Lock On/Off Disulfides Identify the Transmembrane Signaling Helix of the Aspartate Receptor*

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The aspartate receptor of the bacterial chemotaxis pathway regulates the autophosphorylation rate of a cytoplasmic histidine kinase in response to ligand binding. The transmembrane signal, which is transmitted from the periplasmic aspartate-binding domain to the cytoplasmic regulatory domain, is carried by an intramolecular conformational change within the homodimeric receptor structure. The present work uses engineered cysteines and disulfide bonds to probe the nature of this conformational change, focusing in particular on the role of the second transmembrane α-helix. Altogether 26 modifications, consisting of 13 cysteine pairs and the corresponding disulfide bonds, have been introduced into the contacts between the second transmembrane helix and adjacent helices. The effects of these modifications on the transmembrane signal have been quantified by in vitro assays which measure (i) ligand binding, (ii) receptor-mediated regulation of kinase activity, and (iii) receptor methylation. All three parameters are observed to be highly sensitive to perturbations of the second transmembrane helix. In particular, 13 of the 26 modifications (6 cysteine pairs and 7 disulfides) significantly increase or decrease aspartate affinity, while 15 of the 26 modifications (5 cysteine pairs and 10 disulfides) destroy transmembrane kinase regulation. Importantly, 3 of the perturbing disulfides are found to lock the receptor in the “on” or “off” signaling state by covalently constraining the second transmembrane helix, demonstrating that it is possible to use engineered disulfides to lock the signaling function of a receptor protein. A separate aspect of the study probes the thermal motions of the second transmembrane helix: 4 disulfides designed to trap large amplitude twisting motions are observed to disrupt function but form readily, suggesting that the helix is mobile. Together the results support a model in which the second transmembrane helix is a mobile signaling element responsible for communicating the transmembrane signal.

The ability to sense and respond adaptively to changes in the environment is fundamental to all organisms. Bacteria such as Escherichia coli and Salmonella typhimurium acquire chemical information about their environment through a family of homologous transmembrane chemotaxis receptors (reviewed by Stock and Surette (1995), Swanson et al. (1993), Hazelbauer (1992), and Bourret et al. (1991)). The aspartate receptor, a representative of this family, monitors the concentration of aspartate in the periplasmic compartment and transmits this information to the cytoplasm, where it ultimately guides the chemotactic behavior of the cell. The aspartate receptor is a homodimer, in which each 60-kDa subunit possesses two membrane spanning α-helices linking the 18-kDa periplasmic domain to the 36-kDa cytoplasmic domain. The cytoplasmic domain associates with a soluble histidine kinase (CheA) and a coupling protein (CheW), together forming a kinetically stable ternary complex (Schuster et al., 1993; Gegner et al., 1992). This complex carries out an autophosphorylation reaction that is inhibited by aspartate binding to the receptor (Ninfa et al., 1991; Borkovich et al., 1989). Following autophosphorylation, the phosphate moiety is transferred from the ternary complex to a soluble signaling protein (CheY) which diffuses to the flagellar motor, binds, and regulates the transition between two propulsion states.

The present study addresses the mechanism utilized by the aspartate receptor to send a transmembrane signal from the periplasmic ligand-binding site to the cytoplasmic ternary complex. The same signaling mechanism is almost certainly employed by the other chemotaxis receptors, which regulate the same CheA histidine kinase and share extensive sequence identities in their cytoplasmic domains. Moreover, this same mechanism may also be utilized by an even broader class of receptors that includes the chemotaxis receptors as a subset: all members of this class exist as elements of histidine kinase pathways and exhibit nearly identical transmembrane topologies. It would appear that these receptors represent an ancient, highly adaptable signaling motif distributed throughout the prokaryotic world (reviewed by Parkinson (1993) and Spudich et al. (1993)) and recently discovered in eukaryotes as well (Swanson et al., 1994; Alex and Simon, 1994; Ota and Varshavsky, 1993; Chang et al., 1993).

Although a molecular picture of the transmembrane signal remains to be elucidated for the aspartate receptor and its relatives, previous studies have revealed important features. The aspartate receptor signals via an intramolecular conformational change within the dimer, since (i) numerous covalently linked dimers containing an engineered inter-subunit disulfide have been shown to retain transmembrane signaling (Chervitz et al., 1995; Scott and Stoddard, 1994; Stoddard et al., 1992; Lynch and Koshland, 1991; Falke and Koshland, 1987), and (ii) the oligomeric state of the receptor does not change upon addition of ligand (Yeh et al., 1993; Milligan and Koshland, 1998). Since the signal is intramolecular, it must be carried by the transmembrane helices. The first transmembrane helix located at the subunit interface has been shown to play a structural role and does not appear to carry the signal (Chervitz et al., 1995); a similar picture has emerged for the closely related ribose and galactose chemoreceptor (Lee et al., 1994, 1995). Here the goal is to test the hypothesis that the second

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1 S. A. Chervitz and J. J. Falke, unpublished results.
Further mechanistic studies of the aspartate receptor are facilitated by the extensive structural information available for its periplasmic and transmembrane domains, which together generate the transmembrane signal. X-ray crystallography has revealed the structure of the isolated periplasmic domain in both its apo and ligand-occupied conformations (Scott et al., 1993; Yeh et al., 1993; Milburn et al., 1991), in which each subunit of the homodimeric domain is observed to be an elongated four-helix bundle. A structural model has also been developed for the transmembrane region using disulfide mapping (Scott and Stoddard, 1994; Stoddard et al., 1992; Pakula and Simon, 1992; Lynch and Koshland, 1991; Falke et al., 1988), which has revealed the a-helical secondary structure and packing arrangement of the two transmembrane helices provided by each subunit. The first transmembrane helix begins near the receptor N terminus and is believed to be continuous with the first helix (a1) of the periplasmic domain. Similarly, the last helix (a4) of the periplasmic domain is thought continue across the bilayer, yielding the second transmembrane helix. The two transmembrane helices of each subunit are therefore referred to as a1/TM1 and a4/TM2, respectively.

The periplasmic and membrane-spanning regions of the dimer interface are stabilized by extensive contacts between the N-terminal first transmembrane helices of the two subunits (a1/TM1 and a1/TM1') (Scott et al., 1993; Pakula and Simon, 1992; Falke et al., 1988). Disulfide bonds covalently linking these helices over a large fraction of their contact surfaces retain transmembrane signaling, suggesting that structural changes involving the subunit interface and the first transmembrane helix are not required for signal transmission (Chervitz et al., 1995) (also Lee et al. (1995) for the ribose and galactose chemoreceptor).

To probe the role of the second transmembrane helix in signaling, the present study introduces cysteine pairs and disulfides at 17 locations in the periplasmic and transmembrane domains of the receptor, which possesses no intrinsic cysteines. These engineered cysteines and disulfides are designed to (i) perturb or covalently lock the interface between the second transmembrane helix and its adjacent helices, or (ii) force translational or twisting movements of the second transmembrane helix. If this helix serves as a critical signaling element, such perturbations should alter the signal, or even lock the receptor in the "on" or "off" signaling state. To quantitate the effects of engineered cysteines and disulfides on signaling, the receptor-regulated phosphorylation pathway is reconstituted in vitro, providing a direct measure of receptor kinase coupling. The effects of receptor engineering on ligand binding and in vitro receptor methylation are examined as well. The results indicate that the transmembrane signal is highly sensitive to perturbation of the second transmembrane helix. Importantly, these engineered disulfides are found to lock the receptor in the on or off state, in each case by covalently cross-linking the second transmembrane helix to the adjacent first helix of the same subunit. The implications of these results for the mechanism of transmembrane signaling are discussed.

