Transmembrane signalling and the aspartate receptor

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Background: The aspartate receptor is a transmembrane protein that mediates bacterial chemotaxis. The structures of the periplasmic ligand-binding domain reveal a dimer, each subunit with four α-helix bundles, with aspartate binding to one of two sites at the subunit interface. The transmembrane regions of the receptor were not included in these structures.

Results: To investigate the structure of the transmembrane region, we have made a mutant protein with two cross-links, restraining the subunit–subunit interface on both sides of the membrane, and have made an energy-minimized model of the transmembrane region. We demonstrate that the transmembrane helices form a coiled coil which extends from the periplasmic subunit through the membrane. We have constructed a model of the ligand-binding domains with the amino-terminal transmembrane helices.

Conclusions: We draw three conclusions from our model. Firstly, the interface between receptor subunits in the intact receptor consists of an uninterrupted coiled coil. Secondly, this structure rules out several postulated mechanisms of signalling. Thirdly, side chain packing constraints within the helices dictate that local structural changes must be small, but are propagated over a long distance rather than being dissipated locally. Low energy changes in the conformation of side chains are a probable mechanism of signal transduction in the aspartate receptor.

Introduction

Transmembrane receptors are proteins which transmit biochemical signals from the outside to the inside of a cell. Signal transduction is often initiated when a ligand molecule (such as a hormone or nutrient) binds to its specific transmembrane receptor on the cell surface. Two related families of transmembrane receptors, the eukaryotic growth factor receptors (which include the insulin receptor, epidermal growth factor receptor and related tyrosine kinase receptors) [1], and the prokaryotic chemotaxis-mediating receptors found in bacteria (which include the aspartate receptor, serine receptor and others which lack kinase activity) [2], share common structural motifs [3]. In both families the functional receptor possesses a homodimeric structure, and each monomeric subunit contains an extracellular ligand-binding domain, which is connected to an intracellular signalling domain by a single α-helix. (Fig. 1).

The similarity of architecture shared by the two families of receptors suggests that they may also share a common mechanism of signal transduction. This hypothesis is supported by experiments in which a functionally active chimeric receptor was constructed from the ligand-binding domain and transmembrane sequences of the aspartate receptor, coupled to the cytoplasmic portion of the insulin receptor. This fusion yielded a transmembrane receptor whose tyrosine kinase signalling activity is activated by aspartate binding [4]. This observation suggests similarities in the mechanisms of transmembrane signalling in the two types of receptors. However, the crystal structure of the human growth hormone bound to its dimeric receptor [5] indicates a signalling mechanism involving ligand-induced receptor dimerization, an important mechanistic difference from published reports on the bacterial chemotaxis receptors. Thus, the detailed mechanism of transmembrane signalling in the aspartate receptor remains unclear.

The aspartate receptor mediates bacterial chemotaxis behavior by binding aspartate to its external (periplasmic) domain. A conformational change is transduced by the transmembrane helices to the cytoplasmic domain. This leads to modulation of the activity of an autophosphorylating kinase CheA, which binds to the receptor's cytoplasmic domain accompanied by an accessory protein CheW. The crystal structure of the aspartate receptor periplasmic domain reveals that this portion of the receptor is a dimer of four α-helix bundle subunits whose interface is composed primarily of parallel coiled coil contacts between the amino-terminal helices of each subunit [6]. Although the crystal structures of the ligand-binding domain of the aspartate receptor both in the presence and absence of aspartate have been determined [7], no obvious large structural changes were observed upon ligand binding which could be unambiguously related to transmembrane signalling. The mechanism suggested in the original report of the crystal structures of the periplasmic domain proposed a 4° rigid-body rotation of the subunits upon aspartate binding. However, this rigid-body 'pivoting' hypothesis of transmembrane signalling in the aspartate receptor is contradicted by biochemical experiments, which suggest that a conformational change within a single receptor subunit is induced by aspartate binding [8,9].
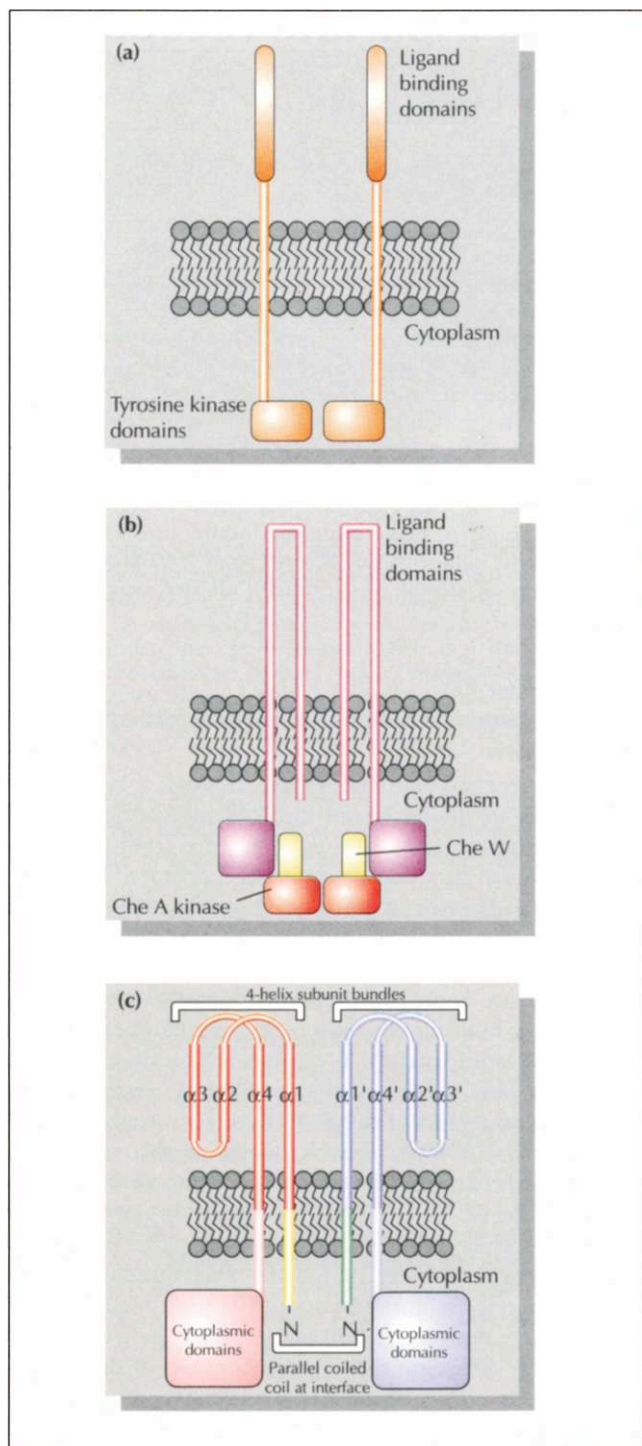
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Fig. 1. Overall topology of the receptor in comparison with growth factor receptors. **(a)** The epidermal growth factor (EGF) receptor has an extracellular ligand-binding domain connected to a cytoplasmic tyrosine kinase domain by a single α-helix. The kinases are ATP-dependent and activated by ligand binding. **(b)** The aspartate receptor has a second, amino-terminal α-helix, which forms the interface of the dimer. The proteins CheW and CheA are required to form an active signalling complex, which is inactivated when aspartate is bound. **(c)** The regions of the aspartate receptor shown in blue and red (different colors for the two monomers) are the only parts for which structures are available. The α-helices are numbered. We have modeled the regions shown in yellow and green as continuations of the parallel coiled coil formed by helices 1 and 1'. The structure of the cytoplasmic domains (light blue and pale red) is unknown.

