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Adaptational modification and ligand occupancy have opposite effects on positioning of the transmembrane signalling helix of a chemoreceptor

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Summary

Sensory systems adapt to persistent stimulation. In the transmembrane receptors of bacterial chemotaxis, adaptation is mediated by methylation at specific glutamyl residues in the cytoplasmic domain. Methylation counteracts effects of ligand binding on functional activities of that domain. Both ligand binding and adaptational modification are thought to act through conformational changes. As characterized for *Escherichia coli* chemoreceptors, a mechanistically crucial feature of the ligand-induced conformational change is piston sliding towards the cytoplasm of a signalling helix in the periplasmic/transmembrane domain. Adaptational modification could counteract this signalling movement by blocking its influence on the cytoplasmic domain or by reversing it. To investigate, we characterized effects of adaptational modification on the position of the signalling helix in chemoreceptor Trg using rates of disulphide formation between introduced cysteines. We utilized an intact cell procedure in which receptors were in their native, functional state. *In vivo* rates of disulphide formation between diagnostic cysteine pairs spanning a signalling helix interface changed as a function of adaptational modification. Strikingly, those changes were opposite those caused by ligand occupancy for each diagnostic pair tested. This suggests that adaptational modification resets the receptor complex to its null state by reversal of the conformational change generated by ligand binding.

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Introduction

Sensory systems adapt to persistent stimulation, restoring a null state even though stimulation persists. This process creates sensitivity to changes in the level of stimulation, rather than simply to the absolute magnitude, and can provide a wide dynamic range of sensing and response. Adaptation in several systems has been linked to covalent modification of a transmembrane receptor protein, for instance the large family of seven transmembrane receptors (Kohout and Lefkowitz, 2003). However, the structural changes by which adaptational modifications restore a null state are little understood. In the current study, we investigated effects of adaptational modification on a structural signalling element in a bacterial chemoreceptor.

Chemoreceptors are central components in the sensory system that mediates bacterial chemotaxis (Fig. 1A). This system has been extensively characterized on the molecular and mechanistic level, particularly in *Escherichia coli* and its relatives (see Hazelbauer, 2004 for an overview; Parkinson *et al*., 2005 for a recent review). Studies in those species have shown that transmembrane chemoreceptors form signalling complexes with an autophosphorylating histidine kinase CheA and a coupling protein CheW. CheA phosphohistidine is the source of aspartyl phosphorylation of response regulator CheY. Phospho-CheY binds to the flagellar rotary motor, causing rotational reversal. These reversals result in tumbles, which change the direction of swimming. The sensory system directs cells towards favourable environments by modulating kinase activity, thus controlling the probability of directional changes.

Chemoreceptors in signalling complexes modulate kinase activity as a function of ligand occupancy and adaptational covalent modification. Attractant occupancy in a ligand-binding site reduces kinase activity and modification increases it (Fig. 1B). The covalent modification is methylation, at four to six glutamyl residues in the receptor cytoplasmic domain. Methylation is catalysed by methyltransferase CheR, demethylation by methylesterase CheB. The esterase has a response regulator domain that acquires a phosphoryl group from phospho-CheA and thus activates the enzyme. Two receptor modification sites are synthesized as glutamines, which are subse-

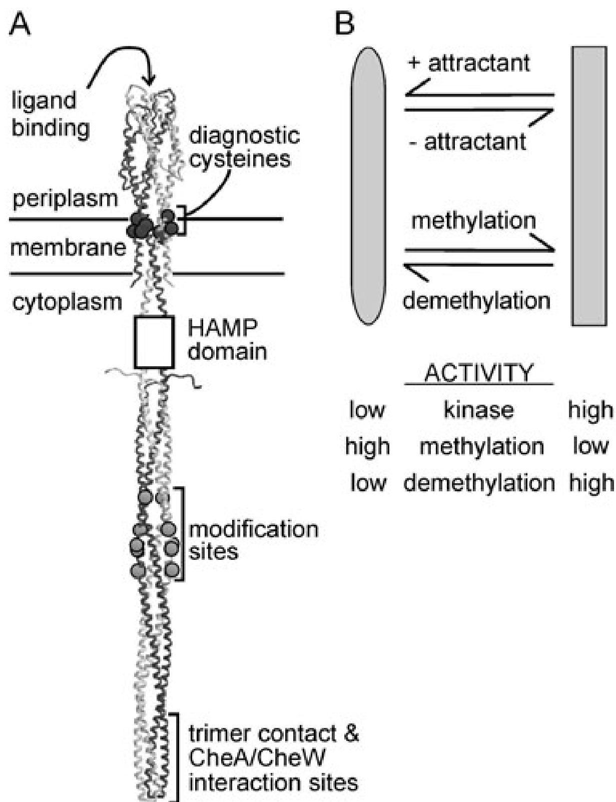

Fig. 1. Chemoreceptor structure and conformational equilibrium.
A. Ribbon diagram of the structure and transmembrane disposition of a modelled chemoreceptor homodimer (Kim *et al*., 1999) indicating positions of diagnostic cysteine pairs and functional sites. As the structure of the HAMP domain is unknown but may not be completely helical, it is represented as a blank box.
B. The postulated equilibrium between receptor conformations and the activities of those conformations.

quently deamidated by CheB to create methyl-accepting glutamates. Glutamines at methyl-accepting sites are functional mimics of methyl glutamates (Park *et al*., 1990; Dunten and Koshland, 1991; Borkovich *et al*., 1992).

