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Detecting the conformational change of transmembrane signaling in a bacterial chemoreceptor by measuring effects on disulfide cross-linking *in vivo*

(four-helix bundle/helical sliding/transmembrane receptors/bacterial chemotaxis)

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ABSTRACT **Transmembrane signaling by bacterial chemoreceptors is thought to involve relative movement among the four transmembrane helices of the homodimer. We assayed that movement by measuring effects of ligand occupancy on rates of oxidative cross-linking between cysteines introduced into neighboring helices of the transmembrane domain of chemoreceptor Trg from *Escherichia coli*. Measurements were done on chemoreceptors in their native environment, intact cells that were motile and chemotactically responsive. Receptor occupancy did not appear to cause drastic rearrangement of the four-helix structure since, among 67 cysteine pairs tested, the same 19 exhibited oxidative cross-linking in the presence or absence of saturating chemoattractant. However, occupancy did cause subtle changes that were detected as effects on rates of cross-linking. Among the seven disulfides appropriate for measurements of initial rates of formation, ligand occupancy had significant and different effects on all three cross-links that connected the two helices within a subunit but had minimal effects on the four that spanned the packing interface between subunits. This constitutes direct evidence that the conformational change of transmembrane signaling involves significant movement within a subunit and minimal movement between subunits, a pattern deduced from several previous studies and now documented directly. Among possible modes of movement between the two helices of a subunit, axial sliding of one helix relative to the other was the conformational change that best accounted for the observed effects on cross-linking.**

Chemotaxis by *Escherichia coli* and *Salmonella typhimurium* is mediated by a family of transmembrane receptor proteins (1, 2). Related proteins, presumably also components of chemotaxis systems, are present in the spectrum of bacterial species (3). In addition, there are many transmembrane sensor proteins, identified in a wide range of bacteria and some eukaryotes, that link environmental stimuli to control of gene expression through paired histidine kinases and phosphorylated response regulators that are homologous to the signal transduction components of the chemotaxis system (1, 4, 5). Hybrid proteins created by fusing the periplasmic and transmembrane domains of a chemoreceptor to the cytoplasmic kinase domain of an environmental sensor are functional, implying a common mechanism of transmembrane signaling for the two receptor classes (6, 7).

Chemoreceptors are homodimers of ≈60-kDa subunits organized in three domains: periplasmic, transmembrane, and cytoplasmic. The periplasmic domain contains the ligand-binding sites, the cytoplasmic domain controls the activity of the noncovalently associated histidine kinase, and the transmembrane domain, containing four helices, two from each subunit, provides the functional connection between the two hydrophilic domains. Structural information is available for chemoreceptor Tar, which mediates taxis toward aspartate by direct binding of this amino acid, and Trg, which mediates response to galactose and ribose by interaction with the ligand-occupied forms of two respective sugar-binding proteins. Analysis by x-ray crystallography of the isolated periplasmic fragment of Tar_S (from *S. typhimurium*) revealed a dimer of two four-helix bundles in which ligand bound across the subunit interface at the membrane distal end, and two long helices from each subunit ($\alpha 1$ and $\alpha 4$) extended over 70 Å to the transmembrane domain (8, 9). Essentially the same structure was determined for the periplasmic fragment of Tar_E (from *E. coli*) (10). The organizations of the transmembrane domain of Tar_E (11) and of Trg_E (12) were deduced from patterns of oxidative cross-linking between introduced cysteines to be very similar structures, loose four-helix bundles in which the most substantial interactions were between transmembrane helix 1 (TM1) and its homologue in the other subunit (TM1′) and between the helices within a subunit (TM1 and TM2). It appears that the four transmembrane helices are uninterrupted extensions of the four long periplasmic helices, creating an extended $\alpha 1$/TM1 and $\alpha 4$/TM2 pair in each subunit.

