# Conformational Changes in the Cytoplasmic Domain of the Escherichia coli Aspartate Receptor upon Adaptive Methylation †

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# Conformational Changes in the Cytoplasmic Domain of the *Escherichia coli* Aspartate Receptor upon Adaptive Methylation†

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ABSTRACT: By using targeted disulfide cross-linking, we have characterized structural changes that the *Escherichia coli* aspartate receptor undergoes upon modification of the four specific residues that are reversibly methylated during sensory adaptation. Cysteine residues were introduced at specific positions either in the cytoplasmic domain or in the periplasmic domain, and the rates of disulfide cross-linking were used to probe for conformational changes upon covalent modification. Conversion of the methylation sites from glutamates to glutamines greatly reduced the rate of disulfide formation between residues 265 and 265′ and residues 250 and 250′ in the cytoplasmic domain but not between residues 36 and 36′ in the periplasmic domain. (Primes are used to indicate the second of the two identical subunits in the homologous dimer.) The covalent modification of the cytoplasmic domain induces conformational changes that are detectable in the cytoplasmic domain but none that are detectable in the periplasmic domain.

Transmembrane signaling is the process by which information is transmitted across the membrane bilayer. Following ligand binding, most receptors transduce the signal by (a) undergoing oligomerization, (b) inducing a conformational change, or (c) opening a channel (*1*−*3*). When transmembrane receptors are exposed to persistent stimuli, covalent modifications diminish the quantity of signal output, a phenomenon called adaptation or desensitization. In chemotactic bacteria, a steady level of attractant results in sensory adaptation that is generated by reversible methylation of glutamate residues located in the cytoplasmic part of chemoreceptors (*4*, *5*). In mammalian receptors, adaptation is effected by reversible phosphorylation of specific residues located in the cytoplasmic part of receptors (*6*, *7*).

In *Escherichia coli*, the aspartate receptor (Tar protein[1]) is one of four homologous transmembrane receptors that mediate the chemotactic response to various attractants and repellents (*8*). Both in the absence and presence of ligand, the aspartate receptor is present in the bacterial inner membrane as a homodimer (*9*). Each 60-kDa subunit is organized into three domains: (a) a periplasmic ligand-recognition domain, (b) a transmembrane domain that consists of two membrane-spanning segments, and (c) a cytoplasmic domain that has several functional subdomains (*10*) (Figure 1).

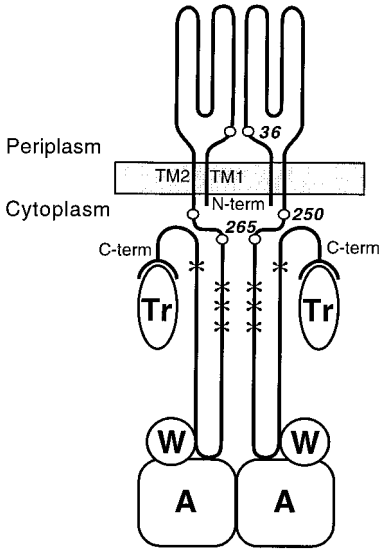


FIGURE 1: Schematic representation of the aspartate receptor. This schematic representation illustrates the positions of the engineered cysteine pairs used in this study (open circles). The periplasmic domain representation is based on its X-ray structure (*23*). The cytoplasmic domain representation is based on sequence analysis and targeted disulfide studies (*10*, *28*). The four methylation sites at positions 295, 302, 309, and 491 are represented as stars. The receptor-associated proteins are the CheR methyltransferase (Tr) (*38*), the CheW coupling protein (W), and the CheA histidine kinase (A) (*39*).

Ligand binding to the periplasmic domain of the aspartate receptor generates a transmembrane signal that induces changes in the cytoplasmic domain (Figure 2B). In turn, this modulates an intracellular phosphorylation cascade involving the receptor-associated protein histidine kinase CheA and the diffusible response regulator CheY (*11*). Evidence for a ligand-induced conformational change in the aspartate receptor comes from several independent experimental approaches. The rate of cross-linking of several

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[1] Abbreviations: Tar protein, protein produced by translation of the *tar* gene; *tar* gene, gene encoding the aspartate receptor; residues 265 and 265′, equivalent residues that belong to two different subunits are distinguished by primes; EEEE, QEQE, and QQQQ, aspartate receptor mutants in which either Glu (E) or Gln (Q) indicate the residues at positions 295, 302, 309, and 491 that are normally converted to glutamate, methylated, and demethylated during in vivo sensing; bp, base pair(s); DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.

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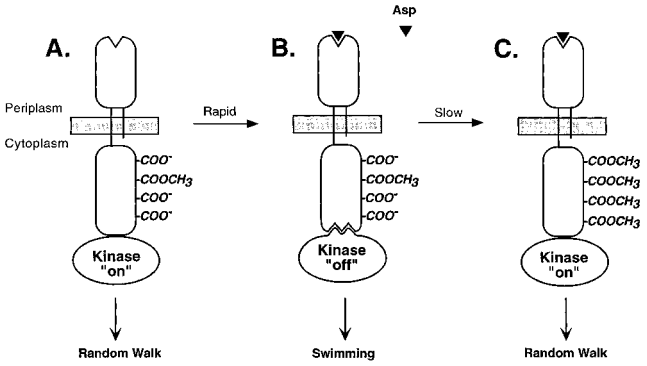


