Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 3391–3395, April 1995
Biochemistry

Transmembrane signaling characterized in bacterial chemoreceptors by using sulfhydryl cross-linking *in vivo*

(transmembrane receptors/protein conformational change/bacterial chemotaxis/helical bundles)

GEOFFREY F. LEE*, MICHAEL R. LEBERT†, ANGELA A. LILLY, AND GERALD L. HAZELBAUER‡

Department of Biochemistry and Biophysics, Washington State University, Pullman, WA 99164-4660

Communicated by Julius Adler, University of Wisconsin, Madison, WI, December 27, 1994

ABSTRACT **Transmembrane signaling by bacterial chemoreceptors is thought to involve conformational changes within a stable homodimer. We investigated the functional consequences of constraining movement between pairs of helices in the four-helix structure of the transmembrane domain of chemoreceptor Trg. Using a family of cysteine-containing receptors, we identified oxidation treatments for intact cells that catalyzed essentially complete sulfhydryl cross-linking at selected positions and yet left flagellar and sensory functions largely unperturbed. Constraining movement by cross-links between subunits had little effect on tactic response, but constraining movement between transmembrane segments of the monomer drastically reduced function. We deduce that transmembrane signaling requires substantial movement between transmembrane helices of a monomer but not between interacting helices across the interface between subunits.**

A family of related transmembrane chemoreceptors mediates chemotaxis in *Escherichia coli, Salmonella typhimurium*, and many other bacteria (1–3). These integral membrane proteins are homodimers (5) organized as diagrammed in Fig. 1. The structure of the periplasmic domain, determined by x-ray crystallography (6), is two four-helix bundles, one from each monomer, that interact along a central axis. The two central helices (α1 and α4) of each bundle are >70 Å long and extend to the membrane (Fig. 1*A*). The organization of the transmembrane domain, deduced from patterns of disulfide formation between introduced cysteines (4, 7) is a bundle of four helical segments (Fig. 1*B*) that appear to be uninterrupted extensions of the four central helices of the periplasmic domain (4, 6–9). The cytoplasmic domain interacts with a kinase and an accessory protein to form a ternary complex that controls the rotational bias of flagellar motors by phosphorylation of a response regulator (10, 11). Sensory adaptation is mediated by methylation of specific glutamyl residues in the cytoplasmic domain of the chemoreceptor (1–3).

How does ligand binding to the periplasmic domain of a chemoreceptor cause a change in the cytoplasmic domain that alters the phosphorylation activity of the ternary complex and ultimately cellular behavior? Several lines of evidence (5, 8, 9, 12–14) indicate that transmembrane signaling in the chemoreceptors involves a conformational change within the stable dimeric protein. In this study we addressed the importance for transmembrane signaling of movement between specific pairs of transmembrane helices. We used sulfhydryl cross-linking to constrain movement between helices in cysteine-containing forms of the chemoreceptor Trg and assessed the ability of the constrained receptors to mediate tactic responses in intact cells.

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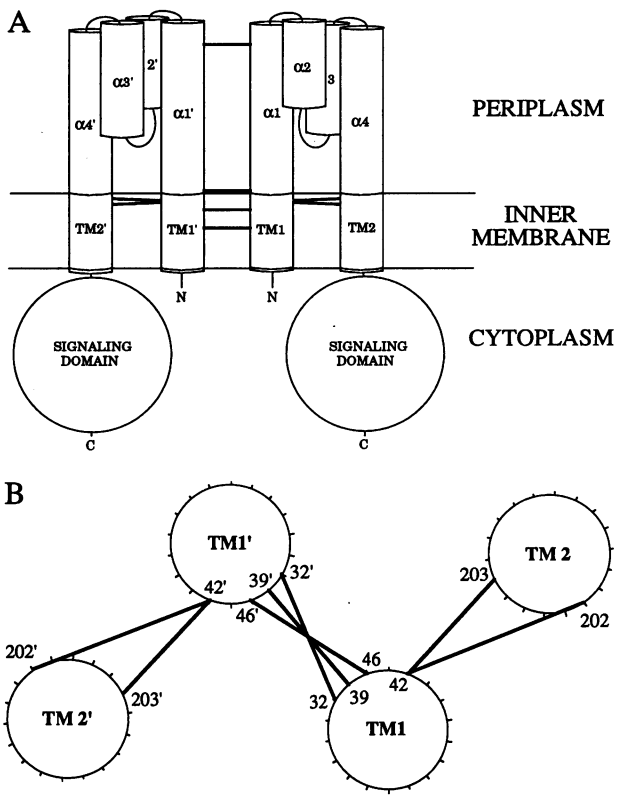