In unstimulated cells, equal rates of methylation and demethylation maintain a dynamic level of partial methylation among the multiple sites, creating intermediate kinase activation. Attractant binding to the receptor periplasmic domain initiates transmembrane signalling that reduces kinase activation by the cytoplasmic domain. Reduced kinase activity lowers cellular phospho-CheY and phospho-CheB. The transmembrane signal also enhances the propensity of the receptor for methylation and reduces propensity for demethylation (Fig. 1B). Together these changes increase receptor methylation until modification balances ligand occupancy, restoring null-state kinase activity and modification propensities. The balance is an adapted state poised to respond to new changes in attractant concentration.

The basic structural unit of a chemoreceptor is a homodimer, organized in large part as extended helical bundles (Fig. 1A). The periplasmic, ligand-binding domain has two four-helix bundles, one from each monomer (Milburn *et al*., 1991). In each bundle, two helices are longer: α1 extends from the first transmembrane helix, TM1, and α4 extends into the membrane as TM2. The transmembrane domain is a loose bundle of two TM1's and two TM2's in which TM1 and TM1′ interact along a packing face between the two subunits and TM2 and TM2′ interact primarily with the other helix in their respective subunits (Pakula and Simon, 1992; Lee *et al*., 1994). As each TM2 exits the membrane into the cytoplasm it is joined to a HAMP domain, a unit thought to contain non-helical structure (Butler and Falke, 1998; Bordignon *et al*., 2005). Distal to the HAMP domain is an extended helical coiled coil (Kim *et al*., 1999). Methyl-accepting glutamates are approximately mid-way between the membrane and the tip of the cytoplasmic domain.

How does ligand binding in the periplasmic domain alter the activities of the cytoplasmic domain? The coupling is thought to be conformational. A large body of data indicates that a mechanistically crucial feature of the conformational change induced by attractant binding is a piston sliding of the α4/TM2 helix towards the cytoplasm (Falke and Hazelbauer, 2001). Thus α4/TM2 is termed a signalling helix. Central to identification of this role were multiple studies that utilized formation of interhelical disulphides between introduced cysteines. Signalling was blocked by disulphides that restricted movement along the α4/TM2–α1/TM1 interface in the same subunit of the receptor dimer, but not by disulphides that restricted movement of α1/TM1–α1′/TM1′ along the subunit interface. This difference identified the helical interface within a subunit as one that needed to shift in conformational signalling and the interface between the subunits as one that did not need to shift beyond the limits of a disulphide bond (Falke and Koshland, 1987; Scott and Stoddard, 1994; Chervitz and Falke, 1995; 1996; Chervitz *et al*., 1995; Lee *et al*., 1995). Multiple lines of evidence indicated that the specific conformational change in the periplasmic and transmembrane domains involved a piston sliding of α4/TM2 towards the cytoplasm relative to a static α1/TM1–α1′/TM1′ interface (Falke and Hazelbauer, 2001). This evidence included the locking of receptor in one or the other extreme of signalling by disulphides that shifted the α4/TM2–α1/TM1 interface (Chervitz and Falke, 1995), analysis of crystal structures of a ligand binding domain with and without bound ligand (Chervitz and Falke, 1995; 1996), changes in spin–spin interactions between spin labels on introduced cysteines (Ottemann *et al*., 1999) and the pattern of ligand-induced changes in rates of disulphide formation between diagnostic cysteine pairs that spanned the α4/TM2–α1/TM1 interface (Hughson and Hazelbauer, 1996; Beel and Hazelbauer, 2001).

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Adaptational methylation counteracts and balances effects of attractant binding on chemoreceptor activity. The mechanism by which modification counteracts effects of the piston movement of the signalling helix is unknown. It might act by blocking or uncoupling its influence on the cytoplasmic domain or by reversing the movement. To address this issue, we utilized the strategy of disulphide formation between diagnostic cysteine pairs that we had used to identify the ligand-induced movement of the signalling helix in chemoreceptor Trg (Hughson and Hazelbauer, 1996; Beel and Hazelbauer, 2001).

