The Receptor Binding Site for the Methyltransferase of Bacterial Chemotaxis Is Distinct from the Sites of Methylation†

Jiongru Wu,‡ Jiayin Li,§ Guoyong Li,‡ David G. Long,§,|| and Robert M. Weis*,‡,§

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

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ABSTRACT: The principal locus for binding interactions between the aspartate and serine receptors of *Escherichia coli* and the methyltransferase was found to be in the last five amino acids of the receptor. The thermodynamic parameters of transferase−receptor interactions were determined by isothermal titration calorimetry. The serine receptor and three C-terminal fragments (C-fragments) of the aspartate receptor consisting of either the last 297, 88, or 38 amino acids gave comparable values for binding ($n = 1$, $\Delta H \approx 13$ kcal/mol, and $K_a \approx 4 \times 10^5$ M^{-1}). Truncating either 16 or 36 amino acids from the C-terminus eliminated observable interactions. Finally the pentapeptide Asn-Trp-Glu-Thr-Phe, which corresponds to the last five amino acids of the receptor and is strictly conserved among the *E. coli* serine and aspartate receptors and the *Salmonella typhimurium* aspartate receptor, was found to have all the binding activity of the full-length receptor and the C-fragments. An *in vitro* methylation assay was used to obtain evidence for the physiological significance of this interaction in which excess peptide was able to completely block receptor methylation. The location of the binding site far from the methylation sites in the primary structure of the receptor suggests that the principle role of this interaction may be to hold the transferase in close proximity to all of the methylation sites. Intersubunit methylation is proposed as plausible consequence of this "controlled proximity" mechanism since the ribose-galactose and dipeptide receptors lack the transferase binding sequence, and appear unable to bind transferase. Intersubunit methylation implies that transferase bound to either the serine or aspartate receptor subunit may catalyze methylation of receptor subunits in a neighboring dimer, including those that have different ligand specificity.

Protein covalent modification is a common regulatory mechanism that induces conformational changes and modulates the strength of binding interactions. In the case of the methylatable chemotaxis receptors from *Escherichia coli* and *Salmonella typhimurium*, the role of receptor methylation in adaptation to chemical stimuli has been firmly established [reviewed in Springer et al. (1979)] even though the specific consequences of covalent modification on receptor structure are not understood. Receptor modification permits the bacterium to adapt to steady chemoeffector concentrations (a reset-to-zero function) so the cell can respond to small deviations from the steady level with greater sensitivity. Modification in the cytoplasmic region of the receptor takes the form of methyl esterification on the γ-carboxyl group of four to five glutamate residues which are found in a methylation consensus sequence, Glu-Glu-X-X-Ala-Ser/Thr (methylated residue underlined; Terwilliger et al., 1983; Kehry et al., 1983; Nowlin et al., 1987; Rice & Dahlquist, 1991). The methylation regions were postulated to be α-helical on the basis of the arrangement of the sites (Terwilliger et al., 1986; Nowlin et al., 1988). Moreover these portions scored high in a structure prediction algorithm for coiled coils (Lupas et al., 1991).

The transfer of methyl groups from *s*-adenosylmethionine to the receptor is catalyzed by a methyltransferase (CheR; Springer & Koshland, 1977), and ester hydrolysis is catalyzed by a methylesterase (CheB; Stock & Koshland, 1978). The bacterium is found in the adapted behavioral state when the concentrations of attractants (and repellents) are steady and when the rates of receptor methylation and demethylation are equal. After a chemotactic stimulus, the number of methyl groups per receptor changes, rising in response to an increase of attractant and falling in response to a decrease, until a new steady-state is achieved. Recently, the enzymatic constants for the transferase have been defined (Simms & Subbramaniah, 1991), and the factors that influence the ability of a site to be methylated have been characterized (Shapiro & Koshland, 1994; Shapiro et al., 1995a,b).

It seems possible that more than one transferase molecule could bind to the receptor simultaneously, in view of the fact that the methylation sites are distributed through the receptor primary sequence and since the cytoplasmic region may adopt an extended conformation (Long & Weis, 1992). A preliminary study of the interaction between the transferase and a cloned cytoplasmic fragment (C-fragment)[1] from the *E. coli* aspartate receptor demonstrated a simple 1:1 interaction (Long & Weis, 1993), indicating that either the C-fragment had a single receptor binding site or that transferase binding to a primary site blocked binding to the secondary sites. To determine the exact nature of transferase binding, isothermal titration calorimetry (ITC) has been used to

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* Address correspondence to the Department of Chemistry. Phone: (413) 545-0464. FAX: (413) 545-4490. Internet: rmweis@chem.umass.edu.

‡ Department of Chemistry.

§ Graduate Program in Molecular and Cellular Biology.

|| Deceased.

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measure the interaction with the intact receptor, a series of receptor fragments, and a synthetic pentapeptide. The binding interaction was localized to the last five amino acids of the serine and aspartate receptor sequence, Asn-Trp-Glu-Thr-Phe. The pentapeptide was found to retain full binding activity and behaved like a competitive inhibitor in receptor methylation assays. The unexpected separation of the transferase binding site from the receptor methylation sites has provided novel information that facilitates a deeper understanding of the methylation process.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals and solvents were reagent grade. IPTG was obtained from BaChem CA (Torrance, CA). Fmoc amino acids were obtained from BaChem CA and PerSeptive Systems (Cambridge, MA). Fmoc-phenylalanine-activated PEG-polystyrene (0.2 mmol/g of resin) was obtained from PerSeptive Systems. [^{3}H]CH_3-*s*-adenosyl-L-methionine (specific activity 64 Ci/mmol, catalog no. NET155H) was obtained from DuPont NEN (Wilmington, DE). Restriction enzymes, T4 DNA ligase, and Klenow fragment were obtained from New England Biolabs (Beverly, MA), and *Taq* DNA polymerase was obtained from Promega (Madison, WI).

Bacterial Strains and DNA Plasmids. The *E. coli dam* strain GM2929 (*dam-13*::Tn*9dcm-6hsdR2refF143*McrA$^-$-McrB$^-$, a gift of Martin Marinus, University of Massachusetts School of Medicine, Worcester, MA) was used as a host strain for the isolation of plasmids where *Cla*I digestions were required. *E. coli* JM103 (*supE thi*-Δ(*lac-proAB*), F′[*traD36 proAB lacI*^{q}]) was used as the host strain for the aspartate receptor C-fragment plasmid pNC189, and RP3808 (relevant genotype: Δ(*cheA-cheZ*)2209 *tsr-1*; Slocum & Parkinson, 1983) was used as the host for pME43, an expression vector for the *S. typhimurium* methyltransferase (Simms et al., 1987). The serine receptor was expressed in its wild type (QEQEE), deamidated (5E), and amidated (QQQQE) forms using the pHSE5 plasmid system (Muchmore et al., 1989; Rice & Dahlquist, 1991) using *E. coli* HCB721 as the host (Conley et al., 1989). The aspartate receptor was expressed using pNT201 (Borkovich et al., 1989) in HCB721.