A series of disulfide cross-linking experiments performed on the intact aspartate receptor have been reported by several research groups [9–12]. Based upon distance constraints imposed by the results of these experiments, and using the existing crystal structures, we hypothesize that the amino-terminal transmembrane helices of the intact aspartate receptor (which were not included in the crystal structure of the periplasmic domain) form a nearly perfect canonical parallel coiled coil of α-helices. We demonstrate the validity of this assertion in two ways. The first demonstration involves constructing and characterizing an aspartate receptor with two simultaneous, site-directed disulfide cross-links located immediately on each side of the lipid bilayer, at sites compatible with the extended parallel coiled coil interface. The simultaneous cross-links covalently link the two α-helices of the parallel coiled coil to each other at both ends of the membrane, so that rigid-body movements between the subunits are severely restrained.

The second demonstration of the validity of our hypothesis involves constructing an energy-minimized model of the aspartate receptor amino-terminal transmembrane region coupled to the ligand-binding domain. This model incorporates all the experimental constraints from our own site-directed cross-linking experiments and those of other groups. Our model of the aspartate receptor amino-terminal transmembrane dimer interface enables us to arrive at conclusions regarding the structure and function of this transmembrane receptor.

Results and discussion
The dimer interface of the aspartate receptor: an uninterrupted parallel coiled coil

The crystal structure of the aspartate receptor periplasmic domain reveals that the receptor interface is composed primarily of parallel coiled coil contacts between residues 32 and 76 of the amino-terminal helices of each subunit. The region between residues 32 and 43 adheres to the canonical conformation. The parallel coiled coil deviates somewhat from ideality, however, as it is distorted locally between residues 43 and 54, where few side chain contacts occur [6].

Single cysteine disulfide cross-links between the parallel helices have been introduced experimentally at position 4 [9], position 36 [10], position 18 [11], and positions 11, 22, 25 and 29 [12], along the receptor subunit interface. All these constructs are physically compatible with the overall structure of the receptor. We can easily demonstrate that all these individual disulfide cross-linking results agree with a structure in which residues 4–36 of the amino-terminal helix of the aspartate receptor form an uninterrupted ideal parallel coiled coil of the type first predicted by Crick [13] and observed in the GCN4 'leucine zipper' peptide structure [14].

In the axial helical projection shown in Fig. 2a, the helices are shown as slightly under-wound (with 3.5

rather than 3.6 to 3.7 residues per turn of the helical wheel) to compensate for the left-handed supercoiling of a parallel coiled coil [15]. This projection makes clear that the cross-linking of cysteines introduced at positions 4, 11, 18, 22, 25, 29 and 36 are all entirely consistent with the assumption that residues 4–43 form an ideal parallel coiled coil. To confirm that these seven disulfide cross-links are each consistent with a parallel coiled coil structure, residues 1–36 of the aspartate receptor were modeled based upon the canonical parallel coiled coil of the GCN4 leucine zipper coordinates [14] with its side chains substituted for those of the aspartate receptor. The

modeled region incorporating the seven disulfide cross-links as constraints was then energy-minimized in X-PLOR 3.1 [16] with little distortion to the canonical parallel coiled coil backbone atoms, demonstrating that these cross-links are compatible with the uninterrupted coiled coil structure hypothesized for the transmembrane region of the aspartate receptor. This energy-minimized model is depicted in Fig. 2b. Although this modeling exercise represents an extreme case, and some distortions might reasonably be expected in such a structure with seven sequential cross-links, the results of the minimization clearly support a coiled coil structure for the
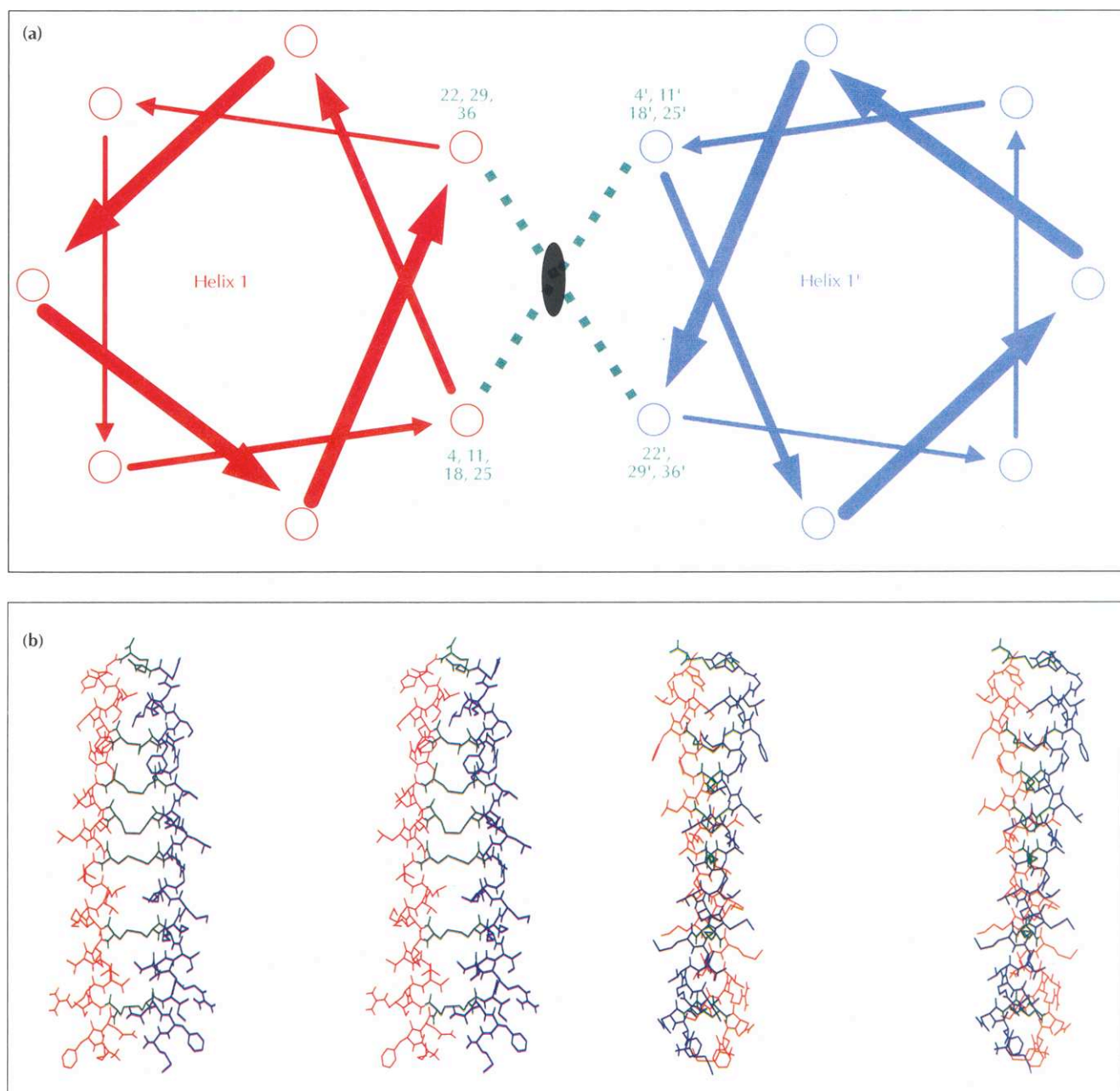