Results

Monitoring effects of adaptational modification on the position of the signalling helix

Does adaptational modification in the chemoreceptor cytoplasmic domain alter the position of the signalling helix in the periplasmic/transmembrane domain relative to the static interface between the subunits of the receptor dimer? Such an alteration could be as subtle as the piston sliding of the signalling helix induced by ligand occupancy (Falke and Hazelbauer, 2001; Peach *et al.*, 2002) and thus it was important to examine receptors in their native state in intact cells. We utilized conditions in which disulphide cross-links can be catalysed in cellular chemoreceptors without significantly perturbing flagellar rotation or control of rotation by the chemosensory system (Lee *et al.*, 1995). In addition, we used cells in which Trg was expressed at a physiological level that preserved receptor localization (Maddock and Shapiro, 1993; Lybarger *et al.*, 2005) and allowed formation of signalling complexes with CheA and CheW (Beel and Hazelbauer, 2001). Previous work had surveyed 14 forms of double-cysteine Trg that exhibited detectable α1/TM1–α4/TM2 cross-links upon exposure to an oxidation catalyst at a concentration that preserved a functional sensory system, and identified four that showed sufficiently high rates of disulphide formation to serve as diagnostic tests of the relative positioning of the two helices (Hughson and Hazelbauer, 1996; Beel and Hazelbauer, 2001). These were at positions 38-202, 38-203, 42-202 and 42-203. As would be expected, these positions were not buried in the hydrophobic core of the lipid bilayer nor near the reducing environment of the cytoplasm. Instead, they bracketed the hydrophobic–hydrophilic boundary of membrane and periplasm (Fig. 1); position 38 is in the lipid bilayer and 42 in the periplasm (Boldog and Hazelbauer, 2004). Each pair forms an intrachain disulphide but no interchain disulphides when intact cells are treated with copper(II)-(*o*-phenanthroline)$_3$ and each reaction rate is differentially altered by ligand-induced transmembrane signalling (Hughson and Hazelbauer, 1996; Beel and Hazelbauer, 2001).

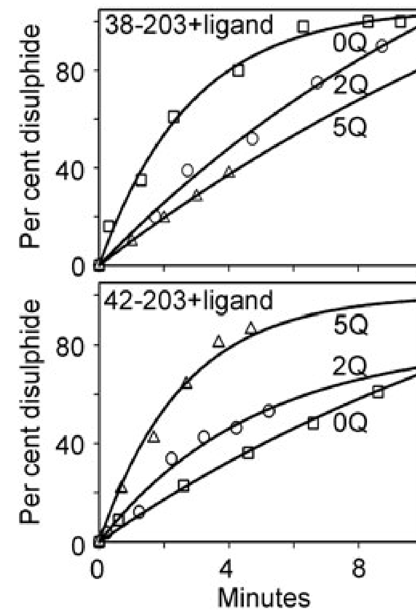

Fig. 2. Time-courses of *in vivo* disulphide formation between diagnostic cysteine pairs. At 0 min, copper(II)-(*o*-phenanthroline)$_3$ was mixed with suspensions of cells containing forms of Trg with the indicated diagnostic cysteine pair and the indicated number of glutamines at sites of adaptational modification, samples taken and immunoblots analysed for extent of disulphide formation. In the examples shown, the cell suspensions contained a saturating concentration of the Trg-linked attractant ribose. Points for different proteins are not necessarily at the same time, reflecting different numbers or times of sampling. Curves are fits to data as described (*Experimental procedures*).

Trg has five methyl-accepting sites (Nowlin *et al.*, 1987; 1988). We created receptors containing one of the four cysteine pairs and 0, 2 or 5 glutamines, the methyl-ester mimic, at the five sites. Each engineered receptor mediated response of otherwise chemotactically wild-type cells to Trg-recognized stimuli, as determined by response to a spatial gradient in the semisolid agar plate assay (Feng *et al.*, 1999) (data not shown). These responses showed that these receptors were functional in a cellular environment in which the modification enzymes could adjust modification at the methyl-accepting sites but the receptors carried the respective diagnostic cysteine pairs. Time-courses of disulphide formation after addition of the oxidation catalyst copper(II)-(*o*-phenanthroline)$_3$ to cell suspensions were determined for each cysteine pair, each level of modification and in the absence or presence of a saturating concentration of the Trg-linked attractant ribose. Representative time-courses are shown in Fig. 2. Rate constants for disulphide formation were determined from such data by curve fitting that considered competing oxidation reactions (Careaga and Falke, 1992). Average, normalized rate constants are displayed in Fig. 3 as a function of receptor modification. We calculated effects on disulphide formation of ligand binding at each level of