Signaling from the ligand-binding site in the periplasmic domain to the cytoplasmic domain is thought to be a conformational change within the stable homodimer (9, 13). Initial comparison of the structures of the periplasmic fragment of Tar_S with and without bound aspartate revealed only a modest difference, a subtle shift along the dimer interface in the relative positions of the two subunits (8). The authors suggested that in the intact receptor this shift could cause a scissors motion between the subunits that, when amplified over the extended length of the receptor, would constitute the transmembrane signal. The idea of signaling as movement between subunits was attractive since ligand binding occurs across the subunit interface (8). However several lines of evidence argue that relative movement within a subunit, between $\alpha 4$/TM2 and $\alpha 1$/TM1, not between subunits, is the central feature of conformational signaling. Transmembrane signaling in Tar or Trg was blocked by disulfide bonds that constrained movement between $\alpha 1$/TM1 and $\alpha 4$/TM2 but not by those that constrained movement between $\alpha 1$/TM1 and $\alpha 1'$/TM1′ (14–17); mutational analysis of Trg showed that disruption of the interface between heterologous transmembrane helices could induce signaling and disruption of the interface between homologous helices could reduce signaling (18, 19); assays of mixed dimers of Tar_S containing one truncated subunit indicated that signaling could occur through a single TM1/TM2 pair (20); some cross-links between TM1 and TM2 locked Tar in a particular signaling state (16, 21); and analyses of the periplasmic fragment of Tar_S provided indica-

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tions of movement of the periplasmic extension of TM2 (22, 23). In the work described herein, we used measurements of disulfide cross-linking between introduced cysteines as a direct assay of the nature and extent of movement between helices in the transmembrane domain of chemoreceptor Trg.

MATERIALS AND METHODS

Strains and Plasmids. Plasmid pGB1 (24) and a series of its derivatives, all of which carried *trg* under the control of the *tac* promoter as well as the genes *lacI*^{q} and *bla*, were introduced into CP553, a strain of *E. coli* K-12 deleted for the four chemoreceptor genes and for *cheR* and *cheB*, but otherwise wild type for chemotaxis and motility (24). pGB1 codes for the wild-type form of Trg that contains a single cysteine, Cys-23. The derivatives coded for a protein without that cysteine (C23S) but with a cysteine introduced at one or two specific positions in the transmembrane segments (12). Each strain was also transformed with the compatible plasmid pAI12, a derivative of pACYC184 carrying *rbsB*, the gene for the ribose-binding protein, constitutively expressed from a vector promoter (25).

***In Vivo* Cross-Linking.** Strains to be used in cross-linking experiments were treated in a manner designed to produce physiologically unperturbed cells. Cells were inoculated from a frozen glycerol stock into 2 ml of tryptone broth containing ampicillin (50 μg/ml); incubated overnight with vigorous agitation at 35°C; inoculated from that culture to $\approx 10^8$ cells per ml in 4 ml of H1 salts medium (26) containing required amino acids at 0.5 mM, 0.4% sodium succinate, ampicillin (50 μg/ml), chloramphenicol (25 μg/ml), and 50 μM isopropyl β-D-thiogalactoside; incubated as above until reaching 2.5×10^8 cells per ml; inoculated from that culture to $\approx 2.5 \times 10^7$ cells per ml in the same minimal medium; incubated as above until reaching 2.5×10^8 cells per ml; and then rapidly cooled in an ice slurry to be kept for use that day. For each experiment, 0.5 ml of cell suspension was placed in each of two 15-ml beakers, ribose was added to one beaker to 13 mM from a 100-fold concentrated stock, and the beakers were incubated at 35°C with vigorous agitation to provide generous aeration. A 40-μl sample ("time 0") was removed, immediately mixed with 10 μl of stop solution/sample buffer [20 mM Tris/8 mM NaH_2PO_4, pH 7.8/12.5 mM EDTA/12.5 mM *N*-ethylmaleimide/1.25% SDS/12.5% sucrose/bromophenol blue (2.5 μg/ml)], and then placed on ice. An equal volume of a 35°C solution of Cu(II)-(*o*-phenanthroline)$_3$ (hereafter Cu-phenanthroline) prepared as described (14) was added to each cell suspension, and samples were removed and processed as for the first sample at regular intervals (in initial tests at 5 and 10 min and for detailed time courses every minute for the first 5 min and at three times in the subsequent 5 min). Samples were boiled for 4 min and stored at −20°C until further processing.