Fig. 2. Representations of the transmembrane parallel coiled coil aspartate receptor subunit interface. (a) An axial helical projection of the parallel coiled coil as viewed from the carboxyl-termini of these helices. The two subunits are denoted by red and blue coloration. The locations of the site-directed disulfide cross-links are highlighted in green. (b) The corresponding stereoviews of the energy-minimized model of the parallel coiled coil transmembrane region, showing the seven cysteine cross-links in green.

transmembrane dimer interface. Therefore, two related models of the transmembrane helices were constructed, based upon either two parallel but non-coiled helices, or a coiled coil structure as described above. Each structure had three simultaneous cross-links imposed at positions 4 (located in the cytoplasm), 18 (in the lipid bilayer) and 36 (in the periplasm). The model for the parallel non-coiled helices was generated in Polygen QUANTA using a pitch of 3.6 residues per turn, and this leads to residue 4 being almost completely out of phase on the helix relative to residues 36 and 18. Refinement of these models leads to substantial distortions of the helix backbone in the non-coiled helices (Fig. 3), suggesting that the disulfide cross-linking data are incompatible with a non-coiled coil structure; this is not entirely surprising when one considers packing constraints upon parallel, bundled helices (Fig. 4). Similar minimization experiments on receptor models with two cross-links at positions 4 and 36 (directly to each side of the lipid bilayer) give similar results; these results were then tested experimentally as described in the next section. For the non-coiled starting models for these minimizations, numerous crossing angles and helical bends were modeled initially to allow for possible cross-link formation; in each case the resulting model was severely strained prior to minimization.

A model of the aspartate receptor periplasmic domains coupled to the amino-terminal transmembrane α-helices was constructed and energy-minimized using residues 23–180 of the refined structure of the aspartate receptor ligand-binding domain [6], and residues 4–36 of the transmembrane region model described above. This combined model (Fig. 5) demonstrates that the parallel coiled coil structure is compatible with both the biochemical cross-linking data and the crystal structure, and is thus likely to be an accurate representation of the

structure of these regions of the intact aspartate receptor as it would appear in the cell membrane.

Restraining the dimer interface of the aspartate receptor with two simultaneous site-directed cross-links

The direct genetic and biochemical evidence against mechanisms of signalling which involve rigid-body rotations or movements along the dimer interface in the membrane fall into two categories. Firstly, there are data from experiments with chimeric receptor species containing one full-length receptor subunit existing as a heterodimer with a second, truncated subunit. Such constructs have been shown to undergo aspartate-induced increases in methylation by the methyl transferase enzyme CheR, indicating that only a single subunit is necessary for transmission of a signal through the membrane [8]. Constructs which have all four trans-membrane sequences intact in the chimeric dimer exhibit signalling behavior very similar to the intact receptor. Constructs including one subunit which is missing all of the carboxy-terminal transmembrane helix and cytoplasmic domain, as well as much of the amino-terminal helix (residues 1–24), signal very weakly. Secondly, receptor species with disulfide cross-links at positions 4, 18 and 36 along the dimer interface (as described above) are fully active in methylation assays, indicating that restraining the distance and geometry between the two monomer amino-terminal helices anywhere along the subunit interface has no effect upon signalling [9–11].

Neither of these sets of experiments, however, is capable of unambiguously rejecting signalling mechanisms which invoke structural rearrangements along the dimer interface. As discussed below, the truncated receptor chimera may be signalling through a mechanism involving pivoting of periplasmic domains and their
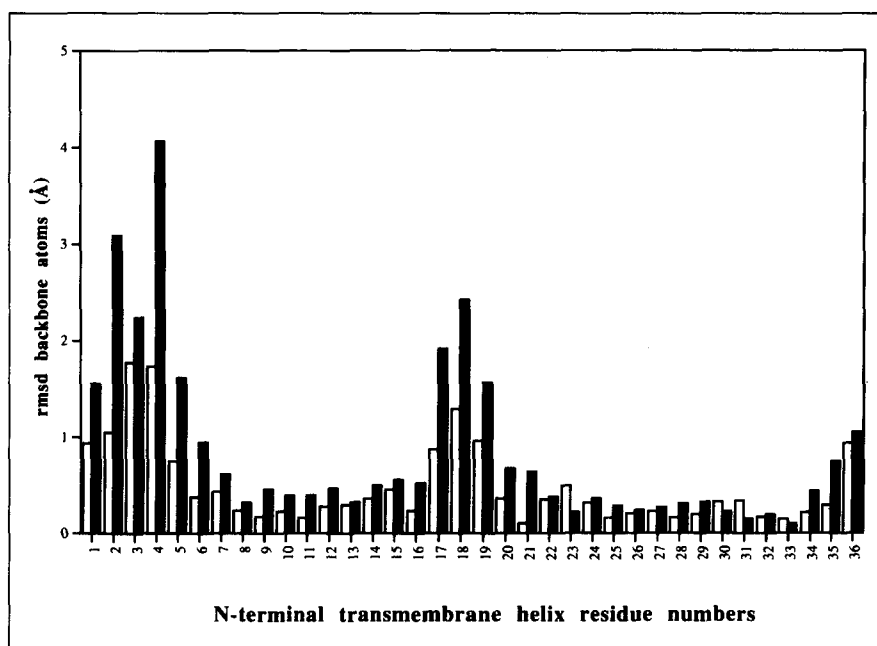