The IPTG-inducible expression vectors pQE30 and pET19b, used to construct plasmids producing histidine-tagged receptor fragments, were obtained from Qiagen Inc. (Chatsworth, CA) and Novagen Inc. (Madison, WI), respectively. pET19b was used with the *E. coli* strain BL21/pLysS lysogenized for λDE3, which expresses T7 polymerase under control of the *lacUV5* promoter. pLysS produces T7 lysozyme, which inhibits the small amount of polymerase present under noninducing conditions (Studier, 1991). pQE30 derivatives were transformed into DH5αF′ competent cells from GlBCO-BRL (Bethesda, MD).

Construction of His-Tagged C-Fragment Expression Vectors. A histidine-tagged form of the 297 amino acid C-fragment from the *E. coli* aspartate receptor (HTCF) was constructed by taking the *Bam*HI-*Eco*RI fragment from pNC189 and ligating it into a modified pQE30 vector using standard methods (Sambrook et al., 1989). A modified form of pQE30 was isolated in which the unique *Eco*RI site upstream of the ribosome binding sequence was eliminated by treatment of the vector with *Eco*RI, followed by a fill-in reaction with the Klenow fragment of DNA polymerase I and ligation. The *Eco*RI site was reintroduced into the polylinker site by ligation of the *Sma*I-digested intermediate with the phosphorylated double-stranded linker GGATTCC (New England Biolabs, Beverly, MA, catalog no. 1020). The *Eco*RI-digested, gel-purified product was re-ligated to produce closed circular DNA, thereby introducing an *Eco*RI site downstream from the *Bam*HI site in the polylinker. Elimination and introduction of sites were determined by restriction analysis. The *Bam*HI-*Eco*RI fragment of pNC189 fragment was ligated with the corresponding vector fragment, and plasmids (pHTCF) were screened by PAGE analysis of transformants for a protein with the expected molecular mass (32.7 kDa).

C-terminus-truncated histidine-tagged C-fragments were constructed using PCR to amplify sequences from the *tar* gene terminated at one end by the unique *Cla*I site within *tar* and at the other by a stop codon. A 164 bp PCR product was generated to introduce a stop codon and truncate the receptor by 36 amino acids (Δ36), and a 224 bp PCR product was generated to truncate the receptor by 16 amino acids (Δ16). Primer sequences for the 5′ *Cla*I site and 3′ stop sites were 5′-GGCATCGATCAAGTCGCATTG-3′, 5′-GCGAATTCTAGGCTGCCAGACGGAACGCGGA-3′ (Δ36), and 5′-GCGAATTCCTAAGCCGGTGGTTGCTCACTGGC-3′ (Δ16), respectively. In addition to the stop codon, the Δ16 and Δ36 primers introduced an *Eco*RI site to facilitate subcloning. The *Cla*I-*Eco*RI-digested PCR products were ligated with the *Eco*RI-*Cla*I fragment of pHTCF to produce the plasmids pHTCFΔ36 and pHTCFΔ16 which expressed the histidine-tagged, truncated C-fragments.

Using a similar PCR strategy, two other plasmids were constructed, one which produced the last 88 amino acids of Tar and the other the last 38 amino acids (pJW88 and pJW38, respectively). Two PCR products were generated using pNC189 as a template with a common 3′ primer (5′-GCGGATCCCCATCAGGCGGCAATGA-3′) and different 5′ primers, one that produced a 325 bp product (5′-CGGATGAACATATGCGTGGCATCGATCCAG-3′) and the other a 174 bp product (5′-CGCGTTCCATATGGCAGCCAGCCCACT-3′). The PCR products were digested with *Nde*I and *Bam*HI, and ligated to the *Nde*I-*Bam*HI fragment of pET19b, which has a T7 *lac* promoter−regulator sequence. C-terminal segments of Tar produced by pJW38 and pJW88 were fused to vector-encoded polypeptide at the N-terminus which was composed of a $(His)_{10}$ tag and an enterokinase cleavage site. The plasmids were screened initially by restriction endonuclease analysis and subsequently checked for the expression of 6.6 kDa (pJW38) and 12.6 kDa (pJW88) polypeptides using tricine−SDS−PAGE (Schägger & von Jagow, 1987).

Peptide Synthesis and Purification. The pentapeptide NWETF was synthesized by solid-phase synthesis (Stewart & Young, 1984; Atherton & Sheppard, 1989) using Fmoc chemistry. A 4-fold molar excess of free acid and coupling

[1] Abbreviations: Tar, aspartate receptor; Tsr, serine receptor; Trg, ribose/galactose receptor; Tap, dipeptide receptor; ITC, isothermal titration calorimetry; IPTG, isopropyl thio-β-D-galactoside; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; C-fragment, 257−553 residue fragment of *E. coli* Tar; HTCF, N-terminal histidine-tagged C-fragment; P88, N-terminal histidine-tagged polypeptide fused to residues 466−553 of *E. coli* Tar; P38, N-terminal histidine-tagged polypeptide fused to residues 516−553 of *E. coli* Tar; NWETF, Asn-Trp-Glu-Thr-Phe pentapeptide; bp, base pair; LB, Luria broth; GFC, gel filtration chromatography.

agent was used relative to the resin-bound acid (phenylalanine, 0.1 mmol). TFFH (Carpino & El-Faham, 1995) and HATU/HOAt coupling chemistries were both used with similar results. The cleavage/deprotection step was carried out in 95% TFA and 5% triethylsilane (10 mL/g of resin) for 2 h. The resin was removed by filtration and washed with 3 × 2 mL TFA. After the addition of 50 mL of diethyl ether, crude peptide was allowed to precipitate overnight at 4 °C. The precipitate was collected by centrifugation (2000*g* for 20 min), washed 3 times with 20 mL of ice-cold ether, and lyophilized to remove all trace of solvent.

The peptide was purified with reverse-phase chromatography using a Waters HPLC equipped with a diode-array detector at room temperature (10 mm × 25 cm, Vydac C18, catalog no. 218-TP1010). Fractions were eluted during a 27%−33% acetonitrile/water gradient (with 0.1% TFA) developed over 12 min at a flow rate of 3 mL/min. Two major products were isolated with significant absorptivity at 280 nm. Only one fraction proved to have binding activity. This fraction was found to have the expected molecular weight (by mass spectrometry) and sequence (by 2D NMR spectroscopy) for the pentapeptide NWETF.

