The Serine Chemoreceptor from *Escherichia coli* Is Methylated through an Inter-Dimer Process†

Jiayin Li,§ Guoyong Li,‡ and Robert M. Weis*,‡,§

Department of Chemistry and Graduate Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, Massachusetts 01003

Received June 23, 1997⊗

ABSTRACT: Covalent modification of receptors is a widespread phenomenon in signal transduction. In the chemosensory system of *Escherichia coli*, the reversible methylation of certain glutamic acid residues in the cytoplasmic domain of receptor homodimers mediates adaptation to stimuli. Here we report that the serine receptor is methylated by an inter-dimer process. Methyltransferase bound to one subunit in a serine receptor homodimer was found to catalyze the addition of methyl groups to a receptor subunit in an adjacent dimer in the membrane. These results demonstrate a role for inter-dimer interactions in transmembrane signaling.

Covalent modification of receptors has been observed among a variety of integral membrane receptor proteins. For example, the epidermal growth factor receptor belongs to the class of receptor tyrosine kinases that possess an intrinsic enzymatic activity that catalyzes receptor autophosphorylation (Heldin, 1995; Ullrich & Schlessinger, 1990). In other cases the catalytic unit resides as a separate protein which binds to the receptor, as in the G-protein-coupled receptor kinases (Premont et al., 1995) and the receptor methyltransferase of bacterial chemotaxis (Wu et al., 1996). In the bacterial system it has been established that the physiological role for receptor methylation is to mediate adaptation to changes in attractant concentration in which the steady-state level of methylation is proportional to the external concentration of attractants [reviewed in Springer et al. (1979)]. The 60 kDa methyl-accepting chemotaxis receptors are reversibly modified by the 31 kDa methyltransferase (CheR) which catalyzes methyl esterification of several glutamate residues in the cytoplasmic region of the receptors, and the 35 kDa methylesterase which catalyzes methyl ester hydrolysis. The glutamate residues are found in two α-helical segments (Figure 1) (Terwilliger & Koshland, 1984; Nowlin et al., 1988), which score high in coiled-coil prediction algorithms (Lupas et al., 1991; Berger et al., 1995). The methylatable residues are predicted to lie on one face in the two helices (Terwilliger et al., 1986).

The functional unit of the methylatable chemoreceptors is generally regarded to be a dimer (Milligan & Koshland, 1988). Evidence for the dimeric arrangement of subunits was obtained from the X-ray structure of the aspartate receptor (Tar)[1] ligand binding domain, in which aspartate was bound at the interface of a dimer of subunits (Milburn et al., 1991; Scott et al., 1993; Yeh et al., 1993; Bowie et al., 1995), and from disulfide-cross-linking studies of the receptor transmembrane segments, in which the predicted arrangement of the transmembrane helices was consistent with a dimeric structure (Pakula & Simon, 1992; Lee et al., 1995). The transmembrane-signaling activity retained in some disulfide cross-linked dimers has also been cited as evidence that the receptor dimer is the functional signaling unit (Milligan & Koshland, 1991; Lynch & Koshland, 1991; Chervitz & Falke, 1995; Chervitz et al., 1995; Lee et al., 1995).

Evidence supporting the involvement of larger clusters of receptor in the signaling process has also been obtained. In chemical cross-linking experiments carried out using whole cells, the principal reaction products were interpreted to be dimers, trimers, and tetramers by their electrophoretic mobility (Chelsky & Dahlquist, 1980). Also receptors were observed by electron microscopy to cluster extensively in patches at the poles of the *Escherichia coli* cell, and the extent of clustering and polar location was promoted by the formation of ternary complexes between receptors and the cytoplasmic signaling proteins, CheW and the kinase, CheA (Maddock & Shapiro, 1993). Furthermore soluble cytoplasmic fragments (C-fragments) of Tar have been shown to cluster in aggregates larger than dimers (Long & Weis, 1992; Seeley et al., 1996). Finally, studies of receptor-stimulated CheA phosphotransferase activity by C-fragments from either Tsr (Ames & Parkinson, 1994) or Tar (Cochran & Kim, 1996) have indicated that clustered fragments are more effective in stimulating CheA activity.

Transferase binds to Tar and Tsr primarily at the carboxyl terminus of the receptor, a region distinct from the sites of methylation (Wu et al., 1996). The absence of this site in both the ribose/galactose and dipeptide receptors of *E. coli* has led us to propose that receptor methylation takes place via an inter-dimer process. We have tested this hypothesis

† Supported by a University of Massachusetts Faculty Research Grant (R.M.W.), a Beckman Foundation Young Investigator Award (R.M.W.), NIH Grant 1 R01 GM53210 (R.M.W.), and the University of Massachusetts Program in Molecular and Cellular Biology (J.L.). This paper is dedicated to Harden M. McConnell on the occasion of his 70th birthday.

* Corresponding author. E-mail: rmweis@chem.umass.edu.
‡ Department of Chemistry.
§ Graduate Program in Molecular and Cellular Biology.

⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1997.

[1] Abbreviations: Tar, aspartate receptor; Tsr, serine receptor; Trg, ribose/galactose receptor; CheR, methyltransferase; PMSF; phenylmethylsulfonyl fluoride; SAM, *s*-adenosyl-L-methionine; Q, glutamine; E, glutamic acid; QEQE, amidation pattern of wt Tsr; 4Q Tsr, E403,493Q double mutant Tsr; SDS, sodium dodecyl sulfate; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; IPCR, inverse PCR.

