We found that the percentage of three-subunit cross-linking products declined with the distance between the reporter sites and the cytoplasmic tip (Table 1). Three cysteine residues in level II yielded high levels of three-subunit cross-linking products, ~10−25% of those obtained with the 366C control site in level I. At the opposite extreme, cysteine residues at level VI yielded at most 3% cross-linking products. Cysteine reporters at intermediate levels yielded cross-linking values between those of the level II and level VI reporters. Although the cross-linking behavior of individual reporter sites depends to some extent on their relative orientations, the consistent decline in cross-linking yield with the distance between the reporter and the tip suggests that interdimer distances within the trimer increase with distance from the tip. These results indicate that the in vivo geometry of receptor trimers is fully consistent with that observed in the crystal structure of the Tsr cytoplasmic domain.

The absence of cross-linking products from the *D345C* reporter [level II (see Table 1)] is consistent with the position of this residue in the crystal structure and suggests that individual dimers may not undergo rotational motions that bring the reporter sites within cross-linking distance. To ask whether attractant stimuli might induce such rotation, we coexpressed Tar-*D345C* and Tsr-T433C receptors (both reporters reside at level II) and stimulated the cells with aspartate or serine immediately before TMEA addition. We did not observe three-subunit products containing Tar-*D345C* subunits (data not shown), which indicates that attractant stimulation does not cause significant rotation of dimers within the trimer.

Effect of Reporter Geometry on Three-Subunit Cross-Linking. In a cell containing Tsr and Tar, two general types of trimers can form: pure trimers, composed exclusively of Tar or Tsr dimers, and mixed trimers containing both types of dimers (Figure 2A). If both receptors carry a cysteine reporter at the same or closely adjacent level, TMEA should generate both pure and mixed two- and three-subunit cross-linking products (*8*) (Figure 2A). Mixed cross-linking products can be detected in low-bisacrylamide gels, in which Tar and Tsr subunits have different mobilities (see below).

TMEA treatment of cells expressing Tar E436C (level II) and Tsr E440C (level III) generated both two- and three-subunit mixed cross-linking products (Figure 2C and Table 2). This result conforms to the trimer arrangement in the Tsr crystal structure, because both the distances between reporters (Table 2) and their geometry (Figure 2B, left panel) are consistent with TMEA-mediated cross-linking.

In contrast, TMEA treatment of cells expressing Tar-V430C (level II) and Tsr-E440C (level III) failed to generate mixed three-subunit cross-linking products (Figure 2C and Table 2). Again, this result is consistent with the arrangement of reporters predicted by the Tsr crystal structure. In a mixed trimer of dimers, the three reporter sites would not have an equilateral arrangement and should not be trapped by the same TMEA molecule (Figure 2B, right panel). However, the calculated distances are compatible with the formation of a two-subunit mixed product (Table 2), and this is what we observed (Figure 2C). This and the previous result with the off-axis *D345C* reporter argue against any substantial rotation of one dimer with respect to the others in the same trimer.

Table 2: Cross-Linking between Cysteine Reporters Located at Close or Distant Levels in Different Receptors^a

position in Tar	position in Tsr	distances^b	Tar−Tsr mixed cross-linking products^c	
			two subunits	three subunits
436 (II)	440 (III)	20.97, 22.73	+	+
430 (II)	440 (III)	19.41, 26.72	+	−
366 (I)	433 (II)	30.61, 31.50	−	−
430 (II)	366 (I)	27.08, 29.82	−	−
436 (II)	447 (V)	27.62, 30.75	−	−

^{a}UU1581 cells expressing cysteine-substituted Tsr and Tar receptors at physiological levels were treated with TMEA, and the cross-linking products were analyzed by immunoblotting with an anti-Tsr antibody that reacts with both receptors. ^{b}Distances between the α-carbon from the corresponding residue in Tar and each of the α-carbon residues in two differently substituted Tsr dimers in a mixed trimer. ^{c}The actual results of the corresponding experiments are shown in Figures 2 and 3, and the presence of mixed cross-linking products is summarized here.

Assessment of Motions between Dimers through Interlevel Cross-Linking. The availability of cysteine reporters at different distances from the receptor tip allowed us to test for vertical motions between dimers within a trimer. We reasoned that two different reporter sites farther from the receptor tip than the previously characterized position 366 should generate two- and three-subunit cross-linking products, provided that both reporters are at the same level. However, reporter sites at different levels, e.g., Tsr molecules with a reporter at level I and Tar molecules with a reporter at level II, should give rise to mixed two- or three-subunit cross-linking products only if dimers undergo displacements relative to one another along the central axis of the trimer (Figure 3A).