FIGURE 2: Schematic illustration of transmembrane signaling and sensory adaptation. For clarity, only one of the two receptor subunits is represented. The unmethylated and methylated glutamates are symbolized by $COO^-$ and $COOCH_3$ moieties, respectively. Histidine kinase is associated to the receptor in both the absence and presence of aspartate as reported by Gegner et al. (*39*). (A) The aspartate receptor is shown with an external ligand-recognition domain, two transmembrane segments, and a cytoplasmic domain that can signal and be reversibly methylated. The receptor is unstimulated and the receptor-associated kinase is active. (B) Transmembrane signaling: upon aspartate binding to the ligand-recognition domain, a conformational change is transmitted to the cytoplasmic domain where it inhibits the activity of the receptor-associated kinase. (C) Sensory adaptation: as a result of aspartate binding, a new steady-state level of methylation is achieved. This brings the receptor back to an "unstimulated" state, even though aspartate remains bound to the protein.

cysteine residues introduced at different locations in the receptor was found to be affected by the presence of aspartate (*12*). A $^{19}F$ NMR study showed that aspartate binding perturbs the resonance of several *p*-fluorophenylalanine probes introduced in the soluble ligand-recognition domain of the receptor (*13*). A model in which the second transmembrane helix (TM2) moves in a piston-type motion upon aspartate binding has been proposed (*14*, *15*). The comparison of the apo and aspartate-occupied structures of the ligand-recognition domain strongly supports this model (*15*).

In response to a persistent stimulus, chemotactic bacteria adapt through the methylation or demethylation of specific glutamate residues located in the cytoplasmic part of their chemoreceptors. The achievement of a new steady state of methylation resets the receptor to zero (*16*) and allows a new chemotactic response (Figure 2C). Methyl groups are transferred from the methyl donor *S*-adenosylmethionine to the specific glutamate side chains by the CheR methyltransferase (*17*) and are hydrolyzed by the CheB methylesterase (*18*). In the aspartate receptor, the methylation sites consist of residues 295, 302, 309, and 491 (*19*), which are encoded in the *tar* gene as two glutamines and two glutamates (receptor designated as QEQE). The glutamine residues are postranslationally deamidated to glutamate by a deamidase activity of the CheB methylesterase prior to methylation (*20*). Reversible methylation has been shown to influence the signaling properties of the receptor by modulating the receptor-associated protein histidine kinase activity (*21*, *22*).

In the past decade, targeted disulfide cross-linking has been used to define several structural attributes of the aspartate receptor (*9*, *12*, *23*−*29*). By introducing a single cysteine codon in the receptor coding gene (e.g., S36C), we obtain a receptor dimer in which two cysteines are located at similar positions in the two subunits (i.e., 36 and 36′) (Figure 1). The rate at which these two cysteines form a disulfide depends on the spatial proximity of the two sulfhydryl groups and the conformational flexibility of the protein (*30*, *31*).

In this study, we introduced cysteine residues either in the cytoplasmic domain (at position 265) or in the periplasmic domain (at position 36). The rates of disulfide formation were measured in the aspartate receptor mutants EEEE, QEQE, and QQQQ to determine how covalent modification affects the conformation. Amidation of the aspartate receptor has previously been shown to cause the same signaling change as methylation (*21*). Both methylation and amidation convert the negatively charged glutamates ($COO^-$) to neutral moieties ($COOCH_3$ or $CONH_2$).

## MATERIALS AND METHODS

*Bacterial Strains and DNA Plasmids*. The *E. coli* strain HCB721 [(*tsr*)7021 *trg*::Tn*10* (*cheA*-*cheY*)::XhoI(Tn*5*)], which was kindly provided by Dr. H. C. Berg, Harvard University (*32*), was used for receptor expression. The *E. coli* strain RP8611 [D*tsr7028* D*(tar-tap)5201zbd::Tn5* D(*trg*)-*100*], which was a gift of Dr. J. S. Parkinson, University of Utah, was used for swarm assays. Plasmid pMK650 contains the wild-type *E. coli tar* gene (*14*).

*Construction and Expression of the Various Receptor Site-Directed Mutants*. All plasmids used in this study are derived from plasmid pSK2 that encodes an *E. coli* aspartate receptor in which all four methylation sites consist of glutamate residues (EEEE) (*33*). Plasmid pSK102 that encodes a receptor in which the four methylation sites are as in the wild-type *tar* gene (i.e., QEQE) was generated by substituting the *Bst*EII-*Pst*I DNA fragment (1200 bp long) of pMK650 for the equivalent fragment in pSK2. Plasmid pSK103 (QQQE) was derived by mutagenesis of plasmid pSK102, using the Quick-change kit (Stratagene, Palo Alto, CA). Subsequently, plasmid pSK105, in which all four methylation sites consist of glutamine residues (QQQQ), was derived by mutagenesis of plasmid pSK103. A second series of plasmids that encode these differentially amidated receptors but contain a cysteine at position 265 were constructed as follows: Plasmid pSK51 (V265C-EEEE) was derived by site-directed mutagenesis of plasmid pSK2. Subsequently, plasmids pSK52 (V265C-QEQE) and pSK55 (V265C-QQQQ) were obtained by substituting the *Bst*EII-*Kpn*I DNA fragment (130 bp long) produced by digestion of pSK51 for the equivalent fragments of pSK102 and pSK105, respectively. Similarly, we generated a third series of plasmids that encode differentially amidated receptors containing a cysteine at position 250 (L250C). Plasmid pSK71 (L250C-EEEE) was derived by site-directed mutagenesis of plasmid pSK2. Subsequently, plasmids pSK72 (L250C-QEQE) and pSK75 (L250C-QQQQ) were obtained by substituting the *Bst*EII-*Kpn*I DNA fragment (130 bp long) produced by digestion of pSK71 for the equivalent fragments of pSK102 and pSK105, respectively. A fourth series of plasmids that encode differentially amidated receptors containing a cysteine at position 36 were generated. Plasmid pSK61 (S36C-EEEE) was derived by site-directed mutagenesis of plasmid pSK2. Plasmids pSK62 (S36C-QEQE) and pSK65 (S36C-QQQQ) were obtained by substituting a *BamHI*−*HpaI* DNA fragment (430 bp long) of pSK61 for the equivalent fragments of pSK102 and pSK105, respectively.