FIG. 1. Diagrams of chemoreceptor organization showing positions of extensive cross-linking *in vivo*. (*A*) Transmembrane (TM) disposition and domain organization. Positions of disulfide cross-links used in this study are shown by bold lines between helices. From top to bottom, the TM1 to TM2 cross-links are 42–202 and 42–203; the α1-TM1 to α1′-TM1′ cross-links are 82–82′, 46–46′, 39–39′, and 32–32′. (*B*) Relative positions and orientation of the transmembrane helices. A view from the periplasm of a model of the transmembrane domain of Trg (4), showing the four helices represented as helical wheels. The bold lines indicate disulfide cross-links.

MATERIALS AND METHODS

Strains and Plasmids. Derivatives of pGB1 (15), which carries *trg* under the control of the *tac* promoter, *lacI*^{q} and *bla*, were introduced into CP177, a strain of *E. coli* K-12 deleted for *trg* but otherwise wild type for chemotaxis and motility (16). The plasmids carried an altered *trg* coding for a product devoid of cysteine (Trg-C23S) or derivatives of that gene coding for proteins with a cysteine introduced at one or two specific

Abbreviations: CCW, counterclockwise; CW, clockwise.

*Present address: Department of Protein Engineering, 460 Point San Bruno Boulevard, South San Francisco, CA 94080.
†Present address: Botanisches Institut der Universität Erlangen, D-91058 Erlangen, Germany.
‡To whom reprint requests should be addressed.

positions (4, 17). All seven altered Trg proteins were functional as assessed by the formation of chemotactic rings in response to gradients of ribose on semisolid agar plates.

***In Vivo* Cross-Linking.** Cells to be assayed for response to ribose were grown to ≈2.5 × 10^8 cells per ml in H1 minimal salts medium (19) containing required amino acids at 0.5 mM, 0.4% ribose, ampicillin at 50 μg/ml, and 50 μM isopropyl β-D-thiogalactoside and, for assays of response to aspartate, were grown in the same medium without isopropyl β-D-thiogalactoside. For oxidation by molecular iodine, a 10-ml beaker containing 2.5 ml of culture was placed on a shaker at 25°C for 5 min, and 2.5 ml of 0.5 mM iodine or water (for control cells) at 25°C was added. After 15 s at 25°C the cells were processed for behavioral assays and for analysis of cross-linking (see below). Oxidation by treatment with Cu(II)-(*o*-phenanthroline)$_3$ (hereafter Cu-phenanthroline) was by a similar procedure at 35°C for 10 min with 0.3 mM oxidant and termination by addition of 10 mM EDTA. A 60 mM solution of the reagent was prepared by mixing 60 μmol of $CuSO_4$, 200 μmol of 1,10-phenanthroline, and 50 μmol of sodium phosphate (pH 7.4) in 1 ml, and a 1:100 dilution was made for use in each experiment. After the termination of either treatment, 0.2 ml of cell suspension was removed and precipitated in 7% (wt/wt) trichloroacetic acid/8 mM *N*-ethylmaleimide/10 mM EDTA for analysis by electrophoresis and immunoblotting to quantify cross-linking efficiency (4). The cellular content of Trg was adjusted by using the inducer isopropyl β-D-thiogalactoside in the minimum amount necessary to allow immunoblot analysis with anti-Trg serum in which Trg would be detected but the other three cross-reacting chemoreceptors present in the host cells would not be visible; this resulted in ≈10^5 Trg polypeptides per cell.

