Structure and Dynamics of Transmembrane Signaling by the *Escherichia coli* Aspartate Receptor†

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ABSTRACT: The structure of the cytosolic extension of the first transmembrane region (TM1) of the *Escherichia coli* aspartate receptor (residues 3, 4, and 5) and conformational changes within that region have been characterized by targeted cross-linking studies and by measurement of the effect of aspartate binding on cross-linking and methylation rates and compared with the periplasmic extension of the same helix. These experiments show that (1) the cytosolic extension of TM1 is helical, with residues 4 and 4′ closest together at the dimer interface; (2) the helix is more solvent-exposed at the cytosolic side of the membrane than on the periplasmic side; and (3) aspartate binding enhances the rate of cross-linking at Cys 4, and the resulting cross-linked receptor displays aspartate-induced transmembrane increases in methylation by the cytoplasmic methylase (the CheR protein). We conclude that aspartate induces a conformational change that does not involve large intersubunit movements that lead to an increase in distance between the cytosolic ends of the first membrane-spanning helices; rather, the motion involved is largely contained within individual subunits, possibly resulting in a small movement between positions 4 and 4′.

The aspartate receptor from the *Escherichia coli* chemotaxis pathway (the Tar[1] protein) is a transmembrane signal transducing protein which is responsible for mediating the detection of a number of extracellular ligands, including aspartate, maltose-binding protein, and various metal and aromatic species. The receptor is a homodimer with two transmembrane domains per subunit, a general structural motif similar to the receptors for LDL, insulin, interleukins, and other ligands (Stoddard et al., 1992). Ligand binding to the external, periplasmic domain is known to cause activation of a single cascade process within the cytoplasm which ultimately causes flagellar reversal (Koshland, 1988; Bourret et al., 1991). Adaptation of the receptor to bound aspartate is caused by methylation of four conserved glutamic acid resiudes in the cytosolic domain of the receptor (Terwilliger et al., 1986). The three-dimensional structure of the ligand-binding and transmembrane portions of the aspartate receptor was deduced from the crystallographic structure of the periplasmic ligand-binding domain (Milburn et al., 1991) and the use of targeted disulfide cross-linking (Lynch & Koshland, 1991). These data showed that helices 1 and 4 within a subunit extend through the membrane as transmembrane region 1 (TM1) and transmembrane region 2 (TM2) as uninterrupted α-helices at least to the cytoplasm–membrane border, forming a bundle of four helices with contacts across the dimer interface formed by TM1 and TM1′. This model was confirmed by Pakula and Simon (1992) through a cross-linking study employing a random cassette mutagenesis method. Mechanisms of signaling that have been proposed involve movement of the two subunits relative to one another in scissors- or seesaw-type motions and/or movements of helices within individual subunits in a piston-type motion (Milligan & Koshland, 1991; Milburn et al., 1991; Stoddard et al., 1992). If helix 1 continues into the cytoplasm, then the presence of these types of ligand-induced movements should be discernible. The purpose of this work was to characterize the structure of the cytoplasmic extension of TM1, look for such movements, and interpret them in terms of transmembrane signaling.

The method of targeted disulfide cross-linking was developed in order to characterize the degree of flexibility and of ligand-induced conformational change involved in transmembrane signaling (Falke & Koshland, 1987; Falke et al., 1988), the oligomeric structure of the receptor (Milligan & Koshland, 1988), and the structure, packing, and interactions between the transmembrane domains of the receptor (Lynch & Koshland, 1991). The application of this technique is particularly useful for receptor molecules, due to the difficulty of crystallizing integral-membrane proteins.

MATERIALS AND METHODS

Mutagenesis of Tar. *E. coli* Tar with glutamic acid at all four cytosolic methylation sites (295, 302, 309, 491) was originally subcloned into the pSL1180 vector (Pharmacia LKB Biotechnology). A modified form of Kunkel's method (Kunkel, 1985) was used to prepare the uracil-containing single-stranded template from this vector. Site-directed mutagenesis was used to produce four mutants with cysteines at positions 3, 4, 5, and 36. The primers were from Operon (Alameda, CA). Between 2 and 20 pmol of phosphorylated primer was annealed to 0.1 μg of template DNA. Dideoxynucleotide sequencing (Sanger et al., 1977) confirmed the mutant sequences.