S0006-2960(97)01510-9 CCC: $14.00

by constructing Tsr molecules that are defective in transferase binding yet have methylatable glutamate residues in the cytoplasmic region (referred to as *substrate subunits*), and have used these substrate subunits in combination with Tsr molecules which are able to bind to the transferase effectively but cannot be methylated (*binding subunits*). As described below, the results of *in vitro* methylation experiments using mixtures of these receptors provide strong evidence that communication between receptor dimers takes place during the methylation process. A preliminary account of this work has been published (Li, 1996; Li et al., 1997).

MATERIALS AND METHODS

Chemicals, Molecular Biology Reagents, and Bacterial Strains. Unlabeled SAM and [^{3}H-*methyl*]SAM (64 Ci/mmol, cat. no. 155H) were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA) and Dupont NEN (Boston, MA), respectively. γ-[^{32}P]ATP (10 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Other chemicals were reagent grade and were obtained from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Restriction endonucleases were obtained from New England Biolabs (Beverly, MA) and *Pfu* DNA polymerase was obtained from Stratagene (La Jolla, CA). DH5αF′competent cells were purchased from GIBCO/BRL (Gaithersburg, MD). HCB721 was kindly provided by H. C. Berg (Harvard University). The *tsr* expression plasmids (pHSe5) coding for Tsr with the wild type pattern of covalent modification (amino acids Q_{297}, E_{304}, Q_{311}, and E_{493}, at the principal methylation sites, abbreviated QEQE) (Rice & Dahlquist, 1991) and the amidated, (E304Q, E493Q) double mutant form of Tsr (abbreviated 4Q) were kindly provided by F. W. Dahlquist (University of Oregon). The *Salmonella typhimurium cheR* expression vector, pME43, was a gift of S. Simms (City University of New York).

Construction of the Tsr Substrate Subunit and Binding Subunit/Substrate Subunit Coexpression Plasmids. The C-terminal truncation (ΔC_{34}) of Tsr was engineered to contain a histidine affinity tag at the C-terminus. The 7.2 kb plasmid (pHSe5) expressing wt *tsr* (Rice & Dahlquist, 1991; Muchmore et al., 1989) was digested with *Hin*dIII and partially digested with *Bsp*EI to isolate a *Hin*dIII−*Bsp*EI fragment cut at the *Bsp*EI site 113 bases in from the 3′ end of the *tsr* open reading frame. The 6.5 kb fragment was gel-purified and ligated with a synthetic adapter to generate the plasmid pJL21. The pJL21 plasmid expresses a truncated Tsr molecule 523 amino acids in length, with residues 1−517 of Tsr fused to the hexahistidine tag (V_{512}AVFR IHHHH HH_{523}). Analysis by SDS−PAGE produced a protein band that migrated with the expected molecular mass (∼56.4 kDa), 3 kDa smaller than the full-length wt Tsr.

A plasmid coexpressing the C-terminally truncated (ΔC_{35}) substrate subunit and the full-length (4Q) binding subunit was produced by fusing wt *tsr* and 4Q *tsr* pHSe5. Both plasmids were digested with *Hin*dIII and were also partially digested with *Bsp*EI. The 6.5 kb *Hin*dIII−*Bsp*EI fragment from the wt *tsr* pHSe5 and the 2.4 kb *Bsp*EI−*Hin*dIII fragment from the 4Q *tsr* pHSe5 were gel-purified and ligated to generate pJL52. pJL52 expressed both full-length 4Q Tsr (551 amino acids), and truncated Tsr (522 amino acids), which consists of residues 1−516 of wild type Tsr (Δ35) and six vector-encoded amino acids ($\underline{V_{512}}$AVFR RKEKP C_{522}, Tsr amino acids underlined). Coexpression of ΔC_{35} substrate subunit Tsr (expected molecular mass, 56.2 kDa) and binding subunit Tsr (molecular mass, 59.2 kDa) was verified by SDS−PAGE.

