Methylation of the Escherichia coli Chemotaxis Receptors: Intra- and Interdimer Mechanisms †

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Methylation of the *Escherichia coli* Chemotaxis Receptors: Intra- and Interdimer Mechanisms†

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ABSTRACT: The mechanism(s) of methylation of the *Escherichia coli* chemotaxis receptors was analyzed by experiments involving the construction of a series of aspartate receptor variants. Truncation of five or more residues from the C-terminal end of the aspartate receptor, which prevents the methyltransferase from binding to the receptor, resulted in very low rates of methylation, indicating that the methyltransferase is activated by binding to the receptor. Coexpression of a receptor variant that is unmethylatable but able to C-terminally bind the methyltransferase resulted in much higher methylation rates for all of the truncated receptors. By preventing the possibility of subunit exchange between receptor variants, we showed that the truncated receptors were methylated via an interdimer mechanism. The interdimer methylation rates of the truncated receptors were found to be 3-fold lower than the methylation rate of the unaltered receptor, suggesting that intradimer methylation as well as interdimer methylation accounts for the methylation of the unaltered receptor. In addition, the presence of the cytoplasmic signaling proteins, which have been shown to cause receptor clustering, did not influence the rates of methylation.

Reversible covalent modification of specific cytoplasmic residues of integral membrane receptors is widespread and plays an essential role in signal transduction. In mammalian receptors, phosphorylation on serine, threonine, or tyrosine residues modulates both excitation upon ligand binding and desensitization (Sibley et al., 1987). In many bacterial receptors, external stimuli trigger a two-component signal-transduction pathway involving an intrinsic or receptor-associated protein histidine kinase (Hess et al., 1988) and a response regulator (Koshland, 1977). Bacterial receptors involved in taxis, which represent a subfamily of the response regulator two-component systems, undergo reversible methylation on specific cytoplasmic glutamate residues. This process, called sensory adaptation, allows the bacteria to adapt to a sustained stimulus (Aswad & Koshland, 1975; Goy et al., 1977; Springer et al., 1979).

In *Escherichia coli*, four homologous transmembrane receptors, encoded by the *tar*,[1] *tsr*, *tap*, and *trg* genes, mediate chemotaxis toward attractants (nutrients) and away from repellents (toxic chemicals) (Adler, 1969). These chemotaxis receptors are present in the inner membrane as 120 kDa noncovalently bound homodimers in both the presence and absence of ligand (Milligan & Koshland, 1988). The four chemotaxis receptors from *E. coli* are members of a large family of bacterial receptors in which the amino acid sequence corresponding to the cytoplasmic domain is highly conserved with the exception of the juxtamembrane domain and the C-terminal tail (Le Moual & Koshland, 1996).

Upon ligand binding to the receptor periplasmic domain, a conformational signal is transmitted through TM2 to the cytoplasmic domain (Mowbray & Koshland, 1987; Falke & Koshland, 1987; Danielson et al., 1994) where it controls two interrelated processes, namely, excitation and sensory adaptation. Excitation is a fast process that controls the direction of flagellar rotation by modulating the autophosphorylation of a receptor-associated protein histidine kinase (CheA) and the subsequent phosphotransfer to a diffusible response regulator (CheY) (Hess et al., 1988). Sensory adaptation is a slower methylation-mediated process that counteracts the excitation process. In the presence of a persistent positive stimulus, several specific cytoplasmic glutamate residues are reversibly methylated. The methyl esterification of the γ-carboxyl groups is catalyzed by the CheR methyltransferase that uses *S*-adenosylmethionine as a methyl donor (Springer & Koshland, 1977). Thereafter, the γ-carboxylmethyl glutamates can be hydrolyzed by the CheB methylesterase (Stock & Koshland, 1978). The *E. coli* aspartate receptor contains four methylation sites that are randomly modified at different rates (Terwilliger & Koshland, 1984; Terwilliger et al., 1986a). The *tar* gene encodes a receptor in which the four methylation sites are translated as two glutamines and two glutamates (Q_{295}, E_{302}, Q_{309}, and E_{491}). The two glutamine residues are posttranslationally deamidated into glutamates by the CheB methylesterase prior to methylation (Kehry & Dahlquist, 1982). These four methylation sites are found in two segments of the protein that are predicted to form α-helical coiled coils (Terwilliger et al., 1986b; Lupas et al., 1991). The methylation status of the receptor has been shown to influence bacteria behavior.

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[1] Abbreviations: *tar*, gene coding for the aspartate receptor; *tsr*, gene coding for the serine receptor; *tap*, gene coding for the dipeptide receptor; *trg*, gene coding for the ribose/galactose receptor; PAGE, polyacrylamide gel electrophoresis; QEQE and QQQQ, the amino acids in the engineered receptors at positions 295, 302, 309, and 491 that are normally converted to glutamates, methylated, and demethylated during *in vivo* sensing by the aspartate receptor in a wild-type bacterial cell [in this study, the glutamates (E) become methylated and the glutamines (Q) remain unchanged]; QEQE-*x*, the truncated receptors have the designation "-*x*" where *x* indicates the number of residues truncated from the C-terminal end; QEQE-0 and QQQQ-0, the notation "-0" indicates that no amino acid residues are truncated and thus the receptor is native for methyltransferase binding; S-S-QQQQ, a disulfide cross-linked receptor is designated as S-S.