Expression of Mutant Receptors and Preparation of Membranes Containing Overexpressed Receptor. All mutant forms of the receptor were expressed in strain RP4080 (cheR$^-$) obtained from J. S. Parkinson (University of Utah). Membranes were prepared as previously described (Foster et al., 1985). For standard cross-linking and spontaneous oxidation studies, a high concentration of the reducing agent dithio-

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[1] Abbreviations: Tar, aspartate receptor; CheR, cytoplasmic methyl transferase; TM1, transmembrane region 1; TM2, transmembrane region 2; DTT, dithiothreitol; β-ME, β-mercaptoethanol; SAM, *S*-adenosylmethionine; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetate.

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threitol (DTT) was used (50 mM during sonication and 5 mM throughout the wash steps and final resuspension) in order to reduce spontaneous disulfide formation prior to incubation with specific oxidation catalysts. The DTT was then removed in a final spin, and the membranes were resuspended directly before cross-linking experiments. For experiments which assessed the susceptibility of specific cysteine mutants to overoxidation, a lower concentration of DTT was used throughout the membrane preparation (2 mM) and removed from the final resuspension buffer (10 mM Tris-HCl, pH 7.5, room temperature, 10% glycerol, 5 mM phenanthroline, 1 mM PMSF, 5 mM EDTA).

Targeted Cross-Linking Used To Measure Relative Distances across the Dimer Interface. Cross-linking rates and steady-state levels for cysteines 3, 4, 5 (cytosol), and 36 (periplasm) were measured for each membrane-bound receptor species by the method previously reported (Lynch & Koshland, 1991) using membrane prepared in the presence of high DTT (5 mM). Briefly, receptor was radiolabeled in the membrane by specific methylation using CheR with [^{3}H]SAM. The membranes were then spun at 200000g for 10 min and resuspended to remove DTT and excess SAM, and samples of the labeled receptor in membranes were then immediately incubated over a time course at 37 °C with copper phenanthroline as catalyst (0.2 mM $CuSO_4$, 0.6 mM 1,10-phenanthroline). The oxidation was stopped at various time points by adding aliquots to a Laemmli sample buffer (without β-mercaptoethanol) with 10 mM NEM and 10 mM EDTA and freezing the solution in liquid nitrogen. Samples were run on a 7.5% nonreducing SDS–acrylamide gel, and bands corresponding to un-cross-linked monomer and cross-linked dimer, 60 and 120 kDa, respectively, were excised and assayed for ^{3}H decay using a scintillation counter. Data are expressed as the ratio of cross-linked vs total receptor as a function of time. The cpm ratio measured for cross-linked receptor (running at 120 kDa) vs total receptor (60 + 120 kDa) directly reflects the percentage of cross-linking.

Oxidation under Varying Conditions To Assess the Environment and Solvent Accessibility of Specific Sites. A second set of cross-linking experiments were performed under different conditions in order to measure the susceptibility of the periplasmic and cytoplasmic regions of TM1 to overoxidation and subsequent cleavage and to examine the uncatalyzed, initial cross-linking rates of sites on either side of the membrane. Membranes prepared from low DTT (2 mM) as described above were oxidized at 37 °C with a higher concentration of catalyst (1.5 mM $CuSO_4$, 4.5 mM 1,10-phenanthroline) and then assayed for cross-linked vs total labeled receptor as a function of time. In a second experiment, membranes prepared in the presence of high DTT were cross-linked simply by incubation at 37 °C in the absence of any catalyst in order to examine whether susceptibility to overoxidation at specific positions (as described above) on the helix correlates with higher rates of spontaneous, uncatalyzed oxidation and disulfide formation.

Measurement of Aspartate-Induced Motions at the Cytosolic End of TM1. We examined two different effects in order to assess the physical and dynamic effects of aspartate binding on the cytosolic extension of TM1: the effect of aspartate binding on the cross-linking of Cys 4 and the effect of cross-linking at position 4 on the rate and level of specific methylation by CheR in the presence and absence of bound aspartate. In order to measure the effect of aspartate binding on cross-linking rates, 10 mM aspartate was added along with copper phenanthroline to membranes prepared from high DTT. Cross-linking was then assayed as described above. In the second experiment, membranes containing receptor with Cys 4 were cross-linked to steady state in the absence of aspartate (yielding membranes which are approximately 50% disulfide cross-linked and 50% free thiol at Cys 4). After the membranes were washed to remove copper phenanthroline, the receptors in the membranes were then methylated at 22 °C in the presence or absence of bound aspartate. At this temperature the receptor containing Cys 4 does not exhibit spontaneous disulfide oxidation or reduction. The methylation reaction was quenched at specific time points by adding aliquots of the membranes to Laemmli buffer (no β-ME) and freezing the solution in liquid N_2. Samples were then run on acrylamide gels, and bands corresponding to cross-linked and to free receptor were excised and counted as described above.