**EXPERIMENTAL PROCEDURES**

Materials—L-[2,3-3H]Aspartic acid (15–50 Ci/mmol) was purchased from Amersham Corp. N-Octyl-β-D-glucoside and ultrapure L-aspartate were purchased from Sigma. All other materials were the same as described previously (Chervitz et al., 1995).

Cloning and Mutagenesis—Site-directed mutagenesis was performed as described previously (Chervitz et al., 1995). Pairs of cysteines substitutions were engineered simultaneously into the Salmonella typhimurium aspartate receptor by including two mutagenic oligonucleotides in the in vitro mutagenesis reactions.

Preparation of Membranes Containing the Aspartate Receptor—Bacterial membranes containing overexpressed wild-type or engineered aspartate receptors were prepared and quantitated as described previously (Chervitz et al., 1995), with the following modifications. Cells were lysed by sonication rather than French pressing, since sonication was found to produce higher yields of receptor. Sonication was performed in an ice-NaCl-water bath using thin-walled plastic centrifuge tubes to facilitate heat dissipation (1 × 3.5 inches, Beckman Ultra-Clear tubes). After removal of unbroken cells, membranes were pelleted and washed in the previously specified buffers by centrifugation at 500,000 × g (95,000 rpm) for 15 min at 2°C in a TLA 100.3 rotor (Beckman) using 3-ml centrifuge tubes. Resuspension of membrane pellets in each buffer was achieved by sonication using a ½-inch microtip at 40% maximum power.

Preparation of Soluble Chemotaxis Components—CheA, CheW, CheY, and CheR were all prepared as described previously (Chervitz et al., 1995).

Reduction and Oxidation of Cysteine-containing Receptors—Reduced or oxidized membrane-bound receptors were generated as detailed elsewhere (Chervitz et al., 1995). Briefly, reduction was achieved using 0.1 mM dithiothreitol, while oxidation was accomplished by the addition of either (i) 0.2 mM Cu(II)-(1,10-phenanthroline), as a redox catalyst in the presence of ambient O2 as the oxidant, or (ii) 1.0 mM I2 as oxidant. Reduction and oxidation reactions were carried out for 20 min at 37°C. For two engineered receptors, N36C,S183C and G39C,F180C, reduction was incomplete after this treatment and thus a higher dithiothreitol concentration (0.2 mM) was employed to increase the yield of reduced receptors.

In Vitro Phosphorylation Reactions and Methylation Reactions—Quantitation of receptor-mediated transmembrane regulation of the CheA kinase was carried out using the in vitro coupled phosphorylation assay as described previously (Chervitz et al., 1995). This assay is sensitive to changes in (i) the ligand-regulated conformational state of the receptor, (ii) the Km, for the formation of the receptor-CheW-CheA ternary signaling complex, and (iii) the Vmax and Km of the kinase activity in the ternary complex. Similarly, the in vitro methylation assay used was to detect methyl esterification of four receptor glutamate side chains by the CheR methyltransferase, as described elsewhere (Chervitz et al., 1995).

Aspartate Binding Assays—The aspartate affinities of wild-type and engineered receptors in their reduced and oxidized states were determined using the competition centrifugation assay of Clarke and Koshland (1979), modified as follows. Binding reactions consisted of receptor-containing membranes (0.7–1.4 μM receptor dimers, final), 25 mM Tris, pH 7.5, with HCl, 25 mM NaCl, 50 mM KCl, 1 mM EDTA, 0.7 mM phenylmethylsulfonyl fluoride and L-[3H]aspartate acid (0.5–16 μM, 150 cpn/ml). Reactions (100 μl) were brief vortexed for 45–50 μl aliquots were added to centrifuge tubes at 24°C containing either 5 μl of water or 5 μl of 200 mM nonradioaland L-Aspate buffered with 25 mM Tris, pH 7.5, with HCl. Reactions were mixed by pipetting and sealed in a room temperature at 24°C for 15 min prior to centrifugation in a TLA 100.2 rotor (Beckman) for 15 min at 40,000 × g (95,000 rpm) at 22°C. Supernatants (40 μl) were transferred into scintillation fluid (Scintiverse II, Fisher), and radioactivity was quantitated in a Packard 1600 TR scintillation counter. The radio labeled aspartate concentrations in the indicated supernatants provided the free and total ligand concentrations, respectively, while the bound ligand concentration was calculated as their difference. The dissociation constant (Kd) was finally ascertained by nonlinear least squares analysis of the relationship between bound ligand (IAsp free) and free ligand (IAsp bound):

\[
\text{IAsp bound} = \frac{I_{\text{Asp,free}}}{K_{d} + I_{\text{Asp,free}}} \quad (1)
\]

where [B] is the total binding site concentration. Control binding experiments using membranes lacking receptors yielded no detectable aspartate binding (data not shown).

Analysis of Engineered Receptors in Vivo—Chemotaxis swarm plates were prepared and analyzed as described elsewhere (Chervitz et al., 1995). When assessing disulfide linkages in vivo, we were also quantitated as described (Chervitz et al., 1995), except that in some cases an additional purification step was included. Such purification was initiated by treating receptor-containing membranes with 1.25% N-octyl-β-D-glucoside on ice for 20 min to selectively solubilize the receptor, followed by centrifugation at 500,000 × g (95,000 rpm) at 2°C. The supernatant (40 μl) was transferred into scintillation fluid (Scintiverse II, Fisher), and radioactivity was quantitated in a Packard 1600 TR scintillation counter. The radio labeled aspartate concentrations in the indicated supernatants provided the free and total ligand concentrations, respectively, while the bound ligand concentration was calculated as their difference. The dissociation constant (Kd) was finally ascertained by nonlinear least squares analysis of the relationship between bound ligand (IAsp bound) and free ligand (IAsp bound):
the receptor and its cross-linked products by SDS-polyacrylamide gel electrophoresis.

Protein Graphics—Coordinates of the periplasmic domain containing the C36–C36 engineered disulfide (Milburn et al., 1991) were displayed on a Silicon Graphics Personal Iris 4D/35 running the Insight II molecular graphics package (BioSym).

Results

Design of the Engineered Cysteine Pairs—To test the hypothesis that the second transmembrane helix (α4/TM2) serves to carry the transmembrane signal, the interactions of this helix with the first transmembrane helix (α1/TM1) and the third helix of the periplasmic domain (α3) were probed by introducing thirteen cysteine pairs and the corresponding disulfide bonds, all targeted to the interfaces between these helices. Four additional cysteine pairs were included to trap twisting motions of α4/TM2 relative to α1/TM1 by disulfide formation. Thus, a total of 17 pairs of cysteines and their corresponding disulfides were engineered individually into the periplasmic and transmembrane regions of the full-length receptor, as illustrated in Fig. 1. The design strategy focused on interactions within a subunit since the interactions of helix α4/TM2 are primarily intra-subunit in nature. Due to the symmetry of the homodimeric structure, each engineered cysteine pair was generated in both subunits of the dimer. Moreover, since the receptor lacks intrinsic cysteines, each cysteine pair was unique.

To simplify the presentation, the following sections focus initially on the 13 interfacial disulfide pairs and disulfide bonds targeted to α4/TM2 and its adjacent helices. Subsequently, the four disulfides designed to trap α4/TM2 twisting motions are examined separately.