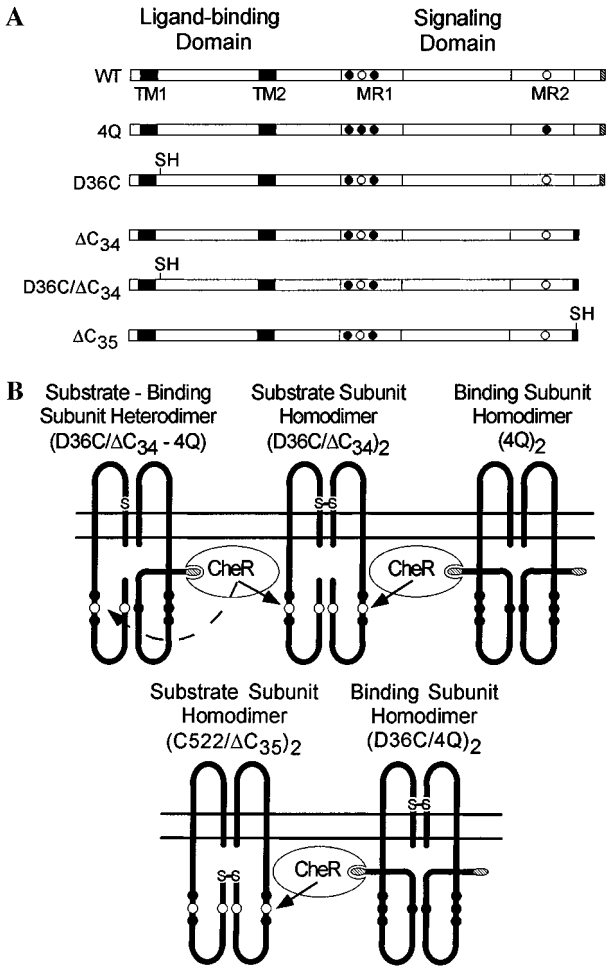

FIGURE 1: Tsr constructs used in this study. (A) Primary structures. TM1 and TM2 indicate the first and second transmembrane segments (two blackened segments on the left), MR1 and MR2 mark the methylation regions, which flank a highly conserved signaling region. The methylatable glutamate residues are represented by open circles, and nonmethylatable glutamine residues are depicted as filled circles. The ΔC_{34} and ΔC_{35} Tsr subunits lack the transferase binding site at the C-terminus (depicted by the stripped segment). Both truncated receptors contain a short vector-encoded (black) segment at the C-terminus. SH represents the D36C substitution and cysteine-522 in ΔC_{35} Tsr. (B) A diagram of Tsr dimers in the membrane. Upper bilayer: inter-subunit methylation depicted as an intradimer process within a heterodimer (dashed arrow at left) and as an inter-dimer process in the middle and at the right (solid arrows); CheR ≡ methyltransferase. Lower bilayer: disulfide bond formation in membranes of coexpressed ΔC_{35} substrate and D36C/4Q binding subunits leads exclusively to homodimers of ΔC_{35} and homodimers of D36/4Q since the active sulfhydryls in each type of subunit are on opposite sides of the membrane bilayer.

To facilitate IPCR site-directed mutagenesis (Hemsley et al., 1989), 4Q *tsr* was moved from pHSe5 to a smaller vector (a pQE30 derivative, Qiagen Corp., Chatsworth, CA) by inserting the 2.4 kb *tsr* fragment produced by *Bam*HI/*Hin*dIII digestion to generate pJL24 (5.8 kb). The synthetic primers used in the PCR reaction were used to alter codon 36 of *tsr* from GAC to TGC and resulted in a cysteine for aspartate substitution (D36C), which was verified by DNA sequencing. The D36C substitution was subcloned into the pHSe5 vectors to produce D36C forms of Tsr which were (otherwise) wild

type (pJL26c), 4Q (pJL27c), or ΔC_{34} (pJL21c). To coexpress cross-linkable substrate subunit (D36C/ΔC_{34}) Tsr and binding subunit (4Q) Tsr, a synthetic *Eco*RI−*Sma*I−*Hin*dIII linker was inserted into the *Hin*dIII site of pJL21c to generate pJL21cL. The 2.4-kb *Eco*RI−*Hin*dIII fragment of pJL24 containing *tsr* was inserted into pJL21cL to create a plasmid (pJL55) that expressed both D36C substrate subunit and binding subunit Tsr.

Membrane Preparations. *E. coli* strain HCB721 (Conley et al., 1989) was used to prepare membranes samples of Tsr as previously described (Lin et al., 1994), except that the final sucrose-gradient step was omitted. HCB721 lacks all the known methyl-accepting chemoreceptors (Tar, Tap, Tsr, Trg), methyltransferase, and the methylesterase, as well as the autophosphorylating kinase CheA, CheW, and CheY; thus the membrane preparations contained Tsr in well-defined states of covalent modification. The Tsr concentration in membrane samples was estimated by comparing the intensity of Coomassie-stained receptor bands on SDS gels to Ni-NTA-purified standards of histidine-tagged Tsr ΔC_{34}, whose concentration was determined by the BCA assay (Smith et al., 1985). Similarly, the ratio of binding subunits to substrate subunits was estimated by comparison to calibrated samples containing either only the binding or the substrate subunit.

Phosphorylation Assay. The procedure of Borkovich et al. (1992) was used to assay Tsr regulation of CheA phosphotransferase activity. Reactions samples of 8 μM membranous Tsr, 0.2 μM CheA, 4 μM CheW, and 30 μM CheY were assayed at 22 °C in 50 mM KCl, 5 mM $MgCl_2$, 50 mM Tris buffer (pH 7.5) with 1 mM dithiothreitol. γ-[^{32}P]ATP (10 Ci/mmol) was added to initiate the reaction (100 μM final concentration), and the ^{32}P-phosphate incorporation into CheY was quantified on SDS gels using a Molecular Dynamics Storm 840 phosphorimager. Phosphate incorporation was measured at 60 s after the start of the reaction.

Methylation Assays. Methyltransferase was purified as previously described (Simms et al., 1987; Wu et al., 1996). *In vitro* methylation assays consisted of 20 μM Tsr in membrane vesicles, 2 μM transferase and 200 μM [^{3}H-*methyl*]-SAM (0.1 Ci/mmol) in 35 μL of buffer (50 mM sodium phosphate buffer, pH 7.5, with 1 mM PMSF). To measure the time course of the methylation reaction, ten μL aliquots were spotted on filter paper (1 cm^2), quenched in 10% TCA, rinsed in methanol, dried, and quantified by scintillation counting (Stock et al., 1984). Methyl group incorporation was found to occur primarily at sites E_{304} and E_{493}. Contribution from the minor site (E_{503}) was, as observed previously (Rice & Dahlquist, 1991), not significant. Estimates of methylation were based on the [^{3}H-*methyl*]SAM specific activity and an assumed 100% recovery of receptors from the reaction.