N-terminal transmembrane helix residue numbers

Fig. 3. Bar graph showing the backbone atom distortion from ideality in helix 1 (residues 1–36) caused by Powell minimization refinement of the initial parallel coiled coil model (white bars) and parallel non-coiled model (black bars) with cysteine disulfide cross-linking constraints imposed at positions 4, 18 and 36. These constraints are better accommodated by the parallel coiled coil model of the amino-terminal transmembrane region.

associated transmembrane helices which then induce a conformational change in individual cytoplasmic domains. Likewise, receptor molecules which are cross-linked at only one position along the dimer interface, regardless of location, still might allow a degree of rotational freedom, which might permit rigid-body rotational rearrangements of the subunit interface. We therefore constructed a version of the receptor which is disulfide cross-linked directly on either side of the lipid bilayer at residues 4 (a cytoplasmic cross-link) and 36 (a periplasmic cross-link). Single disulfide cross-links at both of these sites have been shown previously to allow the receptor to function with wild-type properties, both *in vitro* by methylation assays and *in vivo* in bacterial chemotaxis assays. The effect of cross-linking the dimerization interface on both sides of the membrane is to restrain any conformational changes involving rearrangements or movements along the interface at either end of the receptor. The construct also provided a test of the validity of the modeling results for a receptor with multiple disulfide bonds discussed in the previous section.

A detergent-solubilized form of the receptor (in either octyl-glucoside or LDAO) was purified and cross-linking was quantitated by a combination of non-reducing gel electrophoresis and thiol-trapping, followed by ion-exchange chromatography and native electrophoresis to detect the presence of receptor species which are oxidatively cross-linked at only one of the two cysteine residues. This is an important control, because under denaturing gel electrophoresis conditions used to measure methylation, receptors cross-linked at only one cysteine or both cysteines co-migrate and are indistinguishable. Previous experiments involving cysteine cross-links at each individual position indicate that both residues (4 and 36) can readily form disulfide bonds, and that these reactions can be driven quickly to completion in the presence of an appropriate catalyst, such as copper phenanthroline. However, position 4 is prone to alternative oxidative reactions which form non-cross-linked sulfenic acid groups. Therefore, we found and optimized reproducible and gentle non-catalyzed oxidation conditions under which the receptor can be driven uniformly to a form with both sites fully disulfide cross-linked (see Materials and methods). Proper characterization of this construct demands that there be no detectable receptor which is disulfide cross-linked at neither position, or at only one position. Under our final preparative conditions, receptor which is more than 99 % cross-linked at both cysteine residues was isolated both in purified membranes and in a pure detergent-solubilized system. It should be noted that previous cross-linking experiments [9] of single sites at residues 3, 4, and 5 showed unambiguously that this region of the receptor primary sequence exists in a helical conformation, and that a cross-link will only form, even under highly oxidizing conditions, at the residue which is in proper register to form a disulfide bridge with its mate across the dimer interface (position 4). Therefore, the successful formation of a Cys4/4′, Cys36/36′ double disulfide
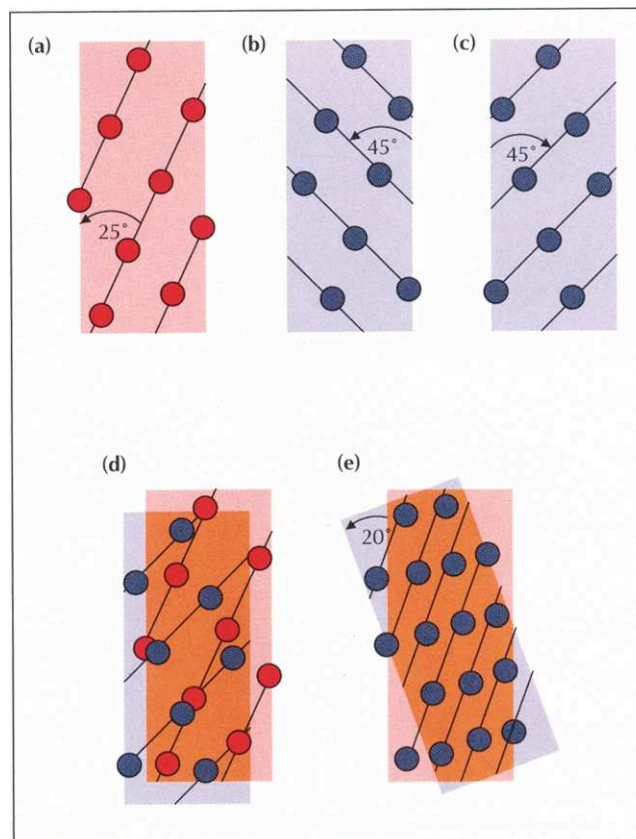

Fig. 4. The 'ridges-into-grooves' packing scheme construction allows one to visualize the geometrical restrictions upon the packing of parallel α-helices at the aspartate receptor subunit interface. **(a)** and **(b)** show identical α-helices (red and blue corresponding to the two aspartate receptor subunits) with ridges from side chains separated by four residues and three residues indicated by red and blue lines respectively. To model the parallel coiled coil interface, one of the helices must be flipped around; **(c)** is identical to **(b)** seen from behind. If the helices are absolutely parallel they cannot form a coiled coil interface as the ridges from one helix will clash with the grooves of the other as seen in **(d)**. However the helices pack properly if they cross approximately 20° from parallel, forming a parallel coiled coil as depicted in **(e)**. From this analysis it is clear that α-helices may pack only at certain discrete angles, and that a pivoting motion between the two helices therefore cannot be accommodated over the length of the helical interface without disrupting helix packing, making rigid-body pivoting or supercoil unwinding energetically highly unfavorable mechanisms of signal transduction. Figure adapted from [13,22–24].

mutant indicates that in the native receptor conformation, both positions are appropriately positioned for disulfide cross-linking.