*Swarm Plate Assays*. The *E. coli* strain RP8611, which lacks the four chemoreceptor genes, was transformed with the three series of plasmids encoding for the differentially amidated receptors with or without a cysteine at positions 36 or 265. Negative control transformants contained the cloning vector alone (pSL1180). Minimal swarm plates contained Vogel−Bonner minimal citrate medium, 0.3% agar, 1% glycerol, 100 $\mu$g/mL of each L-histidine, L-methionine, L-leucine, L-threonine, 50 $\mu$g/mL thiamine, and 100 $\mu$g/mL ampicilin. Aspartate plates consisted of minimal media supplemented with 100 $\mu$M L-aspartate. Swarm plates were inoculated in the center of a Petri dish from an overnight culture grown in Luria broth. After inoculation, swarm plates were incubated at 30 °C. Swarm diameters were measured five or six times over a 12 h period, starting 16 h after inoculation. Swarm rates were defined as the slope of a linear curve fit of the time-course data points.

*Preparation of Membranes Containing Overexpressed Receptor*. One-liter cultures of Luria broth supplemented with ampicillin (100 $\mu$g/mL) and kanamycin (50 $\mu$g/mL) were inoculated with HCB721 cells transformed with various plasmids and grown at 30 °C for 16 h. Cells were harvested by centrifugation and stored at −80 °C. Membranes containing the various cysteine-containing aspartate receptors were prepared essentially as described previously (*34*), with the exception that DTT was added to some of the buffers in order to prevent spontaneous disulfide formation. Cell pellets were resuspended in 20 mL of low-salt buffer (100 mM sodium phosphate, pH 7.0, 10% glycerol, 5 mM EDTA, 10 mM DTT) supplemented with 1 mM PMSF, 1 mM leupeptin, 1 mM aprotinin, and 5 mM 1,10-phenanthroline. Cells were lysed by sonication. Unbroken cells and debris were removed by two low-speed centrifugations (6000*g*, 10 min, SS-34, 4 °C). Membranes were collected by high-speed centrifugation (142000*g*, 60 min, 45-Ti, 4 °C). Membrane pellets were resuspended in 5 mL of high-salt buffer (20 mM sodium phosphate, pH 7.0, 2 M KCl, 10% glycerol, 5 mM EDTA, 5 mM DTT) supplemented with 1 mM PMSF, 1 mM leupeptin, and 5 mM 1,10-phenanthroline. Membranes were pelleted by high-speed centrifugation in a benchtop ultracentrifuge (488000*g*, 10 min, TLA-100.3, 4 °C). A second high-salt wash was carried out. Finally, membranes were resuspended in 2 mL of final buffer (20 mM Tris−HCl, pH 7.5, 10% glycerol, 1 mM DTT) supplemented with 1 mM PMSF, 1 mM leupeptin, 1 mM 1,10-phenanthroline. Membranes were pelleted in a benchtop ultracentrifuge as mentioned above, resuspended in 2 mL of final buffer devoid of DTT and protease inhibitors, aliquoted, and stored at −80 °C.

*Quantification of the Aspartate Receptor in Membranes*. The total amount of protein in membranes was determined by using the BCA protein assay (Pierce, Rockford, IL) in the presence of 0.1% SDS. Receptor-containing membranes were resolved on a 12.5% SDS−PAGE. The Coomassie Blue-stained gels were scanned using a flatbed scanner equipped with a transparent media adapter. The scanner was calibrated using a photographic step tablet (Eastman Kodak, cat. no. 152 3414). Protein bands were quantified using the NIH image program (version 1.61) (http://rsb.info.nih.gov/nih-image). The percentage corresponding to the overproduced receptor was determined, and the receptor concentration was estimated.

*Methylation Assays*. In vitro methylation assays were performed at room temperature, after a 15-min preincubation period, in buffer containing 50 mM phosphate buffer, pH 7.5, 1 mM EDTA, 10% glycerol, and 1mM PMSF, as previously described (*33*). The concentrations of various receptors present in membranes were estimated as described above, and identical amounts of receptors were introduced into the methylation assays. At time zero, a volume of diluted membranes containing the appropriate receptor(s) (about 5 $\mu$M for each receptor species) was mixed with an equal volume of methylation mixture containing 120 $\mu$M [$^3$H]-*S*-adenosylmethionine (1.5 Ci/mmol) and a 1:100 dilution of a crude extract containing the overproduced methyltransferase. Methylation assays were performed in the absence and presence of 100 $\mu$M L-aspartate. At time points, 20 $\mu$L of the reaction was absorbed on a 1-cm-square piece of filter paper and immediately dropped into 10% TCA. Thereafter, the filters were washed twice with 10% TCA and twice with methanol for 10 min each. Finally, the filter papers were air dried, and the radioactivity was counted. Methylation rates were defined as the slope of a linear curve fit of the early time-course data points. They were found to be linear for 5 min.