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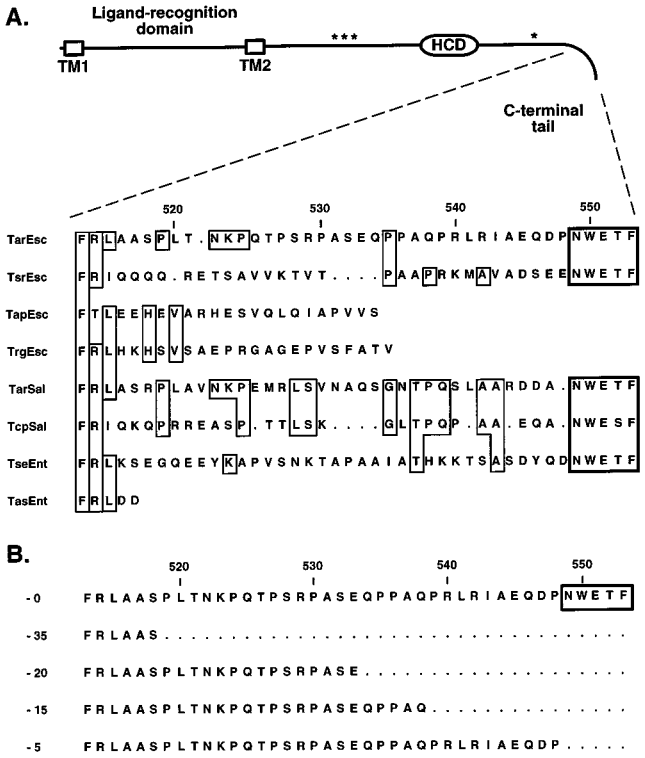

FIGURE 1: (A) Domain organization of the *Escherichia coli* aspartate receptor. TM1 and TM2 indicate the first and second transmembrane domains. Asterisks stand for the four methylation sites. HCD indicates the highly conserved domain or signaling domain. The C-terminal tail of the receptor (residues 513−553) is shown as a curved line. An alignment of the C-terminal tail amino acid sequences of the chemotaxis receptors from *Escherichia coli*, *Salmonella typhimurium*, and *Enterobacter aerogenes* is shown in an expanded view. Conserved residues are boxed. Dots represent gaps. When present, the C-terminal methyltransferase binding site (N-W-E-T-F) is boxed in boldface type. Designation and GenBank accession numbers are as follow: TarEsc, *E. coli* aspartate receptor (J01705); TsrEsc, *E. coli* serine receptor (J01718); TapEsc, *E. coli* dipeptide receptor (D90831); TrgEsc, *E. coli* ribose/galactose receptor (D90782); TarSal, *S. typhimurium* aspartate receptor (J01809); TcpSal, *S. typhimurium* citrate receptor (L06029); TseEnt, *E. aerogenes* serine receptor (M26411); TasEnt, *E. aerogenes* aspartate receptor (M26411). (B) C-terminal amino acid sequences of the native (-0) and various truncated (-*x*) aspartate receptors used in this study.

A completely demethylated receptor (EEEE) inhibits the CheA kinase and promotes smooth swimming. Conversely, a fully methylated or amidated receptor (QQQQ) activates the CheA kinase and causes tumbling (Dunten & Koshland, 1991; Borkovitch et al., 1992).

Truncation of the C-terminal tail of the aspartate receptor has been shown to interfere with sensory adaptation but not with transmission of the ligand-induced signal (Russo & Koshland, 1983). Recently, Weis and co-workers have shown that the CheR methyltransferase binding site is distinct from the sites that get methylated and consists of the last five amino acids of the serine and aspartate receptor C-terminal tails (N-W-E-T-F, Figure 1A) (Wu et al., 1996). Surprisingly, the amino acid sequences corresponding to the C-terminal tail are poorly conserved among chemotaxis receptors. These tails range in size from several to 70 residues with their amino acid sequences particularly rich in proline or glycine residues and are predicted to be mostly random coil (Le Moual & Koshland, 1996). Moreover, only 8 out of the 29 related chemoreceptors contain a C-terminal amino acid sequence that can be related to the N-W-E-T-F methyltransferase binding site. For example, in *E. coli*, the C-terminal pentapeptide is present in the aspartate and serine receptors but absent from the dipeptide and ribose/galactose receptors, suggesting that the last two receptors are unable to C-terminally bind the methyltransferase (Figure 1A). Nevertheless, the ribose/galactose receptor has been shown to undergo reversible methylation (Kondoh et al., 1979; Kehry et al., 1983; Nowlin et al., 1987) and the dipeptide receptor, which exhibits four sequences very similar to the consensus sequence of a methylation site, is believed to be methylated, although this has not been shown experimentally (Krikos et al., 1983). This allowed Wu et al. (1996) to propose that the CheR methyltransferase bound to either the aspartate or serine receptor could methylate adjacent dipeptide or ribose/galactose receptors via an interdimer mechanism. Such interdimer methylation could be facilitated by the clustering at the cell poles of the chemotaxis receptors associated with the intracellular signaling proteins (Maddock & Shapiro, 1993).

This study describes experiments designed to assess the mechanisms of both methyltransferase activation and receptor methylation.

MATERIALS AND METHODS

Bacterial Strains and DNA Plasmids. *E. coli* strain HCB721 [(*tsr*)7021 *trg*::Tn10 (*cheA*−*cheY*)::XhoI(Tn5)], which is deficient in all four chemotaxis receptors and all cytoplasmic chemotaxis proteins except the CheZ phosphatase, was kindly provided by Dr. H. Berg, Harvard University (Wolfe et al., 1988). Plasmid pME43 that overproduces the CheR methyltransferase was a gift from Dr. J. B. Stock, Princeton University (Simms et al., 1987). Plasmids pBR322 and pACYC184 were commercially purchased. Plasmid pSK2 has been described previously (Shapiro & Koshland, 1994).