RESULTS

Mutagenesis, Expression, and Membrane Preparation. Expression experiments showed the highest expression of the Cys 36 mutant in LB media at 37 °C overnight. Receptors containing mutant Cys residues at positions 3, 4, and 5 were expressed in VBC media at 30 °C overnight. Typical yields were 2–3 mg of receptor per 1 g of cells under these conditions. For membranes resuspended and frozen in the presence of high DTT (5 mM), the percentage of the total receptor found to be disulfide cross-linked prior to oxidation experiments was approximately 20% for Cys 3, 4, and 36 and much lower for Cys 5. For membranes prepared in low DTT (2 mM) and stored without DTT, the level of spontaneous oxidative cross-linking increased dramatically for Cys 3 and Cys 4 (to approximately 40–50% of the total receptor) but not for Cys 36.

Oxidative Cross-Linking Used To Measure Relative Distances across the Dimer Interface. Figure 2 depicts the cross-linking time course for oxidation of the four receptor species containing Cys 3, 4, 5, and 36. The membranes were prepared in high DTT (50 mM) and stored at 5 mM DTT. After the membranes were washed to remove DTT and incubated with 0.2 mM Cu^{2+} and 0.6 mM phenanthroline at 37 °C in the absnece of aspartate, the steady-state levels of the disulfide cross-linked Cys 3, 4, and 5 mutants rise to approximately 20%, 50%, and less than 5% of the total receptor population, respectively. Cys 36, treated identically, cross-linked to a final level of greater than 90% of the total receptor population. Of the three cysteine mutants in the cytosolic region of TM1, Cys 4 displayed the fastest initial rate of cross-linking and the highest steady-state level of stable disulfide formation. Cys 3 and Cys 5 cross-link much less readily than Cys 4, with steady-state values of 20% and 4%, respectively, of the total receptor population.

Cross-Linking under More Highly Oxidizing Conditions Used To Assess the Environment and Solvent Accessibility of Specific Sites. After preparation and storage of membranes with lower concentrations of DTT (2 mM washes, 0 mM resuspension), the receptor with Cys 4 displays increased levels of spontaneous disulfide formation, yielding a receptor which was almost 50% cross-linked prior to incubation with copper phenanthroline, as seen at time 0 in the bottom panel of Figure 3. Subsequent incubation of the Cys 4 receptor with elevated concentrations of catalyst (1.5 mM Cu^{2+}, 4.5 mM phenanthroline) produced increased cross-linking which exceeded the nominal steady-state level of disulfide formation (Figure 3). The ratio of cross-linked to total receptor then was observed to decrease, possibly corresponding to further oxidative cleavage of the disulfide bond (see Discussion).