*Disulfide Bond Formation as a Function of the Concentration of Catalyst*. Disulfide cross-linking assays were performed using ambient oxygen as an oxidant and Cu(II)-(1,10-phenanthroline)$_3$ as a catalyst (*12*). Membranes were diluted to a receptor concentration of about 5 $\mu$M with either final buffer or final buffer supplemented with 100 $\mu$M L-aspartate. Membranes were preincubated at 22 °C for 15 min. The catalyst dilutions were prepared by serial dilution of a 3 mM stock solution. Cross-linking was initiated by mixing 10 $\mu$L of preincubated membranes with 10 $\mu$L of catalyst. The reaction was performed at 22 °C and stopped after 6 min by addition of 10 $\mu$L of a solution containing EDTA (50 mM) and NEM (50 mM). SDS−Laemmli sample buffer (without reducing agent) was subsequently added. The samples were boiled and analyzed on a 7.5% nonreducing SDS−PAGE, which was subsequently stained with Coomassie Blue. Three gels were run for each reaction. Each gel was analyzed using a flatbed scanner, as described above. The ratio of the integrated value corresponding to the dimer band versus the sum of the integrated values corresponding to the monomer and dimer bands directly reflects the percentage of cross-linked dimer.

*Initial Disulfide Bond Formation Rates.* The various receptor-containing membranes were preincubated for 15 min at room temperature, in the absence and presence of 100 $\mu$M L-aspartate. Reactions were initiated by mixing equal volumes of preincubated membranes and catalyst solution. For each series of mutants, we determined the concentration of catalyst for which the rate of disulfide formation is linear for at least 5 min. At specific times, aliquots were removed, and the reaction was stopped as described above. The extent of disulfide-linked dimer was determined on a nonreducing SDS−PAGE, as described above. Initial rates were defined as the slope of a linear curve fit of the early time-course data points.

## RESULTS

*Isolation of the Differentially Amidated V265C, L250C, and S36C Receptors.* The differentially amidated aspartate

receptor mutants were produced in the *E. coli* strain (HCB721) that cannot covalently modify the receptors because it lacks both the methyltransferase and the methylesterase genes. Membranes containing these receptors were prepared as described under Materials and Methods. With the exception of the L250C-EEEE receptor (5−10% of total membrane protein), all the receptors were produced at high levels (each receptor representing about 20−30% of total membrane protein). Although the EEEE, QEQE, and QQQQ receptors have almost identical molecular weights, they showed slightly distinct migration rates on SDS−PAGE (Figure 3, lanes 1−3). This phenomenon has been attributed to the different levels of methylation or amidation that characterize the chemotaxis receptors (*22*, *35*). The percentage of disulfide cross-linked receptors prior to in vitro oxidation was found to be very low. Even after a 1-h incubation at 22 °C in the absence of catalyst, most of the V265C receptors were still non-cross-linked (Figure 3, lanes 4−6). However, the three differentially amidated V265C receptors became fully cross-linked after a 1-h incubation at 22 °C in the presence of 250 mM catalyst (Figure 3, lanes 7−9).

*In Vivo Swarm Assays.* To examine the effects of the cysteine substitution at positions 36 and 265, we performed in vivo swarm plate assays using an *E. coli* strain (RP8611) that does not express any of the four chemotaxis receptors but is otherwise functional for chemotaxis genes. Bacteria of this strain were transformed with the various plasmids encoding the differentially amidated wild-type, S36C, and V265C receptors and tested in swarm plate assays (Figure 4). In the absence of aspartate, the swarm rates of bacteria expressing differentially amidated wild-type, S36C, or V265C receptors were very similar (Figure 4). When aspartate is present in the agar, cells containing the wild-type or S36C receptors showed a 4-fold increase in their swarm rates. Likewise, the presence of aspartate increased the swarm rates of cells containing the various V265C receptors, albeit to a lesser extent (2-fold increase) (Figure 4). Cells that do not express an aspartate receptor (null) showed the same very low swarm rate whether or not aspartate was present in the agar (Figure 4). Thus, these data confirm that the S36C mutant receptor is functional for transmembrane signaling (*12*, *27*). They also indicate that the V265C receptor is functional for transmembrane signaling, even though the substitution of a cysteine for a valine at this position reduces by 2-fold the aspartate-induced increase in swarm rates.

In contrast to the EEEE and QEQE receptors, the fully amidated receptor (QQQQ) is an engineered receptor that does not naturally occur, in vivo. Our swarm plate assays showed that cells transformed with the QQQQ receptor respond to the presence of aspartate as well as cells transformed with either the QEQE or EEEE receptors (Figure 4). Thus, these data suggest that the four glutamine residues of the QQQQ receptor are efficiently deamidated by the methylesterase and subsequently methylated by the methyltransferase.