Mutagenesis and Cloning. All DNA manipulations were performed according to standard protocols (Sambrook et al., 1989) unless indicated. The different site-directed mutations were introduced by using the Quick-change kit (Stratagene, Palo Alto, CA). In each case, the mutated region was sequenced and subcloned into the parental plasmid in order to avoid nonspecific mutations. Plasmids pSK102 (QEQE) and pSK105 (QQQQ) were generated by site-directed mutagenesis of plasmid pSK2 (EEEE) (Le Moual and Koshland, manuscript in preparation). To facilitate subsequent subcloning, an *Eco*47III restriction site was introduced 5′ of the *lac* promoter that directs the *tar* gene expression in pSK plasmids. The sequence 350 nucleotides 5′ of the first translated nucleotide was changed from AGCGCA to AGCGCT. The resulting plasmids pSK102-*Eco*47III and pSK105-*Eco*47III were used for further mutagenesis. The four C-terminally truncated mutants (QEQE-35, QEQE-20, QEQE-15, and QEQE-5) were generated by introducing a stop codon at the appropriate location within the coding sequence of pSK102-*Eco*47III. The C36-QQQQ mutant, which contain a single cysteine at position 36 of the receptor coding sequence, was generated by substituting a TGC codon for the original AGC codon of pSK105-*Eco*47III.

In order to coexpress in the same bacteria a methylatable QEQE receptor and an unmethylatable QQQQ receptor, we constructed pACYC184 derivatives harboring the coding sequences of the various QEQE receptors and pBR322

derivatives harboring the coding sequences of the various QQQQ receptors. *Eco*47III−*Hin*dIII DNA fragments (2400 bp long) were isolated from the various pSK102-*Eco*47III and pSK105-*Eco*47III derivatives and ligated with pACYC184 digested with *Hin*cII−*Hin*dIII and pBR322 digested with *Bsa*BI−*Hin*dIII, respectively. pBR322 and pACYC184 derivatives conferred to the bacteria resistance to ampicillin and chloramphenicol, respectively.

Preparation of Membranes Containing Overproduced Receptor(s). One-liter cultures of Luria broth (LB) supplemented with the appropriate antibiotics (kanamycin 50 μg/mL, ampicillin 100 μg/mL, and/or chloramphenicol 35 μg/mL) were inoculated with HCB721 cells transformed with various plasmids and grown at 30 °C for 20 h. Cells were harvested by centrifugation. Membranes containing the overproduced aspartate receptor(s) were prepared essentially as described previously (Foster et al., 1985). Cell pellets were resuspended in 20 mL of low-salt buffer (100 mM sodium phosphate, pH 7.0, 10% glycerol, and 5 mM EDTA) supplemented with 1 mM phenylmethanesulfonyl fluoride (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 rotor, 4 °C). The membranes were collected by high-speed centrifugation (142000*g*, 60 min, 45-Ti rotor, 4 °C). The membrane pellets were resuspended in 2 mL of high-salt buffer (20 mM sodium phosphate, pH 7.0, 2 M KCl, 10% glycerol, and 5 mM EDTA) supplemented with 1 mM PMSF, 1 mM leupeptin, and 5 mM 1,10-phenanthroline. Membranes were pelleted by high-speed centrifugation in a bench-top ultracentrifuge (488000*g*, 10 min, TLA-100.3 rotor, 4 °C). A second high-salt wash was performed. The membranes were resuspended in 2 mL of final buffer (20 mM Tris-HCl, pH 7.5, and 10% glycerol) supplemented with 1 mM PMSF, 1 mM leupeptin, and 1 mM 1,10-phenanthroline. Finally, membranes were pelleted, resuspended in 1.5 mL of final buffer, aliquoted, and stored at −80 °C. Membranes containing the C36-QQQQ receptor were prepared as described above except that DTT was added to the low-salt buffer and to the high-salt buffer at concentrations of 10 and 5 mM, respectively.

Quantitation of Aspartate Receptor in Membranes. Receptors in membranes were quantified according to Chervitz et al. (1995). The total amount of protein in the 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 SDS−12.5% polyacrylamide gels. The Coomassie blue-stained gels were scanned with a laser densitometer and the percentage corresponding to the overproduced receptor 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 1 mM PMSF, as previously described by Shapiro and Koshland (1994). 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 0, a volume of diluted membranes containing the appropriate receptor(s) (about 5 μM for each receptor species) was mixed with an equal volume of methylation mixture containing 132 μM [^{3}H]-*S*-adenosylmethionine (1.5 Ci/mmol) and a 1:100 dilution of a crude extract containing the overproduced methyltransferase (Simms et al., 1987). Methylation assays were performed in the absence and presence of 100 μM L-aspartate. At time points, 20 μL of the reaction was absorbed on a 1 cm square piece of filter paper and immediately dropped in 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 radioactivity was counted. Methylation rates were defined as the slope of a linear curve fit of the early time-course data points. Methylation rates of the QEQE-0 and QEQE-*x* receptors were found to be linear for 5 and 12 min, respectively.

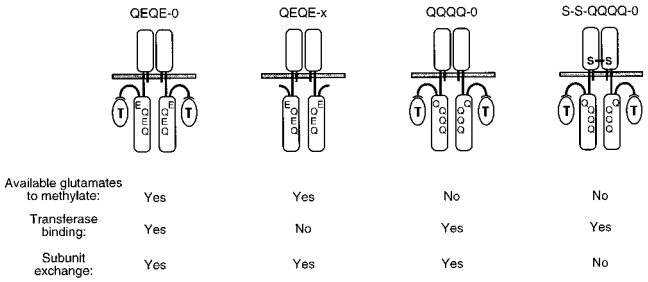

FIGURE 2: Relevant biochemical properties of the aspartate receptor variants used in this study. Methylatable glutamates at positions 302 and 491 are represented as E. Unmethylatable glutamines at positions 295 and 309 are represented as Q. Methylatable and unmethylathable receptors are referred as to QEQE and QQQQ, respectively. The methyltransferase is represented as an ellipse labeled T. Receptors that contain the C-terminal methyltransferase binding site are designated as -0. Truncated receptors that do not bind the methyltransferase are referred as to -*x*, where *x* indicates the number of residues truncated from the C-terminal end. Disulfide cross-linked receptors are designated as S-S.