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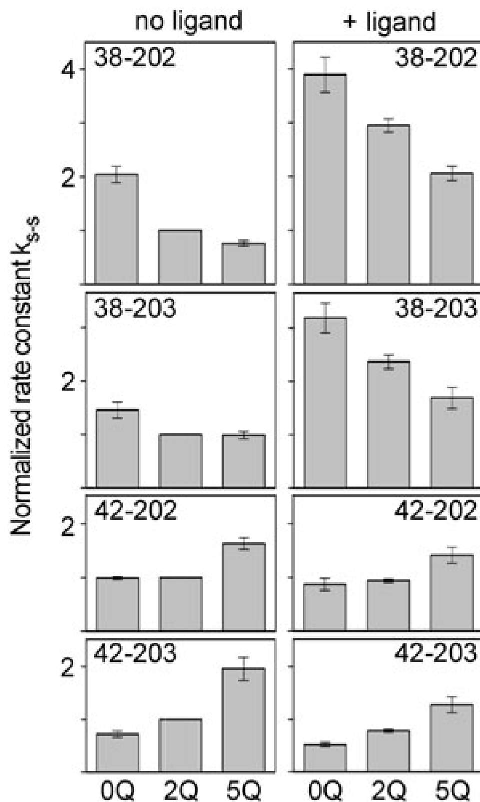

Fig. 3. Rate constants of disulphide formation as a function of diagnostic cysteine pair, receptor modification and ligand. Rate constants (k_{S-S}) were derived (*Experimental procedures*) from data like that in Fig. 2. Values are averages of constants determined in at least five independent experiments, normalized for each diagnostic pair to the value for receptor with two glutamines (2Q) in the absence of ligand. Error bars are standard errors of the mean.

modification (Fig. 4A) and maximal modification in the absence or presence of ligand (Fig. 4B).

Adaptational modification affects the signalling helix

Rate constants of disulphide formation for all four cysteine pairs were altered by changing the extent of receptor modification from 0 to 5 glutamines (Figs 3 and 4B). Rate constants for two-glutamine forms were intermediate or corresponded to the unmodified or fully modified form (Fig. 3). Ligand occupancy changed disulphide rates at all three levels of receptor modification (Fig. 4A), indicating that Trg carrying each combination of cysteines and modification performed transmembrane signalling. For diagnostic pairs 38-202 and 38-203, increased modification (more glutamines) decreased the rate constants of disulphide formation; for 42-202 and 42-203 more glutamines increased the rate constants (Figs 3 and 4B). Strikingly, these effects were opposite those of ligand occupancy (Hughson and Hazelbauer, 1996; Beel and Hazelbauer, 2001) as illustrated in Fig. 4A and B. Disulphide rates enhanced by ligand occupancy were diminished by modification and rates diminished by occupancy were enhanced by modification.

Full appreciation of the data in Figs 3 and 4 requires consideration of effects of individual cysteine substitutions on receptor signalling. *In vivo* signalling assays indicate that cysteines at position 38 or 202 have, respectively, little or no effect on the signalling state of Trg but 203C mimics repellent stimulation and 42C strongly mimics attractant stimulation (Lee *et al.*, 1995). These individual effects are consistent with *in vitro* kinase activation by Trg carrying the respective cysteine pairs (Fig. 5) and provide bases for understanding specific differences among patterns for different pairs. Trg 38C-202C, which is in the same signalling state as wild-type receptor (Fig. 5), exhibited step decreases in disulphide rate constants as glutamines increased from zero to two to five, in the absence or presence of ligand (Fig. 3). In addition, at each level of modification, ligand occupancy increased

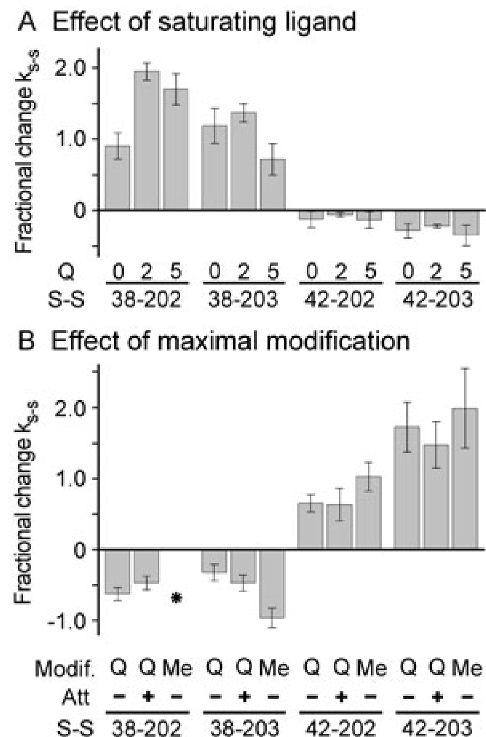

Fig. 4. Effects of saturating ligand and maximal modification on rate constants of disulphide formation. Fractional changes in rate constants of disulphide formation (k_{S-S}) were calculated from the data in Fig. 3 for (A) effects of a saturating concentration of ribose at each level of adaptational modification and (B) effects of maximal modification, either glutamines (Q) or methyl-esters (Me), in the absence (–) or presence (+) of a saturating concentration of ribose. Fractional change was calculated using the relationship: $(k^{alteration} - k^{initial})/k^{initial}$. The asterisk indicates that for 38-202 with maximal methylation the change in rate constant was negative but technical problems prohibited quantification (see text). Error bars are standard errors of the mean.