Locations of the Interfacial Cysteine Pairs—Eight of the 13 interfacial cysteine pairs were introduced into the periplasmic region of the receptor (Fig. 1, A and C). Of these eight periplasmic pairs, six were targeted to the periplasmic contacts between helices α4/TM2 and α1/TM1 (S43C,Y176C; S43C,T179C; G39C,Y176C; G39C,T179C; G39C,S183C; and N36C,S183C), while the remaining two pairs were located at the interface between α4/TM2 and the periplasmic helix α3 (Y130C,L161C and A119C,Y168C). Six of the eight periplasmic pairs were located in the region characterized by the available crystal structures of the apo and ligand-occupied periplasmic domains (Milburn et al., 1991), enabling analysis of their angular and spatial separations as summarized in Table I. All of these pairs but one (G39C,Y176C) exhibited angular separations within the ranges allowed for disulfide bonds in proteins (Careaga and Falke, 1992a, 1992b; Srinivasan et al., 1990; Balaji et al., 1989), indicating that disulfide formation may result from simple translational motions. Moreover, two of the pairs (Y130C,L161C and A119C,Y168C) satisfied an even more stringent condition, exhibiting β-carbon separations within the range allowed for protein disulfides in both the apo and aspartate-occupied conformations of the domain (3.4–4.6 Å) (Careaga and Falke, 1992a, 1992b; Srinivasan et al., 1990; Balaji et al., 1989); these two pairs could form disulfide bonds with little or no movement of the polypeptide backbone. For the remaining four pairs whose separations were characterized, relative translations of the α4/TM2 helix ranging from 0.2 to 4.3 Å were required for disulfide formation.

Turning to the transmembrane region of the receptor, five engineered cysteine pairs were placed at positions previously demonstrated to yield efficient intra-subunit disulfide formation (Pakula and Simon, 1992). These cysteine pairs are predicted to lie within the bilayer, at or near the interface between the two transmembrane helices α4/TM2 and α1/TM1 (S25C,L197C; L21C,L201C; L11C,G211C; M10C,G211C; 4.3 Å were required for disulfide formation.

Discussion

In Vivo Activity of Receptors Containing Interfacial Cysteine Pairs—Di-cysteine containing receptor subunits were constructed by oligonucleotide-directed mutagenesis and were expressed in an E. coli strain lacking the aspartate receptor. All engineered receptors were observed to overexpress and assemble in the cytoplasmic membrane at high levels (5–10% of total membrane protein) with the exception of the M10C,G211C receptor (~5%).

The resulting receptors displayed varying degrees of spontaneous intra-subunit disulfide cross-linking when grown under normal conditions in E. coli, as summarized in Table II. Of the eight interfacial cysteine pairs located in the periplasm, four were nearly fully disulfide-linked in vivo (90–100%: A119C,Y168C; G39C,T179C; G93C,S183C; N36C,S183C). Three periplasmic cysteine pairs were partially cross-linked in
**Engineered Disulfide Studies of the Aspartate Receptor**

### Table I

| Engineered cysteine pairs | Cysteine pair | Interatomic distance $r_{ij}$ (Å) | $\chi^a_i$ (deg.) | $\chi^b_j$ (deg.) | $\eta^a_{ij}$ (deg.) | $\eta^b_{ji}$ (deg.) | $\eta^c_{ij}$ (deg.) |
|---------------------------|--------------|-----------------------------------|------------------|------------------|-------------------|-------------------|-------------------|
| (-) Asp                   | A            | B                                 | (-) Asp          | A                | B                | (-) Asp          | A                | B                |
| (+) Asp                   | A            | B                                 | (+) Asp          | A                | B                | (+) Asp          | A                | B                |

*Calculated from the crystallographic coordinates of Milburn et al. (1991). Tabulated distances ($r_{ij}$) are between the $\beta$-carbons of the indicated residues within a subunit of the dimer. When aspartate binds to the dimer, the symmetry of the two subunits is lost so that parameters are specified for each subunit (A, B). The bound aspartate interacts primarily with subunit A. Values in parentheses indicate the minimum translation required to bring the engineered cysteines within the range required for disulfide formation (3.4 $\leq r_{ij} \leq$ 4.6 Å) (Careaga and Falke, 1992a, 1992b; Srinivasan, 1990; Balaji et al., 1989). Parameters for position 39 (glycine in the native receptor) were determined by substituting an alanine into the structure to provide a $\beta$-carbon at this position. Parameters for positions within or near the transmembrane region could not be determined since this region is absent in the crystal structure, thus no parameters are given for the remaining cysteine pairs employed: G39C,Q178C; G39C,F180C; G39C,Y176C; G39C,S183C; N36C,S183C; S25C,L197C; L21C,L201C; L11C,G211C; M10C,G211C; V7C,G211C. The pseudodihedral angle $\chi^a_i$, $\chi^b_j$, and $\chi^c_{ij}$ were determined using the atomic coordinates for G39C,F180C and G39C,Y176C, respectively. Values in parentheses indicate the minimum rotation required to bring the engineered cysteines within the range required for disulfide formation (60° $\leq \chi^a_i \leq 60°$ and 60° $\leq \chi^b_j \leq 80°$ are forbidden pseudodihedral angles; 60° $\leq \chi^c_{ij} \leq 180°$ are allowed pseudobond angles) (Careaga and Falke, 1992a, 1992b; Srinivasan, 1990; Balaji et al., 1989).

### Table II

| Receptor | Fraction S-S in vivo | Relative swarm rate |
|----------|----------------------|---------------------|
|          | S-S                  | (+) Asp             | (-) Asp             |
| Wild type| 0.0                  | 1.0 ± 0.2           | 1.0 ± 0.2           |
| Y130C,L161C| 0.1                  | 0.6 ± 0.1           | 1.6 ± 0.2           |
| A119C,Y168C| 1.0                  | 1.1 ± 0.1           | 1.1 ± 0.3           |
| S43C,Y176C| 0.6                  | 1.0 ± 0.1           | 0.5 ± 0.2           |
| S43C,T179C| 0.5                  | 0.8 ± 0.1           | 0.4 ± 0.1           |
| G39C,Y176C| 0.5                  | 1.1 ± 0.1           | 0.4 ± 0.1           |
| G39C,Q178C| 0.9                  | 1.0 ± 0.1           | 0.0 ± 0.1           |
| G39C,T179C| 1.0                  | 0.6 ± 0.1           | 0.0 ± 0.1           |
| G39C,F180C| 0.9                  | 0.8 ± 0.1           | 0.0 ± 0.1           |
| G39C,D181C| 0.9                  | 0.7 ± 0.2           | 0.2 ± 0.3           |
| G39C,Q182C| 1.0                  | 1.0 ± 0.1           | 0.1 ± 0.1           |
| G39C,S183C| 0.9                  | 1.0 ± 0.1           | 0.2 ± 0.1           |
| N36C,S183C| 0.9                  | 0.9 ± 0.1           | 0.6 ± 0.1           |
| S25C,L197C| 0.9                  | 1.3 ± 0.1           | 1.8 ± 0.1           |
| L21C,L201C| 0.0                  | 1.1 ± 0.1           | 0.2 ± 0.1           |
| L11C,G211C| 0.0                  | 0.6 ± 0.1           | 1.3 ± 0.2           |
| M10C,G211C| 0.0                  | 1.4 ± 0.1           | 1.5 ± 0.3           |
| V7C,G211C| 0.0                  | 0.7 ± 0.1           | 1.8 ± 0.2           |
| No tar   | na                   | 0.4 ± 0.1           | 0.1 ± 0.1           |

* (-) Asp gives the rate of swarming on minimal agar lacking aspartate, normalized to the swarm rate for the native receptor. (+) Asp gives the increase in the swarm rate generated by the presence of aspartate, calculated as the difference between the swarm rates in minimal agar containing and lacking aspartate, normalized to the native differential rate (0.4 mm/h).