Disulfide Cross-Linking. In vitro disulfide cross-linking was achieved by incubating membranes containing the C36-QQQQ receptor with 1.5 mM $(Cu^{2+})(1,10\text{-phenanthroline})_3$ at room temperature for 1 h, as described in Falke and Koshland (1987). To remove the catalyst, membranes were pelleted by high-speed centrifugation, as described above, and resuspended in a volume of methylation buffer. A second wash was performed. Methylation assays were performed next to cross-linking. Cross-linking was achieved to completion (>95%) and disulfides were stable for at least the time of the experiment, as estimated by immunoblot analysis. Immunoblots were performed using an anti-Tar serum and a goat anti-rabbit IgG coupled to alkaline phosphatase and processed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates.

RESULTS

Rationale. To assess the significance of the C-terminal methyltransferase binding site on receptor methylation, and to determine how the four *E. coli* chemotaxis receptors are methylated, we constructed a series of aspartate receptor variants. As shown in Figure 2, these variants differ in their ability to bind the methyltransferase, to be covalently modified by the methyltransferase, and to exchange subunits.

The rationale in making each of the receptors was as follow (Figure 2): (1) A receptor was made (QQQQ-0) in which all four of the sites that are methylatable *in vivo* (where deamidation, methylation, and demethylation can occur) were converted to nonmethylatable glutamines. This receptor, which was native in the methyltransferase binding site (-0), could activate methylation of other receptors by an

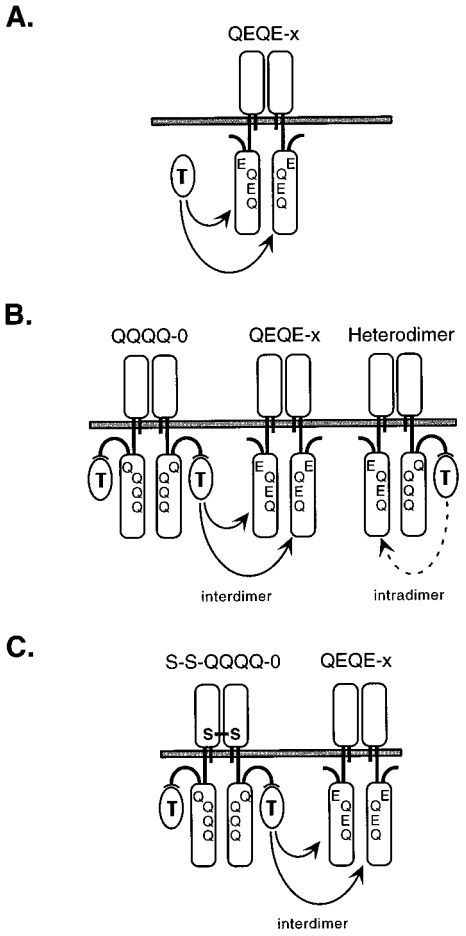

FIGURE 3: Schematic representation of all the possible mechanisms of methylation for the C-terminally truncated QEQE-*x* aspartate receptors. Designations are as described in Figure 2. (A) Truncated QEQE-*x* is the sole chemotaxis receptor present. Receptor methylation can be achieved only by the free methyltransferase. (B) Truncated QEQE-*x* receptor is present together with the QQQQ-0 receptor. Receptor methylation can be achieved via either an inter- or intradimer mechanism. (C) QEQE-*x* receptor is present together with the disulfide cross-linked S-S-QQQQ-0 receptor. Receptor methylation can be achieved only via an interdimer mechanism.

interdimer methylation or by an exchange of subunits (Milligan & Koshland, 1988) followed by an intradimer methylation (Figure 3B). (2) A disulfide cross-linked QQQQ receptor (S-S-QQQQ) was made that could activate only interdimer methylation (Figure 3C). (3) Plenty of time for exchange between subunits occurred during the growth of the bacterial cultures, but disulfide cross-linking was allowed to go to completion in the case of the SS-QQQQ receptor so it was assumed that no QQQQ·QEQE heterodimers remained (Figure 3B,C). (4) Various truncated receptors (QEQE-*x*) (Figure 1B) were made to test whether increasing degrees of truncation had increasing effects on methylation. In contrast to the QQQQ-0 receptor, these QEQE-*x* receptors can be methylated on the two glutamate residues (Van Der Werf & Koshland, 1977). (5) All receptors were produced in bacteria lacking both the methylesterase and the methyltransferase, so they remained unmodified. These receptors were then incubated together with the methyltransferase, *in vitro*, so that methylation could occur on glutamate residues but no demethylation and no deamidation occur.

Methylation Rates of QEQE-x Receptors in the Absence of Any Other Chemotaxis Receptor. HCB721 cells were transformed with the pACYC184-derived plasmids encoding the QEQE-0 and the various QEQE-*x* receptors. Membranes containing the QEQE-0 and QEQE-*x* receptors were prepared as described under Materials and Methods and analyzed by SDS−PAGE. All of the receptors were correctly incorporated into the cytoplasmic membrane and produced at high levels (each receptor representing about 10−15% of total membrane proteins) (data not shown). The initial methylation rates of these various receptors were measured by *in vitro* methylation assays as described under Materials and Methods. As determined previously, the QEQE-0 receptor was highly methylated by the methyltransferase and the presence of aspartate increased the methylation rate by 1.5-fold (Table 1) (Wang & Koshland, 1980). Hereafter, all methylation rates were normalized to that of the QEQE-0 receptor.