The signalling ability of the double disulfide cross-linked receptor construct in membranes was then characterized by assaying for enhanced methylation of the carboxy-terminal domain in response to aspartate binding at the periplasmic side. The basal rate and the magnitude of methylation enhancement was identical for wild-type, Cys4, Cys36, and Cys4/36 cross-linked receptor (Fig. 6). In addition, bacteria expressing the various receptor constructs behave similarly in chemotaxis swarm-plate assays
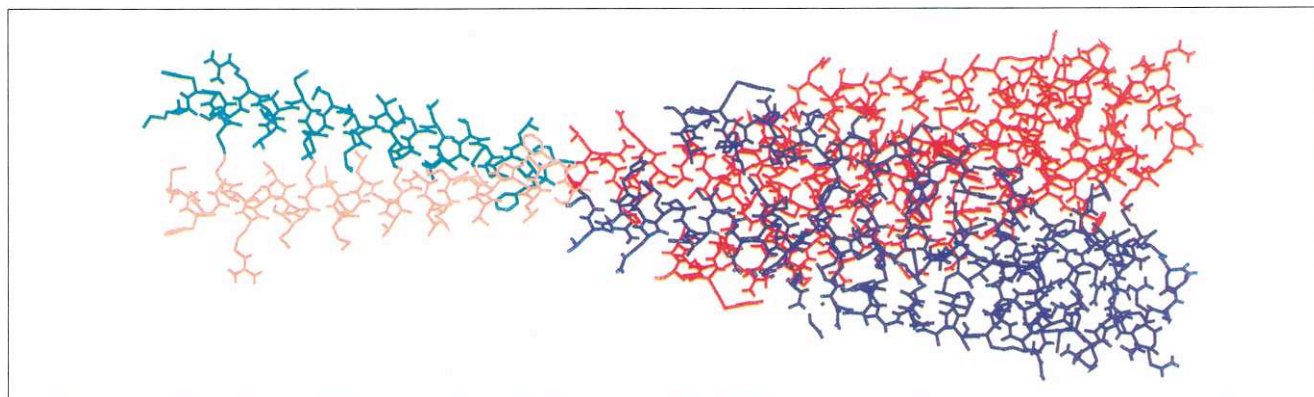
Fig. 5. The refined model of the aspartate receptor ligand-binding domain fitted with the amino-terminal transmembrane coiled coil helices. This model is based upon the combination of all reported biochemical mapping studies of the amino-terminal transmembrane helices by targeted single disulfide cross-links, the successful creation of a functional receptor construct cross-linked at both sides of the membrane (at sites 4 and 36, see Fig. 6 and accompanying text) and modeling and energy minimization studies of possible conformational states of those same helices.

(data not shown), although such assays do not address the *in vivo* oxidation state of the receptor species.

Transmembrane signalling and the receptor coiled coil interface
Structural conclusions
These experiments suggest that the dimer interface of the aspartate receptor, including the first transmembrane sequence, is most likely to be an uninterrupted parallel coiled-coil between residues 4 and 75 (which deviates slightly from ideality between residues 43 and 54 as noted above) as shown in Fig. 5. This structure is most closely approximated by the prototypical coiled coil found in the crystal structure of the 'leucine zipper' dimerization domain of GCN4. The amino-terminal transmembrane region of the aspartate receptor has many

leucine residues (positions 10, 11, 15, 20, 21) and valine residues (positions 8, 12, 14, 17); however the sequence is not a heptad repeat and is clearly not a 'leucine zipper' structure. The coiled coil interface in the aspartate receptor transmembrane region may be described further by calculating the total buried surface area in the coiled coil model ($\sim$1800 Å^2), the number of side chain contacts in the interface (16 or 17 per helix) and the degree of supercoiling twist between residues 4 and 36 ($\sim$100°). These results imply that some rather severe constraints may be placed upon the various previously postulated mechanisms of aspartate receptor transmembrane signalling, because the coiled coil interface is incompatible with the 'pivoting' and 'supercoil unwinding' mechanisms [17,18] described below. The model of the amino-terminal transmembrane helices as a
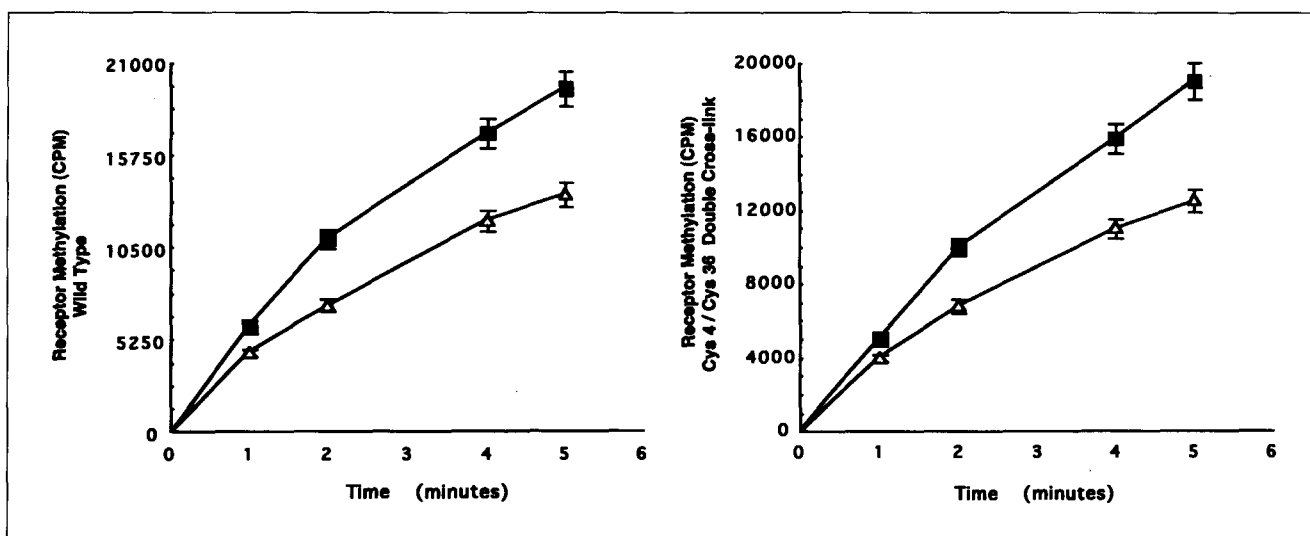

Fig. 6. Methylation of the aspartate receptor in the presence (squares) and absence (triangles) of saturating aspartate. An aspartate-induced increase in methylation of similar magnitude was seen in both the wild-type receptor (left panel) and for a receptor species containing two simultaneous disulfide cross-links between residues 4 and 4' (in the cytoplasm) and residues 36 and 36' (in the periplasmic domain). This double mutation effectively restrains the membrane-spanning interface from conformational changes involving long-range shearing or repacking of the coiled helices.

coiled coil is amenable to deformations no more drastic than shearing motions mediated by low energy torsion angle conformational changes of side chains across the interface, due to the extensive interface of interdigitating side chains between the helices.

Relevance of the structural data to signalling models

The various proposed mechanisms for transmembrane signalling in the aspartate receptor can be summarized briefly as follows (Fig. 7).

The 'pivoting' hypothesis. A small conformational change was reported to be associated with aspartate binding in the form of a 4° pivoting motion between the four helical bundle subunits of the periplasmic domain of the aspartate receptor [7,17]. This observation suggested a simple mechanism for transmembrane signalling in which pivoting of receptor subunits as rigid bodies, changing the positions of the cytoplasmic domains with respect to one another, is responsible for the signalling event (Fig. 7a).

The 'supercoil unwinding' hypothesis. Recognizing that the aspartate receptor periplasmic domain helical bundle subunits form a supercoiled structure, Kim and co-workers [17,18] have also suggested a mechanism in which a partial unwinding of the subunits takes place upon aspartate binding, and the relative change in orientation resulting between the cytoplasmic domains constitutes the transmembrane signalling process (Fig. 7b).