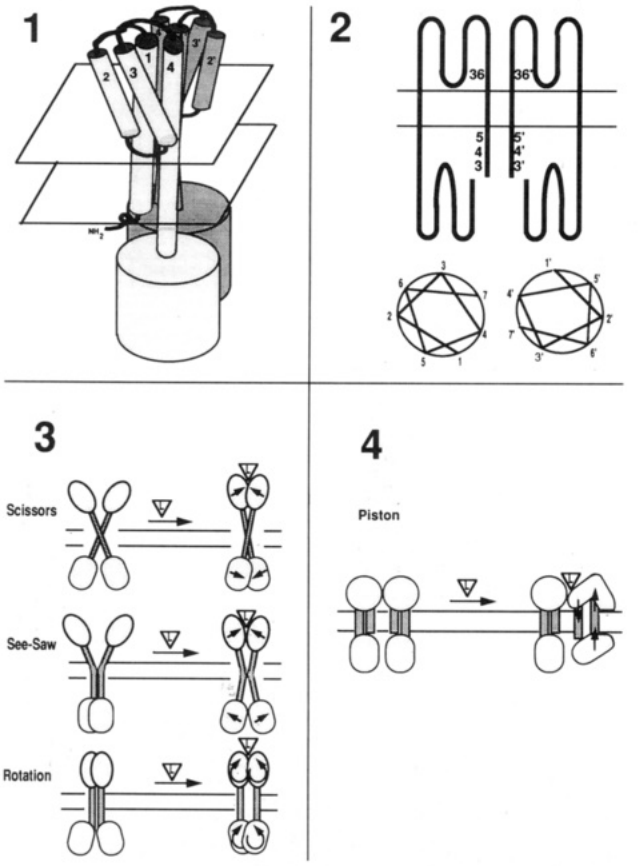

FIGURE 1: Bacterial chemotactic aspartate receptor, distribution of various residues on helices 1 and 1′, and possible mechanisms of ligand-induced conformational changes and signal transduction. The structure of the periplasmic ligand-binding domain of the receptor has been solved by X-ray crystallography, and a model of the transmembrane-spanning helices has been extrapolated on the basis of that structure (panel 1). Placement of a cysteine at position 36 on the periplasmic side of TM1 and 1′ yields a cross-linked receptor species with full activity; creation of cysteines at positions 3, 4, and 5 allows characterization of the structure and relative alignment of those residues and comparison with the extrapolated model (panel 2). Mechanisms for signal transduction have been hypothesized which include rigid-body rearrangements between subunits, causing movement of the cytoplasmic domains relative to each other (panel 3), or conformational changes within individual subunits (panel 4) which might lead to "piston"-like movements of the transmembrane helices and cytoplasmic signaling by individual subunits.

Conversely, the receptor containing Cys 36 showed only a very small increase in spontaneous disulfide formation when membranes were prepared and stored under low DTT concentrations (2 mM washes, 0 mM in storage buffer) as shown in Figure 3 at time 0. During subsequent incubations with copper phenanthroline, the receptor with the Cys 36 mutation asymptotically approaches a high steady-state cross-link ratio when incubated under both high and low concentrations of catalyst (as opposed to the overoxidation of Cys 4 under identical conditions). The observation that the receptor with a cysteine at position 4 is more susceptible to oxidative processes than when the cysteine is on the other side of the membrane (even though Cys 4 does not ultimately cross-link as extensively as Cys 36) led us to examine the rate of spontaneous, uncatalyzed disulfide formation for both species.

Oxidation and disulfide formation of the membrane-bound receptor [prepared in the presence of high DTT (50 mM)] containing Cys 4 or Cys 36 were measured at 37 °C in the absence of all catalyst. For both species of the receptor, cross-link formation proceeded in a linear manner over the duration

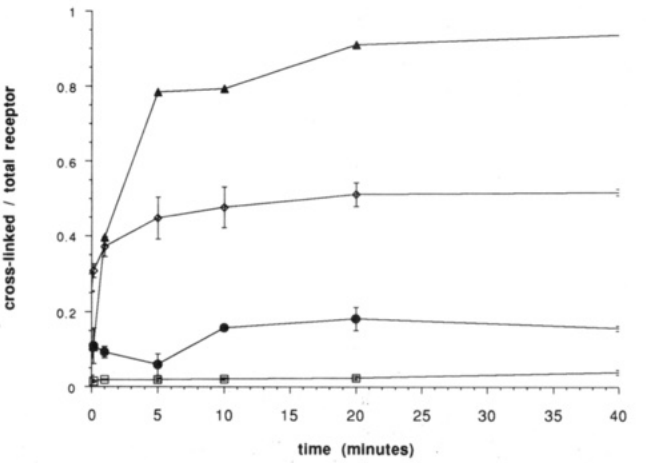

FIGURE 2: Cross-linking of Tar mutants to determine the relative distances across the Tar cytosolic dimer interface. After preparation of membranes from high DTT (5 mM), the membranes were washed, and a low catalyst concentration (0.2 mM $CuSO_4$, 0.6 mM phenanthroline) was used to oxidize Cys 3, 4, and 5 to form disulfide-linked homodimers in the absence of aspartate. Aliquots were removed at various time points, and the reaction was quenched to yield the reaction coordinate of cross-linking (expressed as dpm for cross-linked receptor divided by total receptor dpm). Zero time reflects the fraction of cross-linked receptor before addition of catalyst. Error bars reflect the standard error of the mean. Cys 36 cross-linking (determined under indentical conditions) is shown for comparison. Similar experiments on a faster time scale confirm that Cys 4 displays the fastest initial kinetics of cross-linking of all four residues (data not shown). Symbols: (▲) Cys 36; (◇) Cys 4; (●) Cys 3; (□) Cys 5.