Cysteine pairs were tested for the ability to mediate chemotaxis toward aspartate in vivo by means of a chemotaxis swarm plate assay (Weis and Koshland, 1988; Adler, 1966), as quantitated in Table I. These assays were carried out under normal growth conditions, and no attempt was made to alter the extent of disulfide formation during the assay. Engineered receptors exhibited in vivo activities ranging from no aspartate swarming up to 180% of the native swarm rate. Of the four receptors exhibiting extensive in vivo disulfide formation, two retained significant aspartate chemotaxis (≥ 20% native: A119C,Y168C; N36C,S183C), while one was severely defective in aspartate chemotaxis (< 10% native, G39C,T179C). The remaining nine engineered receptors, which exhibited low levels of in vivo disulfide formation, retained significant aspartate chemotaxis (> 20% native).

Isolation of Membranes, and Redox Treatments of Engineered Receptors—To complement the in vivo data indicating that at least one of the engineered disulfides inhibits receptor signaling, further in vitro analysis was carried out to enable greater redox control of disulfide formation and to eliminate the adaptive methylation of the receptor, which can mask perturbations of the transmembrane signal. Moreover, in vitro studies could identify whether perturbations stem from altered ligand binding, kinase regulation, or receptor methylation.

Receptors were prepared for in vitro studies by isolating E. coli membranes (Foster et al., 1985) containing a given engineered receptor, then reducing or oxidizing the membrane-bound receptor to generate either the free cysteine pair or disulfide bond, respectively. Reduction was carried out using dithiothreitol, while two different oxidation systems were used to drive disulfide formation: (i) oxidation by ambient oxygen catalyzed by Cu(II)-(1,10-phenanthroline), or (ii) oxidation by iodine (Chervitz et al., 1995; Pakula and Simon, 1991; Falke and Koshland, 1987; Kobashi, 1968). The system providing the most complete disulfide formation for a given disulfide pair was determined empirically and used in subsequent studies. Disulfide formation reactions proceeded to 50–100% completion, with most reactions generating over 90% desired product.
Engineered Disulfide Studies of the Aspartate Receptor 24047

| Receptor | Treatment | Fraction S-S | $K_D$ | Relative affinity$^a$ |
|----------|-----------|--------------|-------|----------------------|
| Wild type | (−)       | 0.0          | 3 ± 1 | 1.0                  |
|           | Red       | 0.0          | 4 ± 2 | 1.0                  |
|           | Ox, O$_2$ | 0.0          | 6 ± 3 | 1.0                  |
|           | Ox, I$_2$ | 0.0          | 3 ± 1 | 1.0                  |
| Y130C,L161C | Red   | 0.0          | 12 ± 5| 0.3                  |
|           | Ox, I$_2$ | 1.0          | 5 ± 1 | 0.6                  |
| A119C,Y168C | Red   | 0.0          | 7 ± 1 | 0.6                  |
|           | Ox, I$_2$ | 0.9          | 4 ± 1 | 0.8                  |
| S43C,Y176C | Red       | 0.0          | 1.0 ± 0.6| 4.2       |
|           | Ox, I$_2$ | 1.0          | 1.0 ± 0.1 | 6.4       |
| S43C,T179C | Red       | 0.0          | 0.8 ± 0.1 | 5.0       |
|           | Ox, I$_2$ | 1.0          | 0.9 ± 0.1 | 6.9       |
| G39C,Y176C | Red       | 0.0          | 1.9 ± 0.4 | 2.2       |
|           | Ox, I$_2$ | 1.0          | 1.4 ± 0.2 | 4.5       |
| G39C,Q178C | Red       | 0.0          | 2.3 ± 0.2 | 1.8       |
|           | Ox, I$_2$ | 1.0          | 1.3 ± 0.2 | 4.8       |
| G39C,T179C | Red       | 0.0          | 4.5 ± 1.1 | 0.9       |
|           | Ox, I$_2$ | 1.0          | 1.4 ± 0.5 | 4.7       |
| G39C,F180C | Red       | 0.7          | (1.6 ± 0.7)$^b$ | 2.5       |
|           | Ox, I$_2$ | 1.0          | 0.9 ± 0.4 | 6.7       |
| G39C,D181C | Red       | 0.0          | 1.3 ± 0.4 | 3.0       |
|           | Ox, I$_2$ | 1.0          | 1.5 ± 0.4 | 4.2       |
| G39C,Q182C | Red       | 0.0          | 3 ± 1   | 1.3       |
|           | Ox, I$_2$ | 1.0          | 0.8 ± 0.4 | 7.8       |
| G39C,S183C | Red       | 0.0          | 1.0 ± 0.3 | 4.2       |
|           | Ox, I$_2$ | 1.0          | 2.8 ± 0.7 | 2.3       |
| N36C,S183C | Red       | 0.6          | (3 ± 1)$^b$ | 1.4       |
|           | Ox, I$_2$ | 1.0          | 4 ± 1   | 1.6       |
| S25C,L197C | Red       | 0.0          | 1.3 ± 0.3 | 3.1       |
|           | Ox, I$_2$ | 0.7          | 9 ± 3   | 0.3       |
| L21C,L201C | Red       | 0.0          | 4 ± 2   | 0.9       |
|           | Ox, I$_2$ | 1.0          | 2 ± 1   | 1.6       |
| L11C,G211C | Red       | 0.0          | >30     | 0.1       |
|           | Ox, I$_2$ | 0.5          | 30 ± 20 | 0.2       |
| M10C,G211C | Red       | 0.0          | 3 ± 1   | 1.3       |
|           | Ox, I$_2$ | 0.7          | 2 ± 1   | 4.1       |
| V7C,G211C | Red       | 0.0          | 3 ± 1   | 1.4       |
|           | Ox, I$_2$ | 0.5          | 1.6 ± 0.3 | 4.0       |

$^a$ Relative aspartate affinity is calculated as the ratio ($K_D$ (wild type)/$K_D$ (mutant)) for the same chemical treatment.

$^b$ $K_D$ for the reduced receptor is not well determined due to incomplete reduction.

Aspartate binding was also measurably altered by specific engineered disulfides constraining the α4TM2 helix. One such disulfide which cross-links α4TM2 to the adjacent α1TM1 helix, S25C–L197C, decreased the aspartate affinity 9-fold relative to the reduced state, or 2.8-fold relative to the native receptor. More commonly, disulfides cross-linking the α4TM2–α1TM1 interface tended to enhance aspartate binding; in particular, seven interfacial disulfides provided a 2–6-fold increase in the aspartate affinity relative to the native receptor (G39C–Y176C; G39C–T179C; G39C–S183C; S43C–T176C; G39C–T179C; M10C–G211C; V7C–G211C). These results indicate that the aspartate-binding site can be easily perturbed, but is not easily disrupted, by engineered cysteines and disulfides in the periplasmic and transmembrane domains which constrain the second transmembrane helix. The basis of this unusual coupling between the putative signaling helix and the ligand-binding site is analyzed further under "Discussion."
inhibition of both kinase activation and aspartate regulation (20% of native). In contrast, the receptor containing the A119C,Y168C pair was essentially normal in both measures of kinase signaling (≈70% of native). Based on this limited data, the α3–α4/TM2 interface may be less tightly coupled to the transmembrane signal than the α1/TM1–α4/TM2 interface.