Protein Purification. The 297 amino acid C-fragment of the *E. coli* aspartate receptor was purified as described previously (Long & Weis, 1992). Samples of the Tsr receptor used in titrations were prepared as sonicated whole (inner and outer) membranes of *E. coli* HCB721/pHSE5 according to the method of Foster et al. (1985). For the methylation assays, Tar-containing inner membranes were prepared from HCB721/pNT201 as described previously (Gegner et al., 1992; Lin et al., 1994). The methyltransferase from *S. typhimurium* was purified using the protocol developed by Simms et al. (1987) with minor modifications. *S. typhimurium* transferase was used since the pME43 expression plasmid overproduced transferase *ca*. 1000-fold (Simms et al., 1987), greatly facilitating the isolation of transferase in the quantities required for ITC experiments. *S. typhimurium* methyltransferase is expected to be functionally identical to the *E. coli* transferase given the generally high level of amino acid identity between the chemotaxis proteins of *E. coli* and *S. typhimurium*, approximately 90% between the transferases (Mutoh & Simon, 1986; Simms et al., 1987) and aspartate receptor C-fragments (Russo & Koshland, 1983; Krikos et al., 1983). Homologues between *E. coli* and *S. typhimurium* share greater sequence identity than the members of the *E. coli* receptor family. (For example the *E. coli* aspartate and serine receptor C-fragments have 80% amino acid identity.) The *S. typhimurium* transferase has also been used in methylation studies of the *E. coli* aspartate receptor (Shapiro & Koshland, 1994).

Histidine-tagged C-fragments produced from the IPTG-inducible expression plasmids pHTCF, pHTCFΔ16, pHTCFΔ36, pJW88, and pJW38 were purified with Ni−NTA affinity chromatography (Janknecht et al., 1991; Crowe et al., 1994). For protein purification, cells were grown at 37 °C in 1 L of LB containing 150 μg of ampicillin/mL. When the cultures reached an apparent absorbance of 0.5 at 600 nm, IPTG was added to a final concentration of 1 mM, and the cultures were then grown an additional 4 h before being harvested by centrifugation (3000*g* for 10 min). The cell pellet was resuspended in buffer A (50 mM sodium phosphate, 300 mM NaCl, pH 7.6) using *ca*. 25 mL of buffer for a cell paste obtained from 1 L of culture. The resuspended cells were sonicated at 300 W in 10 one-minute bursts while on ice. Cell debris was removed by centrifugation at 25 000*g* for 20 min. The supernatant was loaded onto an Ni−NTA agarose column at room temperature (1.5 cm i.d. × 4 cm) equilibrated with buffer A. The column was washed with 3 bed volumes of buffer B (buffer A containing 1 mM imidazole) and histidine-tagged protein was eluted with buffer C (buffer A containing 250 mM imidazole). An additional step after the buffer B wash was used in the purification of the polypeptides produced by pJW88 and pJW38 (P88 and P38, respectively) consisting of 4 bed volumes of 8 M urea, 0.1 M sodium phosphate, 10 mM Tris, pH 7.6, followed by 3 bed volumes of buffer B before eluting with buffer C. Protein purity (CheR, CF, HTCF) was assessed by SDS−PAGE. The purity of P88 and P38 was assessed by tricine−SDS−PAGE using 16% polyacrylamide gels with 3% bisacrylamide cross-linker (Schägger & von Jagow, 1987). Fractions containing the C-fragments were pooled and dialyzed against titration buffer before use.

The concentrations of proteins were determined either by the BCA assay (Smith et al., 1985) or by UV−vis absorption spectrometry using the value of ϵ_{280} determined previously for the C-fragment (7500 M^{-1} cm^{-1}; Long & Weis, 1992) and 40 800 M^{-1} cm^{-1} for the transferase which was determined by the method of dry weights (Kupke & Dorrier, 1978). The concentrations of P88, P38, and NWETF, which contained a single tryptophan, were determined by the absorbance at 280 nm using a molar extinction coefficient of 5600 M^{-1} cm^{-1} (Schmid, 1989).

Preparation of Monomeric and Dimeric C-Fragment. Gel filtration chromatography was used to isolate the noncovalent dimeric form of the S461L mutant C-fragment as described by Long and Weis (1992) for ITC experiments between the transferase and either monomeric or dimeric C-fragment. A large activation energy for interconversion between the monomer and dimer forms has been observed (Seeley et al., 1994), so that no significant interconversion was expected during the ITC experiments carried out at 10 °C (*ca*. 2 h). To precisely match the protein concentration in the monomer and dimer samples, a solution of dimer was isolated by GFC and divided into two aliquots, one aliquot was converted to monomer by incubating the solution for 20 min at 30 °C, which was near T_m for dimer dissociation (35 °C) but well below the T_m of denaturation (*ca*. 60 °C; Wu et al., 1995). The solution was then rapidly cooled on ice. GFC was used to determine the dimeric content of the samples before and after ITC.

Isothermal Titration Calorimetry. Most titrations were carried out using a MicroCal MCS titration calorimeter (MicroCal Inc., Northampton, MA) with Observer software for instrument control and data acquisition, although some of the experimental results reported here were obtained with the older generation MicroCal Omega. Instrumentation, software, and data analysis have been described elsewhere (Wiseman et al., 1989; Lin et al., 1994; Li et al., 1995; Li & Weis, 1996). Titration buffer for experiments involving the C-fragments, peptide, and methyltransferase was 20 mM potassium phosphate, 20 mM NaCl, 1 mM EDTA, 1 mM PMSF, pH 7.0. For ITC experiments involving Tsr-containing membranes and transferase, samples were dialyzed against 20 mM sodium phosphate, 20 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10% (v/v) glycerol, pH 7.4. Typically, titrations consisted of either 5 or 10 μL injections in a schedule of 10−30 injections spaced at intervals of 3−4 min and a syringe stirring speed of 500 rpm. The data were

analyzed according to a single-set-of-sites model from which were obtained values of the binding stoichiometry, n (moles of injectant per mole of reactant in the calorimeter cell), the binding enthalpy, ΔH (kcal per mole of injectant), and the association constant, K_a.

Ni−NTA-Based C-Fragment−Pentapeptide Competitive Binding Assay. The competitive interactions between the C-fragment and the pentapeptide were measured directly using Ni−NTA agarose, similar to the assay described previously which measured CheA−CheY interactions (Li et al., 1995). The histidine-tagged C-fragment (HTCF) was captured by Ni−NTA agarose, which was in turn used to bind the methyltransferase. The efficiency of methyltransferase binding activity was assessed as function of pentapeptide (NWETF) concentration. In a typical experiment 720 pmol of HTCF was captured on Ni−NTA agarose (*ca*. 300 μL of swollen resin) suspended in a 600 μL total volume of buffer D (50 mM sodium phosphate, 300 mM NaCl, 1 mM imidazole, 2 mM β-mercaptoethanol, pH 8.0). After being incubated on ice for 1 min the supernatant was removed by centrifugation (1 min at 11 000*g*), and the pellet was washed twice with 1.0 mL of buffer D, once with 1.0 mL of buffer E (50 mM sodium phosphate, 40 mM NaCl, 5 mM imidazole, 2 mM β-mercaptoethanol, pH 8.0), and then resuspended in 600 μL of buffer E. The imidazole concentration in buffer E was found to prevent transferase binding and not to elute C-fragment. The HTCF−Ni−NTA suspension was divided into 100 μL aliquots. After the supernatant was removed following centrifugation, 30 μL of a transferase−pentapeptide mixture was added. Various ratios of peptide to transferase (0, 1, 10, and 100, using 100 pmol of transferase in buffer E) were incubated with HTCF−Ni−NTA on ice for 1 h. After centrifugation the supernatant was removed (S fraction), the resin was washed once with 1 mL of buffer E, and the bound proteins were eluted from the pellet (P fraction) with 30 μL of buffer F (buffer E with 250 mM imidazole). 10 μL aliquots of the S and P fractions were analyzed by SDS−PAGE.