Accordingly, we assessed interlevel cross-linking between reporters at levels I and II, whose distances from the tip differ by ~30 Å (Table 1). We expressed Tsr-S366C (level I) or Tsr-T433C (level II) in cells that contained chromosomally encoded levels of Tar-*S366C*, treated with TMEA, and analyzed the cross-linking products by immunoblotting (Figure 3B). As expected, mixed cross-linking products were detected when both receptors carried the 366C reporter (Figure 3B, left). However, no mixed products were seen when the cysteine reporter in Tsr resided at level II (Figure 3B, right). It is important to note that Tsr-T433C is incorporated into mixed trimers of dimers, as evidenced by the decrease in the level of pure Tar cross-linking products as the level of Tsr expression increases and by the fact that pure Tsr three-subunit products are evident only at the highest level of Tsr expression. Thus, the absence of mixed cross-linking products indicates that even though the marked receptors can form mixed trimers, TMEA cannot trap receptor subunits that have reporter residues at different levels. We conclude that, within an assembled trimer, there are no movements of dimers relative to one another that are sufficiently large to allow cross-linking across reporter levels.

In a reciprocal experiment, we expressed Tsr-S366C (level I) or Tsr-T433C (level II) in cells expressing Tar-*V430C* (level II) from

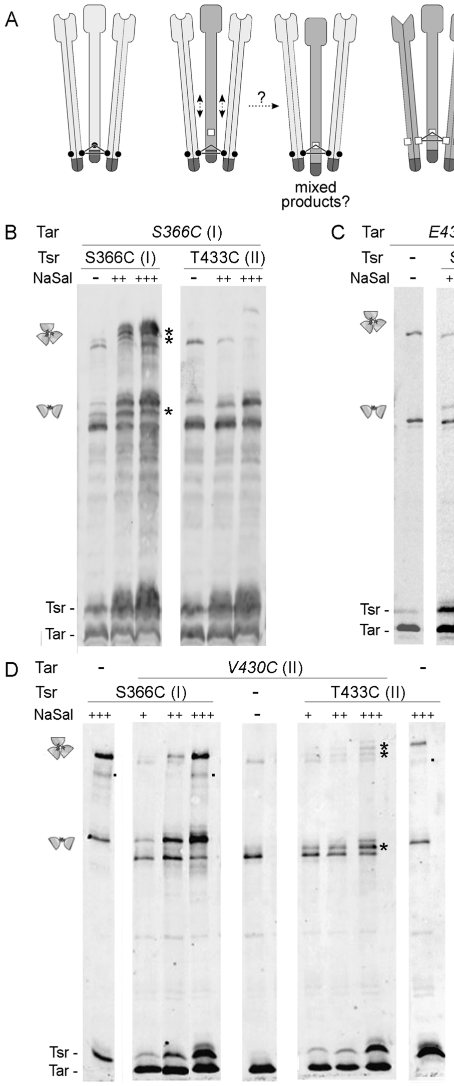

FIGURE 3: Inter-receptor cross-linking between reporters located at distant levels. (A) Schematic representation of a cross-linking experiment in which two different receptors bear cysteine reporters at distant levels. Individual dimers represent Tsr (light gray) or Tar (dark gray) molecules, as in Figure 2A. Cysteine substitutions on Tsr are represented by small circles and those on Tar by small squares, situated at a different level. TMEA treatment of cells expressing Tsr molecules bearing a cysteine residue at one level and Tar molecules bearing a cysteine reporter at a different level will give only cross-linking products of the pure type (Tsr- or Tar-only) unless a displacement of some dimer with respect to the others occurs along the central axis of the trimer. (B−D) Strains expressing single cysteine-substituted Tar and Tsr receptors, and lacking other chemotaxis proteins, were treated with TMEA, analyzed by SDS−PAGE in long, 9% acrylamide gels, and visualized by immunoblotting with an anti-Tsr antibody that reacts with both receptors. Tsr expression was induced at 0 (−), 0.15 (+), 0.30 (++), or 0.60 μM sodium salicylate (+++). Tar expression, when driven from the pPA818 derivatives, was induced at 10 μM IPTG. Mixed cross-linking products are denoted with asterisks. Small dots on the right side of some lanes indicate cross-reacting proteins or receptor degradation products. (B) UU1613 cells (carrying a chromosomal copy of Tar-*S366C* under native promoter control) transformed with pCS12 Tsr-S366C or pCS12 Tsr-T433C were grown to midlog phase under different induction conditions and treated with the TMEA cross-linker. (C) UU1581 cells carrying pPA818 Tar-6His-*E436C* and pCS12 Tsr-S447C were grown to midlog phase under different induction conditions and treated with TMEA. (D)UU1581 cells carrying pPA818Tar-6His-*V430C* and pCS12 Tsr-S366C or pCS12 Tsr-T433C were grown to midlog phase under different induction conditions and treated with TMEA.