Effect of Interfacial Disulfides on Transmembrane Kinase Regulation—Disulfide formation was used to examine the sensitivity of transmembrane kinase regulation to covalent cross-links between α4/TM2 and its adjacent helices, serving either to (i) lock together a specific helix-helix interface or (ii) lock a translational motion of α4/TM2. Only 1 of the 11 engineered disulfides located along the α4/TM2–α1/TM1 interface (N36C–S183C) retained significant activity in the reconstituted phosphorylation complex (≈20% of native kinase activation and aspartate regulation, Figs. 1 and 2 and Table IV). Four other disulfides at the α4/TM2–α1/TM1 interface retained detectable kinase activation (≈20% of native) but essentially destroyed regulation by ligand (≤10% of native) even at an aspartate concentration sufficient to saturate the ligand-binding site: S43C–T179C; G39C–S183C; S25C–L197C; and L21C–L201C. The remaining six interfacial disulfides between α4/TM2 and α1/TM1 virtually eliminated both kinase activation and aspartate regulation (≤10% of native): S43C–Y176C; G39C–Y176C; G39C–T179C; L11C–G211C; M10C–G211C; and V7C–G211C.

The two engineered disulfides covalently linking the α4/TM2–α3 interface both produced significant changes in signaling (Fig. 1, Table IV). The Y130C–L161C disulfide restored normal phospho-signaling to this receptor, which was substantially inhibited in the reduced state. In contrast, the A119C–Y168C disulfide substantially reduced regulation of the kinase by aspartate, although significant ligand regulation still remained (30% of native).

Of particular interest were three perturbing disulfides, all cross-linking α4/TM2 helix to the adjacent α1/TM1 helix, which appeared to lock the receptor signaling state. The S25C–L197C disulfide both restored and locked the signal in the fully on state. As expected for a lock on disulfide, S25C–L197C substantially decreased the aspartate affinity (10-fold relative to the reduced receptor, or 3-fold relative to native; Table III), and maintained full kinase activation even in the presence of saturating aspartate (Table IV, Fig. 2). Such behavior is expected for a disulfide which traps the apo-conformation of the receptor, since it is this conformation which activates the kinase. The trapped conformation appears to be somewhat perturbed, however, since it exhibits a higher than normal in vitro methylation rate (see below). The G39C–T179C disulfide, by contrast, prevented kinase activation and increased the aspartate affinity 4-fold relative to native, as expected for a receptor locked in the off or ligand-occupied conformation. A number of other disulfides exhibited less dramatic lock off behavior: S43C–Y176C is the next best example.

In principle, the effects of lock on and off disulfides can be reversed by reduction. Such reversibility was confirmed for the periplasmic G39C–T179C and S43C–Y176C lock off disulfides, for which kinase activation was restored by reduction with dithiothreitol (data not shown). For the S25C–L197C lock on disulfide, however, reduction could not be accomplished except under denaturing conditions. This resistance to reduction likely stems from the buried location of this disulfide within the bilayer, which could decrease accessibility to the polar dithioretol molecule, or increase the energy barrier for the ionic disulfide exchange reaction.

Alongside the engineered disulfide results provide additional evidence for the critical coupling between the α4/TM2 helix and the transmembrane signal, which is particularly sensitive to disulfide linkages along the α4/TM2–α1/TM1 interface. Such sensitivity appears to be specific to interfaces involving α4/TM2, since disulfides linking the α1/TM1–α1/TM1’ helix contacts at the subunit interface are, by comparison, relatively nonperturbing (Chervitz et al., 1995).

Effect of Interfacial Cysteines and Disulfides on Receptor Methylation—The in vitro methylation assay, which is a sensitive indicator of native receptor structure (Lynch and Koshol, 1991; Effroy and Koskol, 1994), was used to further characterize the engineered receptors. In this assay the methyltransferase enzyme CheR catalyzes the methyl esterification of specific receptor glutamates in a reaction stimulated by the trapped conformation appears to be somewhat perturbed, however, since it exhibits a higher than normal in vitro methylation rate (see below). The G39C–T179C disulfide, by contrast, prevented kinase activation and increased the aspartate affinity 4-fold relative to native, as expected for a receptor locked in the off or ligand-occupied conformation. A number of other disulfides exhibited less dramatic lock off behavior: S43C–Y176C is the next best example.

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effect of engineered cysteines and disulfides on in vitro phosphorylation

| Receptor  | Condition | Fraction S-S | Relative phosphorylation rate | Maximum activity | Aspartate regulation |
|-----------|-----------|--------------|-------------------------------|------------------|----------------------|
|           |           |              | (-) Aspartate (WT (-) Asp) | (+) Aspartate (WT (+) Asp) | (WT) (WT -) Asp) |
| WT        | (-)      | 30 ± 2       | 1.0 ± 0.3                    | 1.0              | 1.0                  |
| WT        | Red      | 30 ± 2       | 1.0 ± 0.3                    | 1.0              | 1.0                  |
| WT        | OX (O2I2)| 30 ± 2       | 1.0 ± 0.3                    | 1.0              | 1.0                  |
| Y130C, L161C | Red | 0.0 ± 0.1   | 6.0 ± 0.4                    | 1.7 ± 0.3        | 0.2                  |
| Y130C, L161C | Ox-I2 | 1.0 ± 0.1 | 40 ± 20                      | 1.2 ± 2.2        | 1.4                  |
| A119C, Y168C | Red | 0.0 ± 0.1   | 29 ± 5                      | 1.3 ± 0.2        | 1.0                  |
| A119C, Y168C | Ox-I2 | 1.0 ± 0.1 | 25 ± 8                      | 2.7 ± 0.5        | 0.8                  |
| S43C, Y176C | Red | 0.0 ± 0.1   | 11 ± 1                      | 1.0 ± 0.6        | 0.4                  |
| S43C, Y176C | Ox-O2 | 0.9 ± 0.1 | 1 ± 1                       | 0.9 ± 0.4        | 0.04                 |
| S43C, T179C | Red | 0.0 ± 0.1   | 6.0 ± 0.4                    | 1.7 ± 0.3        | 0.2                  |
| S43C, T179C | Ox-O2 | 1.0 ± 0.1 | 4 ± 1                       | 1.3 ± 0.4        | 0.2                  |
| G39C, Q178C | Red | 0.0 ± 0.1   | 13 ± 3                      | 1.1 ± 0.4        | 0.4                  |
| G39C, Q178C | Ox-O2 | 1.0 ± 0.1 | 1 ± 0.5                     | 1.0 ± 0.5        | 0.04                 |
| G39C, T179C | Red | 0.0 ± 0.1   | 30 ± 10                     | 1.3 ± 0.5        | 1.1                  |
| G39C, T179C | Ox-O2 | 1.0 ± 0.1 | 0.6 ± 0.4                   | 0.8 ± 0.4        | 0.02                 |
| G39C, F180C | Red | 0.5 ± 0.2   | 3 ± 1                       | 1.3 ± 0.6        | 0.1                  |
| G39C, F180C | Ox-O2 | 1.0 ± 0.1 | 2.0 ± 0.5                   | 2 ± 1            | 0.1                  |
| G39C, D181C | Red | 0.0 ± 0.1   | 13 ± 1                      | 1.7 ± 0.6        | 0.4                  |
| G39C, D181C | Ox-O2 | 1.0 ± 0.1 | 2 ± 0.4                     | 1.6 ± 0.8        | 0.1                  |
| G39C, S183C | Red | 0.0 ± 0.1   | 12 ± 4                      | 1.1 ± 0.3        | 0.4                  |
| G39C, S183C | Ox-O2 | 1.0 ± 0.1 | 3 ± 1                       | 1.0 ± 0.4        | 0.1                  |
| N36C, S183C | Red | 0.4 ± 0.1   | 11 ± 4                      | 1.2 ± 0.2        | 0.4                  |
| N36C, S183C | Ox-O2 | 1.0 ± 0.1 | 14 ± 2                      | 1.6 ± 0.2        | 0.5                  |
| S25C, L197C | Red | 0.0 ± 0.1   | 3 ± 1                       | 2 ± 1            | 0.1                  |
| S25C, L197C | Ox-I2 | 0.9 ± 0.1 | 37 ± 3                     | 30 ± 1           | 1.2                  |
| L21C, L201C | Red | 0.0 ± 0.1   | 0.5 ± 0.4                   | 0.7 ± 0.2        | 0.02                 |
| L21C, L201C | Ox-I2 | 0.8 ± 0.1 | 6 ± 2                       | 3 ± 1            | 0.2                  |
| L11C, G211C | Red | 0.0 ± 0.1   | 25 ± 5                      | 2.1 ± 0.2        | 0.8                  |
| L11C, G211C | Ox-O2 | 0.7 ± 0.1 | 2.0 ± 0.5                   | 1.0 ± 0.4        | 0.1                  |
| M10C, G211C | Red | 0.0 ± 0.1   | 3 ± 1                       | 1.3 ± 0.3        | 0.1                  |
| M10C, G211C | Ox-O2 | 1.0 ± 0.1 | 2.0 ± 0.3                   | 1.0 ± 0.2        | 0.1                  |
| V7C, G211C | Red | 0.0 ± 0.1   | 6 ± 2                       | 1.4 ± 0.3        | 0.2                  |
| V7C, G211C | Ox-O2 | 0.5 ± 0.1 | 2 ± 0.4                     | 1.4 ± 0.4        | 0.1                  |