Quantification of Cross-Linking. Samples from cross-linking experiments were analyzed by SDS/polyacrylamide gel electrophoresis in the absence of reducing agents and examined by immunoblot analysis using anti-Trg serum and treatment with goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad) followed by 4-chloro-1-naphthol (3). The extent of cross-linking for each sample was quantified by measuring the intensity of staining of the cross-linked and monomeric species using a Molecular Dynamics scanning densitometer and the analysis program IMAGEQUANT (version 3.2). A value for percent cross-linking was calculated by dividing the intensity of the cross-linked species by the sum of intensities of the two species and multiplying by 100. Analysis was done using immunoblots in which staining intensities were linearly related to the amount of protein.

RESULTS

Oxidative Cross-Linking as a Probe for Conformational Change. This laboratory had previously used patterns of oxidative cross-linking between introduced cysteines to deduce the three-dimensional organization of the transmembrane domain of chemoreceptor Trg (12). If the conformational change of transmembrane signaling involves relative movements of the helices within the four-helix organization of this domain, then cross-linking between particular cysteine pairs on neighboring helices might be affected by ligand occupancy. The pattern of those effects could reveal the nature of the signaling change. However, as observed for the periplasmic fragment of Tar$_S$ (8, 9), conformational movements within a chemoreceptor could be relatively subtle and thus might be best detected in the most unperturbed native state. For this reason, we utilized a procedure for treating intact cells of *E. coli* with the oxidation catalyst Cu-phenanthroline in which disulfide cross-links could be induced between cysteine pairs in the transmembrane domain of a chemoreceptor without significantly perturbing the cellular functions relevant to receptor action, flagellar rotation, and the control of that rotation by the chemosensory system (14). With this procedure, we determined the effect of ligand occupancy on cross-linking between pairs of cysteines in which the two members of the pair were in different helices of the four-helix structure of the transmembrane domain of Trg. To investigate possible movements between homologous helices in the two subunits of the native homodimer, we tested our comprehensive collection of 54 single-cysteine forms of Trg (12), each with one cysteine per polypeptide chain, located at each of the possible positions in TM1 (30 total) or TM2 (24 total). In such proteins, the native dimer contains two cysteines, one at each of the corresponding positions in the two subunits. To investigate possible movement of heterologous helices within a subunit or between subunits, we tested 13 double-cysteine forms of Trg, each with a cysteine in both TM1 and TM2. These 13 were previously identified by screening combinations of cysteines in the two helices for those that formed TM1-TM2 or TM1-TM2′ cross-links (12). Cysteine-containing proteins were produced from appropriate plasmid-borne genes in host cells that lacked chromosomal copies of the four chemoreceptor genes and the genes for the receptor-modifying methyltransferase and methylesterase. This avoided possible influences of adaptational modification or of cross-talk with other chemoreceptors (27). In wild-type cells, the concentration of ribose-binding protein in the periplasm is not sufficient to saturate Trg, even with a saturating concentration of ribose and maximal induction of the binding protein (28). We introduced into each strain a second plasmid carrying the gene for the ribose-binding protein. This resulted in a 5- to 10-fold increase in content of binding protein, thus providing maximal occupancy of Trg in the presence of excess ribose.

Effects of Ligand Occupancy on Formation of Disulfide Cross-Links. We screened the 67 cysteine-containing forms of Trg for cross-linking *in vivo* in the absence and presence of saturating amounts of ribose, using the strongest treatment with the oxidation catalyst Cu-phenanthroline (0.3 mM for 10 min) that allowed unperturbed flagellar and sensory function. Catalyst was added to cells actively growing in minimal medium and samples were taken before, and at intervals after, this addition. The extent of oxidative cross-linking for each sample was determined by SDS/polyacrylamide gel electrophoresis in the absence of reducing agent and immunoblot analysis with an anti-Trg serum, and the intensity of stained bands representing monomer and cross-linked dimer was measured. In the absence of receptor stimulation, 19 cysteine-containing receptors exhibited cross-linking, ranging from just detectable to complete. The *in vivo* cross-links observed were all between cysteines located near each other in the model of the Trg