Membrane samples containing cysteine-mutant Tsr were either methylated in the presence of 3 mM DTT to prevent disulfide bond formation between Tsr subunits (reducing conditions), or after formation of disulfide bonds between receptor subunits. Disulfide bonds were allowed to form for 30 min using ambient O_2 concentration with 0.5 mM Cu^{2+}(1,10-phenanthroline)$_3$ as a catalyst [as described by Chervitz and Falke (1995)]. Under these conditions, cross-linking was greater than ninety percent complete. Methylation reactions involving cross-linked receptor were carried out in the absence of DTT and in the presence of the Cu^{2+}-(1,10-phenanthroline)$_3$ used to catalyze disulfide bond formation, which did not interfere with receptor methylation for this amount of Cu^{2+}(1,10-phenanthroline)$_3$. Increasing the concentration of catalyst did not increase the cross-link formation, but a detectable decrease in the methylation rate was observed (data not shown). Thus 0.5 mM Cu^{2+}(1,10-phenanthroline)$_3$ was used to maintain stably cross-linked receptor without interfering with the methylation reaction.

Specific methyl group incorporation into the monomeric and disulfide-cross-linked dimeric Tsr was determined with SDS−PAGE to resolve cross-linked dimer from monomer, and methylated receptor from unmethylated receptor. Reactions (10 μL total volume) were carried out in phosphate buffer with membranes containing 10 μM methylatable receptor, 2 μM transferase, 200 μM SAM. For analysis by SDS−PAGE, aliquots 8 μL in size were quenched by mixing with SDS nonreducing sample buffer, boiled for 3 min and separated on a 7% polyacrylamide, 2.7% bisacrylamide SDS gels. Methylation was assessed qualitatively by a characteristic increase in the mobility of the receptor band caused by methylation (DeFranco & Koshland, 1980). In this case the methylation reaction was allowed to incubate overnight before the receptor bands were visualized with Coomassie stain. For experiments in which the rate of methylation was determined, [^{3}H-*methyl*]SAM (0.1 Ci/mmol) was used and the labeled Tsr was then resolved by SDS−PAGE. The gels were stained with Coomassie for 5 min, destained for 10 min, the Tsr monomer and dimer bands were excised, and [^{3}H-*methyl*] group incorporation was quantified by scintillation counting. Receptor bands from reactions without transferase were subtracted as background. The extents of methylation reported were based on the specific activity of [^{3}H-*methyl*] groups and an assumed 100% recovery of methylated receptor.

RESULTS

Strategy for Testing Inter-Dimer Methylation. Figure 1 depicts the two types of "substrate subunits" of Tsr in which the wt pattern of covalent modification (QEQE) was retained. Based on the results of a previous study which localized the principal site of interaction between the transferase and the receptor (Wu et al., 1996), these subunits of Tsr were expected to be unable to interact effectively with the transferase since either 34 or 35 amino acids were removed from the C-terminus. These proteins are referred to as ΔC_{34} and ΔC_{35}, respectively (Figure 1A). SDS−PAGE of the plasmid-expressed ΔC Tsr indicated that it was truncated in the expected fashion, by migrating more rapidly than the full-length Tsr. "Binding subunits" are full-length Tsr molecules that retain the transferase binding site but are poor substrates because the four principal sites of methylation are unmethylatable glutamine residues (4Q) (Figure 1A). To test the hypothesis of inter-dimer methylation as it is depicted in Figure 1B, substrate subunits and binding subunits were coexpressed in the same membrane. A cysteine residue was introduced so that either the substrate subunit, binding subunit or both could be induced to form disulfide cross-linked homodimers. Under reducing conditions, heterodimers of substrate subunits and binding subunits may form (as depicted in Figure 1B at the left) leading to the possibility of receptor methylation occurring either by an inter-subunit−intradimer process (dashed arrow) or by an inter-dimer

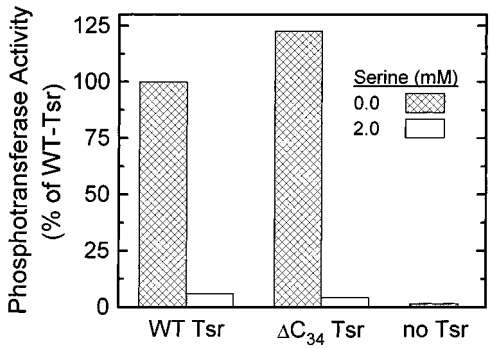

FIGURE 2: Transmembrane signaling activity of full-length and truncated Tsr assayed by the CheA phosphotransfer reaction. Steady-state levels of CheY phosphorylation (as the percentage of wild type) are shown for the wild type (WT), the substrate subunit (ΔC_{34}) Tsr, and the receptor minus control. Serine was either absent or present at 2 mM.

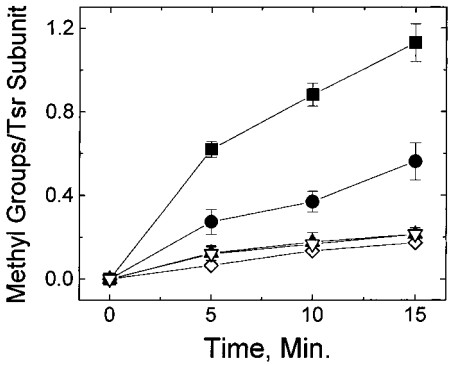

FIGURE 3: Tsr methylation measured by [^{3}H-*methyl*] group incorporation. Membrane samples with 3D concentrations of 20 μM wt Tsr (■); 20 μM ΔC_{35}/QEQE (substrate subunit) Tsr, and 5 μM 4Q (binding subunit) Tsr in the same membrane (●); 20 μM ΔC_{35}/QEQE Tsr mixed with 5 μM 4Q Tsr (▽); 20 μM 4Q Tsr (◇), or 20 μM ΔC_{35}/QEQE Tsr (▲). Error bars are standard deviations of three separate experiments.

process (solid arrow). Under oxidizing conditions the substrate subunits will be present only as disulfide linked homodimers (e.g. the center dimer at the top of Figure 1B) which then obliges the methylation reaction to occur by an inter-dimer process (solid arrows).