The 'plunger' hypothesis. Milligan and Koshland [8] subsequently challenged the 'pivoting' hypothesis by constructing a receptor composed of one intact subunit and one subunit lacking three-quarters of the cytoplasmic domain, and found that this construct nevertheless was methylated and demethylated in an aspartate-dependent fashion. Based upon these experimental results, mechanisms have been proposed in which transmembrane signalling takes place due to conformational changes within individual receptor subunits upon aspartate binding, such as a 'plunger' motion of individual carboxy-terminal helices within the four-helix bundle of the receptor subunits (Fig. 7c).

The 'pistons' hypothesis. Pakula and Simon [19], and Milligan and Koshland [8] proposed a model to reconcile the apparent disparity between an apparent ligand-induced rigid subunit rotation and normal signalling by truncated receptor chimera and cross-linked receptor species. In this model, pivoting of the periplasmic domains induces an independent change within each of the cytoplasmic signalling domains via a mechanism similar to that of a piston (Fig. 7d). This latter model therefore does not require both monomers to be intact.

Upon dimerization, the GCN4-p1 leucine zipper forms a nearly ideal parallel coiled coil; the left-handed

superhelical twist is approximately 90° over 45 Å (about 31 α-helical residues) [18]. If we assume, based upon the aspartate receptor periplasmic domain crystal structures and the disulfide cross-link results, that the amino-terminal helix persists uninterrupted from at least residue 4 to 75, an ideal coiled coil comprising the receptor dimer interface would contain about 213° of superhelical twist over 71 residues. However, dimer dissociation will become difficult much beyond 60 residues or 180° of parallel coiled coil, because the entwined helices will effectively be locked together like a cable of two component wires. Therefore the region of non-ideal coiled coil in the aspartate receptor dimer interface, in which the coiled coil is somewhat under-wound and the individual helices bow out slightly from one another [6], may be necessary to allow subunit dissociation to take place. Formation of a four-helical bundle and a parallel coiled coil place conflicting requirements upon the curvature of the amino-terminal helix in this region of superhelical distortion. The locally under-wound region of the amino-terminal helix is hence stabilized by four-helical bundle contacts.

On the basis of the disulfide cross-linking data we assume that residues 4–36 of the amino-terminal helix, which do not contribute to the four-helical bundle of the periplasmic ligand-binding domain, form a nearly ideal coiled coil. Such an arrangement of helices produces completely rigid super-secondary structure (eg: keratin and fibrinogen). Therefore this region would not be capable of participating in a pivoting motion between the aspartate receptor subunits unless a motion takes place between the two α-helices which necessitates repacking of their interdigitated side chains. Such a dissociation of the receptor is also ruled out by disulfide cross-linking data [9,10] which demonstrate that disulfide cross-linking at positions 36 and 4 does not disrupt receptor signalling. In essence, any rotational conformational change or rigid-body motion propagated along the receptor interface must involve extensive side-chain repacking and switching between different interdigitating configurations, at an energetic cost which would preclude the rapid changes in receptor signalling states involved in chemotaxis. Pivoting, if it takes place at all, must therefore be in the form of a motion about a centrally located rigid core formed by the closely packed amino-terminal helices of the two receptor subunits.

A multiply cross-linked aspartate receptor having disulfide links at two or more of the positions (4, 11, 18, 22, 25, 29, 36) should, according to this analysis, be compatible with a coiled coil structure and produce a receptor incapable of signalling according to the 'pivot' and 'supercoil unwinding' hypotheses, but should still be active if signalling involves movements which maintain the tight packing of the subunit interface through the membrane. If the amino-terminal helices indeed form a rigid coiled coil in the intact aspartate receptor, then the other three helices of the periplasmic
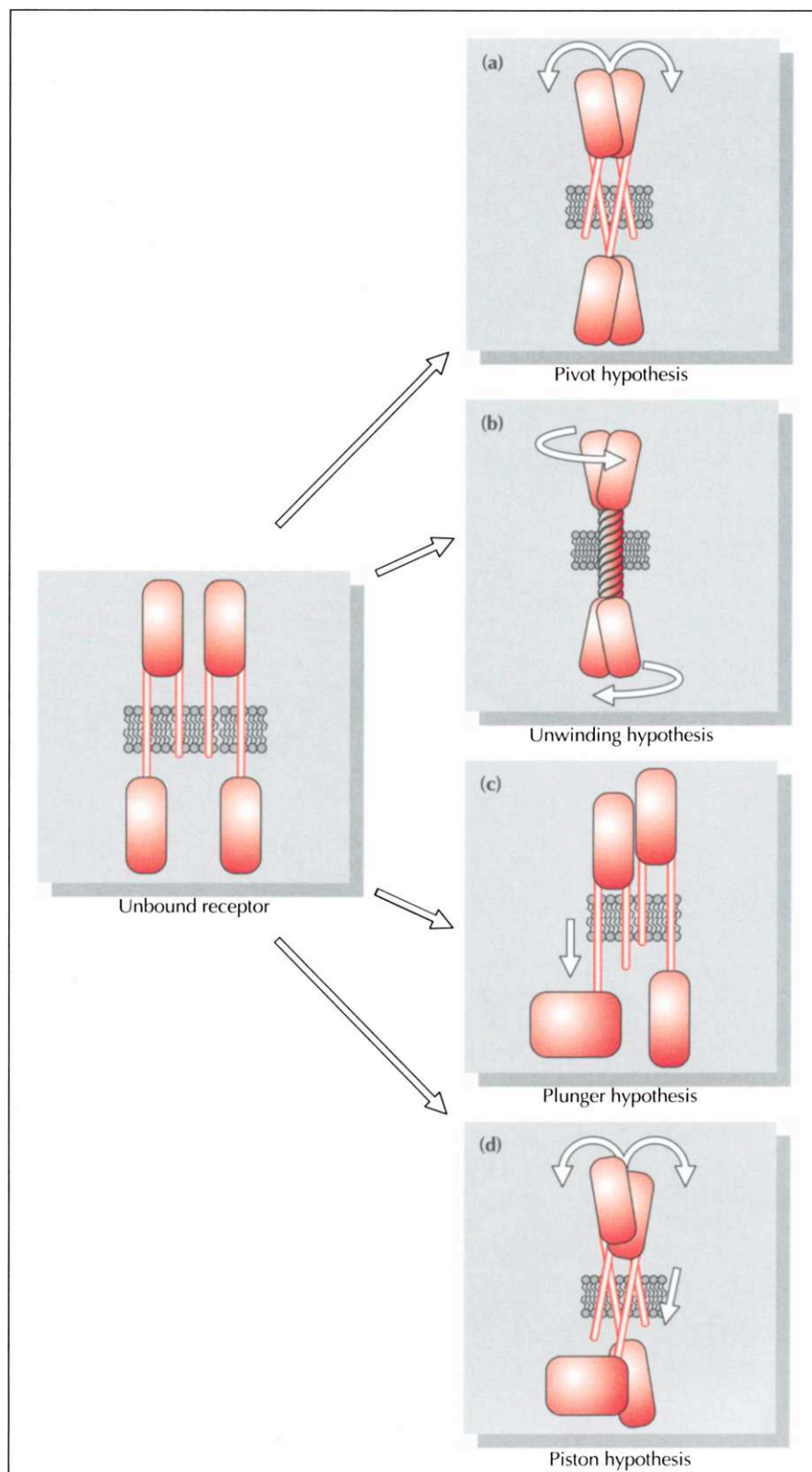
Fig. 7. Hypothesized mechanisms of signal transduction. **(a)** The rigid-body pivoting hypothesis, in which the subunits of the aspartate receptor pivot inward upon binding aspartate. **(b)** The supercoil unwinding hypothesis, in which the parallel coiled coil would somehow change its degree of supercoiling upon aspartate binding. **(c)** The plunger hypothesis, in which a conformational change induced by aspartate binding pushes (or pulls) a transmembrane α-helix through the membrane interface, inducing a conformational change asymmetrically in one of the subunits. **(d)** The piston hypothesis in which mechanisms (a) and (c) are combined to reconcile apparently disparate observations.