*In Vitro Methylation Assays.* To assess further the functionality of these mutants, we measured the initial rates of methylation of the various EEEE and QEQE receptors as described under Materials and Methods (the QQQQ receptors cannot be methylated in vitro). As determined previously, the EEEE and QEQE S36C receptors were methylated at rates that are very similar to those of the corresponding wild-

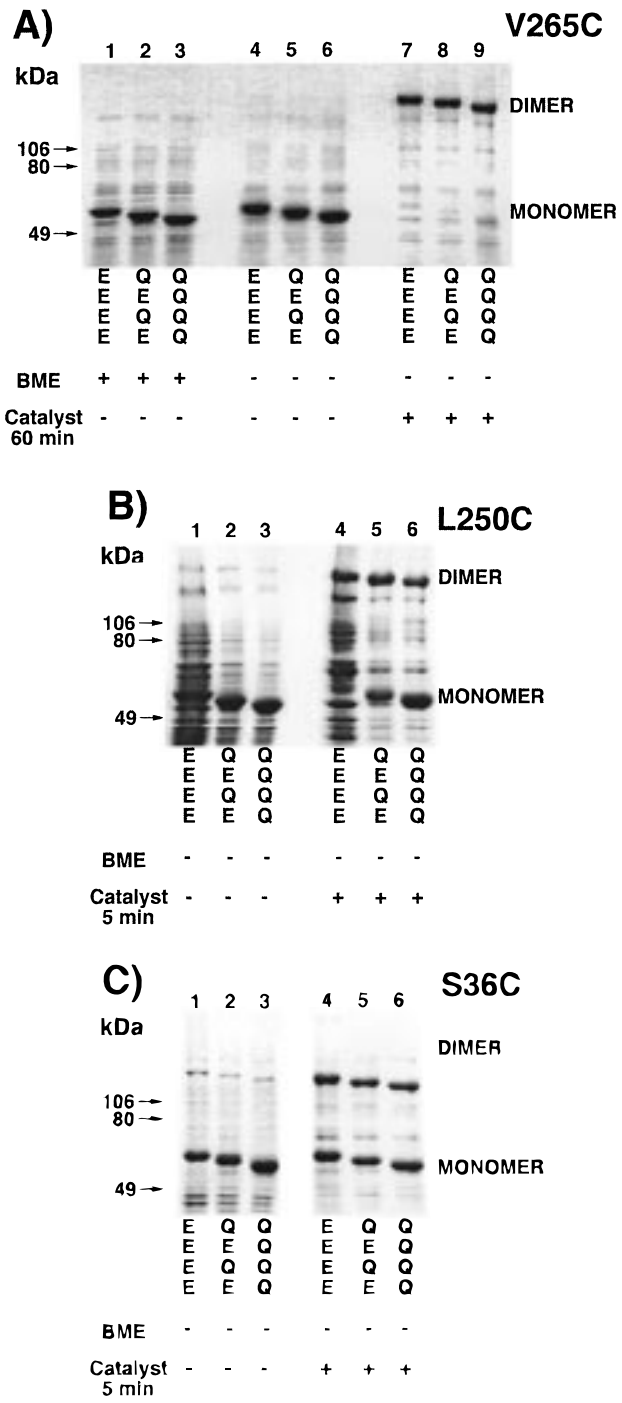


FIGURE 3: Analysis of the EEEE, QEQE, and QQQQ V265C receptor-containing membranes. All of the receptors were expressed in *E. coli* HCB721, which lacks all chemotaxis genes including the methyltransferase and methylesterase genes. The EEEE, QEQE, and QQQQ V265C mutant receptors can be distinguished by migration through an SDS−PAGE. The receptor-containing membranes were prepared as described under Materials and Methods. The samples of lanes 1−3 contained 2-mercaptoethanol. The samples of lanes 4−6 were incubated in the absence of catalyst for 1 h at 22 °C prior to the addition of Laemmli loading buffer supplemented with EDTA and NEM. The samples of lanes 7−9 were incubated in the presence of 250 $\mu$M catalyst for 1 h at 22 °C prior to the addition of Laemmli loading buffer supplemented with EDTA and NEM. Figure 3A is V265C, 3B is L250C, and 3C is S36C.

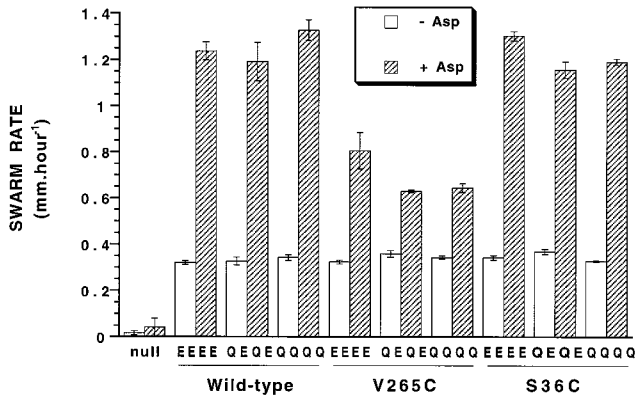


FIGURE 4: In vivo chemotactic behavior of bacteria overexpressing the EEEE, QEQE, and QQQQ wild-type, S36C, and V265C receptors. RP 8611, an *E. coli* strain lacking the four chemoreceptors, was transformed with either plasmids encoding for the different variants of the aspartate receptor or the cloning vector alone (null). Swarm assays were performed as described under Materials and Methods. Swarm rates were the slopes of the linear fit of the time-course data points. Error bars show the standard deviation of three determinations.