Table 1: Methylation Rates of the QEQE-0 and QEQE-*x* Aspartate Receptors in the Absence of Any Other Receptor[a]

receptor	−Asp	+Asp
QEQE-0	1.00 ± 0.05	1.43 ± 0.12
QEQE-35	0.04 ± 0.01	0.12 ± 0.02
QEQE-20	0.03 ± 0.01	0.08 ± 0.01
QEQE-15	0.04 ± 0.01	0.12 ± 0.01
QEQE-5	0.03 ± 0.01	0.10 ± 0.01

[a] Methylation rates were determined as described under Materials and Methods and were normalized with respect to the rate of the QEQE-0 receptor in the absence of aspartate (6 pmol of methyl groups min^{-1} for 5 μM receptor dimer). Methylation rates are the slopes of a linear fit of the time-course data points. Each rate is the average of three or more determinations and is shown along with the standard deviation.

As shown in Table 1, the methylation rates of all of the four truncated QEQE-*x* receptors, where *x* is greater or equal to 5, were very low but above background. These methylation rates were about 3% of that observed for the QEQE-0 receptor. Regardless of the extent of the C-terminal truncation (−5, −15, −20, or −35 residues), the four QEQE-*x* receptors were methylated at essentially the same low rates, so increasing the degree of truncation beyond −5 had little or no effect. The presence of aspartate increased the methylation rates of all the QEQE-*x* receptors by about 3-fold (Table 1). Since truncation of the C-terminal methyltransferase binding site had a 97% decrease in the methylation rate, these data indicate that the interaction between the receptor C-terminus and the methyltransferase is crucial for high receptor methylation. As schematized in Figure 3A, these truncated QEQE-*x* receptors, which are the sole chemotaxis receptors present in HCB721 membranes, can only be methylated by the free methyltransferase. The very low methylation rates obtained for the QEQE-*x* receptors are consistent with the fact that no interaction was observed between the free methyltransferase and the C-terminally truncated cytoplasmic domain of the receptor (Wu et al., 1996). The interaction between the receptor C-terminus and the methyltransferase could be necessary to hold the methyltransferase in close proximity of the sites being methylated and/or to allosterically activate the methyltransferase. Moreover, the finding that the presence of aspartate increases the methylation rates of the truncated QEQE-*x* receptors can be explained by an aspartate-induced conformational change affecting the methylation unit.

Methylation Rates of QEQE-x Receptors in the Presence of QQQQ-0 Receptor. To test the hypothesis of whether methylation can take place between adjacent receptors, we

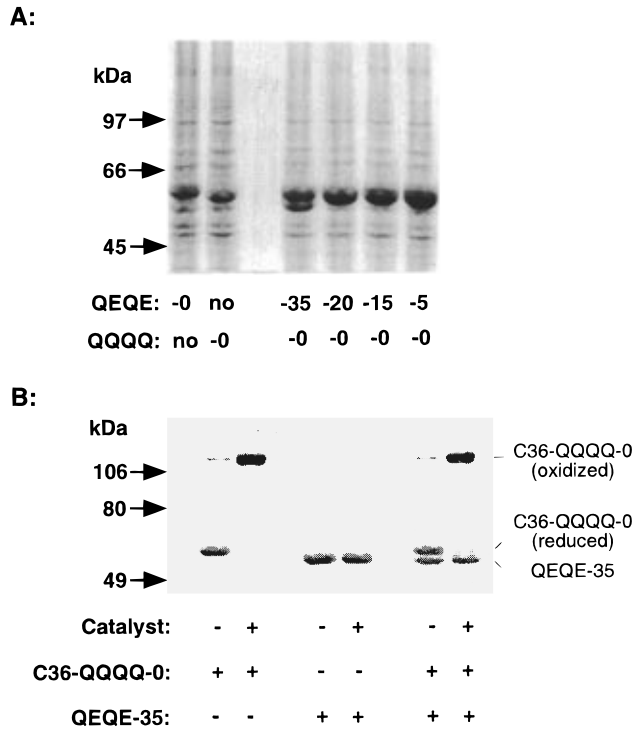

FIGURE 4: Analysis of the various coexpressed aspartate receptor variants. Membranes were prepared as described under Materials and Methods. Similar amounts of membranes (1.5 μL) were solubilized in sample buffer, heated at 90 °C for 3 min, and resolved on a nonreducing SDS−7.5% polyacrylamide gel. (A) SDS−polyacrylamide gel of membranes that contain a QEQE variant together with a QQQQ variant. (B) Immunoblot analysis of membranes that contain the C36-QQQQ-0 variant, the QEQE-35 variant, or the QEQE-35 variant together with the C36-QQQQ-0 variant. Disulfide cross-linking was achieved to completion (>95%) by incubating the various membranes with 1.5 mM cartalyst at room temperature for 1 h.

coexpressed in the same cells the various QEQE-*x* receptors together with the QQQQ-0 receptor (Figure 2), using the pACYC184-derived plasmids and the pBR322-derived plasmid. These two compatible plasmids present in the cell at approximately the same copy number are expected to produce equimolar amounts of the two receptor variants. HCB721 cells were cotransformed with both plasmids, and membranes containing both receptors were prepared and analyzed by SDS−PAGE. As shown in Figure 4A, coexpression of two receptor variants resulted in approximately twice as much of the total amount of receptor in membranes. Moreover, the two receptor variants were expressed at approximately equimolar concentrations, as estimated by laser densitometry.