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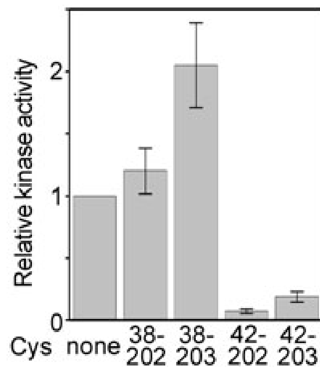

Fig. 5. Kinase activation *in vitro*. Forms of Trg containing the indicated cysteines and two glutamines, at the two sites of adaptational modification designated by wild-type *trg*, were tested for ability to activate the kinase activity of CheA in standard assay conditions. In those conditions, essentially no disulphides form between any diagnostic pair, so the data are for cysteine-substituted but not cross-linked receptors. In analysing each experiment, kinase activities were normalized to those generated by Trg with no cysteines. Values are averages of duplicate determinations on at least three independent membrane preparations. Error bars are standard errors of the mean.

the propensity for disulphide formation (Fig. 4A). For Trg 38-203 the repellent-mimicking properties of the 203C substitution shifted the receptor towards the kinase-activating conformation also favoured by adaptational modification (Fig. 5). In the absence of ligand, adding two modifications reduced cross-linking between 38 and 203 but adding more modifications had no additional effect (Fig. 3), implying that the combination of the repellent-mimicking 203C substitution and two glutamines placed the receptor close to one extreme of the conformational equilibrium (Fig. 1B) and thus additional glutamines had little additional effect. Consistent with this interpretation, saturating attractant shifted Trg away from that extreme and thus progression from zero to two to five glutamines resulted in step decreases in propensity for disulphide formation (Fig. 3).

Cysteine at position 42 shifts the receptor equilibrium strongly towards a signalling (Lee *et al*., 1995), kinase-inhibiting (Fig. 5) conformation. This strong shift meant that ligand binding, which shifts the equilibrium in the same direction, had little additional effect on conformation of Trg with cysteines at 42 and 202 (Figs 3 and 4A). In addition, propensity for disulphide formation for this receptor was not increased by addition of two glutamines but only by five, implying that the strong effect of 42C in this receptor required more than two modifications to shift the receptor equilibrium detectably (Fig. 3). Combining 42C with 203C, which shifts the receptor towards the kinase-activating conformation (Fig. 5), resulted in a receptor for which each step of modification increased cross-linking propensity and for which attractant occupancy reduced cross-linking at each level of modification (Fig. 3). These patterns implied that the counteracting effect of 203C on the conformational equilibrium of a 42C-containing receptor was sufficient to poise Trg 42C-203C in a state in which each combination of modification and attractant occupancy could discernibly shift the conformational equilibrium.

Glutamines are useful mimics of methyl esters at sites of adaptational modification, but it was important to determine directly the effects of methylation on the positioning α1/TM1–α4/TM2. It is not possible to control the specific extent or positions of receptor methylation, but we were able to create a cellular condition in which a significant proportion of Trg was maximally methylated (see *Experimental procedures*) and determine rate constants of disulphide formation for this receptor species. *In vivo*, maximal methylation altered rate constants in the same pattern observed for glutamines: decreased for 38-202 and 38-203; increased for 42-202 and 42-203. These changes could be quantified for all but 38-202, for which the decrease was substantial as seen by the reduced rate of appearance of the disulphide cross-linked form, but incompletely methylated forms of the receptor interfered with quantification (see *Experimental procedures*). Figure 4B shows the fractional change in rate constant of disulphide formation for the other pairs in five-methyl Trg, compared with rates for 5Q and unmodified receptor. Effects of methylation were to some extent greater than for amidation, consistent with previous comparisons of methyl esters and amides at modification sites (Park *et al*., 1990; Borkovich *et al*., 1992). The less extensive effect of amidation explains the data for 38-202 and 38-203 (Fig. 3) in which increased amidation reduced the rate constant of disulphide formation for ligand-occupied receptor but did not restore it to the value for unoccupied receptor.

Discussion

We used disulphide formation between diagnostic cysteine pairs to investigate *in vivo* the effects of adaptational modification on the position of the chemoreceptor signalling helix. Rates of disulphide cross-linking were increased or decreased for all four diagnostic pairs, indicating that modification at methyl-accepting sites in the cytoplasmic domain changed the position of the signalling helix in the distant periplasmic and transmembrane domains. Strikingly, adaptational modification had the opposite effect from ligand occupancy at every diagnostic position. Ligand occupancy generates a piston sliding of the signalling helix towards the cytoplasm (Falke and Hazelbauer, 2001). The opposite effects of adaptational modification imply that the signalling helix slides in the opposite direction. Thus adaptation appears to reverse the piston movement induced by ligand binding. This sug-

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gests that the mechanism by which adaptational modification resets the receptor complex to its null state is a reversal of the conformational change generated by ligand binding.