Notably, five of the disulfides at the α4TM2-α4TM1 interface caused receptor overmethylation both in the absence and presence of aspartate: G39C–T179C, S25C–L197C, L21C–L201C, L11C–G211C, and M10C–G211C (Table V). As discussed above, the G39C–T179C disulfide was especially interesting because it appears to lock the receptor in its off state, which is normally stabilized by ligand binding. In the presence of aspartate the maximum methylation rate of this receptor was 1.3-fold faster than native, while in the absence of aspartate its rate was 4.1-fold that of the apo-native receptor, supporting the conclusion that this disulfide locks a conformation resembling the ligand-induced off state. Another lock off disul-
fide, S43C-Y176C, failed to yield overmethylation but its maximum methylation rate, which was 0.6-fold that of native, did not respond to aspartate: such methylation parameters are consistent with a locked conformation close to a native signaling state. In contrast, the 1.5-10-fold overmethylation caused by the S25C-L197C, L21C-L201C, L11C-G211C, and M10C-G211C disulfides appeared to stem from perturbations of the receptor structure, since none of these disulfides caused the increase in ligand affinity characteristic of the locked off receptor (Table III).

Engineered cysteine pairs and disulfides at the a4TM2-a3 interface exhibited considerably smaller effects on receptor methylation. Only the A119,Y168C cysteine pair generated a significant perturbation, increasing the maximum methylation rate 2.4-fold (Table V). Formation of a disulfide bond between this cysteine pair largely restored the normal methylation parameters.

Although usually in agreement, the methylation and phosphorylation assays yielded conflicting results in several cases (Tables IV and V and Fig. 3). For example, three engineered cysteine pairs or disulfides (S43C,T179C; V7C,G211C; and L21C-L201C) which severely inhibited kinase activation or regulation (≤10% of native) were observed to retain significant signaling in the methylation assay (40-150% native methyla-

TABLE V  Effect of engineered cysteines and disulfides on in vitro methylation

| Receptor | Condition | Fraction S-S | Relative methylation rate | Maximum activity | Aspartate regulation |
|----------|-----------|--------------|---------------------------|------------------|---------------------|
|          |           |              | (-) Aspartate             | (WT) Aspartate    | (WT)                |
|          |           |              | (WT) (-) Asp              | (WT) (-) Asp      |                     |
| WT       | (-)       | NA           | 1.0 ± 0.4                 | 3.3 ± 1.3        | 1.0                 |
| Red      | NA        | 1.0 ± 0.4    | 3.3 ± 1.3                 | 1.0              | 1.0                 |
| Ox (O2)  | NA        | 1.0 ± 0.4    | 3.3 ± 1.3                 | 1.0              | 1.0                 |
| Y130C,L161C | Red        | 0.0 ± 0.1    | 1.6 ± 0.4                 | 4.0 ± 1.0        | 1.2                 |
| Ox (O2)  | 1.0 ± 0.1 | 0.9 ± 0.2    | 5 ± 1                      | 1.4              | 1.7                 |
| A119C,Y168C | Red        | 0.0 ± 0.1    | 3.1 ± 0.8                 | 8.0 ± 2.0        | 2.4                 |
| Ox (I2)  | 1.0 ± 0.1 | 1.3 ± 0.3    | 4.7 ± 1.2                 | 1.4              | 1.2                 |
| S43C,Y176C | Red        | 0.0 ± 0.1    | 2.2 ± 0.6                 | 4 ± 2            | 1.2                 |
| Ox (O2)  | 0.9 ± 0.1 | 1.5 ± 0.9    | 2 ± 2                      | 0.6              | 0.1                 |
| S43C,T179C | Red        | 0.0 ± 0.1    | 3.2 ± 0.2                 | 5.1 ± 0.5        | 1.5                 |
| Ox (O2)  | 1.0 ± 0.1 | 1.0 ± 0.3    | 1.0 ± 0.2                  | 0.3              | 0.0                 |
| G39C,Y176C | Red        | 0.0 ± 0.1    | 1.3 ± 0.9                 | 2 ± 2            | 0.7                 |
| Ox (O2)  | 1.0 ± 0.1 | 0.5 ± 0.3    | 0.7 ± 0.7                  | 0.2              | 0.2                 |
| G39C,Q178C | Red        | 0.0 ± 0.1    | 1.2 ± 0.3                 | 2.1 ± 0.5        | 0.6                 |
| Ox (O2)  | 1.0 ± 0.1 | 0.20 ± 0.04  | 0.3 ± 0.1                  | 0.1              | 0.4                 |
| G39C,T179C | Red        | 0.0 ± 0.1    | 1.5 ± 0.2                 | 4.0 ± 0.8        | 1.2                 |
| Ox (O2)  | 1.0 ± 0.1 | 4.1 ± 1.2    | 4.4 ± 0.8                  | 1.3              | 0.03                |
| G39C,F180C | Red        | 0.5 ± 0.2    | 0.8 ± 0.4                 | 1.3 ± 0.8        | 0.4                 |
| Ox (O2)  | 1.0 ± 0.1 | 0.3 ± 0.1    | 0.2 ± 0.1                  | 0.1              | 0.0                 |
| G39C,D181C | Red        | 0.0 ± 0.1    | 2.6 ± 0.9                 | 4 ± 2            | 1.2                 |
| Ox (O2)  | 1.0 ± 0.1 | 1 ± 1        | 1 ± 1                      | 0.4              | 0.1                 |
| G39C,Q182C | Red        | 0.0 ± 0.1    | 0.9 ± 0.1                 | 0.8 ± 0.1        | 0.2                 |
| Ox (O2)  | 1.0 ± 0.1 | 0.5 ± 0.1    | 0.3 ± 0.2                  | 0.1              | 0.0                 |
| G39C,S183C | Red        | 0.0 ± 0.1    | 0.7 ± 0.3                 | 1.3 ± 0.3        | 0.4                 |
| Ox (O2)  | 1.0 ± 0.1 | 0.5 ± 0.2    | 0.5 ± 0.3                  | 0.1              | 0.0                 |
| N36C,S183C | Red        | 0.4 ± 0.1    | 2.5 ± 0.9                 | 3.5 ± 0.7        | 1.1                 |
| Ox (O2)  | 1.0 ± 0.1 | 1.9 ± 0.2    | 2.9 ± 0.8                  | 0.9              | 0.2                 |
| S25C,L197C | Red        | 0.0 ± 0.1    | 4 ± 2                      | 5 ± 2            | 1.5                 |
| Ox (I2)  | 0.9 ± 0.1 | 6 ± 2        | 12 ± 5                     | 3.5              | 0.4                 |
| L21C,L201C | Red        | 0.0 ± 0.1    | 1.0 ± 0.1                 | 1.5 ± 0.2        | 0.5                 |
| Ox (I2)  | 0.8 ± 0.1 | 3.2 ± 0.2    | 5.1 ± 0.1                  | 1.5              | 0.3                 |
| L11C,G211C | Red        | 0.0 ± 0.1    | 4 ± 1                      | 7 ± 3            | 2.2                 |
| Ox-O2    | 0.7 ± 0.1 | 7 ± 3        | 8 ± 2                      | 2.5              | 0.04                |
| M10C,G211C | Red        | 0.0 ± 0.1    | 5.8 ± 0.5                 | 6.1 ± 0.8        | 1.8                 |
| Ox-O2    | 1.0 ± 0.1 | 10 ± 4       | 14 ± 5                     | 4.1              | 0.2                 |
| V7C,G211C | Red        | 0.0 ± 0.1    | 0.7 ± 0.3                 | 2.0 ± 0.8        | 0.6                 |
| Ox-O2    | 0.5 ± 0.1 | 3 ± 1        | 3 ± 1                      | 1.0              | 0.1                 |