As shown in Table 2, the methylation rates of the four QEQE-*x* receptors coexpressed together with the QQQQ-0 receptor were much higher than the methylation rates of the same QEQE-*x* receptors expressed alone (Table 1). The latter could only be methylated by the free methyltransferase, since no binding sites were present to activate the methyltransferase (Figure 3A). The presence of the QQQQ-0 receptor increased the methylation rates of the QEQE-*x* receptors by 9−15-fold in the absence of aspartate and by 6−9-fold in the presence of aspartate (Table 2). As shown schematically in Figure 3B, this large increase in the methylation rates of the QEQE-*x* receptors induced by the presence of the QQQQ-0 receptor is consistent with both interdimer methylation involving the two distinct homodimers and intradimer methylation involving the formation of heterodimers.

Methylation Rates of QEQE-x Receptors in the Presence of S-S-QQQQ-0 Receptor. To discriminate between these two possibilities, we prevented the formation of heterodimers and the possibility of intradimer methylation by specifically disulfide cross-linking the C36-QQQQ-0 receptor (Figure 3C). The various QEQE-*x* receptors were coexpressed together with the C36-QQQQ-0 receptor. Disulfide cross-linking of the C36-QQQQ-0 receptor was performed on membranes, as described under Materials and Methods, before the methylation assays were performed. Disulfide cross-linking was achieved to completion, since more than 95% of the cysteine-containing receptors migrate as 120 kDa dimers in nonreducing SDS−PAGE followed by immunoblotting (Figure 4B). The disulfide cross-linked dimers were stable for at least 24 h (data not shown).

As shown in Table 2, the methylation rates of the four QEQE-*x* receptors present in membranes with the S-S-QQQQ-0 receptor were still much higher than the methylation rates of the QEQE-*x* receptors expressed alone (Table 1). The presence of the S-S-QQQQ-0 receptor increased the methylation rates of the QEQE-*x* receptors by 7−12-fold in the absence of aspartate and by 3−6-fold in the presence of aspartate (Table 2). Altogether, these data show that aspartate receptors impaired in methyltransferase binding (QEQE-*x*) are efficiently methylated, via an interdimer mechanism, by adjacent aspartate receptors (QQQQ-0 or S-S-QQQQ-0) that are able to C-terminally bind the methyltransferase (Figure 3C).

Disulfide cross-linking of the QQQQ-0 caused an average decrease of the methylation rates of the QEQE-*x* receptors of 27% in both the presence and absence of aspartate (Table 2). It would be logical to assume that this 27% decrease in methylation rates of the QEQE-*x* receptors is attributable to intradimer methylation occurring within heterodimeric receptors (Figure 3B). Alternatively, this 27% decrease could also be a direct effect of disulfide cross-linking. To test this second possibility, we measured the methylation rates of the reduced and oxidized C36-QEQE-0 receptor. Whereas the methylation rate of the reduced C36-QEQE receptor was identical to that of the QEQE receptor, we observed a 25% decrease in the methylation rate of the C36-QEQE receptor upon cysteine oxidization, in both the presence and absence of aspartate (data not shown). Thus, the 27% decrease in methylation rates of the QEQE-*x* receptors upon disulfide cross-linking of the C36-QQQQ-0 receptor could also be the direct consequence of disulfide cross-linking. This would suggest that cysteine oxidization in the periplasmic domain of the QQQQ-0 receptor influences the methyltransferase bound to the cytoplasmic domain. Moreover, this would support a model in which the position and/or the activity of the enzyme is finely tuned by conformational signals.

Methylation Rate of Unaltered QEQE-0 Receptor. Our data clearly indicate that methylation can occur via an interdimer mechanism. However, the rates of interdimer methylation shown in Table 2 remain about 3-fold slower than the methylation rate of the unaltered aspartate receptor (QEQE-0) expressed alone (Table 1). One possibility is that the various truncations at the C-terminus of the receptor altered its structural integrity. This is most unlikely, since all of the four truncated receptors are fully functional for signaling to the associated kinase, as we determined by

Table 2: Methylation Rates of the Various QEQE-0 and QEQE-*x* Aspartate Receptors[a]

	in the presence of QQQQ-0 receptor				in the presence of S-S-QQQQ-0 receptor			
receptor	−Asp	*x*-fold[b]	+Asp	*x*-fold[b]	−Asp	*x*-fold[c]	+Asp	*x*-fold[c]
QEQE-35	0.36 ± 0.01	9	0.72 ± 0.05	6	0.29 ± 0.03	7	0.42 ± 0.05	3
QEQE-20	0.43 ± 0.01	14	0.72 ± 0.06	9	0.28 ± 0.01	9	0.52 ± 0.03	6
QEQE-15	0.37 ± 0.02	9	0.63 ± 0.09	6	0.33 ± 0.01	8	0.50 ± 0.02	4
QEQE-5	0.46 ± 0.03	15	0.87 ± 0.02	9	0.35 ± 0.02	12	0.57 ± 0.02	6

[a] Methylation rates were determined as described in Table 1. [b] *x*-Fold increase induced by the presence of QQQQ-0 receptor. [c] *x*-Fold increase induced by the presence of S-S-QQQQ-0 receptor.

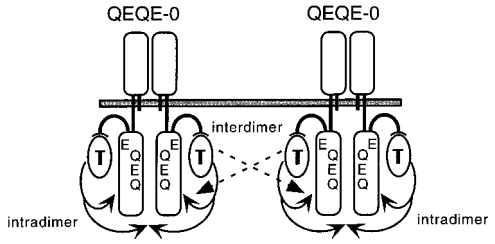

FIGURE 5: Schematic representation of the possible mechanisms of methylation for the unaltered QEQE-0 aspartate receptor. Designations are as decribed in Figure 2. The QEQE-0 receptor is the sole chemotaxis receptor present in membranes. Receptor methylation is achieved via a combination of intra- and interdimer mechanisms.