domain must move slightly with respect to the central amino-terminal α-helix of the dimer interface in order to accommodate a pivoting motion between the periplasmic domains of the receptor. This of course implies a conformational change within an aspartate receptor subunit.

Constraints upon conformational changes imposed by parallel and antiparallel α-helical packing: a re-evaluation of the crystal structures.

If one overlays the α-carbons of only the most well ordered amino-terminal residues from one subunit of the periplasmic domain with and without aspartate bound

(residues 54–75, rather than the entire amino-terminal helix, which is slightly perturbed in the region of Cys36), one actually finds the largest movement to occur in helix 4 within a single subunit (with a root mean square deviation of about 1.5 Å), rather than between the two identical subunits as originally described. In addition, small conformational changes at the aspartate binding sites in the original crystal structure of the aspartate-bound receptor have been reported subsequent to the original report of the crystal structures. Specifically, the receptor fragment in the crystal structure was observed to bind aspartate at only one site per dimer. Recent biochemical evidence indicates that the *Salmonella* aspartate receptor exhibits partial negative cooperativity of ligand-binding and the *E. coli* version of the receptor exhibits almost complete negative cooperativity [20]; conformational changes induced by binding of aspartate in site 1 cause the shape of the unoccupied ligand-binding site to be diminished relative to either of the sites in the uncomplexed, asparate-free receptor [6,20,21]. Arg64 in the unoccupied site rotates into the binding cleft, Ser68 protrudes further into the site, and Tyr149 shifts 1.9 Å laterally so that an aspartate ligand can no longer be accommodated sterically in the binding cleft. These small conformational changes in turn may also be correlated both with small movements between helices within each receptor subunit, and with long-distance rearrangements of side chain conformations as described below.

The transmission of conformational changes in tightly packed α-helices such as a coiled coil, and other protein interfaces and interiors in general, results from an inherent limit on the ability of α-helices to accommodate a conformational perturbation locally, according to an analysis of conformational changes induced within insulin molecules by different crystal packing forces [22]. This limit is imposed by geometrical packing restrictions on the α-helices (for example the 'knobs-into-holes' and 'ridges-into-grooves' packing of coiled coils [13] and four-helix bundles [24]); larger conformational changes would require repacking as illustrated in Fig. 4 (which would probably force dissociation of the four-helix bundle or receptor monomers in our case). The existence of this limit upon the local dissipation of helix movements results in propagation of a perturbation over long distances. This perturbation of the dimer interface presumably is responsible for a motion of α-helix 4 (the carboxy-terminal helix in the aspartate-binding domain) with respect to the central rigid coiled coil within each aspartate receptor subunit. However, the same small side chain conformational changes propagated over long distances (which may be responsible for mediating movement of helix 4 with respect to the rigid amino-terminal coiled coil) may also be capable of inducing a concatenation of small side chain conformational changes. This could lead to an alteration in the structure of the cytoplasmic domain (connected to the carboxy-terminal end of helix 4 extending through the membrane) upon aspartate binding. The latter changes

would therefore be ones which occur within the receptor subunit (and therefore would accommodate Milligan and Koshland's results [8]) and would be a direct result of the side chain conformational changes which take place in the aspartate binding site upon ligand binding. The small conformational changes within the periplasmic domains which take place about the rigid amino-terminal coiled coil and the movement of helix 4 relative to this rigid core would thus be seen as two inexorably linked manifestations of a single correlated set of packed helix side chain conformational changes. These changes are prevented from dissipating locally due to the extensive packing interactions within the four-helix bundles, as well as the packing interactions between them which constitute the parallel coiled coil interface.

Biological implications

The aspartate receptor belongs to a class of cell-surface signal-transducing proteins which transmit a signal to the cytoplasm. The class includes receptors which mediate bacterial chemotaxis, as well as receptors which mediate metabolic functions, growth, differentiation and division in eukaryotic cells.

The crystal structure of the extracellular domain of the aspartate receptor has been solved in the absence and presence of aspartate. Comparison of these structures does not give a clear indication of the mechanism of signalling in this transmembrane receptor. This is probably because the transmembrane regions and the internal signalling domain were not present in the crystal structure. However, ongoing biochemical experiments, such as site-directed disulfide cross-linking between receptor subunits, reveal additional structural information. By placing the results of these experiments in the context of the known crystal structures, we have constructed a model of the aspartate receptor in which the interface between the two subunits is an uninterrupted parallel coiled coil of approximately 71 amino acid residues per helix.

Based upon this structure, we argue that the mechanism of aspartate receptor transmembrane signalling cannot involve large conformational changes between the receptor subunits because such movements cannot be accommodated by the coiled coil interface without causing disruption of the helical packing arrangement. Similarly, mechanisms involving large conformational changes within a subunit of the receptor are unlikely because they would necessitate disruption of packing within the subunit. The most likely mechanism of signal transduction, therefore, is one in which side chain conformational changes induced by the binding of aspartate are propagated over the length of the helix because the

tight helical packing of the protein prevents local accommodation of such structural perturbations. Such a mechanism may be a common feature of many receptors that signal through conformational changes and consist of one or two transmembrane helices.

Materials and methods

Site-directed double cross-linking of the aspartate receptor
Receptor with glutamic acid at all four cytoplasmic methylation sites (295, 302, 309, 491) was originally subcloned, mutagenized in separate constructs at positions 4 and 36, and expressed in strain RP4090 (JS Parkinson, University of Utah) as described previously [9–11]. A double mutant construct was then created by restriction digest and religation of the two plasmid constructs, making use of a unique restriction site conveniently located between the two cysteine-encoding codons. The double mutant, and similar expression vectors encoding wild-type, Cys4 and Cys36 forms of the receptor, were then transformed into strain RP4080, (provided generously by JS Parkinson, University of Utah). All forms of the receptor were overexpressed in liter cultures in Luria broth and membrane suspensions or pure detergent preparations were purified as previously described.