Behavioral Assays. Samples of cells from *in vivo* cross-linking experiments were treated to shear their flagella, tethered to a glass surface using antibodies to flagellin, and observed at 35°C by video microscopy (20). In an initial 2-min viewing period of each field, cells in three randomly placed boxes, 5 cm on a side, were used to determine (*i*) rotational competency, calculated from the percentage of cells that were rotating (at least one complete revolution); and (*ii*) rotational balance of rotating cells, defined by four classes: reversing with a clockwise (CW) or counterclockwise (CCW) bias or rotating exclusively one direction or the other. These two measurements provided experimentally relevant parameters for judging the physiological state of the experimental cells. For mock-treated cells, our tethering procedure resulted in 33% of cells exhibiting rotation, a value similar to that for untreated cells (many cells are attached at more than one point and thus cannot rotate) and which we assigned a rotational competency of 1.0. Cultures treated with high levels of oxidant or catalyst exhibited no rotating cells, whereas ones treated with optimized conditions for oxidation mediated by Cu-phenanthroline or iodine exhibited average rotational competencies of 0.97 and 0.82, respectively. Thus the aspects of cellular physiology important for flagellar function were minimally perturbed by the oxidation procedures, and the ultimate target of receptor action, the flagellar motor, was not significantly altered. The ratio between CW and CCW flagellar rotation reflects a finely tuned balance among stoichiometries and activities of the multiple components of the chemosensory system as well as the level of proton motive force. If our oxidation conditions had affected the general chemosensory or motility systems severely, there would have been dramatic shifts in rotational bias. We observed only moderate shifts. Populations of rotating control cells were almost all reversing, with 60 to 80% biased to CW rotation; after oxidation, a small fraction rotated exclusively CCW, and approximately half the reversing cells were CW-biased. The only significant exception was Trg-A82C as described in *Results*.

After the 2-min period, cells were stimulated by addition of buffer or buffer plus attractant. The percentage of cells that responded by exclusively CCW rotation after attractant addition was determined, and the magnitude of the response was measured as the time after stimulation with an attractant at which 50% of the cells were no longer rotating exclusively CCW minus the comparable time after addition of buffer alone (usually a few seconds).

RESULTS

Oxidative Cross-Linking *in Vivo*. A powerful strategy for analysis of protein structure and function is the use of disulfide cross-links between cysteines introduced at specific positions in a protein by oligonucleotide-directed mutagenesis (4, 5, 7–9, 12, 13, 21). We aimed to broaden the applications of this strategy by identifying conditions in which cross-linking could be done *in vivo*. Specifically, we hoped to oxidize cysteine pairs in a bacterial membrane protein by treatments that allowed cells to remain functional. This aim was pursued by using our collection of ≈70 cysteine-containing Trg proteins in which one or two cysteines per subunit of the homodimeric receptor are present in the transmembrane segments (4) or one cysteine per subunit is present near the ligand-binding site (17). We found conditions in which treatment of intact cells with the oxidant molecular iodine or the oxidation catalyst Cu-phenanthroline resulted in disulfide formation between specific cysteine pairs to extents that ranged from detectable to essentially complete and yet preserved flagellar function and chemotactic response in the treated cells (see *Materials and Methods*). In these optimized conditions, 6 of the 70 cysteine pairs could be cross-linked nearly to completion, yet none of the pairs exhibited significant cross-linking in the absence of the specific oxidation treatment (Fig. 2 and Table 1). The position of these cross-links in the receptor structure is diagrammed in Fig. 1. For one of the six pairs (46–46′), either treatment resulted in extensive cross-linking; for others only iodine (32–32′ and 39–39′) or Cu-phenanthroline (82–82′, 42–202, and 42–203) was sufficiently active. We presume that in these finely balanced, optimized conditions, particular features of accessibility and local chemistry for each cysteine pair resulted in the rather specific requirements for extensive oxidation. As would be expected for the selectivity of the oxidation conditions, the sulfhydryls of each cysteine pair were in close spatial proximity in the deduced structure of the transmembrane domain of Trg (ref. 4 and Fig. 1*B*) or in the modeled structure of the periplasmic domain of Trg based on the crystallographically determined structure of the analogous domain of Tar_s (6).