Table 3: Methylation Rates in the Absence and Presence of the Cytoplasmic Signaling Proteins[a]

receptor(s)	−CheW/CheA	+CheW/CheA
QEQE-0	1.00	1.06
QEQE-35/QQQQ-0	0.39	0.39

[a] Methylation rates were determined in the absence of aspartate as described in Table 1. Membranes containing the appropriate receptor(s) were incubated for 1 h at room temperature in the presence or absence of equimolar amounts of CheW coupling protein and CheA protein histidine kinase. The CheA and CheW proteins were expressed and purified according to Borkovich and Simon (1991).

reconstituting the signaling pathway *in vitro* (data not shown). It appears that the QEQE-0 receptor is methylated via a combination of both inter- and intradimer mechanisms (Figure 5). Assuming that the methyltansferase is as efficient when bound to either the QQQQ-0 or the QEQE-0 receptor and knowing that interdimer methylation approximates to 40% of the total rate, we estimate the proportion of intradimer methylation as 60% (Figure 5).

Receptor Methylation in the Presence of Intracellular Receptor-Associated Proteins. Because receptor clustering induced by the presence of the CheW coupling protein and the CheA histidine kinase (Maddock & Shapiro, 1993) could favor interdimer methylation, we measured the methylation rate of the unaltered QEQE-0 and that of the truncated QEQE-35 receptor coexpressed with the QQQQ-0 receptor in the presence and absence of equimolar amounts of CheW and CheA. Before the methylation assays were performed, membranes containing the various receptors were incubated in the presence of CheW and CheA at room temperature for 1 h in order to allow both ternary complex formation and clustering (Gegner et al., 1992; Maddock & Shapiro, 1993). Contrary to our expectations, neither the methylation rate of the QEQE-0 receptor nor that of the QEQE-35 receptor was affected by the presence of the cytoplasmic receptor-associated proteins (i.e., CheW and CheA) (Table 3). These data may indicate that receptor clustering has no effect on the rate of receptor methylation.

DISCUSSION

The present study clarifies the mechanism of methylation of the *E. coli* chemotaxis receptors. The main finding of the study is that C-terminally truncated aspartate receptors impaired in methyltransferase binding (QEQE-*x*), which are barely methylated in the absence of any other chemoreceptor, become efficiently methylated when present in membranes together with an aspartate receptor variant able to C-terminally bind the methyltransferase (QQQQ-0). Our cross-linking experiments rule out any possibility of heterodimer formation and show that these truncated receptors are methylated via an interdimer mechanism. We also found that the rates of interdimer methylation of the truncated receptors were lower than the methylation rate of the unmodified aspartate receptor (QEQE-0), suggesting that the latter receptor may be methylated via both intra- and interdimer mechanisms. These data support the conclusion of Weis and co-workers (Wu et al., 1996) on the importance of the C-terminal methyltransferase binding site and detail the roles of inter- and intradimer methylation.

Methyltransferase Binding to the Receptor C-Terminus Is Required for Efficient Methylation. Previous work demonstrated that the amino acid sequence NWETF, which is present at the C-terminus of the *E. coli* aspartate and serine receptors, constitutes the methyltransferase binding site (Figure 1A) (Wu et al., 1996). By showing that C-terminally truncated aspartate receptors are barely methylated in the absence of any other chemoreceptor, our results demonstrate the functional importance of the interaction between the methyltransferase and the receptor C-terminus. Two possibilities could account for the necessity of this protein−protein interaction for efficient methylation. One possibility is that the methyltransferase is allosterically activated upon binding to the receptor C-terminus. Another possibility is that methyltransferase binding to the receptor C-terminus is necessary to hold the enzyme in close proximity of the sites that get methylated.

Ligand-Induced Conformational Change within the Methylation Unit. Our data provide strong evidence for a conformational change within the cytoplasmic methylation unit of the receptor upon aspartate binding to the periplasmic domain (Table 1). The increase of the methylation rate of the QEQE-0 receptor upon aspartate binding (Wang & Koshland, 1980) is attributed to a conformational change that is transmitted through the membrane from the extracellular ligand-recognition domain to the cytosolic part of the receptor (Mowbray & Koshland, 1987). In the cytoplasmic domain of the receptor, this change in structure could affect the methylation sites. In addition, the finding that there is a methyltransferase binding site opens the possibility that the change caused by aspartate binding affects the activity or the positioning of the methyltransferase. Since the QEQE-*x*

receptors do not bind the methyltransferase, the conformational change cannot be transmitted to the methyltransferase through the C-terminal tail in that case. Consequently, the aspartate-induced change must affect the conformation of the methylation unit in this case and probably in other cases as well.

Methylation of QEQE-x Receptors via an Interdimer Mechanism. The conclusion of this study that extends the previous work of Wu et al. (1996) is that the truncated QEQE-*x* receptors that do not bind the methyltransferase can be methylated by the methyltransferase bound to the C-terminus of adjacent receptors, via an interdimer mechanism (Figure 3C). Whereas the large increase in methylation rate observed for the QEQE-*x* receptors in the presence of QQQQ-0 receptor can be interpreted by an exchange of subunits followed by an intradimer methylation (Figure 3B), the very similar increase in methylation rate observed for the QEQE-*x* receptor in the presence of S-S-QQQQ-0 receptor is not open to such ambiguous interpretation and clearly indicates the existence of interdimer methylation (Figure 3C).