Identifying the conformational change of adaptation

The data in Figs 3 and 4 demonstrate that adaptational modification changed rates of disulphide cross-linking across the interface between signalling helix α4/TM2 and its neighbour α1/TM1. Rates of oxidative cross-linking between cysteine sulphhydryls can be altered by changes in one or more parameters: distance, orientation, local chemical environment or dynamics. For cysteines located in or near the membrane on the four-helix receptor structure, the first three changes would likely occur through relative movement of helices and the fourth by modulation of such movements. As α1/TM1 is part of the stable central axis of subunit interaction in the receptor dimer (Falke and Hazelbauer, 2001) and thus is unlikely to move, the movement(s) that caused changes in disulphide formation across the interface between α1/TM1 and α4/TM2 were likely to involve movements of the latter helix. Any such movement, whether direct or involving dynamics, could be described by reference to four basic motions: (i) normal or (ii) parallel to the plane of helical interaction; (iii) helical rotation or (iv) piston sliding along a long helical axis. Could one of these motions be the primary contributor to the pattern of changes we observed as the receptor was modified? A movement of α4/TM2 towards or away from α1/TM1 either normal or parallel to the plane of interaction would be expected to increase or decrease rates of disulphide formation for all four diagnostic pairs, but instead two rates increased and the other two decreased (Figs 3 and 4). A variation on these movements, tilting of α4/TM2 relative to α1/TM1 could result in differential effects on pairs that were separated along the long axis of the helices, but the four diagnostic pairs are very near one another. Thus possibilities 1, 2 or the tilting variation do not provide a satisfying explanation of the data (Fig. 6A). The same is true of helical rotation. As seen in Fig. 6A, a rotation of α4/TM2 that would move position 202 farther from position 38, and thus decrease disulphide formation. However, this rotation would also move 202 farther from position 42, yet the data show an increase in the 42-202 cross-linking rate with increased modification. The same contradiction occurs for the 38-203 and 42-203 pairs. In contrast, a piston sliding towards the periplasm of α4/TM2 along its long axis relative to α1/TM1 can account for all of the data (Fig. 6B). Thus we conclude that the signalling helix α4/TM2 is moved by adaptational modification as diagrammed in Fig. 6B. Of course, effects on rates of disulphide formation do

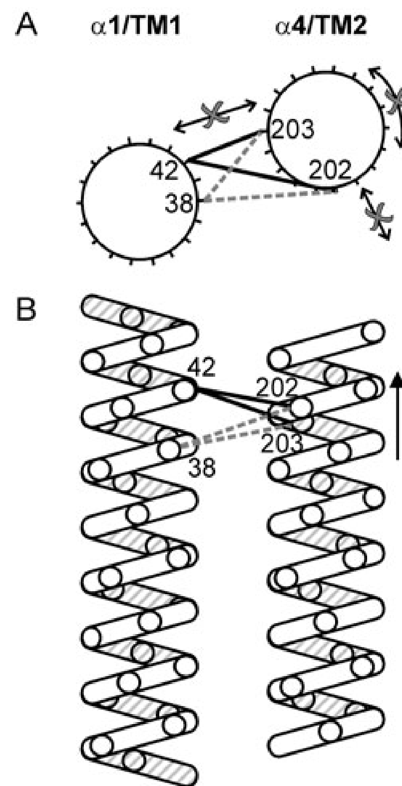

Fig. 6. Alternative explanations for effect of adaptational modification on disulphide formation. Diagrams of the helices of one subunit in a Trg dimer are shown normal (A) and parallel (B) to the cytoplasmic membrane. Orientations of helices and residues are based on cross-linking moments (Lee and Hazelbauer, 1995). Positions of introduced cysteines are indicated by residue numbers and disulphides between diagnostic cysteine pairs by solid lines (rate constant increased by adaptational modification) or dashed lines (rate constant decreased by adaptational modification). Arrows indicate possible modes of motion between the helices (see text). Those covered by an 'X' cannot account for the data.

not provide information about the molecular details of this movement, but with the data available the best model of the adaptational conformational change is this simple piston movement. In any case, it is interesting to note that the membrane-distal end of the signalling helix is positioned near the ligand-binding site, where binding initiates its piston movement. An opposite sliding induced by adaptational modification could thus alter affinity for ligand. Such changes have been observed for apparent affinities determined by the relationship of ligand concentration to kinase activity for signalling complexes *in vitro* (Bornhorst and Falke, 2000; Li and Weis, 2000).