* Isolated membranes were treated as described under “Experimental Procedures”: no treatment (−); reduction by dithiothreitol (red); oxidation (ox) by molecular iodine (I2) or oxygen (O2).

† Rate of in vitro receptor methylation by the methyltransferase CheR in the absence or presence of ligand, relative to the rate observed for the unliganded wild-type receptor subjected to the same chemical treatment (1.6 pmol of methyl groups min⁻¹). All rates are normalized to the same receptor concentration (3 μM dimer). WT, wild type.

‡ Rate of in vitro receptor methylation by CheR in the presence of aspartate, relative to the rate observed for the aspartate-occupied wild-type receptor subjected to the same chemical treatment (1.6 pmol of methyl groups min⁻¹ for 3 μM receptor dimer).

§ Effect of aspartate on the rate of in vitro receptor methylation by CheR, relative to the effect of aspartate on the wild-type receptor subjected to the same chemical treatment (3.3-fold up-regulation upon aspartate addition). Calculated as the ratio ((rate (+) Asp)/rate (−) Asp) = 1/2.3, yielding a range between 0 and 1 for no aspartate regulation or wild-type regulation, respectively.
tions demonstrate that large-amplitude motions of the all four reactions approached completion (Table IV). The latter where three fell within the region defined by the crystal structure (G39C, Q178C; G39C, F180C; G39C, D181C; G39C, Q182C). All motion was a twisting motion of the two cysteines within sufficient proximity for disulfide formation; in each case the simplest motion capable of bringing the cysteine pairs were all outside the limits allowed for disulfide formation is facilitated by several periplasmic proteins during or after protein synthesis and assembly (Darby and Creighton, 1995). Similarly, when disulfide formation was driven by oxidation of receptor-containing membranes in vitro, all four reactions approached completion (Table IV). The latter observation demonstrates that large-amplitude motions of the a4TM2 helix are features of the fully folded and assembled receptor.

Despite the efficiency with which they were formed, the rotational disulfides caused significant perturbation of receptor activity. In the native cellular environment, where all four cysteine pairs were primarily in their disulfide-linked form, the corresponding engineered receptors were each defective in mediating aspartate chemotaxis during the in vivo swim assay (0–20% native activity, Table II). When the effects of these disulfides were further analyzed in vitro, all four were observed to (i) increase aspartate affinity (4–7-fold native, Table II), (ii) essentially destroy both maximum kinase activation and its regulation by aspartate (≤10% of native, Table IV), and (iii) severely reduce either the maximum methylation rate or its regulation by ligand, or both (≤10% of native, Table V). It follows that the receptor conformations trapped by these disulfides represented significant deviations from normal signaling states.

DISCUSSION

The present study has characterized the transmembrane signaling behavior of seventeen separate intra-subunit cysteine pairs and their corresponding disulfides engineered into the bacterial chemotaxis aspartate receptor, a representative of a growing class of receptors which regulate histidine kinase pathways. The effects of these cysteines and disulfides on ligand binding and transmembrane kinase regulation support the model presented in Fig. 4, in which the second transmembrane helix, designated a4TM2, plays a critical role in transmembrane signaling. The model proposes that a4TM2 is a mobile signaling element and that movements of this helix carry the transmembrane signal. Earlier evidence for this picture was provided by 35S NMR studies of the isolated ligand-binding domain, which revealed perturbations of the a4 helix triggered by aspartate binding (Danielson et al., 1994). Similar conclusions have been reached in independent studies examining signaling perturbations caused by (i) specific mutations in the a4TM2 helix of the aspartate receptor (Jeffery and Koshland, 1994), and (ii) engineered disulfides constraining the a4TM2 helix of the related ribose and galactose receptor (Lee et al., 1995). Further evidence obtained in the full-length aspartate receptor can now be summarized as follows.

As expected for a crucial signaling element, most engineered cysteines and disulfides involving helix a4TM2 are observed in the present study to perturb ligand binding and/or transmembrane kinase regulation. The packing interface between a4TM2 and the adjacent first transmembrane helix, a1TM1, was probed by eleven engineered cysteine pairs and the corresponding disulfides, as illustrated in Figs. 1C and 4. The majority of the resulting modifications (5 cysteine pairs and 7 disulfides) were observed to significantly alter ligand-binding affinity. Moreover, most of the modifications (5 cysteine pairs and 10 disulfides) essentially destroyed either transmembrane kinase activation or regulation of the kinase by saturating concentrations of aspartate, or both of these measures of the transmembrane signal. Thus, the a4TM2-a1TM1 packing interface appears to be tightly coupled to receptor function. In contrast, a previous study of the a1TM1-a1/TM1' packing interface
and in vitro methylation, as expected for receptors locked in the ligand-occupied or off state. These disulfides appear to trap conformations resembling true receptor signaling states. Moreover, the locked state can be restored to normal ligand regulation by simple reduction of the disulfide. One disulfide cross-linking \( \alpha_{4}/TM2 \) to \( \alpha_{1}/TM1 \), namely N36C–S183C, retained kinase activation and aspartate regulation, suggesting that this disulfide lies at a special location which is able to accommodate the aspartate-induced movement of \( \alpha_{4}/TM2 \) via the modest flexibility of the disulfide linkage. (Protein disulfide bonds exhibit a range of \( \beta \)-carbon separations spanning 1.2 Å (Careaga and Falke, 1992a, 1992b; Srinivasan et al., 1990; Balaji et al., 1989).