The data reported in Table 3 show that the presence of the intracellular receptor-associated proteins (CheW and CheA) that are reported to be responsible for receptor clustering (Maddock & Shapiro, 1993) did not increase the rate of interdimer methylation of the QEQE-35 receptor by the methyltransferase bound to the QQQQ-0 receptor. One possibility is that receptor clustering does not affect the mechanism of receptor methylation. Another possibility is that the washed membranes that we used for *in vitro* methylation assays do not contain a soluble or membrane-associated protein that could be necessary for receptor clustering. A third possibility is that the overexpression level of the receptors could have been so significantly high that receptor clustering is mimicked in the absence of the signaling proteins.

Methylation of Unaltered QEQE-0 Receptor. Our data suggest that the unaltered aspartate receptor (QEQE-0) may be methylated via a combination of intra- and interdimer mechanisms (Figure 5). Intra- and interdimer methylation were estimated at 60% and 40%, respectively. However, these estimates may greatly vary depending on at least three factors. Although it has been shown that the methyltransferase has the same affinity for the QEQE and QQQQ receptors (Wu et al., 1996), we cannot rule out the possibility that the amidation or methylation status of the receptor affects methyltransferase activity by changing its position with respect to the methylation sites and/or by modulating its catalytic activity. Another factor that could affect the proportion between intra- and interdimer methylation is the different accessibility of the methylation sites. Whereas the methylation sites of the QEQE-*x* receptor are most likely readily accessible to the methyltransferase bound to an adjacent QQQQ-0 receptor (Figure 3C), the methylation sites of the QEQE-0 receptor are likely to be masked by the methyltransferase bound to the same receptor and, consequently, less accessible to the methyltransferase bound to an adjacent receptor (Figure 5). As a consequence, intradimer methylation would be expected to be favored with respect to interdimer methylation. A third factor that could modify the proportion between intra- and interdimer methylation is the entropic advantage of intradimer methylation over interdimer methylation. Indeed, intradimer methylation that takes place within a monomolecular complex in which the distance between the methylation sites and the methyltransferase active site is minimized is likely to be faster than interdimer methylation. These three factors could largely favor one mechanism over the other.

In Vivo Methylation in E. coli. Our data that were obtained using variants of the *E. coli* aspartate receptor can be easily extrapolated to the other three *E. coli* chemotaxis receptors. The serine receptor that possess a C-terminal methyltransferase binding site like the aspartate receptor (Figure 1A) is most likely methylated via a combination of intra- and interdimer mechanisms, as proposed for the unaltered QEQE-0 aspartate receptor. Although the dipeptide and ribose/galactose receptors undergo reversible methylation, they lack the C-terminal methyltransferase binding site (Figure 1A). *In vivo*, these two receptors are most likely methylated by the methyltransferase bound to the adjacent serine or aspartate receptors, via an interdimer mechanism. Since heterologous chemotaxis receptors do not exchange subunits (Milligan & Koshland, 1988), the possibility of intradimer methylation within heterodimeric receptors is precluded. Thus, methylation of the dipeptide and ribose/galactose receptors would strictly depend on the presence of the aspartate and/or serine receptors in the same *E. coli* cells. This hypothesis is strongly supported by previous studies. *E. coli* cells have been shown to be nonchemotactic when either the dipeptide or ribose/galactose receptor is the sole receptor present at the cell surface (Manson et al., 1986; Yamamoto et al., 1990). However, normal chemotactic behavior can be restored by the additional presence of either the serine or aspartate receptor (Hazelbauer & Engström, 1980; Manson et al., 1986). In addition, the amounts of dipeptide and ribose/galactose receptors are about $^{1}/_{10}$ those of aspartate and serine receptors (Stewart & Dahlquist, 1987). This differential expression of the four *E. coli* chemotaxis receptors would be expected to favor interdimer methylation of the dipeptide and ribose/galactose receptors by maximizing the probability of productive interaction with the methyltransferase bound to the aspartate or serine receptors.

Methylation in Other Chemotactic Bacteria. The large majority of the receptors involved in taxis appear to undergo reversible methylation. Whereas the amino acid sequences corresponding to the receptor methylation sites are conserved among bacterial species, the C-terminal methyltransferase binding site (N-W-E-T-F) appears to be absent from many receptors involved in taxis (Le Moual & Koshland, 1996). Our data suggest that interdimer methylation of a chemotaxis receptor that lacks a methyltransferase binding site is only possible if a heterologous receptor containing a C-terminal methyltransferase binding site is present in the cell. Consequently, at least one of the several chemotaxis receptors present in each bacterial species should possess a C-terminal methyltransferase binding site. Indeed, both of the two characterized *Salmonella typhimurium* chemoreceptors and one of the two characterized *Enterobacter aerogenes* receptors contain a C-terminal methyltransferase binding site (Figure 1A). Closely related sequences are also found at the C-terminus of the *Rhodobacter sphaeroides* McpA and *Caulobacter crescentus* McpA receptors (Ward et al., 1995; Alley et al., 1992). Interestingly, the *Rhizobium meliloti* ORF1 receptor, which has been shown to not be methylated, contain a somewhat different C-terminal sequence (G-W-R-R) (Greck et al., 1995). However, C-terminal related

sequences are absent from any chemotaxis receptors from many bacterial species (Le Moual & Koshland, 1996). For example, in *Bacillus subtilis*, none of the six chemotaxis receptors sequenced to date contains a C-terminal methyltransferase binding site. One possibility is that methylation mediated by the CheR methyltransferase involves different mechanisms. This hypothesis is strongly supported by the recent finding that two additional proteins (CheC and CheD) are involved in the *B. subtilis* methylation process (Rosario et al., 1995; Rosario & Ordal, 1996).

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