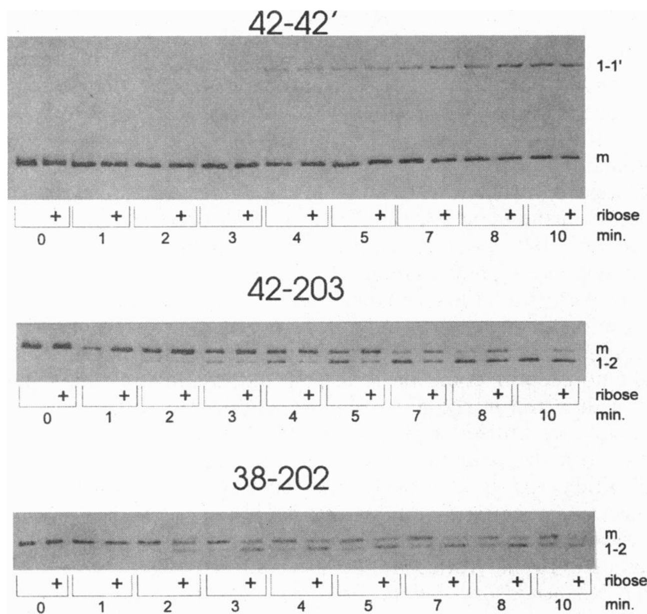

FIG. 1. Examples of immunoblot analysis of effects of ligand occupancy on oxidative cross-linking *in vivo*. Samples from a suspension of intact cells taken just before and at various times after addition of Cu-phenanthroline were examined by SDS/polyacrylamide gel electrophoresis and immunoblot analysis with anti-Trg serum. For each sampling time (shown as a value in minutes), material from two parallel cell suspensions was loaded in adjacent lanes, the first in the absence and the second in the presence (+) of a saturating amount of ribose. Experiments displayed were for cells containing Trg with a cysteine at positions 42 (*Upper*), positions 42 and 203 (*Middle*), or positions 38 and 202 (*Lower*). Only the relevant segments of the immunoblots are shown. Positions are indicated for monomeric Trg devoid of cross-links, apparent M_r ≈60,000 (M); the Trg dimer cross-linked between cysteines in TM1 and TM1′, apparent M_r ≈ 120,000 (1–1′); and the Trg monomer cross-linked between cysteines in TM1 and TM2, apparent M_r ≈ 55,000 (1–2).

transmembrane domain derived from patterns of cross-links observed *in vitro* (12). The reactions in intact cells were restricted, with the exception of one just detectable 2–2′ reaction, to the half of the transmembrane domain nearest the periplasm, a pattern consistent with an influence of the reducing environment of the cytoplasm. In the presence of ribose the same 19 proteins, but no additional ones, exhibited cross-linking. Thus effects of ligand occupancy on the relative placement of the four helices in the transmembrane domain appeared relatively subtle. Subtle changes might be detected by effects on the rate of cross-linking. However, among the 19 cross-linked receptors, 8 exhibited very low extents of cross-linking, precluding reliable determination of rates. Three proteins, containing four cysteines in the dimer, exhibited two types of cross-linked species (1–1′ and 1–2′). A detailed examination of one of these revealed complex kinetics in the appearance of the cross-linked species, again precluding a reliable analysis, and thus the three were not considered further. One other receptor (Trg-G200C) was substantially defective in receptor function (18) and thus was not an appropriate candidate for analysis. For the remaining 7 cysteine-containing receptors, we identified a catalyst concentration equal to or less than the functionally benign value at which cross-linking increased approximately linearly over a period of several minutes, allowing determination of initial rates in the presence and absence of ligand. For the seven proteins, only a single type of cross-linked species (1–1′ or 1–2) was formed, making interpretation straightforward. Example immunoblots are shown in Fig. 1.