Receptor Methylation. Assays of transferase activity used purified transferase with receptor-containing membranes and [^{3}H]SAM as substrates. In the transferase inhibition assays, the reaction mixtures contained 10 μM Tar, 2 μM transferase, 160 μM [^{3}H]SAM, and inhibitor (0.1 μM to 6 mM NWETF) in a total reaction volume of 20 μL (100 mM potassium phosphate, pH 7.0). The extent of methylation was determined after 12 min. of reaction by applying a 15 μL aliquot to 1 cm^2 of Whatman filter paper and quenching the reaction in 10% TCA solution. The paper squares were washed twice in 10% TCA, rinsed in methanol, dried, and analyzed for [^{3}H]methyl group incorporation by scintillation counting using the methanol diffusion assay (Chelsky et al., 1984). The percent transferase activity was calculated relative to samples without inhibitor (full activity) after subtracting as background the counts from controls without transferase. Percent activity (A) data as a function of inhibitor concentration were fitted to a simple model based on competitive inhibition.

$$A = 100\frac{K_M + [S]}{(1 + [I]/K_I)K_M + [S]} \quad (1)$$

where K_M, K_I, [S], and [I] are the Michaelis constant for methylation reaction, the dissociation constant for inhibitor binding, and the substrate and inhibitor concentrations, respectively. Initial rate measurements of transferase activity were also made according to the protocol of Simms et al. (1987).

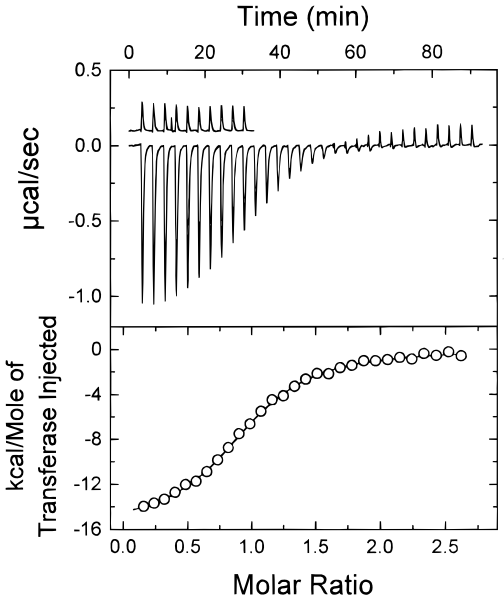

FIGURE 1: Isothermal titration calorimetry of the wild type C-fragment with the methyltransferase. The top trace in the top panel is titration data from a control experiment consisting of ten 10 μL injections of a 0.6 mM transferase solution into buffer at 27 °C, and the bottom trace depicts thirty 10 μL injections of transferase into a solution of the wild type C-fragment (experimental parameters are summarized in Table 1, expt 1). In the bottom panel the background-subtracted, integrated, and normalized data are plotted (○), and a fit derived from the single-set-of-sites model is drawn through the data.

RESULTS

Methyltransferase Forms a 1:1 Complex with the Receptor. ITC was used to establish the 1:1 interaction between the methyltransferase and the receptor. Representative data are plotted in Figure 1 which shows a titration of 26 μM wild type C-fragment with a solution in 0.28 mM transferase at 27 °C. The raw titration data in Figure 1A were integrated and normalized to yield ΔH as a function of the molar ratio of transferase to C-fragment. These data were fitted to the single-set-of-sites model which returned values for n, ΔH, and K_a of 0.96, 15.8 kcal/mol and 4.1 × 10^5 M^{-1}, respectively. The results of this titration (expt 1) as well as a number of others are summarized in Table 1, e.g., a titration in which the transferase was titrated with the C-fragment (expt 2), produced the same values of the thermodynamic parameters within experimental error. Since the C-fragment contained all of the known sites of methylation, the n-value obtained from these experiments provided evidence that the transferase did not bind to each of the individual sites of methylation simultaneously.

Numerous point mutations in the aspartate receptor cytoplasmic domain have been shown to affect the signaling properties of the receptor (Mutoh et al., 1986), in essence locking it into one of two signaling states (either kinase-activating or kinase-inhibiting; Borkovich & Simon, 1990). Several C-fragments containing such point mutations (E301K, T311I, V346M, V433I, A436V) were titrated with the transferase, and it was found that the mutations had no significant effect on the C-fragment−transferase interactions (Table 1, expts 3−7). The values of n, ΔH, and K_a were

Table 1: Thermodynamic Parameters for Titrations of Methyltransferase with Full-Length Receptor and C-Fragments[a]

	protein (concentration)							
expt no.	reaction cell (μM)	syringe (mM)	temp (°C)	n	K_a ($M^{-1} \times 10^{-5}$)	ΔH (kcal/mol)	$\Delta G°$ (kcal/mol)	$\Delta S°$ (eu)
1	CF_{WT} (26)	CheR (0.28)	27.0	0.96	4.1	−15.8	−7.7	−26.9
2	CheR (28)	CF_{WT} (0.41)	28.0	1.08	4.1	−14.1	−7.7	−21.2
3	CF_{E301K} (43)	CheR (0.60)	27.0	0.68	2.5	−16.1	−7.4	−29.0
4	CF_{T311I} (47)	CheR (0.60)	27.0	1.04	3.4	−14.9	−7.6	−24.4
5	CF_{V346M} (47)	CheR (0.60)	27.0	0.94	3.7	−14.3	−7.6	−22.0
6	CF_{V433I} (39)	CheR (0.60)	27.0	0.97	3.0	−15.6	−7.5	−27.1
7	CF_{A436V} (12)	CheR (0.28)	27.0	1.17	6.6	−15.0	−8.0	−23.4
8	Tsr_{5E} (39)	CheR (0.52)	29.4	0.86	4.1	−10.8	−7.8	−10.0
9	Tsr_{WT} (40)	CheR (0.54)	29.5	0.85	3.9	−10.3	−7.7	−8.4
10	Tsr_{4Q1E} (42)	CheR (0.54)	29.5	0.90	4.8	−11.8	−7.6	−13.0

[a] n, K_a, and ΔH were obtained from the single-set-of-sites fit to the data. $\Delta G°$ ($= -RT \ln K_a$) and $\Delta S°$ ($= [\Delta H - \Delta G°]/T$) were calculated accordingly. See Materials and Methods for buffer composition.

generally within experimental error of one another and of the wild type C-fragment.