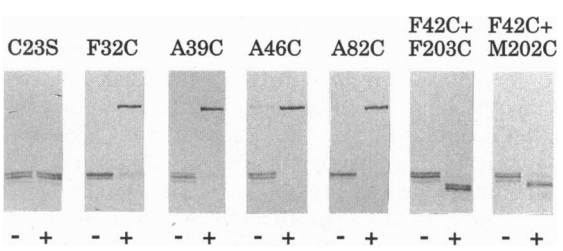

FIG. 2. Immunoblot analysis of disulfide cross-linking. Samples of cells exposed to oxidation conditions (+) or mock-treated (−) were analyzed by SDS/10% polyacrylamide gel electrophoresis in the absence of reducing agent and with immunoblotting by use of anti-Trg serum. The relevant mutational substitutions in the respective Trg proteins are indicated above the electrophoretic patterns. Monomeric Trg migrates at an apparent M_r of 60,000 as a family of electrophoretic bands representing a distribution of methylated forms. Cross-links between TM1 and TM1′ or between TM1 and TM2 result in forms that migrate with apparent M_r values of ≈120,000 and 55,000, respectively.

Table 1. Oxidation treatments and subsequent cellular responses to ribose

Trg	Oxidation treatment* 25°C	Oxidation treatment* 35°C	Cross-link, % ± SEM	Response to 0.5 μM ribose, s ± SEM
C23S	Mock (3)		0 ± 0	71 ± 12
	I_2 (3)		0 ± 0	117 ± 4
		Mock (2)	0 ± 0	105 ± 10
		CuP (2)	0 ± 0	100 ± 13
F32C	Mock (2)		0 ± 0	39 ± 12
	I_2 (2)		77 ± 5	61 ± 21
A39C	Mock (2)		0 ± 0	97 ± 16
	I_2 (2)		91 ± 1	117 ± 5
A46C	Mock (3)		7 ± 1	72 ± 9
	I_2 (2)		85 ± 2	110 ± 22
		Mock (2)	10 ± 2	67 ± 12
		CuP (3)	95 ± 2	55 ± 27
A82C		Mock (4)	2 ± 1	17 ± 2
		CuP (3)	89 ± 3	75 ± 49
F42C/ M202C		Mock (4)	0 ± 0	52 ± 5
		CuP (4)	82 ± 2	3 ± 1†
F42C/ F203C		Mock (5)	0 ± 0	62 ± 7
		CuP (5)	91 ± 0	4 ± 1†

*Number in parenthesis indicates number of independent trials, each with ≈20 cells.

†Approximately one-third of the cells showed no detectable response (18).

Transmembrane Signaling by Cross-Linked Receptors. We examined the activity of cross-linked receptors by assaying chemotactic responses of cells tethered by their flagella to a glass surface in a flow chamber (22). Unstimulated cells alternate frequently between CCW and CW rotation, reflecting the action of the flagellar motor. Addition of chemoattractant results in a rapid shift to exclusively CCW rotation, a behavior that persists for a time proportional to the extent of receptor occupancy (22). For any particular attractant, response depends on the relevant chemoreceptor. For instance, cells lacking chemoreceptor Trg, which mediates tactic response to ribose by recognition of sugar-occupied, ribose-binding protein, do not respond to ribose (19), and the duration of the response is reduced significantly if cells contain an altered version of Trg defective in recognition or signaling (16, 20). We tested strains harboring the cysteine-containing Trg proteins shown in Fig. 2 for sensitivity to stimulation by a concentration of ribose that evoked, for the wild-type receptor, a response of approximately half-maximal duration. Experiments were done with parallel samples of cells, one oxidized and the other mock-treated, and the extent of cross-linking was determined for each sample (Table 1). For control cells containing Trg-C23S, a receptor lacking cysteine, neither oxidation treatment reduced the observed response. Cross-links between four cysteine pairs, 32–32′, 39–39′, 46–46′, and 82–82′, which joined the subunits of the receptor dimer (Fig. 1), resulted in no significant reduction in response, but cross-links between two cysteine pairs, 42–202 and 42–203, which joined segments within a subunit (Fig. 1), reduced receptor function dramatically (Table 1). Because the six experimental strains were isogenic except for the specific cysteine codons and yet oxidation reduced response to ribose for only two strains, it seemed that the defective responses were consequences of the specific cross-links rather than of general effects of oxidation on Trg at residues other than cysteines or on other components of the chemosensory and motility systems. If that were the case, all strains should have been equally affected.