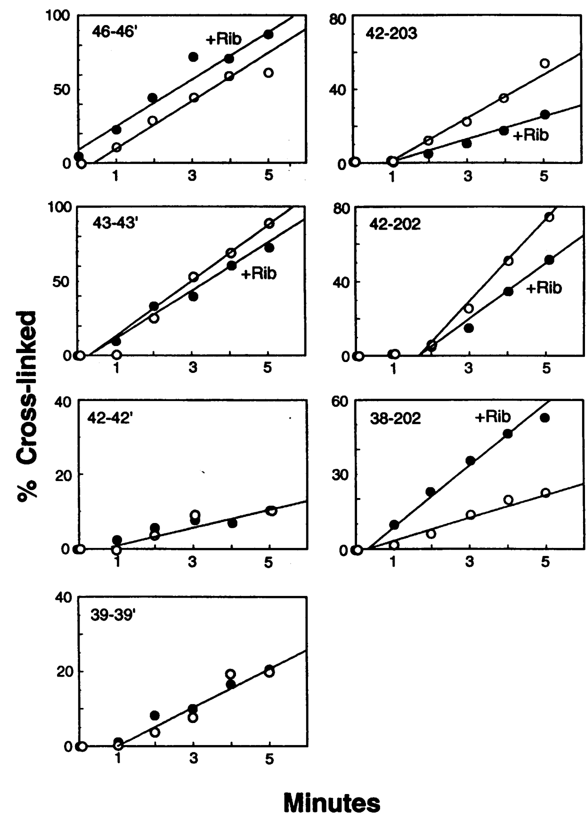

FIG. 2. Representative time courses of cross-linking. The intensity of bands on immunoblots like those shown in Fig. 1 were quantified by densitometry and percent cross-linking was calculated for each sample.

We performed multiple independent experiments, examining the effect of ligand occupancy on rates of cross-linking for the seven cysteine pairs, and obtained consistent results. Representative time courses are shown in Fig. 2. Fig. 3 displays for each cysteine pair the mean fractional change in initial rate of cross-linking resulting from ligand occupancy as well as standard errors of those means. Ligand occupancy had different effects on different cross-linking reactions. The rate of one

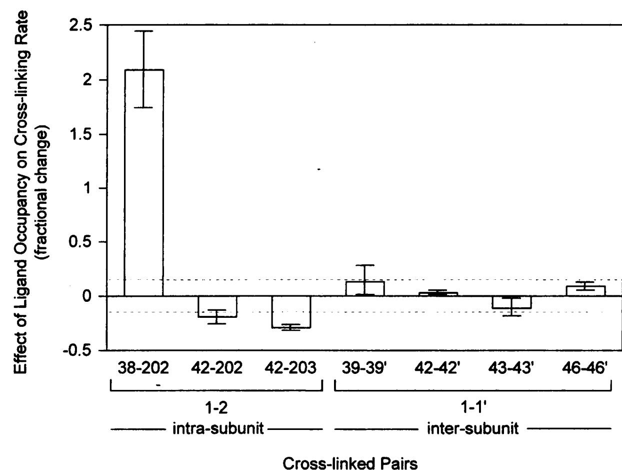

FIG. 3. Effect of ligand occupancy on intrasubunit and intersubunit cross-links. Initial rates were determined by lines fitted to data points, as shown in Fig. 2, greater than 0 and less than the values at which the increase of cross-linking with time was no longer linear for at least five experiments like those shown in Fig. 2. The mean fractional changes in initial rates of cross-linking in the presence of saturating amounts of ribose are shown. Error bars indicate the SEM.