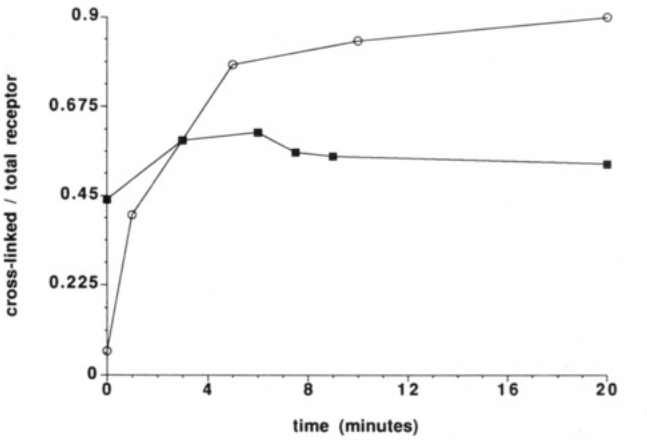

FIGURE 3: Effect of catalyst and DTT concentration on cross-linking of Cys 36 and Cys 4. Preparation of membranes in the presence of lower concentrations of DTT (2 mM during washes, 0 mM in final storage buffer) results in significantly greater spontaneous disulfide formation at residue 4 (see time 0 as compared to Figure 2) but not at residue 36 on the opposite side of the membrane. Subsequent oxidation with increased catalyst concentration seems to involve Cys 4 but not Cys 36 in oxidative processes leading to increase cross-link formation followed by cleavage, as indicated by the nonasymptotic approach to equilibrium. Separate experiments with varying incubation times confirm the transient rise and fall of cross-linked Cys 4 (data not shown). Symbols: (○) Cys 36; (■) Cys 4.

of the time course as shown in Figure 4. The correlation coefficient to a straight-line fit was over 90% for both receptor species. Cys 4 spontaneously cross-linked at a rate twice that of Cys 36, implying a lower activation energy for formation of a disulfide bond at residue 4 than at residue 36.

Measurement of Aspartate-Induced Motions at the Cytosolic End of TM1. The aspartate effect on the cytoplasmic extension of TM1 was explored by (1) measuring the time-dependent cross-linking of Cys 4 with and without bound aspartate and (2) examining the effect of a Cys 4–Cys 4′

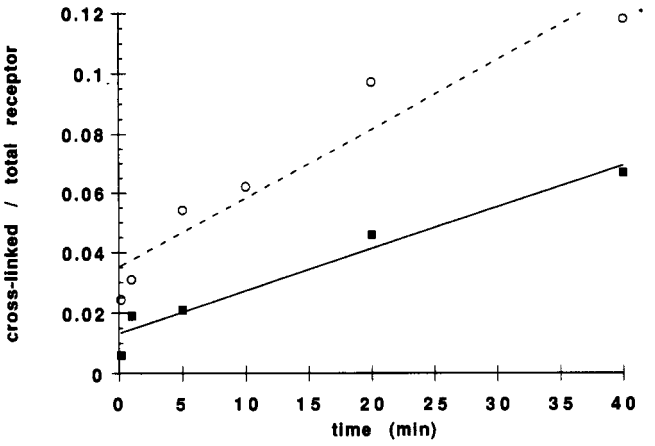

FIGURE 4: Rate of spontaneous cross-linking of Cys 4 and Cys 36. A cross-linking reaction time course was performed in the absence of both catalyst and aspartate. The time course began upon incubation at 37 °C. The rate of uncatalyzed cross-linking is determined from the slope of the time course reaction coordinate and shows that Cys 4 spontaneously forms disulfide cross-links at a rate approximately twice that of Cys 36, even though the Cys 36 cross-links to a higher final level (Figure 2). Symbols: (○) Cys 4; (■) Cys 36.

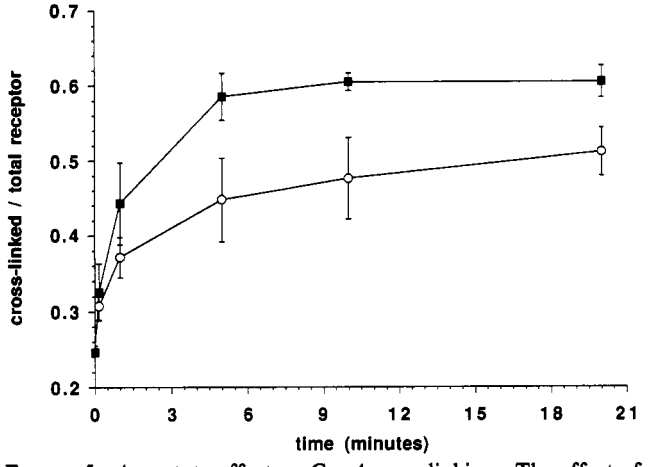

FIGURE 5: Aspartate effect on Cys 4 cross-linking. The effect of aspartate at 10 mM concentration on the rate and extent of disulfide formation at residue 4 was measured in order to assess the possibility that aspartate binding produces an intersubunit movement of the two residues away from one another, as opposed to a conformational change within individual subunits. The initial rate of cross-linking at this postion is approximately 20% faster in the presence of bound aspartate, with a higher final level of cross-linking as shown. Longer incubation also leads to higher final levels of cross-linking in the presence of aspartate than in its absence. Symbols: (■) +aspartate; (○) −aspartate.

disulfide bond on the rate of specific receptor methylation by CheR in the presence and absence of bound aspartate. As seen in Figure 5, addition of 10 mM aspartate prior to cross-linking of the receptor containing Cys 4 produces an increase in both the initial rate and final level of disulfide formation at that position. Cross-linking in the presence of aspartate proceeded to almost 60% completion compared to 50% in the absence of aspartate. This implies that (i) formation of a cross-link at residue 4 produces a structural change which is in some way compatible with or relieved by the motion induced by aspartate binding or (ii) aspartate binding enhances the dynamics of cross-linking by increasing the percentage of the dimeric receptor in solution.