We pursued this issue by testing oxidized and mock-treated cells for response to aspartate, a chemoattractant recognized by an independent chemoreceptor, Tar, which lacks cysteine. For all six strains, the relevant oxidation treatment caused no significant reduction in response. Thus the dramatically reduced responses to ribose seen for oxidized cells containing Trg-F42C, M202C or Trg-F42C, F203C were specific for the constraints introduced by the cross-links. Oxidation treatments resulted in modest (10–30%) increases in the duration of the response to aspartate or, in one case (for cells containing Trg-A82C), a 2-fold increase. We presume that these increases reflected subtle perturbations, caused by oxidation, that would affect all tactic responses in those cells, and thus for each strain in matched experiments, we expressed the activity of a cross-linked receptor as the ratio of the response mediated by the cross-linked protein to the response mediated by the same protein in the absence of cross-linking and normalized the relative response to ribose to the comparable relative response to aspartate, producing the values shown in Fig. 3. These values represent specific effects of the particular disulfide cross-links in Trg on receptor function. Because of the multiple steps involved in calculating the normalized ratios, no error bars are shown.

For Trg-A82C and, to a lesser extent, Trg-F32C, cross-linking increased normalized response time. However, before cross-linking, those cysteine-containing receptors mediated reduced responses, ≈20% and 45%, respectively, of the duration mediated by the control protein devoid of cysteine. The reduced activity of Trg-A82C has been described (20). Responses mediated by the two receptors were increased by cross-linking but were still less than the responses mediated by the control protein. We surmise that the presence of cysteine at position 32 or 82 deformed the receptor sufficiently to reduce activity and that creation of the respective disulfide bonds significantly counteracted or corrected those deformations. The 82–82′ cross-link also altered the steady-state balance between CCW and CW rotation of the flagellar motor. We had determined the effects of oxidation treatments on patterns of rotation as a way to assess possible general effects on the sensory and motility systems. In general, oxidation resulted in little change or a moderate shift (up to 30%) toward a CCW bias (see *Materials and Methods*). The exception was the strain containing Trg-A82C. After treatment with Cu-phenanthroline, >80% of cells exhibited CCW-biased rota-

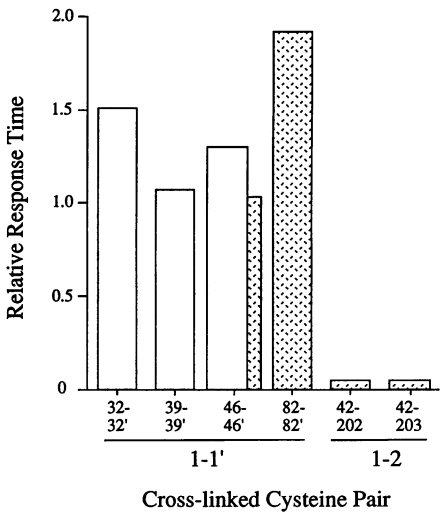

FIG. 3. Effects of cross-links on receptor function. Responses to a temporal gradient from 0 to 0.5 μM ribose of tethered cells containing Trg proteins cross-linked by disulfides between the indicated cysteine pairs are displayed as ratios of the response mediated by the cross-linked protein to the response mediated by the same protein without the cross-link, normalized to the same ratio for response to aspartate, an attractant recognized by the independent receptor Tar. Open and stippled bars represent experiments in which the oxidation treatment was with molecular iodine or Cu-phenanthroline, respectively.

tion, 34% rotating exclusively CCW. The specificity of this shift implied that it was the consequence of the particular position of the cross-link. Because residue 82 of Trg is within the region corresponding to the ligand-binding site of Tar_s (5), it may be that the cross-link brings $\alpha 1$ and $\alpha 1'$ slightly closer together, as does aspartate binding to Tar_s, and the static, rather than dynamic, nature of this shift results in a subtle, but persistent, change in the signaling state of the receptor, as has been observed for chemoreceptor Tsr as the result of mutational substitutions in the comparable region of the receptor structure (23).