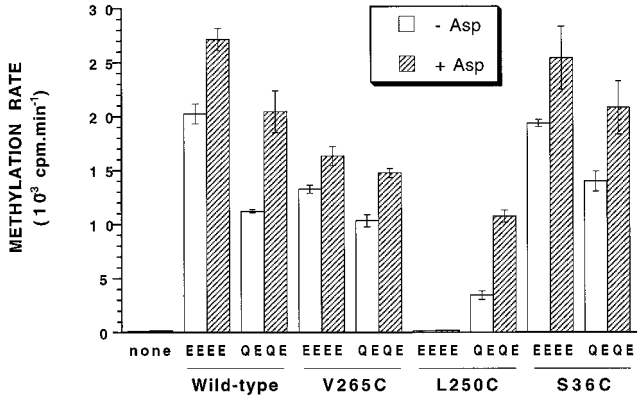


FIGURE 5: In vitro methylation rates of the EEEE and QEQE wild-type, S36C, V265C, and L250C receptors. Methylation assays were performed as described under Materials and Methods using receptor-containing membranes. The rates of methylation are the slopes of a linear fit of the time-course data points. Each rate is the average of three determinations and is shown along with the standard deviation.

type receptors (Figure 5) (*12*, *27*). For both the wild-type and S36C receptors, the presence of aspartate increased the rate of methylation by about 1.5-fold (Figure 5) (*12*, *27*). Although the EEEE and QEQE V265C receptors were methylated at slightly slower rates than the wild-type and S36C receptors, the presence of aspartate increased their rates of methylation by 1.2- and 1.4-fold, respectively (Figure 5). These data are in good agreement with the in vivo swarm plates assays shown in Figure 4 and confirm that the S36C and V265C receptors are functional for transmembrane signaling.

In contrast, the substitution of a cysteine for a leucine at position 250 has some drastic effects on the rates of methylation of the EEEE and QEQE mutants. The EEEE L250C receptor was not significantly methylated (Figure 5). The QEQE L250C receptor was methylated at a rate that is 3-fold slower than the QEQE wild-type receptor and its rate of methylation is increased by 3-fold in the presence of aspartate (Figure 5). These data suggest that the three-dimensional structure of these receptors (especially the EEEE

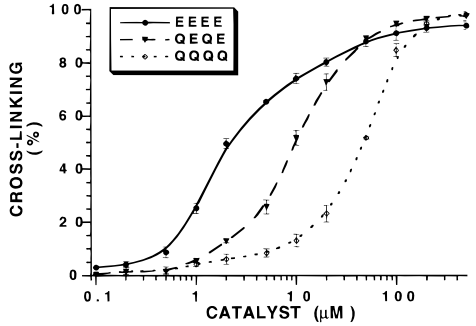


FIGURE 6: Disulfide cross-linking of the EEEE, QEQE, and QQQQ V265C mutant receptors as a function of the concentration of catalyst. Samples of receptor-containing membranes were preincubated in the absence of aspartate and subjected to oxidation by oxygen in the presence of increasing amounts of (CuII)(1,10-phenanthroline)$_3$ as described under Materials and Methods. After a 6-min reaction time, samples were taken into an equal volume of Laemmli loading buffer supplemented with EDTA and NEM, boiled, and loaded on a 7.5% SDS−PAGE. The gels were stained with Coomassie Blue and dried between cellophane sheets. The densities of the dimeric and monomeric protein bands were measured as described under Materials and Methods for calculation of the percentage of cross-linking.

receptor) may be somehow distorted by the L250C mutation. However, the alteration must be minor because the QEQE L250 receptor is methylated and functional for transmembrane signaling, as shown by the fact that its rate of methylation is increased by the presence of aspartate.

*Disulfide Cross-Linking at Position 265 Depends on the Level of Amidation of the Receptor.* In this study, we used the V265C mutant because it has been shown to cross-link very rapidly (*28*). Thus, it is most likely that residues 265 from one subunit and 265′ from the other subunit are in close proximity at the subunit interface (Figure 1). Moreover, residue 265 is located 30 residues upstream from the first methylation site and is predicted to be located at the N-terminus of the methylation domain (*10*). Thus, we assumed that a cysteine at position 265 would be a very good probe to detect conformational changes within the cytoplasmic domain of the receptor.

First, we measured the disulfide formation for the three differentially amidated V265C receptors as a function of the concentration of catalyst. As shown in Figure 6, the EEEE, QEQE, and QQQQ forms of the V265C mutant showed different patterns of cross-linking. The EEEE mutant required a lower concentration of catalyst than either the QQQQ mutant or, to a lesser extent, the QEQE mutant to reach 50% dimer formation. To quantify this effect, we introduced the notation $DC_{50}$ (dimerization concentration 50) that corresponds to the concentration of catalyst necessary for a receptor to reach 50% dimer formation during the time of the experiment (6 min). The $DC_{50}$ values increased with the level of amidation of the receptor (Figure 6). Whereas the $DC_{50}$ value of the V265C-EEEE receptor was $2.3 \pm 0.2$ mM, the $DC_{50}$ values of the V265C-QEQE and V265C-QQQQ receptors were $10 \pm 0.7$ and $46 \pm 1$ mM, respectively (Figure 6). From these data, it appears that the $DC_{50}$ value doubles with each glutamine substituted for a glutamate. Taken together, these data indicate that disulfide cross-linking at position 265 depends more strongly on the level of covalent modification of the receptor than on the presence of aspartate.