In order to confirm this result, we measured the rate of methylation of the receptor (both cross-linked and un-cross-linked at Cys 4) by CheR and examined the effect of aspartate binding on those rates. As seen in Figure 6, the receptor containing Cys 4 shows an aspartate-induced increase in methylation in both the cross-linked and un-cross-linked states, implying that aspartate binding does not induce a large separation of residues 4 and 4′ (see Discussion).

DISCUSSION

In an attempt to characterize the transmembrane structure of the aspartate receptor and the mechanism of signal transduction, we have conducted experiments aimed at examining the cytosolic extension of the first transmembrane domain (TM1); these data complement previous work regarding the periplasmic ligand-binding domain (Milburn et al., 1991) and the membrane-spanning helices (Lynch & Koshland, 1991). The results presented above characterize the cytosolic region of TM1 in the following areas: (1) the structure of the transmembrane helix TM1 upon exiting the lipid bilayer and entering the cytosol and the relative distances between residues at the dimer interface in that region, (2) accessibility of the cytosolic extension of TM1 to solvent, as compared to the periplasmic region of the same helix, and (3) aspartate-induced structural changes which are transduced through the membrane and into the cytosol, leading into the chemotaxis signal cascade.

Extension of helices 1 and 1′ from the periplasmic domain through the transmembrane domain and then into the cytosol predicts a distance of 5.8 Å between the α-carbons of residues 4 and 4′ (the shortest predicted intersubunit distance in residues 1–7) and substantially longer distances for 3 and 3′ and for 5 and 5′ (Figure 1). This model was based on extension of the first and fourth helices in the four-helix bundle structure of the periplasmic ligand-binding domain, producing a model of the membrane-spanning dimer interface which consists of helices 1, 1′, 4, and 4′ from the two receptor subunits.

Modeling of these adjacent helices was performed by extending the crystal structure through the transmembrane domains (residues 1–25 and 180–205) according to the previously elucidated rules for the packing of side chains from parallel helices (Chothia et al., 1981), leading to a structure which can be described as a "bundle" of four helices, with the primary interactions between TM1 and TM1′ (residues 1–25 from each subunit). These two helices run in the same direction through the membrane, packing into a structure with a interhelix angle of approximately 20° and a very slight left-handed twist around one another (approximately a 25° twist in eight turns of a helix spanning 43 Å, from residue 1 to residue 30). This structural model was energy-minimized using XPLOR 2.1 (Milburn et al., 1991), leading to a final model for transmembrane helix packing and intersubunit distances across the dimer interface which agree with targeted cross-linking data measured both within the middle of the lipid bilayer (at residues 18 and 18′; Lynch & Koshland, 1991) and at the entry into the cytoplasm of helices 1 and 1′ as shown in the current studies. Specifically, this model predicts that position 4 in the cytosolic extension of transmembrane helices 1 and 1′ should be considerably closer than positions 3 and 5 (Figure 1) and that cysteine residues placed individually at each of those three sites should provide differing rates and levels of disulfide formation and confirm or refute the extrapolation of the transmembrane domains.

The results shown in Figure 2 indicate that the Cys 4 mutant cross-links faster and to a greater extent than either the Cys 3 or the Cys 5 receptor species, indicating an advantage in stability of the 4–4′ cross-link over the 3–3′ and 5–5′ bond. This is most easily explained by a model for helices 1 and 1′ in the cytoplasm which places residues 4 and 4′ in closer