Figure 2 shows the receptor-stimulated CheA phosphotransferase activity in a reconstituted system consisting of Tsr, CheW, and CheA. Mixtures formed with the (ΔC_{34}/QEQE) substrate subunit Tsr catalyzed CheY phosphorylation at a rate comparable to mixtures with wt Tsr, and like wt, the CheA phosphotransferase activity stimulated by the substrate subunit was fully inhibited by saturating levels of serine. These data demonstrate that the C-terminal truncation did not significantly affect the regulation of CheA phosphotransferase activity and are in agreement with the *in vivo* signaling properties exhibited by a similar truncated form of *S. typhimurium* Tar (Russo & Koshland, 1983; Koshland et al., 1983).

Methylation of Substrate Subunits is Facilitated by Binding Subunits. The data plotted in Figure 3 were obtained from *in vitro* methylation assays of Tsr-containing membranes, purified methyltransferase, and the methyl-donor, SAM. They demonstrate that the methyl-group incorporation into the ΔC_{35} substrate subunits is enhanced significantly when binding subunits are present in the same bilayer membrane (●), relative to the background level exhibited either by membranes containing only the substrate subunit (▲), binding

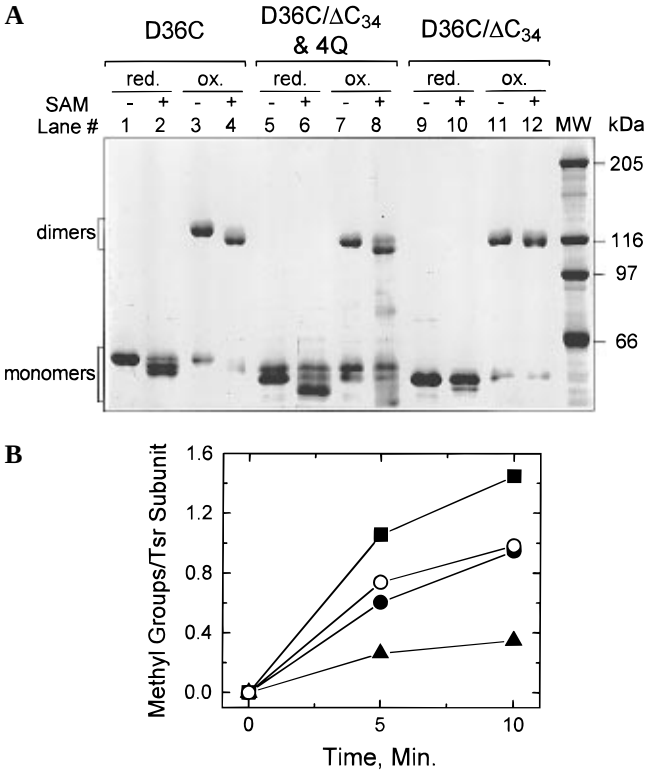

FIGURE 4: Inter-dimer methylation. (Upper Panel) Gel mobility shift assay of Tsr methylation. The Coomassie-stained SDS gel of receptor monomers and cross-linked dimers in unmethylated and (faster-migrating) methylated forms. D36C Tsr is shown in lanes 1−4, D36C/ΔC_{34} (substrate subunit) Tsr coexpressed with 4Q (binding subunit) Tsr is shown in lanes 5−9, and D36C/ΔC_{34} Tsr alone is in lanes 9−12. The more rapidly and more slowly migrating bands in lane 5 are the substrate and binding subunits, respectively. Note that the 4Q binding subunit in lane 5 migrates more rapidly than the wt (QEQE) Tsr (lane 1) since amidation (like methylation) increases electrophoretic mobility. Samples were incubated either with (+) or without (−) 200 μM SAM (even and odd lanes, respectively). (Lower Panel) Methyl group incorporation into monomeric and cross-linked dimeric Tsr. Dimeric full-length D36C Tsr (■), dimeric D36C/ΔC_{34} substrate subunit coexpressed with 4Q binding subunit (●), monomeric D36C/ΔC_{34} substrate subunit coexpressed with 4Q binding subunit (○), and dimeric D36C/ΔC_{34} substrate subunit alone (▲).

subunit (◇), or mixtures of membranes containing only the substrate subunit and only the binding subunit (▽). The lower methylation rate of coexpressed substrate subunits relative to rate of methylation of an equivalent amount of wt Tsr (■) was attributed to the 1 to 4 molar ratio of binding to substrate subunits. In contrast to the situation for membranes containing wt Tsr, not all of the substrate subunits were expected to be in close proximity to receptor-bound transferase and thus the substrate subunits were not expected to be methylated with equivalent efficiency in the coexpressed samples.

Methylation Occurs by an Inter-Dimer Process. To distinguish between methylation within heterodimers consisting of one substrate and one binding subunit (inter-subunit−intradimer), and methylation between homodimers of substrate subunits and homodimers of binding subunits (inter-dimer), disulfide-cross-linked dimers of substrate subunits were formed by the introduction of the D36C substitution. Figure 4A shows the results of a methylation experiment detected by SDS−PAGE. Methylation has been observed to increase the electrophoretic mobility of receptors in SDS gels (DeFranco & Koshland, 1980), so it was used to assess