Receptor Covalent Modification Has No Detectable Effect on Transferase Binding. The sites of methylation must be brought into the transferase active site during the methylation process and thus it might be expected that the methylation level may significantly effect the strength of transferase−receptor interactions. To test this possibility, K_a for the transferase−receptor interaction was measured as a function of the level of covalent modification using the full-length serine receptor (Tsr). Membranous samples of Tsr were prepared from the transferase-minus, esterase-minus strain HCB721 so that receptors were uniformly modified. Thus HCB721 transformed with one of three *tsr* expression vectors produced Tsr in one of three different levels of covalent modification. Previous studies have shown that replacing glutamic acid (E) with glutamine (Q) by genetic methods has a similar effect on receptor signaling properties as methylation (Dunten & Koshland, 1991; Borkovich et al., 1992). Titrations were carried out with Tsr that was completely deamidated (demethylated) at the five sites of methylation (5E), wild type receptor (which is synthesized in a partially amidated form, QEQEE), and amidated receptor (4Q1E). The results of these titrations are summarized in Table 1 (expts 8−10). The binding interaction was specific for the Tsr protein since no binding was detected in control experiments with membranes lacking the serine receptor (Li & Weis, 1996). Covalent modification had no observable effect on either n or K_a, and thus the modifiable glutamates appeared not to be strongly involved in binding. ΔH of transferase binding to Tsr in membranous samples was smaller than the value for the Tar C-fragment by an experimentally significant margin. The reason for this discrepancy is not understood, but it may be due to the differences in the buffer composition of the membranous samples and the soluble C-fragment, although receptor-specific differences (Tar *vs* Tsr) cannot be ruled out.

The similarity in the association constant of transferase binding to the intact receptor and to the C-fragment demonstrated that the residues between the end of the second transmembrane segment and the beginning of the C-fragment (residues 203−256) were not essential for binding. The similarity in K_a also seemed to indicate that transferase binding was unaffected by any changes in the properties of the cytoplasmic region as a result of being separated from the membrane-bound N-terminal portion of the receptor. As a substrate for methylation the C-fragment and the intact receptor are significantly different, the C-fragment has been found to be a poor substrate in methylation assays (Mowbray et al., 1985; A. Pautsch and R. M. Weis, unpublished observations), yet, as the data presented here indicate, the C-fragment retains full binding activity.

C-Fragment Dimerization Has No Effect on Transferase Binding. The receptor is generally believed to function as a dimer, and it thus might be expected that receptor dimerization could significantly affect the transferase−receptor binding interaction. Our previous studies of C-fragment clustering equilibria (Long & Weis, 1992; Wu et al., 1995) enabled us to isolate the S461L C-fragment either as noncovalent dimers or as monomers so that the effect of dimerization on the receptor−transferase interactions could be tested directly. Before titrations with the S461L C-fragment, the dimer fraction was isolated by GFC (85% dimer by GFC), and a monomer sample was generated by gently heating an aliquot of the dimer sample (70% monomer by GFC). GFC was also used to verify that the distribution between monomer and dimer did not change significantly over the course of the titration. Titrations of the monomer and dimer samples produced values of n, K_a, and ΔH that were within experimental error of one another (data not shown), providing strong evidence that the binding site was unchanged by dimerization. Also ligand binding had no observable effect on transferase binding, since titrations of intact Tsr were unaffected by the presence of 1 mM serine in the buffer (data not shown). Since it appeared that transferase binding was unaffected by ligand-induced changes in receptor conformation as well as subunit interaction, we sought to find the minimum portion of the receptor that was still able to bind to the transferase.

Transferase Binds to the C-terminus of the Receptor. Russo and Koshland (1983) and Koshland et al. (1983) observed that the C-terminus of the *S. typhimurium* aspartate receptor was essential for the adaptation of bacteria to stimuli. Specifically it was observed that eliminating 35 amino acids from the C-terminus resulted in a receptor that was still able to bind aspartate *in vitro* and to generate responses to aspartate stimuli *in vivo* but was unable to facilitate adaptation. This receptor was also found to be poorly methylated (Koshland et al., 1983). Thus the last 35 amino acids either were important for maintaining the conformation of the receptor so that it could function as a substrate of the transferase or were directly involved in binding to the transferase. To distinguish between these possibilities, the cloned receptor fragments depicted in Figure 2 were generated and tested for transferase−receptor interactions. As described above, full-length Tsr and the 31 kDa aspartate

Table 2: Localization of Aspartate Receptor−Methyltransferase Interactions Using Receptor Fragments[a]

	protein (concentration)							
expt no.	reaction cell (μM)	syringe (mM)	temp (°C)	n	K_a ($M^{-1} \times 10^{-5}$)	ΔH (kcal/mol)	$\Delta G°$ (kcal/mol)	$\Delta S°$ (eu)
11	297 a.a. CF (28)	CheR (0.35)	28.0	0.84	4.4	−12.1	−7.8	−14.5
12	CheR (43)	CFΔ36 (1.2)	28.0		(no binding detected)			
13	CheR (28)	CFΔ16 (0.60)	28.0		(no binding detected)			
14	P88 (15)	CheR (0.35)	28.0	0.96	3.8	−12.2	−7.7	−14.8
15	P88 (25)	CheR (0.35)	28.0	1.14	2.8	−12.8	−7.5	−17.7
16	P38 (25)	CheR (0.35)	28.0	0.90	3.4	−12.4	−7.6	−15.7
17	P38 (13)	CheR (0.35)	28.0	0.89	3.8	−12.1	−7.7	−14.6
18	NWETF (15)	CheR (0.35)	28.0	0.78	5.5	−13.6	−7.9	−18.9

[a] Experimental values were estimated as described in the legend of Table 1.

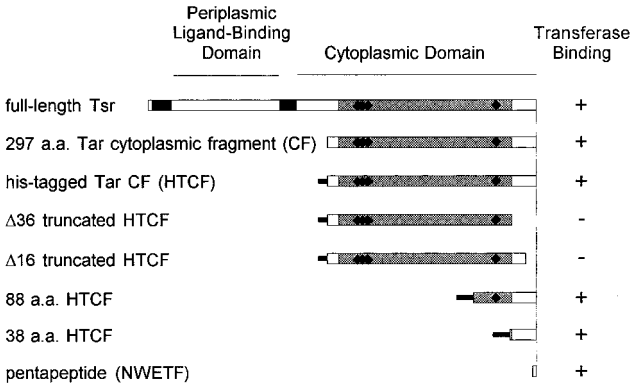

FIGURE 2: Primary structure of the methylatable chemotaxis receptors and the receptor fragments used in transferase binding experiments. At the top, the full-length serine receptor is depicted (551 amino acids), in which black boxes are used to mark the two transmembrane domains that flank the periplasmic ligand-binding domain, and the cross-hatched box is used to mark a region of high amino acid identity (ca. 85%) in the cytoplasmic domain. The sites of methylation (as identified in the aspartate receptor) are marked by filled diamonds. Vector-encoded polypeptide, which includes the N-terminal histidine affinity tag, is indicated on the receptor fragments by a narrow black bar located at the left end. Transferase binding (+) or the lack thereof (−) is indicated on the right.

receptor C-fragment exhibited equivalent ability to bind transferase. To facilitate rapid purification of the cloned receptor fragments, a histidine affinity tag was fused to the N-terminus. This histidine-tagged version of the 297 amino acid C-fragment was found to bind to the transferase with comparable thermodynamic parameters (Table 2, expt 11), thus demonstrating that the affinity tag did not interfere with transferase binding. To verify that C-terminal amino acids were essential for transferase interaction two truncated C-fragments were generated, one lacking 36 amino acids (Δ36) and the other lacking 16 amino acids (Δ16). As the integrated titration data in Figure 3A show, neither the Δ36- nor the Δ16-truncated C-fragments exhibited any detectable interaction with the transferase. These results demonstrated that the portion of the receptor in close proximity to the C-terminus is required for transferase binding.