Table 1: Initial Rates of Disulfide Cross-Linking of the Differentially Amidated V265C Mutant Receptors[a]

| | % dimer min$^{-1}$ | | ratio | ratio |
|---|---|---|---|---|
| | −Asp | +Asp | (+Asp/−Asp) | (Q/E) |
| C265-EEEE | 11.2 ± 0.8 | 11.2 ± 0.6 | 1.00 | 1.00 |
| C265-QEQE | 3.9 ± 0.3 | 3.3 ± 0.5 | 0.85 | 0.35 |
| C265-QQQQ | 0.22 ± 0.1 | 0.19 ± 0.1 | 0.86 | 0.02 |

[a] Reactions were performed as described under Materials and Methods, in the presence of 2.5 $\mu$M catalyst. Values are the mean of three experiments and are shown along with the standard deviation.

Table 2: Initial Rates of Disulfide Cross-Linking of the Differentially Amidated L250C Mutant Receptors[a]

| | % dimer min$^{-1}$ | | ratio | ratio |
|---|---|---|---|---|
| | −Asp | +Asp | (+Asp/−Asp) | (Q/E) |
| C250-EEEE | 35.3 ± 1.5 | 36.0 ± 1.6 | 1.01 | 1.00 |
| C250-QEQE | 20.9 ± 2.1 | 22.9 ± 1.1 | 1.09 | 0.59 |
| C250-QQQQ | 15.5 ± 0.4 | 29.5 ± 2.0 | 1.90 | 0.43 |

[a] Reactions were performed as described under Materials and Methods, in the presence of 250 $\mu$M catalyst. Values are the mean of three experiments and are shown along with the standard deviation.

*Initial Rates of Cross-Linking at Position 265.* To characterize further the differences observed in disulfide cross-linking for the differentially amidated V265C receptors, we measured the initial rates of disulfide cross-linking for the EEEE, QEQE, and QQQQ V265C receptors. As shown in Table 1, the rate of disulfide formation largely depends on the amidation level of the receptor. The fastest rate of disulfide formation was obtained for the fully deamidated receptor (EEEE). The slowest rate (1/50 of the rate of the EEEE receptor) was obtained for the fully amidated receptor (QQQQ). The partially amidated receptor (QEQE) showed an intermediate rate of cross-linking ($^1/_4$ of the rate of the EEEE receptor). In contrast, the rates of disulfide formation of the three V265C differentially amidated receptors were barely affected by the presence of 100 $\mu$M L-aspartate (Table 1). The divergent rates of disulfide cross-linking observed for the EEEE, QEQE, and QQQQ V265C mutant receptors (Table 1) are consistent with the different rates of cross-linking observed as indicated by the levels of oxidation catalyst for these mutant receptors (Figure 6). Taken together, these data indicate that the level of amidation of the receptor strongly influences the reactivity of cysteine 265. Because disulfide cross-linking has been shown to depend mainly on the distance between the two reactive cysteines (*30*, *31*), these data are best explained by the fact that the differentially amidated forms of the receptor are in different conformations.

*Initial Rates of Cross-Linking at Position 250.* To discover whether covalent modification affects the rate of cross-linking at other positions in the cytoplasmic domain, we measured the initial rates of disulfide cross-linking of the EEEE, QEQE, and QQQQ L250C receptors. As shown in Table 2, the rates of disulfide formation at position 250 also depend on the amidation level of the receptor, albeit to a lesser extent than at position 265. In good agreement with our data concerning position 265, the fastest and slowest rates of disulfide formation at position 250 were obtained for the EEEE and QQQQ receptors, respectively (Table 2). Whereas the presence of aspartate did not appreciably increase the already fast rates of disulfide formation of the EEEE and QEQE L250C receptors, it increased the rate of disulfide formation of the QQQQ L250C by about 2-fold (Table 2). Taken together, these data suggest that the conformational change induced by covalent modification affects at least the juxtaposed portions of the two cytoplasmic domains, and the cysteine substitutions chosen at random are good indicators of this change.

Table 3: Initial Rates of Disulfide Cross-Linking of the Differentially Amidated S36C Mutant Receptors[a]

| | % dimer min$^{-1}$ | | ratio | ratio |
|---|---|---|---|---|
| | −Asp | +Asp | (+Asp/−Asp) | (Q/E) |
| C36-EEEE | 10.6 ± 0.3 | 14.3 ± 0.4 | 1.35 | 1.00 |
| C36-QEQE | 10.2 ± 0.9 | 15.3 ± 0.2 | 1.50 | 0.96 |
| C36-QQQQ | 11.3 ± 0.3 | 18.5 ± 0.3 | 1.64 | 1.06 |

[a] Reactions were performed as described under Materials and Methods, in the presence of 25 $\mu$M catalyst. Values are the mean of three experiments and are shown along with the standard deviation.

*Initial Rates of Cross-Linking at Position 36.* To discover whether the conformational change induced by covalent modification is transmitted from the cytoplasmic domain to the periplasmic ligand-recognition domain, we made differentially amidated receptors (EEEE, QEQE, and QQQQ) containing a cysteine at position 36 (S36C) (Figure 1). We measured the initial rates of disulfide formation for these three mutants (Table 3). In contrast to what was observed for the cysteine at position 265, no significant difference was observed in the rates of disulfide cross-linking of the S36C differentially amidated mutants (Table 3). Thus, these data may suggest that the conformational change that takes place in the cytoplasmic domain of the receptor is not transmitted to the periplasmic ligand-recognition domain. Despite their insensitivity to cytoplasmic modification, the rates of disulfide formation of the S36C mutant receptors depend on the presence of ligand, as demonstrated, for example, by the 1.5-fold increase in the rates of disulfide cross-linking in the presence of 100 $\mu$M L-aspartate (Table 3).