Previous cross-linking studies of sites 4 and 36 show that while the periplasmic cross-link (position 36) can be completely driven to the disulfide state by a variety of methods, the cytoplasmic cysteine (position 4) is susceptible to oxidation to non-cross-linked, sulfinic acid derivatives under the same conditions. Under more gentle cross-linking conditions, however, Cys4 also cross-links very slowly to completion. In this study, therefore, cross-linking was carried out by preparing membranes in high dithiothreitol, then washing and incubating under low catalyst concentrations (0.2 mM copper phenanthroline) or even under oxygen-saturated conditions in the total absence of oxidative catalyst. We benefitted in this regard by the fact that the double cross-linked form of the receptor appears to cross-link at residues 4 and 36 in a cooperative manner: the rates of disulfide formation for both sites are much faster at these mild conditions than for either residue in the single mutant constructs. This appears to be caused by a simple proximity effect due to quenching of monomer exchange once an initial disulfide has been formed at either position, and is further evidence in favor of the coiled coil model in that the two cross-linking positions must be mutually compatible in the intact aspartate receptor to obtain this cooperative effect.

After the various receptor species were cross-linked as described, they were incubated and specifically methylated with CheR and tritium-labeled S-adenosyl methionine (SAM) over a time course of 5 min in the presence and absence of 10 mM aspartate as described previously [9–11]. Receptor signalling is indicated by an aspartate-dependent increase in methylation due to transmission of a conformational change to the cytoplasmic signalling domain (and to the methylation sites) which is responded to by the CheR methyl transferase as part of the habituation response of the chemotaxis pathway. The reaction was quenched at multiple time points and aliquots were run on a 7.5 % denaturing gel. Bands corresponding to cross-linked receptor were excised and assayed for tritium decay using a scintillation counter.

In order to assure that the double cysteine mutant receptor construct used in the aspartate-induced methylation assay was actually in the double disulfide cross-linked form, purified receptor and membrane extracts containing overexpressed receptor were assayed for total disulfide formation by free thiol trapping, using a basic procedure first described by Creighton [25]. After the cross-linking reaction had been carried out to its putative completion, the receptor was incubated with 0.1 M iodoacetate, which converts all free cysteine side chains to acidic groups. After dialysis, the receptor population was analyzed on isoelectric focusing gels, native gels, and on mild anion-exchange columns. As a control, unoxidized cysteine-containing receptor samples and wild-type (cysteine-less) receptor were also treated as described. Under these conditions, receptor containing acidified free thiols are resolved from those receptors containing no cysteine residues or those species with completely formed disulfide bonds. This assay was important for ensuring that neither site was incompletely cross-linked, and also for initially finding the appropriate conditions for oxidation of Cys4 and Cys36.

Modeling the transmembrane region
The transmembrane region of the dimeric aspartate receptor, consisting of residues 1–36 and their symmetry pairs, was modeled based upon the fact that the site-directed disulfide cross-linking data were completely consistent with the assumption that this region of the receptor forms a canonical parallel coiled coil. The model was constructed using the polypeptide backbone of the leucine zipper parallel coiled coil [14] and the *Salmonella* aspartate receptor side-chains using the Biopolymer and Builder modules of the graphics display program Insight II (version 2.2.0, Biosym Technologies, CA) to modify the GCN4 coordinates. Because the helices in the leucine zipper structure are only 31 amino acids long, extended helices were first constructed by superimposing two copies of the leucine zipper backbone atoms upon one another in X-PLOR 3.1 and then refining them using 500 cycles of Powell minimization [16].

To test that this refined model was indeed consistent with the disulfide cross-linking data, cysteines at positions 4, 11, 18, 22, 25, 29 and 36 were individually and collectively introduced (with their symmetry pairs) and these models were again refined both by conventional Powell minimization and by simulated annealing with backbone atoms constrained. Because the energy function used for refinement in X-PLOR 3.1 contains no terms corresponding to hydrophobic interactions which favor maintaining surface contacts, and no attempt was made to model the membrane environment surrounding the coiled coil helices, simulated annealing tended to drive the two helices apart in the absence of disulfide cross-links as there were no energy terms to compensate for van der Waals repulsive interactions between the protein surfaces. Thus the backbone atoms were constrained to their initial positions, resulting in refinements indistinguishable from conventional Powell minimization. The classical forcefield energy function used for minimization in X-PLOR 3.1 contains harmonic potential energy terms for covalent bond length deviation, bond angle and torsion angle (dihedral and planar) geometric deviation, and a non-bonding van der Waals interaction potential. Ionic charge interactions, hydrophobic interactions and solvent effects were not modeled in our refinements. The parameters used for calculating the forcefield were obtained from the X-PLOR 3.1 file parhcsdx.pro which includes bond length and angle parameters derived from Cambridge Data Base model structures. The harmonic force constant for the disulfide bond in this

parameter set, for example, is estimated at 500 kcal mol^{-1}, dominating the non-bonded potential by approximately two orders of magnitude. Therefore our models are dominated by constraints governing the stereochemistry of the side chains and backbone atoms; distortions from ideality induced by disulfide bond cross-linking indicate the degree to which the initial model and disulfide cross-links are incompatible based overwhelmingly upon bond length and angle geometries.

Finally, a model based upon ideal but non-coiled parallel α-helices containing cross-links at positions 4, 18 and 36 was similarly constructed using a pitch of 3.6 residues per turn. This model, which initially has residue 4 almost completely out of phase on the helix relative to residues 36 and 18, was refined and compared with a refined model of the parallel coiled coil containing the same three cross-links. The non-coiled model, upon refinement, leads to substantial distortions of the helix backbone in the non-coiled helices (Fig. 3), suggesting that the disulfide cross-linking data are incompatible with a non-coiled coil structure. Both models contain a significant distortion from non-ideality at position 4 upon refinement. However, the magnitude of this distortion in the case of the non-coiled helices is twice that of the coiled coil helices, and a significant distortion occurs at position 18 in the case of the non-coiled helices (Fig. 3).

Modeling residues 4–180 of the aspartate receptor
A model of the aspartate receptor periplasmic domains coupled to the amino-terminal transmembrane α-helices was constructed and energy-minimized using the refined structure of the aspartate receptor ligand-binding domain [6], residues 23–80, and residues 4–36 of the transmembrane region model described above. The two structures were grafted together by least-squares superposition of residues 23–36 and their symmetry pairs in the two structures using the same procedure in X-PLOR 3.1 as described above.

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