To determine whether or not the C-terminus is itself directly involved in binding, two his-tagged polypeptides, which consisted of either the last 88 or 38 amino acids of the receptor were expressed (Figure 2), purified, and titrated with transferase. As Figure 3B shows, these fragments exhibited high-affinity, saturable binding. Fits to the single-set-of-sites model returned values of n, ΔH, and K_a that were indistinguishable from the values obtained for the 31 kDa aspartate receptor C-fragments and provided convincing proof that the transferase binding interaction is found within a region of the receptor sequence distinct from the methylation sites.

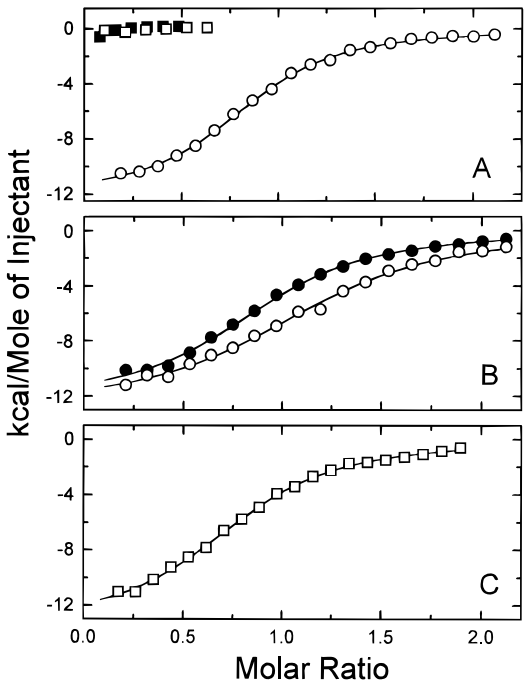

FIGURE 3: Integrated titration heats of the transferase and receptor fragments. Closed and open symbols are the experimental data, and the lines drawn through the points are fits derived from the single-set-of-sites model. A: Histidine-tagged (297 aa) C-fragment titrated with transferase (○, expt 11). Transferase titrated with the Δ36-truncated C-fragment (□, expt 12) and the Δ16-truncated C-fragment (■, expt 13). B: Titration of the P88 (○, expt 15) and P38 (●, expt 17) C-terminal histidine-tagged polypeptides with transferase. C: Titration of NWETF (□, expt 18) with transferase.

To identify the minimum segment of the receptor needed for transferase binding, a pentapeptide (NWETF) corresponding to the last five amino acids of *E. coli* and *S. typhimurium* Tar (Krikos et al., 1983; Russo & Koshland, 1983), and *E. coli* Tsr (Boyd et al., 1983; Rice & Dahlquist, 1991) was synthesized and titrated with the transferase. As the integrated, normalized enthalpy data and fit in Figure 3C demonstrated, the pentapeptide was fully competent to bind to the transferase.

Pentapeptide Blocks Transferase Binding to the C-Fragment. Evidence that the pentapeptide bound to the same site on the transferase as the receptor was obtained in competitive titration experiments in which the transferase was first saturated with an excess of the pentapeptide (Figure 4A). When the pentapeptide−transferase complex was then titrated with the 31 kDa C-fragment, no detectable interaction was observed, i.e., these injections produced no significant heat change. This result was expected for competitive binding even though some displacement of pentapeptide by C-fragment may have occurred, since $\Delta\Delta H$ of the displace-

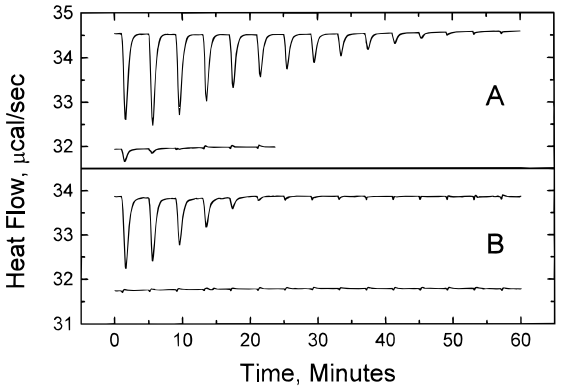

FIGURE 4: Competitive titrations of transferase−ligand complexes with C-fragment or peptide. All titrations depicted were carried out at 28 °C using injection volumes of 5 μL. Panel A, top trace: Fifteen injections of 0.35 mM pentapeptide (NWETF) into 20 μM transferase. Panel A, bottom trace: Six injections of 0.32 mM C-fragment into a reaction cell solution consisting of 11 μM transferase and 39 μM NWETF. Panel B, top trace: Fifteen injections of 0.32 mM C-fragment into 11 μM transferase. Panel B, bottom trace: Fifteen injections of 0.35 mM pentapeptide into a reaction cell solution consisting of 6 μM transferase and 35 μM C-fragment.

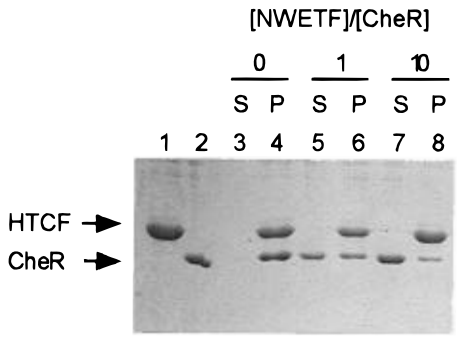

FIGURE 5: Polyacrylamide gel data demonstrating competitive binding between the C-fragment and the NWETF pentapeptide. Lanes 1 and 2, standard samples of HTCF and methyltransferase (CheR). Lanes 3−8 show the effect of NWETF on transferase binding to HTCF. S and P refer to the supernatant fraction, which represents protein not bound to the Ni−NTA agarose, and the pellet fraction, which represents the protein bound to Ni−NTA agarose. The molar ratios of NWETF to transferase were 0 (lanes 3 and 4), 1 (lanes 5 and 6), and 10 (lanes 7 and 8).

ment reaction was predicted, within experimental accuracy, to be zero. Similar results were obtained when the transferase was first saturated with C-fragment and the complex was titrated with pentapeptide (Figure 4B). From these data it was possible to rule out the existence of two independent binding sites on the transferase for the C-fragment and the pentapeptide.