Taken together, data from this study and from studies of the effects of ligand occupancy (Beel and Hazelbauer, 2001; Falke and Hazelbauer, 2001) suggest a simple model for ligand-induced signalling and the compensatory effect of adaptation (Fig. 7C): an increase in ligand occupancy slides the signalling helix towards the cytoplasm

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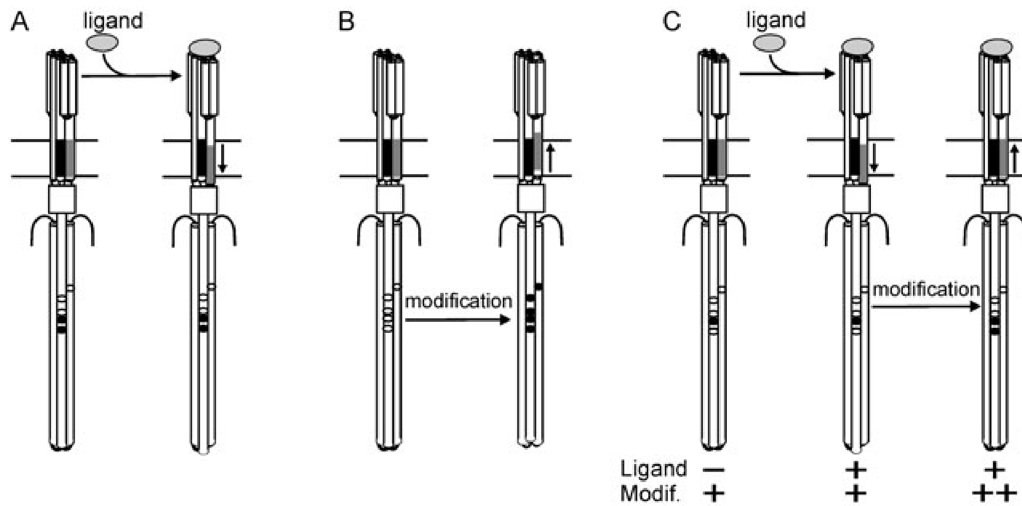

Fig. 7. Opposite and opposing effects of adaptational modification and ligand binding on the signalling helix. Cartoons of receptor homodimers diagram effects of ligand occupancy (A) and adaptational modification (B) on the position of signalling helix α4/TM2 relative to static helix α1/TM1 and the sequence of attractant-induced signalling and adaptation (C). TM1 is shaded black and TM2 grey. Arrows indicate the direction of the piston movement of the signalling helix.

and adaptational modification moves that helix back to its unstimulated position, restoring the null state of conformational signalling. In this model adaptation is not a blocking or uncoupling of the influence of the transmembrane conformational change on the cytoplasmic domain, but a direct reversal of the primary conformational effect of ligand binding. For both ligand-induced signalling and adaptation, effects on conformation can be considered a shift in the equilibrium between the two receptor states diagrammed in Fig. 1B. Thus the low-kinase-activity state would have the signalling helix shifted most extremely towards the cytoplasm (Fig. 7A) and the high-kinase state would have it most extremely towards the periplasm (Fig. 7B). There could be structural limits on the extent to which the signalling helix slides. As discussed in the detailed consideration of the data in Fig. 3, indications of such limits are provided by minimal effects of increased adaptational modification of receptors shifted towards one or the other conformational extreme by specific amino acid substitutions.

Effects of adaptational modification beyond the signalling helix

The work reported here did not address effects of adaptational modification on the conformation of the cytoplasmic domain. Both ligand binding in the periplasmic domain and modification at sites in the cytoplasmic domain alter the activities and thus presumably the conformation of the cytoplasmic domain. The ligand-induced conformational change may be different in the cytoplasmic domain than in the periplasmic and transmembrane domains (Falke and Hazelbauer, 2001). If this were the case, then the HAMP domain could function to transform the piston movement of the signalling helix into the alternative conformational change. Whatever the details, our data indicate that adaptational modification in the cytoplasmic domain alters the transmembrane/periplasmic domain. Thus conformational transformations mediated by the HAMP domain must be bidirectional, allowing modification-induced changes in the cytoplasmic domain to create the piston sliding of the signalling helix.

The initial conformational change induced by ligand binding is the piston movement of a signalling helix within a subunit of a receptor homodimer (Falke and Hazelbauer, 2001). Thus in our studies of structural consequences of adaptational modification, we focused on the position of that helix within a subunit. However, receptor homodimers can form higher order structures, trimers of dimers (Kim *et al*., 1999; Francis *et al*., 2004; Studdert and Parkinson, 2004), hedgerows (Park *et al*., 2006) and clusters (Maddock and Shapiro, 1993; Bray *et al*., 1998; Weis *et al*., 2003). Higher order organization influences ligand-induced signalling (Sourjik and Berg, 2002; Sourjik, 2004) and adaptation (Li and Hazelbauer, 2005). Our results were obtained using *in vivo* conditions in which Trg forms trimers (Studdert and Parkinson, 2004) and clusters (Lybarger *et al*., 2005), and thus the changes we detected occurred in homodimers contained in these higher order structures. The modification-induced conformational change we detected in the periplasmic and transmembrane domains might create extended changes within a trimer of dimers or a receptor array, but documentation of such putative effects requires different experimental approaches.