Table 1: Serine Responses of Truncated and Cross-Linked Receptors

receptor	methylation rate[a] no serine	methylation rate[a] 2 mM serine	ratio[b] 2 mM serine/no serine	response[c] % wild type
wild type	1.00 ± 0.04 (3)	1.66 ± 0.12 (3)	1.66 ± 0.14	100
D36C (red.)	1.17 ± 0.05 (3)	1.25 ± 0.02 (3)	1.06 ± 0.05	9
D36C (ox.)	1.04 ± 0.06 (3)	1.24 ± 0.06 (3)	1.20 ± 0.09	30
D36C/ΔC_{34} + 4Q (red.)	0.64 ± 0.05 (4)	0.78 ± 0.10 (3)	1.22 ± 0.19	33
D36C/ΔC_{34} + 4Q (ox.)	0.57 ± 0.06 (5)	0.61 ± 0.06 (3)	1.08 ± 0.15	12
ΔC_{35} + 4Q (red.)	0.44 ± 0.05 (4)	0.72 ± 0.02 (3)	1.64 ± 0.18	97
ΔC_{35} + 4Q (ox.)	0.42 ± 0.02 (3)	0.65 ± 0.03 (3)	1.56 ± 0.10	85
ΔC_{35} + D36C/4Q (red.)	0.44 ± 0.02 (3)	0.49 ± 0.09 (3)	1.10 ± 0.21	15
ΔC_{35} + D36C/4Q (ox.)	0.42 ± 0.02 (3)	0.48 ± 0.02 (3)	1.16 ± 0.07	24

[a] Rates were determined as the methylation level at 5 min and were normalized with respect to the rate for wt Tsr in the absence of ligand (0.12 methyl group/receptor/min). Values are the mean of three or more separate experiments, as noted by (*n*), and uncertainties are expressed as the standard error of the mean. [b] Uncertainties in the ratio were determined by error propagation using the standard errors of the mean. [c] The percent response was calculated relative to wt Tsr as 100 × (ratio − 1.00/0.66).

the ability of truncated, disulfide-cross-linked Tsr to serve as a substrate of the transferase. The mobility shifts in Figure 4A were observed only when Tsr and transferase were incubated with SAM (+), demonstrating that the shifts were a result of methylation. The D36C substitution in wt Tsr did not affect its ability to function as a substrate for the transferase either in the non-cross-linked form (lanes 1 and 2) or as a disulfide-cross-linked dimer (lanes 3 and 4). The ΔC_{34} substrate subunit, 3 kDa smaller than the full-length binding subunit, was resolved as a more rapidly migrating band (lane 5). When the D36C/ΔC_{34} substrate subunit was coexpressed with the binding subunit and incubated with transferase and SAM, the mobility of the substrate subunit increased in a manner consistent with methylation, both in the non-cross-linked (compare lane 5 with 6), and dimeric (compare lane 7 with 8) forms. These data are strong evidence of inter-dimer methylation, since the methylation of substrate subunit expressed without binding subunit was significantly less efficient (lanes 9−12).

The methylation efficiencies of noncross-linked and cross-linked substrate subunits were compared by excising the monomer and dimer bands from SDS gels following a methylation reaction (*ca.* 60 and 120 kDa, respectively, Figure 4A). Methyl group incorporation into the D36C cross-linked dimers of substrate subunits (Figure 4B) was facilitated by the presence of binding subunits in the same bilayer membrane (●) relative to membranes containing only the substrate subunit (▲). Notably, the inter-subunit disulfide bond had no significant influence on the methylation of substrate subunits; receptor monomers excised from the SDS gel following a reaction carried out under reducing conditions (○) produced the same levels of methylation as the cross-linked dimer. These data demonstrate that inter-dimer methylation occurs efficiently, and provide evidence that inter-dimer interactions are an essential part of transmembrane signaling.

Serine Stimuli Increase the Inter-Subunit Methylation Rate. Serine was found to significantly increase the rate of Tsr methylation by the inter-subunit mechanism (Table 1). Two different binding subunit, substrate subunit pairs were investigated: the (4Q) binding subunit coexpressed with either the ΔC_{34} or the ΔC_{35} substrate subunit. In both groups, 2 mM serine increased the rate of methylation by experimentally detectable levels. The stimulatory effect of serine was largest with the ΔC_{35} substrate subunit. In this group the response to serine was similar to wild type Tsr (*ca.* 90% of the wild type response, Table 1), whether the ΔC_{35} substrate subunits were cross-linked (at Cysteine-522) or not. The D36C substitution was found to generally suppress the stimulatory effect of all the Tsr constructs irrespective of the subunit into which the substitution was made (i.e. the ΔC_{34} substrate subunit, the 4Q binding subunit or wt Tsr), irrespective of cross-linking (oxidizing conditions) or not (reducing conditions). Although the effect of serine was significantly smaller for the D36C receptor samples, the methylation rates were nevertheless observed to be greater in the presence of serine. Suppression of serine stimulation in Tsr by the D36C substitution has not been observed to occur to the same extent in Tar (Falke & Koshland, 1987; Chervitz et al. 1995); for which the extent of aspartate stimulation more closely resembled wt Tar. The differences between Tsr and Tar might either reflect differences in the assay conditions, or that the requirements for retaining activity in the cross-linked forms of Tsr and Tar are different. In this respect the effects of cross-linking on inter-dimer interactions as well as the dimer structure should be considered.

When all the data are considered together, one is led to the plausible conclusion that receptors are methylated by an inter-dimer process under physiological conditions: (1) the inter-dimer methylation process is efficient *in vitro* (*cf.* Figure 4B and Table 1), (2) attractant-stimulated increases in the methylation rate are retained in cross-linked receptors (Table 1; Falke & Koshland, 1987; Lynch & Koshland, 1991; Stoddard et al., 1992; Scott & Stoddard, 1994; Chervitz et al, 1995; Chervitz & Falke, 1995), and (3) the presence of Tar and Tsr appears to be required for effective Trg-mediated taxis in gradients of ribose (Springer et al., 1977; Engström & Hazelbauer, 1980; Yamamoto et al., 1990).