Direct evidence of the competitive nature of NWETF binding was obtained in a Ni−NTA affinity assay. Histidine-tagged CF (HTCF) was captured on Ni−NTA agarose through a specific high-affinity interaction of the N-terminal histidine tag with Ni−NTA. Figure 5 summarizes the results of the assay. Through its specific interaction with HTCF, the transferase was removed from the supernatant fraction and was found exclusively in the Ni−NTA agarose pellet (lanes 3 and 4). When a 1:1 ratio of peptide and transferase was incubated with HTCF bound to Ni−NTA agarose, transferase was found to partition equally between the supernatant (i.e., not bound to HTCF) and the pellet (lanes 5 and 6). A 10:1 ratio of peptide to transferase prevented the majority of the transferase from binding to the HTCF (lanes 7 and 8), and larger ratios of peptide to transferase

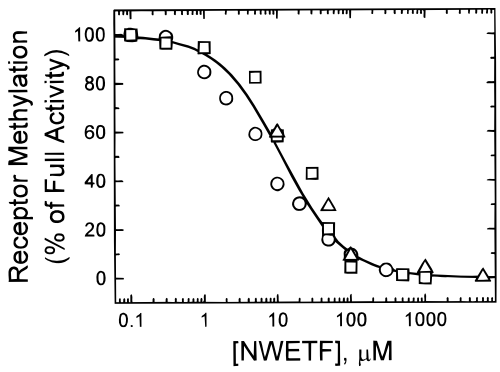

FIGURE 6: Percent methyltransferase activity as a function of pentapeptide concentration. The normalized, background-corrected percent transferase activity was determined in three separate experiments (□, ○, △).

completely blocked binding (not shown). These data, with the results of the competitive titration experiments, provided strong evidence that pentapeptide and C-fragment binding was competitive.

Pentapeptide Blocks Transferase-Mediated Receptor Methylation. Evidence for the physiological significance of the pentapeptide binding interaction was obtained in methylation assays. Figure 6 is a plot of the percent activity of the transferase as a function of the inhibitor concentration, in which the data are shown from three separate experiments using pentapeptide as the inhibitor. Transferase inhibition was essentially complete when the pentapeptide concentration was 100-fold greater than the receptor concentration (10 μM), demonstrating that the pentapeptide functioned as a competitive inhibitor. A quantitative analysis of the data based on a simple model of competitive inhibition (eq 1) fit the data well. In this analysis the receptor concentration and K_M were fixed at 10 and 2.5 μM, respectively, where K_M was approximated by $1/K_a$ of the transferase−Tsr interaction (= 2.6 μM, determined from this work) which was in agreement with the value of K_M (2.1 μM) obtained by Simms and Subbaramaiah (1991). The nonlinear least-squares fit of the data in Figure 6 returned a value of 2.3 μM for K_I, which was in generally good agreement with $1/K_a$ of the pentapeptide−transferase interaction (1.8 μM, expt 18). Using the constraint that K_I must be equal to K_M (so that the Tsr concentration was the only experimentally-determined parameter in the fit) a similar value for K_I (1.8 μM) was obtained. Additional evidence that the peptide exhibited the properties of a competitive inhibitor was obtained in initial rate measurements of transferase activity (data not shown).

DISCUSSION

This study has revealed an unexpected mode of interaction between the receptor and the methyltransferase. The principal determinant of the binding interaction between the receptor and the methyltransferase has been localized in the last five amino acids, away from the sites of methylation in the primary sequence of the receptor. This motif suggests that the principal feature of the binding interaction may be to hold the transferase in proximity to, and in an appropriate orientation for, productive interaction with all of the methylation sites. When this binding motif is considered with the known effect of attractant binding on increasing the rate of receptor methylation (Bogonez & Koshland, 1985), it may be reasonably concluded that the receptor has become a better substrate for the enzyme, which is the result of changes in

either receptor conformation, dynamics, subunit association, or a combination of these factors. This mechanism of regulation can be contrasted to the methylesterase, in which phosphorylation of the regulatory domain on CheB produces a dramatic difference in the activity of the enzyme toward its substrate (Lupas & Stock, 1989). The formal possibility that the transferase is allosterically regulated through its binding interaction with the C-terminal peptide cannot be ruled out, but this possibility appears to be unlikely since the binding interaction was not found to be thermodynamically linked to factors that are expected to influence an allosteric interaction (attractant binding, receptor dimerization, receptor fragmentation).

Commonly-Used Strategy for Multiply-Modified Receptors? Transmembrane receptor proteins are known to be covalently modified at a number of distinct sites on the polypeptide, and thus an enzyme which catalyzes covalent modification of the receptor must be able to recognize several sites on the receptor. For example Rhodopsin is phosphorylated on three residues at the C-terminal tail of the protein after the transmembrane helix VII (Ohguro et al., 1993). In this situation the kinase is also held in close proximity to its substrate by binding to the loop between helices V and VI of Rhodopsin (Palczewski et al., 1991). It thus appears that the close proximity between modifying enzymes and multiply-modified (i.e., phosphorylated or methylated) receptors is frequently achieved by providing a binding site for the enzyme distinct from the sites of modification. As pointed out above, this kind of binding interaction may facilitate effective interaction of the enzyme with all of the covalently-modified sites.

Intersubunit Methylation of the Chemotaxis Receptors. Another unexpected feature of this binding motif was found when all the known sequences of the methylatable chemotaxis receptors in *E. coli* and *S. typhimurium* were compared. The NWETF sequence is found at the C-terminus of the *E. coli* and *S. typhimurium* aspartate receptors and the *E. coli* serine receptor (Krikos et al., 1983; Russo & Koshland, 1983; Boyd et al., 1983; Rice & Dahlquist, 1991). The *S. typhimurium* citrate receptor (Tcp) is terminated in a closely related sequence, NWESF (Yamamoto and Imae, 1993). However, neither the dipeptide (Tap) nor the ribose/galactose (Trg) receptors of *E. coli* end in a sequence resembling the NWETF "consensus" sequence for transferase-binding (Krikos et al., 1983; Bollinger et al., 1984).

These observations led us to hypothesize the existence of an intersubunit methylation process in which a receptor subunit can be methylated by a transferase molecule bound to an adjacent receptor subunit (Figure 7). This unorthodox hypothesis is supported by two sets of observations, a substantial portion of which is in the published literature.

(1) Cells do not adapt to ribose and galactose stimuli when Trg is the sole receptor in the cell, an observation which is consistent with defective methylation of Trg under these conditions (Hazelbauer & Engström, 1980; Yamamoto et al., 1990). These cells are also defective in chemotaxis for ribose in the capillary assay (Springer et al., 1977). Chemotaxis toward dipeptides is similarly defective when Tap is the sole receptor (Manson et al., 1986). The chemotactic defect toward ribose is eliminated when either Tar or Tsr is present in addition to Trg, which is consistent with intersubunit methylation of Trg by methyltransferase bound to either Tar or Tsr. The observation that methylation of Trg is generally more efficient when either Tar or Tsr is present supports this interpretation (Kondoh et al., 1979; Yamamoto et al., 1990).