We determined dose–response relationships for the two cysteine-containing receptors in which disulfide cross-links had dramatic effects (Fig. 4). In the absence of cross-links, these relationships were much like those observed for wild-type receptors (16, 19, 20). This result indicates that even though the two altered receptors each differed from the wild type by three amino acid replacements, function was little perturbed. However, once cross-linked, the proteins were essentially inactive, even when stimulated with ribose at concentrations that evoked maximal response of cells containing receptors devoid of cross-links.

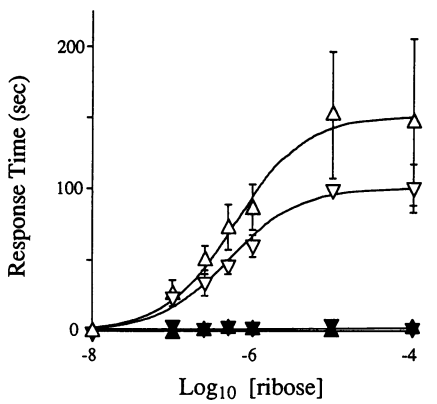

FIG. 4. Effects of cross-links on dose–response relationships. Responses to temporal gradients from 0 to the indicated ribose concentrations of tethered cells containing Trg-F42C + M202C (▽) or Trg-F42C + F203C (△) after mock treatment (open symbols) or oxidation treatment with Cu-phenanthroline (solid symbols). The data are averages of two independent experiments; SEMs are indicated by error bars for those points for which values exceeded the symbol size.

DISCUSSION

Assessing Transmembrane Signaling *in Vivo*. The fundamental activity of a sensory receptor is transmembrane signaling, but little is known about the molecular mechanisms of this process. We found that transmembrane signaling by a chemoreceptor of *E. coli* required significant movement between the transmembrane segments within a receptor monomer but not between the central, interacting segments of the two subunits (Fig. 5). This observation is likely to be relevant to mechanisms of signaling by related chemotactic and phototactic receptors found in a wide variety of bacteria (24) and by the large family of related transmembrane environmental sensors found in prokaryotes (1, 3) and eukaryotes (25). A special feature of this work was that movement-constraining cross-links were created in a transmembrane receptor without removing the protein from its natural environment and without disrupting significantly the general sensory behavior of the treated cells. This approach is a biochemical analog of mutational analysis. The consequences of specific and defined changes in a single protein were assessed by characterizing intact, functioning cells. The changes were not genetic alterations that introduced alternative side chains but rather were chemical couplings of specific positions that limited relative

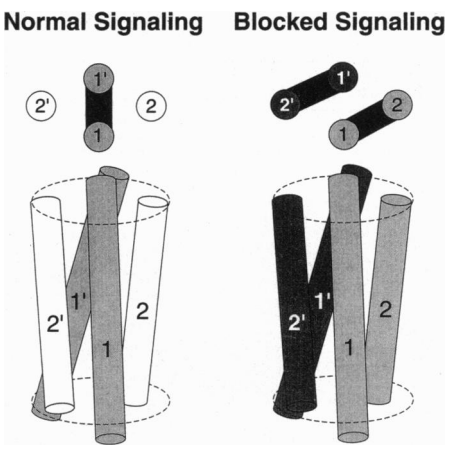

FIG. 5. Consequences of restricting movement between helical pairs in the transmembrane domain. The results of this study are summarized by using diagrams of the deduced, 3-dimensional structure of the transmembrane domain of chemoreceptor Trg (4). The upper and lower diagrams show, respectively, views of the four transmembrane helices from the periplasm (see Fig. 1*B*) and in the plane of the membrane. Helical pairs restricted in their relative movement by disulfide cross-links are indicated by common shading in both diagrams and by connecting bars in the upper diagrams.

movement between elements of secondary structure. What are the constraints imposed by this specific chemical coupling? The distances between alpha carbons of disulfides in proteins with known, high-resolution structures range from 3.8 to 6.8 Å (26). Thus relative movement of the alpha carbons of a cross-linked cysteine pair, and correspondingly of the units of secondary structure in which they are contained, would be no more than a few Å.