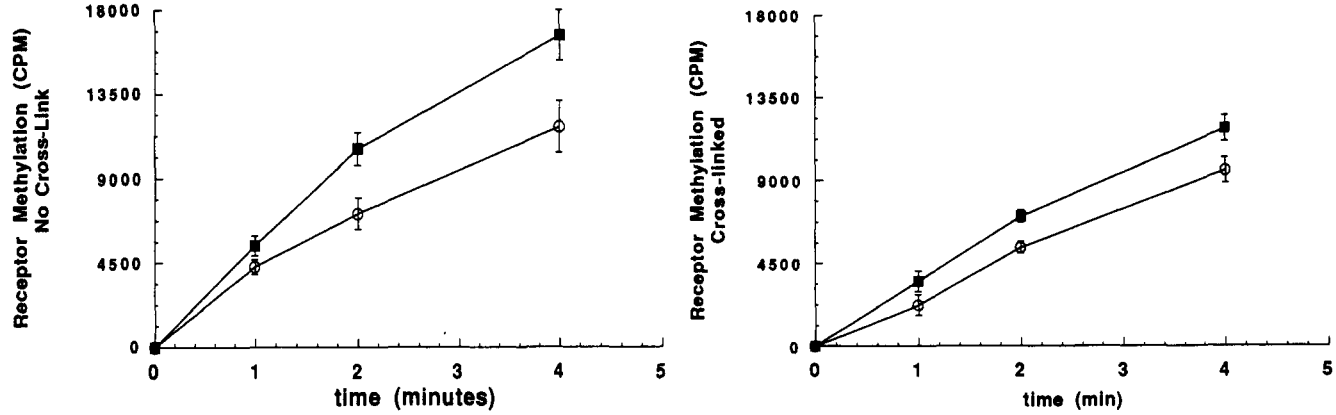

FIGURE 6: Methylation of Cys 4. Membrane containing a mixture of cross-linked and un-cross-linked Cys 4 was specifically methylated by cell extract containing methyl transferase (CheR) with [^{3}H]SAM in the presence (10 mM) or absence of aspartate. An aspartate-induced increase in methylation was seen in both the un-cross-linked and cross-linked forms of the receptor (left and right panels, respectively). The difference in absolute methylation levels between the cross-linked and un-cross-linked protein species is due to different amounts of receptor used in the assays. Symbols: (■) +aspartate; (○) -aspartate.

proximity to one another than residues 3–3′ or 5–5′ as shown on the helical wheel in Figure 1; the proximity of residues 4 and 4′ at the dimer interface is therefore the principal factor governing the greater rate and extent of disulfide formation at that position. We interpret the cross-linking of these three residues as consistent with the helical structure and relative alignment of the residues at the cytosolic end of the first transmembrane domain as proposed by Milburn et al. (1991). It is interesting to note, however, that even though Cys 4 cross-links to a greater extent than Cys 3 or Cys 5 as shown by the steady-state level of cross-linking for all three mutants in Figure 2 (confirming previous modeling efforts), this site does not appear to cross-link as extensively as Cys 36 (located in the periplasm and used in the initial structure solution of the ligand-binding domain). The most straightforward explanation of this observation is that the Cys 4–4′ is structurally or chemically less stable than Cys 36–36′. This may imply that while helices 1 and 1′ apparently retain their helical structure through the lipid bilayer and into the cytosol (and form the bulk of the dimer interface along their entire length), they may diverge slightly from the closely packed structure which is observed in the structure of the periplasmic domain and which leads to extremely stable cross-link formation at position 36. This hypothesis is reinforced by recent crystal structures of the wild-type periplasmic domain (without a disulfide as Cys 36) which does, in fact, show the helices diverging slightly as they approach the transmembrane domain (Yeh et al., in preparation).

Close examination of Figure 2 also shows that Cys 4 appears to cross-link initially at a faster rate than Cys 36, even though the final level of disulfide formation is lower for all the cytoplasmic sites. This observation becomes even more intriguing when we observe that the level of spontaneous cross-link formation for Cys 4 (and to a lesser extent Cys 3) increases significantly when membranes are prepared with lower concentrations of the reducing agent DTT present but that Cys 36 is relatively unaffected by the concentration of DTT during membrane purification. This can be seen in the time = 0 point in Figure 3. Such a result, combined with the observation in Figure 2 that Cys 4 displays the fastest initial disulfide formation, indicates that oxidation of the thiol groups on helices 1 and 1′ actually occurs more rapidly on the cytosolic side of the membrane than at the periplasmic side (which might be accounted for by differing accessibility to solvent, oxygen, and catalyst) but that this fast process is countered by lower stability of the resulting disulfide bonds (which is most readily explained by differences in distances and geometries between various residues).