a compatible plasmid and analyzed the cross-linking products after TMEA treatment (Figure 3D). Again, when both receptors bore reporter sites at the same level (in this case, level II), mixed cross-linking products were seen (Figure 3D, three right panels). In contrast, with reporter sites at different levels, no mixed cross-linking products were evident (Figure 3D, three left panels). The presence or absence of mixed cross-linking products is most apparent in the two-subunit region of the gel, because the corresponding pure cross-linking products (Tar−Tar or Tsr−Tsr) have clearly different mobilities, whereas in the three-subunit region, the cross-linking products for the combination Tar-*V430C* and Tsr-S366C migrate close together and are less easily distinguished by their positions. Note that cross-linked samples of each receptor alone were included in adjacent lanes to show the positions of the pure products. Those positions depend greatly on the location of the cysteine residue.

We also assessed interlevel cross-linking between reporters residing at levels II and V, whose distances from the tip differ by 20 Å (Table 1). Again, no mixed two- or three-subunit cross-linking products could be detected in cells expressing Tar-*E436C* and Tsr-S447C (Figure 3B). We conclude from these experiments that the dimers in a trimer do not move sufficiently far relative to each other to cross-link a cysteine residue at level I to one at level II, or a cysteine residue at level II to one at level V. Given the distances between the corresponding α-carbons in the Tsr crystal structure (Table 2), the absence of two-subunit products argues against the occurrence of significant dimer−dimer movements parallel to the trimer axis.

Effect of Signaling State on TMEA Cross-Linking Efficiency in Trimers of Dimers. In our original characterization of the Tsr-S366C reporter, we observed that its efficiency of TMEA cross-linking was not influenced by the presence of CheA and/or CheW (at physiological levels), by the receptor's methylation state, or by an attractant stimulus (*8*). These results indicate that position 366 in the axial subunits of a trimer, immediately above the trimer contact region, remains essentially stationary over a range of signaling conditions.

To ask whether cysteine reporters farther from the trimer contact region might move closer or farther apart or change their relative orientation in response to an attractant or repellent stimulus, we analyzed the TMEA cross-linking patterns of receptors with reporter sites at various levels. Cells expressing the marked receptors were treated with various stimuli for 5 s just before the addition of the TMEA reagent. We found that attractant stimuli did not cause an appreciable difference in cross-linking efficiency for any of the reporters (data not shown). However, glycerol (a repellent stimulus) caused a reproducible increase in cross-linking efficiency for reporters located at levels II−VI (Figure 4). This finding suggests that the dimers within a trimer move closer together in response to an osmotic stimulus, a behavior previously observed in YFP-tagged receptor molecules by Vaknin and Berg (*24*, *25*).

To determine whether this apparent compression of the trimer reflected the signaling state of the receptors, we introduced the reporter T433C (level II) into Tsr molecules bearing mutations that mimicked different methylation states (QEQE, QQQQ, and EEEE) or that locked receptor output in the kinase-off [A413V (*4*)] or kinase-on [L237R (*26*) or M259T (Q. Zhou et al., submitted)] signaling state. All mutant receptors exhibited a cross-linking efficiency similar to that of the wild-type receptor (data not shown). All mutant receptors also exhibited the glycerol-enhanced cross-linking behavior observed in the