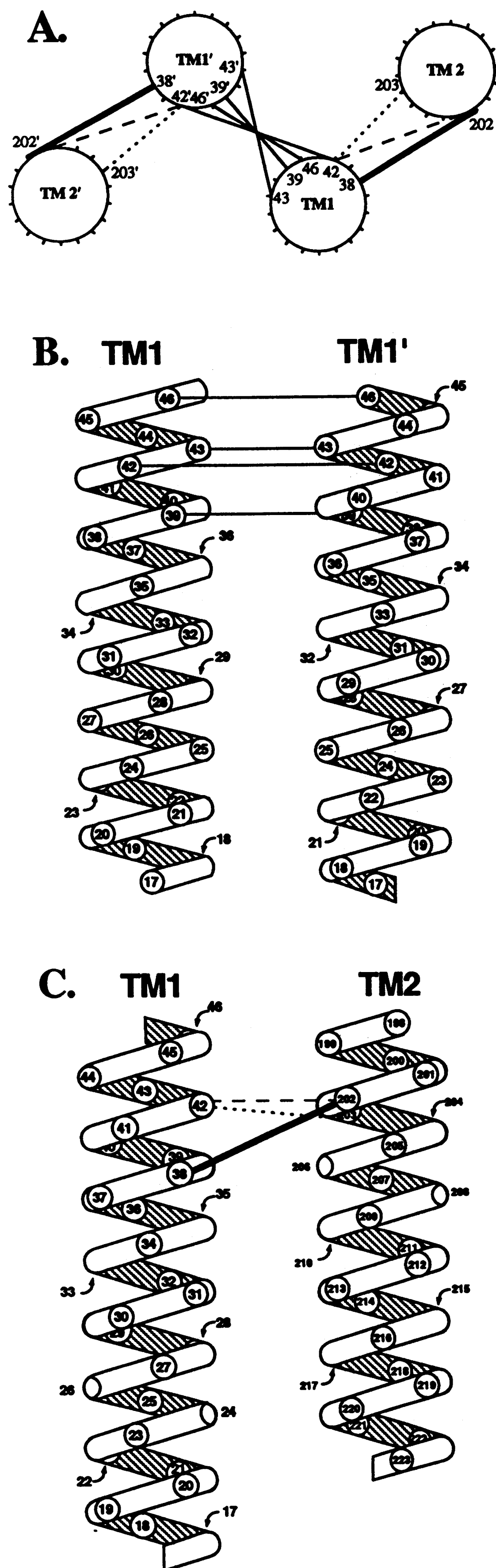

FIG. 4. Positions of cross-links assayed for effects of ligand occupancy. Three views of the deduced structure of the transmembrane domain of Trg (12) are shown. These are a view from the periplasm toward the surface of the cytoplasmic membrane (*A*), the relative orientation of TM1 and TM1′ of the two different subunits (*B*), and the relative placement of TM1 and TM2 within a subunit (*C*). Effects of ligand occupancy on initial rates of cross-linking are symbolized by a thick line (substantial increase), thin lines (no substantial effect), a dashed line (significant but moderate reduction), or a dotted line (substantial decrease).

reaction was substantially increased, the rates of two were significantly decreased, and the rates of the others were hardly altered. This diversity indicated that addition of chemoattractant did not change cross-linking rates by some common, thus possibly artefactual, effect on the oxidation chemistry we utilized to assess protein conformation. Thus we concluded that the differences in cross-linking reflected specific differences between the ligand-occupied and ligand-free conformations of the receptor. We chose a threshold of 0.15 fractional change to identify significant effects (see boundaries indicated in Fig. 3), in large part because apparent effects below that threshold had standard errors of a magnitude almost as large as the apparent effect itself (Fig. 3). By this criterion, significant effects of ligand occupancy on rates of cross-linking were specifically correlated with the interface across which the reaction occurred (Fig. 4). Ligand occupancy had a significant effect on all three intrasubunit cross-links (TM1-TM2) but on none of the four intersubunit reactions (TM1-TM1′).

DISCUSSION

Detecting the Conformational Change of Transmembrane Signaling. We used *in vivo* measurements of oxidative cross-linking between introduced cysteines to detect a structural difference between the transmembrane domain of a ligand-occupied and a ligand-free chemoreceptor. This difference is likely to reflect the conformational change of transmembrane signaling. Our data indicate that the change is relatively subtle, is greatest between helices within a subunit, and is modest at best between subunits. A conformational change could alter rates of cross-linking between sulfhydryls by affecting one or more parameters: distance, orientation, local chemical environment, or dynamics. For cysteines located in the four-helix structure of the transmembrane domain, the first three effects would likely be mediated by relative movements among the transmembrane helices and the fourth effect by modulation of such movements. Thus our considerations of transmembrane signaling within the transmembrane domain will focus on helix movements. A substantial body of data had previously implicated movement within a subunit as a crucial feature of transmembrane signaling by bacterial chemoreceptors (14–23). Our assessment of the structural organization of the transmembrane domain of Trg in the ligand-occupied and ligand-free state provides direct evidence for such intrasubunit movement. There is a satisfying complementarity between the results of our current study and our characterization of signaling by receptors constrained by specific interhelical disulfide cross-links (14). We previously observed that Trg proteins cross-linked across the TM1-TM1′ interface by disulfides between positions 46–46′, 39–39′, or 32–32′ were functionally unperturbed, and this same TM1-TM1′ interface, assessed using some of the same cysteine pairs, exhibited minimal changes in cross-linking rates upon ligand occupancy. Conversely, in the previous study, Trg proteins constrained by TM1-TM2 cross-links between positions 42–203 or 42–202 were incapable of signaling, and in our current study the rates of cross-linking between cysteines at these positions as well as one other pair spanning the TM1-TM2 interface were significantly altered by ligand binding.