Evidence that the \( \alpha_{4}/TM2 \) helix is a mobile element within the receptor structure is provided by disulfide trapping results. In the present work, four disulfide bonds designed to trap \( \alpha_{4}/TM2 \) rotations about its long axis were generated. These disulfides, which all disrupted kinase regulation, were observed to form rapidly and with high efficiency, despite the fact that helix \( \alpha_{4}/TM2 \) must twist about its long axis approximately 90° to 180° relative to the \( \alpha_{1}/TM1 \) helix to bring the engineered disulfides into sufficient proximity for disulfide formation (Fig. 1A). Moreover, previous disulfide trapping studies detected long-range, intramolecular collisions between the \( \alpha_{4}/TM2 \) and \( \alpha_{4}/TM2' \) helices within the same receptor dimer (Pakula and Simon, 1992; Falke and Koshland, 1987). Such dramatic movements indicate that the \( \alpha_{4}/TM2' \) helix is highly mobile in the plane of the bilayer, or that this helix spontaneously unravels at a rate sufficient to yield the observed long-range collisions. It is not yet clear whether this mobility is essential for signaling, or simply represents random fluctuations away from the important signaling conformations.

Interestingly, the coupling between the ligand-binding site and the \( \alpha_{4}/TM2 \) signaling helix appears to be remarkably plastic. At first glance, the structure of the periplasmic domain suggests that the ligand-binding site would be tightly coupled to the \( \alpha_{4}/TM2 \) helix, since nearly 60% of the contacts between the receptor and the bound aspartate involve residues at the N terminus of \( \alpha_{4}/TM2 \) (positions Y-149–T-154; the remainder of the binding pocket consists of three arginine side chains located at the C-terminal end of \( \alpha_{1}/TM1 \) and \( \alpha_{1}/TM1' \)) (Milburn et al., 1991). Yet in all cases where engineered cysteine pairs or disulfides were observed to severely disrupt kinase regulation by perturbing the \( \alpha_{4}/TM2 \) signaling helix (a total of 20 examples in the present study), the inhibition arose from blockage of the transmembrane signal to the kinase, not from failure to bind aspartate. Surprisingly, most of these perturbations actually enhanced aspartate binding. For instance, the four disulfides designed to trap extreme twisting motions of \( \alpha_{4}/TM2 \) all disrupted the transmembrane signal but yielded 4–7-fold increases in aspartate affinity. Such “negative coupling” between ligand binding and signaling suggests that aspartate binding must carry out thermodynamic work to move the \( \alpha_{4}/TM2 \) helix into a different signaling position. In such a picture, perturbations which uncouple the \( \alpha_{4}/TM2 \) helix from the ligand-binding site would increase the aspartate-binding affinity.

At least two molecular explanations can be proposed for the observed plasticity of the coupling between aspartate binding and the signaling helix. (i) The linkage between the aspartate-binding site and the distal regions of \( \alpha_{4}/TM2 \) may contain a flexible hinge which allows dissipation of certain helix perturbations without destroying the affinity of the site. Such a hinge would need to be sufficiently rigid in the motional coordinate triggered by ligand binding, but could be flexible in other coordinates. Structural features which might yield such flexibility include a local distortion of the \( \alpha_{4}/TM2 \) helix generated by

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**Fig. 4. Locations of engineered disulfides which retain, restore, or lock the transmembrane signal.** Shown is a schematic model of the periplasmic and transmembrane regions of the receptor dimer (note that perspectives have been altered for clarity). The indicated disulfide bonds retain or restore aspartate-triggered transmembrane regulation of kinase activity (solid bar; >20% native) or lock the receptor in the on (open bar) or off (stippled bar) signaling state. Formation of a disulfide between the remaining cysteine pairs essentially destroys transmembrane kinase regulation (fine bars; <10% native). Only disulfides located at the interfaces of adjacent helical faces are shown; for simplicity, disulfides are shown in just one of the two symmetric subunits, and disulfides designed to trap \( \alpha_{4}/TM2 \) twisting motions are omitted. The model proposes that the transmembrane signal originating in the ligand-binding site is generated by an undefined movement of the \( \alpha_{4}/TM2 \) helix relative to the subunit interface, which communicates the ligand-binding event to the cytoplasmic domain and its associated histidine kinase.

Within the dimer contact region has indicated that this interface is more weakly coupled to kinase regulation (Chervitz et al., 1995). Finally, the \( \alpha_{4}/TM2-\alpha_{3} \) interface examined in the present study exhibited an intermediate susceptibility to perturbation; half of the cysteine pairs and disulfides tested within this interface yielded moderate effects on ligand binding or kinase regulation (only 2 cysteine pairs and 2 disulfides were tested, however). Altogether, the available evidence indicates that the \( \alpha_{4}/TM2 \) helix is tightly coupled to the transmembrane signal, while the \( \alpha_{1}/TM1 \) helix plays a simple structural role in dimer stabilization.

Perhaps the strongest evidence that the \( \alpha_{4}/TM2 \) helix is the critical signaling element is provided by disulfides observed to block the signaling state on or off. Three disulfides were observed to lock the signaling state of the receptor, in each case by covalently cross-linking the helices within the same subunit. The S25C–L197C disulfide appeared to stabilize a conformation similar to the apo-conformation or on state, yielding decreased ligand affinity as well as maximum kinase activation both in the absence and presence of saturating ligand. In contrast, the G39C–T179C and S43C–Y176C disulfides exhibited enhanced ligand affinity, as well as ligand-insensitive kinase inhibition
Pro-153 within the binding site, or three Gly residues near the site which presumably weaken the helix (Gly-157, Gly-162, and Gly-166). Alternatively, (ii) the aspartate affinity may be dominated by electrostatic interactions with the three arginines on α1TM1 and α1TM1′ (Arg-64, Arg-69, and Arg-73). According to the latter model, the contacts observed between α4TM2 and the bound aspartate are of secondary importance for aspartate affinity but would be required for generating the conformational work resulting in the transmembrane signal. There is genetic and biochemical evidence demonstrating that the aforementioned arginines on α1TM1 and α1TM1′ are critical for aspartate affinity (Wolff and Parkinson, 1988; Mowbray and Koshland 1990) and that the N terminus of α4TM2 plays a key role in aspartate chemotaxis (Lee and Imae, 1990). However, the importance of the N-terminal end of α4TM2 for aspartate affinity has not been studied directly.

Altogether, in vitro and in vivo studies of the aspartate receptor and its relatives strongly implicate the second transmembrane helix as the element which carries the transmembrane signal across the bilayer (this study; Chervitz et al., 1995; Lee et al., 1995; Jeffery and Koshland, 1994; Danielson et al., 1994). This signal could be a simple movement of the α4TM2 helix induced by ligand binding, which could serve to “switch” the cytoplasmic domain between its kinase activating and inactivating conformations. The nature of the helix movement triggered by ligand binding remains to be elucidated.

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