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Conformational changes of signalling and adaptation

Studies of chemoreceptor Trg in its native environment as part of a functional signalling system of an intact cell had identified the initial conformation change induced by ligand occupancy as a piston sliding of the signalling helix α4/TM2 towards the cytoplasm (Hughson and Hazelbauer, 1996; Beel and Hazelbauer, 2001), a conclusion consistent with a large body of observations about signalling in this and other chemoreceptors of *E. coli* and *Salmonella* (Falke and Hazelbauer, 2001). Using the same experimental strategy, we have now shown that adaptational modification, which functionally balances changes induced by ligand occupancy, appears to reverse the piston sliding of the signalling helix. This suggests that the central mechanism of sensory adaptation by chemoreceptor covalent modification is direct reversal of the ligand-induced conformational change. The direct reversal mechanism is simple and elegant. It emphasizes the central role of helical sliding for intramolecular signal transduction in chemoreceptors.

Experimental procedures

Strains and plasmids

CP553 is a strain of *E. coli* K12 that contains deletions of *tsr*, *tap*, *trg, tar*, *cheB* and *cheR* (Burrows *et al*., 1989). pGB1 is a derivative of pBR322 containing *trg* under the control of a *tac* promoter, *lacI^q*, and *bla* (Burrows *et al*., 1989). We used PCR mutagenesis and recombination of restriction fragments to create respective forms of *trg* coding for receptors with one of the four diagnostic cysteine pairs (Beel and Hazelbauer, 2001) and either 0, 2 or 5 glutamines at the sites of adaptational modification or with glutamates at all modification five sites and an extension at its carboxyl terminus of the final 19 residues of chemoreceptor Tsr, ending with the modification-enhancing pentapeptide (Feng *et al*., 1999). The 2Q form had Trg's native arrangement of two glutamines, Q312 and Q319, and three glutamates, E305, E311 and E501. All constructs were verified by DNA sequencing. pAI12 (Iida *et al*., 1985) and pRAR1 (Nara *et al*., 1996) are derivatives of pACYC184 carrying, respectively, constitutively expressed *rbsB*, coding for ribose-binding protein, and constitutively expressed *cheR*.

Oxidative cross-linking in vivo

Procedures for determination of rate constants of disulphide formation between diagnostic cysteine pairs were as described (Beel and Hazelbauer, 2001), using CP553 harbouring one or two plasmids. For each diagnostic cysteine pair we used a concentration of the catalyst that provided rates of disulphide formation that were accurately measured over a time-course of ~10 min (Beel and Hazelbauer, 2001). Thus, although there are substantial differences in propensities for disulphide formation among the four pairs, the rate constants we measured in our experimental conditions were within a sixfold range. A disulphide formed between the two cysteines in the same polypeptide chain constrains the denatured length of the cross-linked protein and thus results in more rapid migration in SDS polyacrylamide gel electrophoresis, corresponding to an apparent molecular mass of ~55 kDa, a difference easily resolved in an immunoblot from the unconstrained receptor chain, which migrates near its native molecular mass of ~60 kDa (Lee *et al*., 1994). The percentage of chains with disulphide cross-links at times after addition of the oxidative catalyst copper(II)-(o-phenanthroline)$_3$ was determined by densitometry of immunoblots. Curve fitting to the time-course data was based on the approach of (Careaga and Falke, 1992), fitting percentage cross-linking as a function of time of exposure to oxidation catalyst with the application 'Exponential rise to maximum/single exponent/2 parameter' of SigmaPlot 7.0 (Systat Software) to calculate the rate constant of oxidative cross-linking independent of competing reactions that oxidize sulphhydryls to sulphinic or sulphonic acids. For experiments in which the number of glutamines was varied, we used strains harbouring the appropriate derivative of pGB1 which provided the form of Trg to be tested and pAI12 which provided sufficient ribose-binding protein to create close to saturating levels of ligand (sugar-occupied binding protein) in the presence of excess ribose (Burrows *et al*., 1989; Yaghmai and Hazelbauer, 1993). For experiments comparing Trg with no methylation and maximal methylation, we used strains harbouring pGB1 derivatives coding for diagnostic-cysteine-containing forms of Trgt (Feng *et al*., 1999), which carries the modification-enhancing pentapeptide at its carboxyl terminus. A high proportion of maximally methylated Trg was achieved by introduction of pRAR1, which produced sufficient CheR to create extensively methylated populations of Trgt in which 60–80% of the receptor population was maximally methylated, as assessed by electrophoretic position on an SDS polyacrylamide gel (Nowlin *et al*., 1987; 1988). The disulphide cross-linked form of maximally methylated Trg appeared as a more rapidly migrating band in SDS polyacrylamide gel electrophoresis. That band and the band of unreacted protein could be quantified and thus extent of disulphide formation calculated except for the 38-202 pair. For this pair, partially methylated, cross-linked protein co-migrated with completely methylated, not cross-linked receptor.

Other assays

Assays of chemotactic ring formation (Feng *et al*., 1999) *in vivo* and kinase activation *in vitro* (Barnakov *et al*., 1998) were performed as described.

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