DISCUSSION

Inter-subunit covalent modification is a widespread phenomenon in receptor biology. For example, the growth factor receptors are regarded to autophosphorylate by inter-subunit processes (Heldin, 1995; Ullrich & Schlessinger, 1990), and the IgE receptor complex on mast cells is probably modified by transphosphorylation (Pribluda et al., 1994). In both cases receptor aggregation can accelerate the rate of phosphorylation. For the process of inter-dimer methylation reported here, the extensive aggregation of the bacterial receptors (Chelsky & Dahlquist, 1980; Maddock & Shapiro, 1993) and the observation that C-fragment trimers and tetramers are

more effective in stimulating CheA activity (Cochran & Kim, 1996) point to a role for receptor clusters larger than dimers in the signaling process.

A New Subunit Interface? Inter-dimer methylation has the novel feature that it takes place across a putative interface between dimers of the cytoplasmic domain, an interface which is distinct from that observed between monomers in the crystal structure of the ligand-binding domain (Milburn et al., 1991). Even interactions between two receptor homodimers of different ligand specificity appear to have physiological significance, since the ribose/galactose and dipeptide receptors are predicted to require either Tar or Tsr in the membrane to be methylated efficiently (Wu et al., 1996). Evidence cited in support of heterologous inter-dimer methylation are observations which indicate that Tar and Tsr are required for adaptation to stimuli detected by the ribose/galactose receptor (Springer et al., 1977; Engström & Hazelbauer, 1980; Yamamoto et al., 1990). Also consistent with this prediction are preliminary results of binding experiments in which transferase is cosedimented with receptor-containing membrane vesicles. Relative to the transferase binding activity of vesicles containing wt Tsr, both Trg-containing and ΔC_{34} Tsr-containing vesicles exhibited markedly lower levels of binding (X. Shan and R. M. Weis, unpublished observations).

Although the probable subunit stoichiometry in the ternary receptor:CheW:CheA complex is 1:1:1 (Gegner et al., 1992), the total number of subunits in the signaling unit is not known. In the absence of a high resolution structure of the cytoplasmic portion of the receptor complex, the nature of these interactions is not known precisely, although the data presented here together with published reports (Chelsky & Dahlquist, 1980; Long & Weis, 1992; Maddock & Shapiro, 1993; Cochran & Kim, 1996; Seeley et al., 1996) are in support of subunit interactions extending beyond the dimeric state. These data are suggestive of models for transmembrane signaling in which both homologous (e.g., receptor−receptor, CheA−CheA) and heterologous (e.g., CheA−CheW−receptor) interactions occur across an interface between receptor dimers. In such models, both ligand binding and methylation could modulate the phosphotransfer activity of CheA in the receptor−CheW−CheA ternary complex by influencing these interactions.

Global versus Receptor-Specific Effects. Inter-dimer methylation may be relevant to the phenomenon of heterologous desensitization found in eukaryotic signal transduction pathways, e.g., in which receptor-stimulated production of second messenger has a desensitizing effect on receptors of different ligand specificity (Dohlman et al., 1991). The similar phenomenon in bacteria is "global feedback" in which the changes in methylesterase activity that occur in response to a specific attractant stimulus act to a certain degree on all receptors irrespective of ligand specificity (Sanders & Koshland, 1988). In contrast, inter-dimer methylation is a localized phenomenon which occurs on the surface of the membrane in the cytoplasm, and may simply be a proximity effect which serves to keep the transferase near its substrate, the sites of methylation on the receptor. Assuming that ligand-specific adaptation requires the greatest change in the methylation level on the receptor to which the ligand binds, then this may happen if the receptor is a better substrate for the transferase when ligand is bound. The mechanism for this is not known precisely, and thus may involve changes either in receptor conformation, the extent of clustering, or both. The various models of conformational change are discussed exclusively in the context of changes within the receptor dimer (Milligan & Koshland, 1991; Stoddard et al., 1992; Kim, 1994; Scott & Stoddard, 1994; Chervitz & Falke, 1996; Gardina & Manson, 1996; Tatsuno et al., 1996). The process of inter-dimer methylation indicates that interactions between dimers should also be examined.

ACKNOWLEDGMENT

The authors thank Dr. Wenhong Lu and Dr. Steven A. Williams of Smith College for assistance in DNA sequencing and Dr. Michael D. Manson for a critical reading of the manuscript.