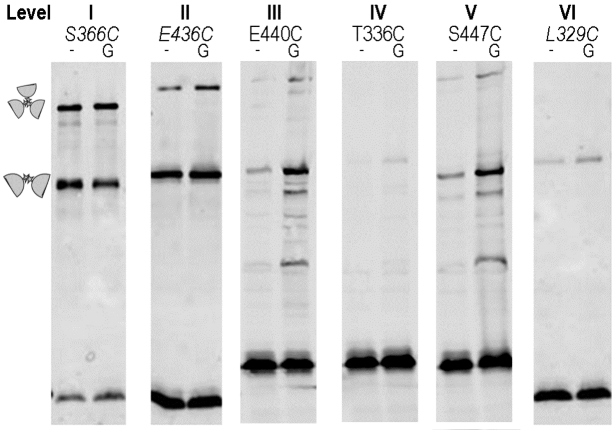

FIGURE 4: Effect of glycerol stimulation on cross-linking efficiency. UU1581 cells carrying pPA818 (*Tar-6His*) or pCS12 (Tsr) derivatives with single-cysteine substitutions were grown to midlog phase under conditions of physiological induction of receptors (10 μM IPTG for Tar and 0.45 μM sodium salicylate for Tsr), washed twice, and resuspended in phosphate buffer. TMEA treatment was performed 10 s after water (−) or 1 M glycerol (G) had been added to the cell suspension.

wild-type receptor. Thus, it appears that a strong osmotic stimulus compresses trimers of dimers, but mutations that "lock" the receptor in different signaling states cannot block this response. It is unclear, therefore, whether the glycerol effect is related to receptor signaling.

DISCUSSION

Native Organization of MCP Trimers of Dimers. Our in vivo cross-linking results are consistent with a native arrangement of receptors in trimers of dimers that are held together at their cytoplasmic tips and splay apart toward the membrane. Our findings do not support proposed alternative arrangements in which the cytoplasmic domains of neighboring receptors are roughly parallel to each other (*15*, *16*). This conclusion is based on a systematic decline in three-subunit TMEA cross-linking efficiency with reporter sites situated at increasing distances from the cytoplasmic tip of the receptor molecule.

The presence or absence of CheA and CheW had no effect on cross-linking efficiency for any of the reporter sites used in this study. This observation suggests that cross-linking yield reflects interactions between dimers within a trimer of dimers rather than intertrimer interactions, because higher-order interactions between receptors would probably be enhanced by the presence of the scaffolding proteins required to form a stable and tight cluster of chemoreceptors (*27*, *28*).

The clear absence of cross-linking between receptors carrying cysteine residues at different levels indicates that trimer-stabilizing interactions of the receptor tips do not permit large vertical displacements of one receptor dimer relative to another. This lack of large-scale movement holds even in the absence of CheA and CheW, a condition in which dimer exchange between trimers is highly dynamic (*18*).

Effect of Stimuli and Signaling State on Relative Distances between Receptor Dimers. Attractant stimuli had no significant effect on cross-linking efficiency for any reporter site tested. This result argues against significant stimulus-induced rotational movements of dimers within the trimer, which would have been expected to decrease the yield of three-subunit cross-linking products for reporters facing the central trimer axis. In the case of Tar carrying the off-axis reporter *D345C*, we did not observe any three-subunit cross-linking product for Tar alone or in combination with Tsr molecules carrying cysteine substitutions at the same level, either in the absence or in the presence of an attractant stimulus. This behavior also argues against rotational motions of dimers within a trimer, regardless of signaling state.

Glycerol, a repellent stimulus, caused a moderate but reproducible increase in cross-linking efficiency at most reporter positions (mainly from level II to VI), indicating that trimers may be compressed during repellent signaling, as previously suggested by the homo-FRET studies of Vaknin and Berg (*24*, *25*). However, this glycerol-induced compression of the trimer does not seem to reflect the signaling state of the receptor, because receptors mutationally locked in the kinase-on state or with activating glutamine residues at all four methylation sites did not exhibit increased cross-linking efficiency. Moreover, glycerol stimuli still enhanced the cross-linking efficiencies of mutationally activated receptors, suggesting that the effect of glycerol on trimer structure is different from the conformational change associated with kinase activation.

In summary, our in vivo receptor cross-linking studies support the trimer-of-dimers geometry revealed by the crystal structure of the Tsr signaling domain. Further, there appear to be no large vertical or rotational shifts between the members of a trimer during chemotactic signaling. We conclude that stimulus-induced changes in the conformation or dynamic behavior of trimer-based signaling teams fall below the resolution of in vivo cross-linking methods. In contrast, several studies have reported stimulus-dependent cross-linking effects that probably reflect changes in the arrangement or relative orientation of trimers in a receptor signaling cluster (*29*−*31*). How relatively small structural changes within trimers lead to seemingly larger changes in the organization of receptor arrays is the key to understanding the mechanism of cooperative signal amplification by chemoreceptors but remains an open question.

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