Cross-links between cysteines in the two subunits should prohibit dissociation of the receptor dimer into independent monomers. Four of the six disulfides that formed extensively *in vivo* joined the two subunits, yet each of these cross-linked receptors was effective in mediating sensory response and thus was not significantly perturbed for transmembrane signaling. Studies *in vitro* have indicated that the predominant form of the chemoreceptors is dimeric, no matter what the sensory state (5, 14). Our work extends the *in vitro* observations and demonstrates clearly that chemoreceptors constrained to remain as dimers *in vivo* are fully functional.

Features of Conformational Signaling. Constraint by disulfide cross-links at any one of four positions along the central axis of subunit interaction (Fig. 1) caused little perturbation of receptor function *in vivo*; thus little movement along this interface is required in the process of signaling, and presumably little movement occurs even in the unconstrained receptor. Any particular cross-linked position might have been near the fulcrum of an otherwise substantial movement between the central helices, but the range of positions at which constraining cross-links did not perturb receptor activity (Fig. 1*A*) implies that functionally significant movement between the TM1 and TM1′ helices, as well as between their periplasmic extensions $\alpha 1$ and $\alpha 1'$, is no greater than the few Å allowed by a disulfide bond. In contrast, cross-links in the transmembrane domain between the two segments of the same subunit made the receptor inactive. The evidence indicates that inactivation was the result of restrictions on movement, rather than trapping of the protein in a distorted state. As noted above, the sulfhydryls of the cross-linked pairs used in these studies are positioned near each other in the three-dimensional structure of the receptor and thus should be formed with minimal distortion. All six pairs formed disulfides rapidly and extensively *in vitro* (4) and *in vivo* (this study). Disulfide cross-links between distant sulfhydryls that are thought to trap chemoreceptors in

distorted, inactive conformations occur more slowly than those that link nearby sulfhydryls and leave the receptor active (12, 13). In the current study, 6 cysteine pairs out of 70 combinations exhibited extensive cross-linking *in vivo*, but only 2 cross-links of the 6 blocked receptor function.

The features we have identified for conformational signaling by Trg can be related to other observations. The crystallographically determined structures of the periplasmic domain of Tar_s with or without bound ligand differed by only a 4° rotation and a 1.4-Å displacement of the two subunits along the $\alpha 1$–$\alpha 1'$ axis (6, 14). Such a subtle movement would be predicted in the presence of a constraining intersubunit cross-link; in fact, the two orientations occurred in crystals of protein containing a disulfide cross-link between positions analogous to 46–46′ in Trg (6), as well as in protein devoid of cross-links (14). A truncated form of Tar_s, consisting of a dimeric periplasmic domain and monomeric transmembrane and cytoplasmic domains, exhibited transmembrane signaling in an *in vitro* methylation assay (27), an observation consistent with our conclusion that the substantial conformational change of transmembrane signaling occurs within a subunit. Genetic analysis by cysteine scanning of the transmembrane segments of Trg indicated that signaling involved movement between the two transmembrane segments within a subunit and was optimal when stable interactions were maintained across the interface between the subunits (28). Cysteine-containing forms of Tar_s, cross-linked by disulfides along the subunit interface within the ligand-binding region or in or near the transmembrane domain, exhibited signaling activity in an *in vitro* methylation assay (8, 9, 12) as well as in an *in vitro* phosphorylation assay (S. A. Chervitz and J. J. Falke, personal communication), observations consistent with our characterization *in vivo* of cross-linked forms of Trg. In addition, ^{19}F-NMR studies of the periplasmic domain of Tar_s have provided indications of ligand-induced movements in helix $\alpha 4$, the periplasmic extension of TM2, but not in $\alpha 1$, the extension of TM1 (29). In summary, the published observations relevant to the issue of conformational signaling by the transmembrane and periplasmic domains of bacterial chemoreceptors are consistent with or support our conclusion that transmembrane signaling involves significant movement within a subunit and little movement in the interface between the subunits. Movement between specific transmembrane helices combined with stable interactions between others may be a central feature in transmembrane signaling by a wide range of transmembrane receptors.

This work was submitted in partial fulfillment of the requirements for a Ph.D. in Biochemistry at Washington State University by G.F.L. This study was supported by Grant GM29963 from the National Institutes of Health (to G.L.H.). G.F.L. was supported in part by Biotechnology Training Grant T32 GM08336 from the National Institutes of Health, and M.R.L. was supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinshaft.

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