REFERENCES

Ames, P., & Parkinson, J. S. (1994) *J. Bacteriol. 176*, 6340−6348.
Berger, B., Wilson, D. B., Wolf, E., Tonchev, T., Milla, M., & Kim, P. S. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 8259−8263.
Borkovich, K. A., Alex, L. A., & Simon, M. I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6756−6760.
Bowie, J. U., Pakula, A. A., & Simon, M. I. (1995) *Acta Crystallogr. D 51*, 145−154.
Chelsky, D., & Dahlquist, F. W. (1980) *Biochemistry 19*, 4633−4639.
Chervitz, S. A., & Falke, J. J. (1995) *J. Biol. Chem. 270*, 24043−24053.
Chervitz, S. A., & Falke, J. J. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 2545−2550.
Chervitz, S. A., Lin, C. M., & Falke, J. J. (1995) *Biochemistry 34*, 9722−9733.
Conley, M. P., Wolfe, A., Blair, D. F., & Berg, H. C. (1989) *J. Bacteriol. 171*, 5190−5193.
Cochran, A. G., & Kim, P. S. (1996) *Science 271*, 1113−1116.
DeFranco, A. L., & Koshland, Jr., D. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2429−2433.
Dohlman, H. G., Thorner, J., Caron, M. G., & Lefkowitz, R. J. (1991) *Annu. Rev. Biochem. 60*, 653−688.
Falke, J. J., & Koshland, D. E., Jr. (1987) *Science 237*, 1596−1600.
Gardina, P. J., & Manson, M. D. (1996) *Science 274*, 425−426.
Gegner, J. A., Graham, D. R., Roth, A. F., & Dahlquist, F. W. (1992) *Cell 70*, 975−982.
Hazelbauer, G. L., & Engström, P. (1980) *Nature 283*, 98−100.
Heldin, C.-H. (1995) *Cell 80*, 213−233.
Hemsley, A., Arnheim, N., Toney, M. D., Cortpassi, G., & Galas, D. G. (1989) *Nucleic Acids Res. 17*, 6545−6551.
Kim, S.-H. (1994) *Protein Sci. 3*, 159−165.
Koshland, D. E., Jr., Russo, A. F., & Gutterson, N. I. (1983) *Cold Spring Harbor Symp. Quant. Biol. 48*, 805−810.
Lee, G. F., Burrows, G. G., Lebert, M. R., Dutton, D. P., & Hazelbauer, G. L. (1994) *J. Biol. Chem. 269*, 29920−29927.
Lee, G. F., Lebert, M. R., Lilly, A. A., Hazelbauer, G. L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3391−3395.
Li, J. (1996) Ph.D. Thesis, University of Massachusetts, Amherst, MA.
Li, J., Wu, J., Li, G., & Weis, R. M. (1997) *Biophys. J. 72*, A123.
Lin, L.-N., Li, J., Brandts, J. F., & Weis, R. M. (1994) *Biochemistry 33*, 6564−6570.
Long, D. G., & Weis, R. M. (1992) *Biochemistry 31*, 9904−9911.
Lupas, A., Van Dyke, M., & Stock, J. (1991) *Science 252*, 1162−1164.
Lynch, B. A., & Koshland, Jr., D. E. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10402−10406.
Maddock, J. R., & Shapiro, L. (1993) *Science 259*, 1717−1723.
Milburn, M. V., Privé, G. G., Milligan, D. L., Scott, W. G., Yeh, J., Jancarik, J., Koshland, Jr., D. E., & Kim, S.-H. (1991) *Science 254*, 1342−1347.
Milligan, D. L., & Koshland, Jr., D. E. (1988) *J. Biol. Chem. 263*, 6268−6275.
Milligan, D. L., & Koshland, Jr., D. E. (1991) *Science 254*, 1651−1654.

Muchmore, D. C., McIntosh, L. P., Russell, C. B., Anderson, D. E., & Dahlquist, F. W. (1989) *Methods Enzymol. 177*, 44−73.
Nowlin, D. M., Bollinger, J., & Hazelbauer, G. L. (1987) *J. Biol. Chem. 262*, 6039−6045.
Pakula, A. A., & Simon, M. I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4144−4148.
Premont, R. T., Inglese, J., & Lefkowitz, R. J. (1995) *FASEB J. 9*, 175−182.
Pribluda, V. S. Pribluda, C., & Metzger, H. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11246−11250.
Rice, M. S., & Dahlquist, F. W. (1991) *J. Biol. Chem. 266*, 9746−9753.
Russo, A. R., & Koshland, D. E., Jr. (1983) *Science 220*, 1016−1020.
Sanders, D. A., & Koshland, Jr., D. E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8425−8429.
Scott, W. G., & Stoddard, B. L. (1994) *Structure 2*, 877−887.
Scott, W. G., Milligan, D. L., Milburn, M. V., Privé, G. G., Yeh, J., Koshland, Jr., D. E., & Kim, S.-H. (1993) *J. Mol. Biol. 232*, 555−573.
Seeley, S. K., Wittrock, G. K., Thompson, L. K., & Weis, R. M. (1996) *Biochemistry 35*, 16336−16345.
Simms, S. A., Stock, A. M., & Stock, J. B. (1987) *J. Biol. Chem. 262*, 8537−8543.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76−85.
Springer, M. S., Goy, M. F., & Adler, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3312−3316.
Springer, M. S., Goy, M. F., & Adler, J. (1979) *Nature 280*, 279−284.
Stock, J. B., Clarke, S., & Koshland, Jr., D. E. (1984) *Methods Enzymol. 106*, 310−321.
Stoddard, B. L., Bui, J. D., & Koshland, Jr., D. E. (1992) *Biochemistry 31*, 11978−11983.
Tatsuno, I., Homma, M., Oosawa, K., & Kawagishi, I. (1996) *Science 274*, 423−425.
Terwilliger, T. C., & Koshland, Jr., D. E. (1984) *J. Biol. Chem. 259*, 7719−7725.
Terwilliger, T. C., Wang, J. Y., & Koshland, D. E., Jr. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6707−6710.
Ullrich, A., & Schlessinger, J. (1990) *Cell 61*, 203−212.
Wu, J., Li, J., Li, G. , Long, D. G., & Weis, R. M. (1996) *Biochemistry 35*, 4984−4993.
Yamamoto, K., Macnab, R. M., & Imae, Y. (1990) *J. Bacteriol. 172*, 383−388.
Yeh, J. I., Bieman, H.-P., Pandit, J., Koshland, D. E., Jr., & Kim, S.-H. (1993) *J. Biol. Chem. 268*, 9787−9792.

BI971510H