Effects of ligand occupancy on cross-linking between introduced cysteines were first reported as part of a pioneering study of Tar_S (29) and later in studies of Tar_E (30, 31), using

protein contained in isolated membranes or detergent/lipid mixtures. Those *in vitro* studies included cysteine pairs near or in the transmembrane segments of Tar but were limited to intersubunit cross-links, and this did not provide information about the relative magnitude of effects within or between subunits. Our investigation of Trg *in vivo* included both intrasubunit and intersubunit cross-links, and thus it was possible to identify the principle consequence of ligand occupancy to be movement within a subunit, between the two transmembrane helices TM1 and TM2.

Deducing Features of the Conformational Change of Signaling. Can our data distinguish among possible types of signaling movement between TM1 and TM2? Any such movement could be described by reference to four fundamental motions: radial movement normal to the plane of helix interaction that would move helices together or apart, sheer movement parallel to the plane of helix interaction that would move helices together or apart, axial movement that would slide one helix relative to the other, or rotational movement that would rotate one helix relative to the other. These possibilities are relevant whether the conformational change be a straightforward mechanical motion or a more sophisticated control of dynamics (32). Could any single motion be the primary contributor to the changes in cross-linking rates we observed? With only three relevant cross-links across the TM1-TM2 interface, conclusions must necessarily be preliminary, and of course the actual motion might include more than one motion. If there were a relative movement of TM2 away from or toward TM1 in a direction normal to their plane of interaction (radial movement) or in a direction parallel to their plane of interaction (sheer movement), then rates of oxidative cross-links between the two helices should be affected in the same way for all cysteine pairs. The rates of the three TM1-TM2 cross-links were affected differently by receptor occupancy, one was substantially increased, one significantly decreased, and one marginally decreased (Fig. 4). This pattern is not consistent with simple radial or sheer movement. Tilting, a variation on these movements, could result in opposite effects on different cross-links along a helical interface if the positions of the cross-links were separated along the long axis and the tilt axis were between them. However, the TM1-TM2 cross-links affected by ligand occupancy were very close to each other, involving identical or closely positioned residues. Thus radial or sheer movements or their tilting variants do not appear to be the conformational change detected by our cross-linking measurements. Relative rotation of TM2 and TM1 could account for the two major effects we observed on rates of cross-linking but not for the modest one. As seen in Fig. 4*A*, a clockwise rotation of TM2 (or the opposite movement of TM1) would move position 202 closer to position 38 and position 203 farther from 42, changes consistent with the respective effects, a rate increase, and a rate decrease. Yet such a rotation would also bring position 202 closer to position 42, inconsistent with the moderate decrease in cross-linking rate caused by ligand occupancy. Because the observed effect of ligand occupancy on the rate of cross-linking positions 42 and 202 is modest, rotation cannot be definitively eliminated as a possible signaling movement. However, axial sliding of TM2 toward the cytoplasm (or the opposite movement of TM1) could account for all observed effects: position 202 would move closer to position 38 and farther from position 42, and position 203 would also move away from position 42, corresponding to the observed increase in cross-linking between positions 202 and 38 and the decreases observed for the other two pairs (Fig. 4*C*). If the unstimulated orientation were as shown in Fig. 4*C*, with positions 202 and 203 bracketing residue 42, then sliding could even account for the smaller effect of occupancy on the cross-link between positions 42 and 202 in relation to the cross-link between positions 42 and 203. Sliding toward the cytoplasm of TM2 (or the opposite movement of TM1) would move position 202 past the closest alignment with position 42 to a position only a little more distant than its original placement, but simply increase the separation of position 203 from position 42. It is interesting that a recent model of transmembrane signaling by Tar_S suggested axial sliding of TM2 toward the cytoplasm as part of the signaling motion (32). This model was based in part on a comparison of the structures of the periplasmic fragment of Tar_S with and without bound aspartate in which an alternative way of superimposing the structures resulted in an apparent shift of $\alpha 4$ toward the membrane in the ligand-bound state, a movement consistent with the sliding of TM2 toward the cytoplasm as suggested by our data. Whatever the molecular details of chemoreceptor signaling, the effects of ligand occupancy documented in these studies identify axial sliding of TM2 relative to TM1 as the best candidate for the conformational change of transmembrane signaling.

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