While exploring these observations further, we determined that use of higher concentrations of copper phenanthrolene in time-dependent oxidation studies of these receptors results in a transient increase in the cross-link formation at position 4 above the final levels seen in Figure 2, followed by a decrease back to steady state. This effect is not observed at position 36, where increased catalyst concentration only serves to increase the rate of disulfide formation. The pattern of increase and subsequent decrease in cross-linking at position 4 is probably due to increased oxidative activity in the presence of high catalyst, which might cause spontaneous oxidative cleavage at position 4:

$$\underset{\text{free thiol}}{\text{–SH + HS–}} \rightarrow \underset{\text{disulfide}}{\text{–S- - -S–}} \rightarrow \underset{\text{sulfenic acid}}{\text{[–S–OH + HO–S–]}} \rightarrow$$

$$\underset{\text{sulfinic acid}}{\text{–SO}_2\text{H + HO}_2\text{S–}} \rightarrow \underset{\text{sulfonic acid}}{\text{–SO}_3\text{H + HO}_3\text{S–}}$$

As a separate measurement of the susceptibility of sites 4 and 36 to oxidation, we determined the kinetics of spontaneous oxidation and disulfide formation of Cys 4 (in the cytoplasm) and Cys 36 (in the periplasm) in the *absence* of catalyst. As seen in Figure 4, Cys 4 displays a rate of spontaneous disulfide formation which is approximately twice that of Cys 36 in the absence of copper phenanthrone, which is in agreement with the kinetic results discussed above, providing confirmation that Cys 4 (the optimal residue for disulfide formation on the cytoplamic end of helices 1 and 1′) is more susceptible to oxidizing species than its counterpart in the periplasm (Cys 36) but does not form an extremely stable disulfide bond. This situation is an excellent example of kinetic vs thermodynamic control of chemical reactivity.

In addition to providing a model for the structure of the transmembrane regions of the helix (a model which is confirmed by cross-linking studies), the crystal structure of the periplasmic ligand-binding domain of the receptor in the presence and absence of bound aspartate indicates that ligand binding produces a small rotation of the two monomers relative to one another by approximately 4°. This minimal motion, if truly indicative of the signaling mechanism for the receptor and if transmitted across the membrane, would produce an increase in the distance between residues 4 and 4′ from approximately 6 Å to over 12 Å. A larger motion would cause an even larger movement of helices 1 and 1′ away from

one another in the cytosol. In order to assess the feasibility of this model, we measured the effect aspartate binding on the cross-linking of Cys 4 and 4′ (cross-linking should *decrease* as a result of aspartate binding if a scissors-type motion actually increases the distance between these residues by a significant amount). In addition, we also measured the effect of creating a disulfide bond at position 4 on the specific methylation rate of the enzyme in the presence and absence of bound aspartate (the wild-type receptor shows an increase in specific methylation by CheR after aspartate binds; if binding of this ligand causes a movement of residues 4 and 4′ away from one another, then imposition of a disulfide cross-link at that position should prevent that particular conformational change and eliminate the aspartate-induced methylation rate increase).

As seen in Figure 4, bound aspartate enhances the formation of a disulfide bond at residue 4 in a manner which is similar to the effect previously observed for the cross-linking of Cys 36 (Falke & Koshland, 1987), implying either an aspartate-induced stabilization of the cross-linked species or possibly a dynamic effect on the rate of dimer/monomer exchange which might also lead to enhanced cross-linking. This stabilization implies aspartate-induced structural changes within the cytoplasmic dimer interface involving either a *decrease* in distance between positions 4 and 4′, a favorable realignment between these residues for disulfide bond formation upon aspartate binding, or possibly little or no movement of residues 4 and 4′, rather than a movement away from one another.

In a second experiment which was designed to complement the results of aspartate binding on cross-link formation at Cys 4, we measured the effect of aspartate binding on the methylation rate of both the cross-linked and un-cross-linked receptor. The wild-type receptor (no cysteine residues or disulfide cross-links) shows an increase in the rate of methylation of the cytosolic domain at four glutamic residues by CheR as a result of aspartate binding. As shown in Figure 5, the receptor with a cysteine at residue 4 displays an increase in methylation after aspartate binding in both the cross-linked and un-cross-linked state. Although the aspartate-induced changes appear to be slightly different in magnitude, the measurable increase in methylation of the cross-linked species upon aspartate binding would seem to rule out the possibility of a large movement of residues 4 and 4′ away from one another when aspartate binds. This result implies no loss of functionality upon cross-linking at position 4 or, more specifically, very little restraint or distortion of the receptor's structure and dynamics which might prevent the aspartate-induced conformational change which leads to signal transduction. Thus, based on (1) the aspartate-induced increase in the Cys 4 cross-linking rate and equilibrium and (2) the aspartate-induced increase in methylation in both the cross-linked and un-cross-linked Cys 4 receptor, we conclude that aspartate induces some conformational change that does not involve large intersubunit movements that may lead to an increase in distance between the cytosolic ends of the first membrane-spanning helices; rather, the motion involved may be largely contained within individual subunits, manifesting on the quaternary protein structure level as a small movement between positions 4 and 4′.

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