## DISCUSSION

This study indicates that the *E. coli* aspartate receptor undergoes conformational changes upon covalent modification of each of the four specific methylation sites located in its cytoplasmic domain. We found that the extent and rapidity of disulfide cross-linking of cysteines introduced in the cytoplasmic domain (V265C or L250C) depend on the level of covalent modification of the receptor (EEEE, QEQE, and QQQQ). Previous studies had shown that methylation and amidation were equivalent (*21*), probably because they neutralize the negative charge. This provides strong evidence for structural changes in the cytoplasmic domain of the aspartate receptor. In contrast, we found that the rate of disulfide cross-linking of a cysteine introduced in the periplasmic domain (S36C) is insensitive to the modification level of the cytoplasmic domain of the receptor. Thus, methylation-induced structural changes are either not transmitted to the periplasmic domain or too small to be detectable in this part of the receptor.

We first assessed the consequence of cysteine substitution on the receptor functions. In good agreement with previous studies, we found that receptors containing a cysteine at position 36 retain the ability to signal (Figures 4 and 5) (*12*,

*27*). By performing in vivo swarm assays and in vitro methylation assays, we showed that receptors with a cysteine at position 265 also retain the ability to signal (Figures 4 and 5). Thus, neither transmembrane signaling nor sensory adaptation is greatly affected by the substitution of a cysteine for a serine at position 36 or for a valine at position 265. In contrast, the lower expression level of the EEEE L2650C mutant and its inability to be methylated suggest that the structure of this particular mutant may be altered (Figure 5).

The main finding of this study is that the rate of in vitro disulfide formation at position 265 strongly depends on the modification level of the receptor (Figure 6, Table 1). The reactivity of cysteine 265 decreases when the receptor is covalently modified. This result is consistent with the idea that the proximity of the two reactive cysteines within a dimer and/or the frequency of structural fluctuations is changed upon covalent modification of the receptor cytoplasmic domain. Regardless of the factor(s) responsible for this change in reactivity at position 265, these data provide strong evidence for modification-induced conformational changes. Our data indicate that these conformational changes are proportional to the level of receptor covalent modification. Indeed, the rate of cross-linking of the partially amidated receptor (QEQE) is intermediate between the rates of cross-linking of the fully deamidated receptor (EEEE) and the fully amidated receptor (QQQQ) (Table 1). These results are in good agreement with behavioral studies showing that increased covalent modification of the receptor is correlated with an increased tumbling signal (*21*).

In good agreement with the work of Chen and Koshland (*28*), we found that the presence of aspartate only slightly affects the rate of cross-linking at position 265 (Table 1). One possibility is that residue 265 is not affected by ligand-induced structural changes. This is most unlikely, because the cross-linking rate of a cysteine introduced at the neighboring position (i.e., position 264) strongly depends on the presence of aspartate (*28*). A second possibility is that the aspartate-induced structural changes at position 265 are so slight that they are not detected by our procedure. Indeed, previous studies have suggested that the magnitudes of the aspartate-induced structural changes in both the ligand-recognition domain and the transmembrane domain are quite small (*12*, *23*, *36*). A third possibility is that residues 265 and 265′ are already so close that the further conformational change due to aspartate binding has no effect on the rate of cross-linking.

The fact that the rate of disulfide formation at position 250 depends on the level of amidation provides additional evidence for a modification-induced conformational change in the cytoplasmic domain of the receptor. This change of conformation induced by covalent modification most likely affects the entire cytoplasmic domain. Upon complete amidation, the rates of disulfide formation of the V265C and L250C receptors were slowed by 50- and 2.3-fold, respectively (Tables 1 and 2). These values may suggest that the modification-induced conformational change is greater at position 265 than at position 250. Alternatively, this may suggest that position 265 is more flexible than position 250. The latter explanation is strongly supported by prediction studies that suggested that V265 is located in a loop between the linker and the methylation domains, whereas L250 is located in the middle of the helix α6 (*10*) (designated as α5 in ref 29).

In contrast to cysteine 265, the reactivity of a cysteine introduced at position 36, within the periplasmic domain, depends on the addition of aspartate but not on the level of covalent modification of the receptor (Table 3). This result suggests that structural changes induced by covalent modification are not transmitted from the cytoplasmic domain to the ligand-recognition domain. Alternatively, the structural changes induced by covalent modification may be transmitted across the membrane but may be too small to be detectable. Both hypotheses are strongly supported by the previous finding that covalent modification of the receptor cytoplasmic domain has little effect on aspartate binding in the periplasmic domain (*21*).

On the basis of previous studies showing that the effect of aspartate is to draw the two subunits closer (*9*, *28*) and the fact that methylation counteracts the effect of aspartate by modulating the activity of the receptor-associated protein histidine kinase (*22*), we postulate that the effect of methylation is to move the two subunits further apart. Because the periplasmic ligand-recognition domain does not appear to be affected by the methylation-induced structural changes, we may assume that only the cytoplasmic domains move further apart. This assumption is consistent with the findings that the isolated cytoplasmic domain possesses a highly dynamic structure in solution (*37*) and that the effect of methylation is to reverse the effect of the aspartate-induced change. These results demonstrate the conformational effects induced in the receptor by itself. It is, of course, possible that these changes are modified in the Che A−Che W complex that is found in vivo.

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