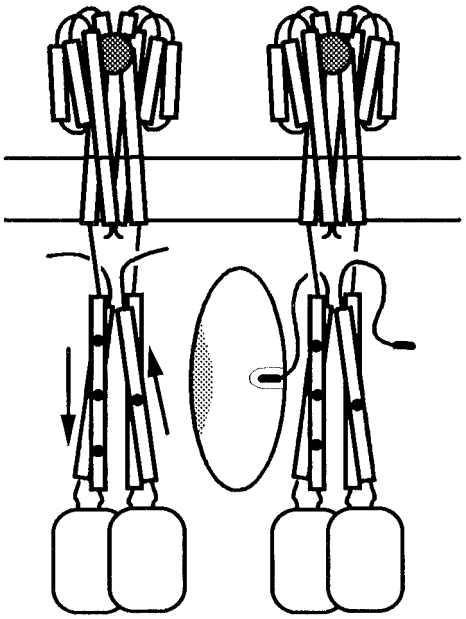

FIGURE 7: Working model for transferase binding to methylatable receptors. The structure of the receptor (Stock et al., 1992) is based on the X-ray structure of the periplasmic binding domain (Milburn et al., 1991) and the prediction of antiparallel coiled-coils in the cytoplasmic region of the receptor (Stock et al., 1992). Arrows indicate the N- to C-terminal direction of the helices. The separation between the receptor-binding and (shaded) active sites on the transferase and the conformation of the receptor polypeptide between the last methylation site and the C-terminus (-NWETF) are speculative and are depicted to allow productive interaction of the transferase active site with all the methylation sites.

(2) When receptors which are expected to bind the transferase are truncated at the C-terminus, the bacteria containing these receptors exhibit anomalous adaptive behavior. A 35 amino acid truncation from *S. typhimurium* Tar resulted in a receptor that could detect aspartate stimuli *in vivo*, but the cell was unable to adapt to the stimuli when it was the sole receptor in the cell (Russo & Koshland, 1983). This receptor was also poorly methylated *in vivo* (Koshland et al., 1983). Furthermore the interaction with the transferase appears to be very specific for the last residues of the receptor under physiological conditions. In a plasmid construct of the *tcp* gene, Yamamoto and Imae (1993) inadvertently replaced the codon for phenylalanine-547 with a codon for leucine-547 and added a codon for alanine-548 which resulted in the production of a Tcp variant terminating in the sequence NEWSLA_{548}-COOH. This mutant form of Tcp was put back into an *E. coli* strain lacking all four methylatable chemotaxis receptors, and although it was able to respond to attractant stimuli (citrate), it was unable to adapt. The wildtype *tcp* gene facilitated both response and adaptation to citrate stimuli when it was reintroduced into the same strain (Yamamoto & Imae, 1993). The simplest interpretation of these results is that the interaction with the transferase was disrupted by replacing Phe with Ala-Leu, so that the receptor could not be methylated, and thus the bacterium was unable to adapt to stimuli transmitted through the Tcp receptor. Finally, the *in vitro* methylation of a Tsr molecule, which was truncated at the C-terminus by 35 amino acids, was facilitated by full-length Tsr in the same membrane (J. Li and R. M. Weis, unpublished observations), providing evidence in direct support of an intersubunit methylation mechanism.

The chemotaxis receptors are all likely to be functionally dimeric given the level of homology in the receptor family and the observation that aspartate binds to a dimer interface in the crystal structure of the *S. typhimurium* Tar ligand-binding domain (Milburn et al., 1991). In view of the observation that receptor heterodimers do not form (Milligan & Koshland, 1988), the preceding discussion implies that the methylation reaction is not just an intersubunit reaction but an interdimer reaction, which may occur between receptors with different ligand specificity (e.g., transferase bound to a subunit in a Tar homodimer may methylate a Trg subunit in a Trg homodimer).

Physiological Significance of Intersubunit Methylation. Both Trg and Tap are found in small proportions relative to Tar and Tsr (ca. 10%; Hazelbauer & Engström, 1981; Slocum & Parkinson, 1983). Provided that receptor dimers assort randomly in the bilayer membrane of the cell, Trg and Tap dimers would more frequently encounter receptors that are able to bind to the transferase. Interdimer methylation may thus be the predominant mechanism for methylation of Tap and Trg under physiological conditions. However, the presence of the binding tail is not an absolute requirement for methylation. *In vivo* methylation of the Trg receptor has been observed in the absence of Tar and Tsr (Park & Hazelbauer, 1986a,b; Yaghmai & Hazelbauer, 1992), although both the level of methylation and the increase in the methylation level in response to an attractant stimulus are reduced (G. L. Hazelbauer, personal communication). We have observed that a Tsr fragment (amino acids 290−470; Ames & Parkinson, 1994), which lacks the transferase binding sequence and does not bind to transferase in ITC experiments, is methylated *in vitro*, albeit with quite low efficiency (D. G. Long and R. M. Weis, unpublished observations). Rabbit tropomyosin is also methylated in a similar, adventitious manner. It contains two potential sites of methylation (based on the identification of the methylation consensus sequence at two locations), yet it does not bind the methyltransferase with high affinity (D. G. Long and R. M. Weis, unpublished observations). Although transferase binding to the C-terminal segment does not appear to be necessary for receptor methylation either *in vivo* or *in vitro*, the binding segment nevertheless has genuine physiological significance, as bacteria do require the presence of receptors with the transferase binding site in order to adapt on a physiologically-relevant time scale (Hazelbauer & Engström, 1980; Russo & Koshland, 1983; Yamamoto & Imae, 1993).

Working Model for Receptor Methylation. These concepts have been incorporated into a working model for transferase binding and receptor methylation. Since detailed structural information of the receptor cytoplasmic region is presently lacking, the conformation for this region is a speculative model based on predictions of coiled-coils (Lupas and Stock, 1991; Stock et al., 1992). As the model in Figure 7 depicts, the methylation sites are found in both helices of an antiparallel coiled-coil. It seems plausible that the transferase active site will dock onto one of the two helices during the process of methylation. A significant amount of flexibility is thus required to accommodate the docking of the transferase onto all the methylation sites. Deuterium-exchange NMR experiments have indicated that the C-fragment exhibits a large degree of conformational flexibility (Seeley et al., 1996). In view of this observation and the position of the transferase binding site with respect to the sites of methylation, it may be possible for the transferase to make contact with both methylation segments while the transferase is bound to the receptor.

Although the arrangement between the transferase binding site and the methylation sites permits productive interaction of the enzyme active site with all of the sites of methylation, the extent to which receptor methylation is a process that occurs exclusively between receptor dimers is not known. Inefficient methylation of receptor C-fragments which are fully competent to bind the transferase would seem to indicate that the reaction is not an intrasubunit event. Further experimentation is required to clarify this issue.

Summary. This study has provided novel information about the interaction between the methyltransferase and its substrates, the methylatable receptors. The major portion of the binding interaction was found to reside at the C-terminus, composed of no more than the last five residues. Since only the aspartate and serine receptors in *E. coli* possess the transferase "binding tail", interdimer methylation was postulated in which the ribose/galactose and dipeptide receptors are methylated by transferase molecules bound to either the aspartate or serine receptor. The extent to which receptor methylation is solely an interdimer process remains to be determined.

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