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In Vitro Methylation of Bacterial Chemotaxis Proteins: Characterization of Protein Methyltransferase Activity in Crude Extracts of Salmonella typhimurium

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A specific in vitro assay was developed for the protein carboxyl methyltransferase that is involved in the chemotactic behavior of Salmonella typhimurium. This cytosolic enzyme catalyzes an S-adenosyl-L-methionine-dependent methyl esterification of glutamyl residues on a class of 60,000-dalton inner-membrane proteins. The activity was found to display a pH optimum of 6.5 and be sensitive to the concentration of salts in the assay medium. No detectable activity was found towards a variety of other proteins which serve as substrates for mammalian and other bacterial carboxyl methyltransferases. This assay was used to quantitate the methylation of the 60,000-dalton methyl-accepting proteins in response to chemoeffectors. Small but reproducible concentration-dependent changes in the initial rates of in vitro methylation were observed with chemotactic attractants and repellents. The specific methyltransferase activity was found to be absent in several mutants in flagellar synthesis (*fla*$^-$), suggesting that the synthesis of this enzyme is coordinately regulated with that of flagellin and basal bodies. The hydrodynamic properties of the enzyme in crude extracts were determined by gel filtration and sucrose velocity gradient centrifugation, and a native molecular weight of 41,000 was calculated from these data.

Key words: Salmonella typhimurium, methylation, chemotaxis, flagellar synthesis

Reversible protein methylation reactions appear to play a central role in bacterial chemotaxis (for reviews see [1, 2]). The sensory response of S typhimurium and Escherichia coli to gradients of attractants and repellents causes the formation and hydrolysis of methyl esters on glutamyl groups of a class of 60,000-dalton membrane proteins [3, 4]. These membrane proteins function in the relay of sensory information to the flagella

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[5, 6] and probably include the receptors for the amino acid attractants aspartate and serine [7]. The level of methylation of these proteins rises in proportion to the chemoattractant concentration [8] and inhibition of methylation alters the adaptive response [9]. The level of methylation is regulated to the activities of at least two enzymes. Springer and Koshland [10] have identified a methyltransferase activity in Salmonella with the *cheR* gene product. This enzyme catalyzes the formation of a γ-glutamyl methyl ester from S-adenosyl-L-methionine on the 60,000-dalton membrane proteins. A methylesterase activity has been identified with the Salmonella *cheX* gene product and the Escherichia *cheB* gene product [11]; this enzyme catalyzes the hydrolysis of the protein-bound methyl ester to methanol.

The purpose of the work described here is to extend the previous in vitro studies of the methyltransferase [10, 12] by developing more quantitative procedures, characterizing the enzyme, and examining the effect of attractants and repellents.

MATERIALS AND METHODS

Materials

S-Adenosyl-L-[methyl-^{3}H]-methionine (6–12 Ci/mmole) and L-[methyl-^{3}H]-methionine were obtained from Amersham or New England Nuclear. [^{14}C]methanol (3.4 mCi/mmole) was a New England Nuclear product. Adrenocorticotropic hormone (Grade I, porcine) and S-adenosyl-L-methionine iodide (88%) were purchased from Sigma. 3-Methyl-1-butanol (98%) was obtained from Aldrich; toluene was from Eastman.

Bacterial strains: S typhimurium wild-type strain ST1 (ATCC 29595) and *cheR*$^-$ strain ST1038 (ATCC 29596) have been described previously [9, 10].

Methods

Preparation of a membrane fraction as a substrate for the *cheR* methyltransferase. A crude membrane fraction containing 60,000-dalton proteins with a full complement of methyl acceptor sites was prepared from cells lacking *cheR* methyltransferase activity. These cells (strain ST1038) were grown with aeration at 37°C in nutrient broth (Difco, 8 gm/liter), NaCl (5 gm/liter), and thymine (30 μg/liter). In late log phase (OD_{650nm} = 0.8 to 1.0) cells were harvested by Sharples centrifugation and stored at −20°C. Typically, 100 gm of frozen cell paste from 100 liters of culture was resuspended in 300 ml of standard buffer (0.1 M $NaPO_4$, 1 mM EDTA, pH 7.0) and sonicated for 6 min at full power with the standard 1/2 in probe of a Heat Systems Model W-220F cell disrupter. The sample was kept below 10°C in a rosette cooling cell immersed in a stirred ice bath during this procedure. Whole cells were removed by centrifugation at 7,500g for 15 min at 4°C in a GSA rotor of a Sorvall RC5 centrifuge. This whole-cell fraction was resuspended in 100 ml buffer and sonicated as before; the supernatant (containing membranes) after low-speed centrifugation was combined with the supernatant from the initial sonication. Membranes were pelleted from this mixture by centrifugation at 236,000g for 30 min at 4°C in a Ti45 rotor of a Beckman L5-75 ultracentrifuge. The membrane fraction was resuspended in 160 ml of buffer by passage through a 4-in, 16-gauge needle. Whole cells were spun out as before and membranes collected by ultracentrifugation. Membranes were washed twice in this fashion.

The final membrane pellet, consisting of cytoplasmic membrane, outer membrane, and cell wall material, was resuspended in 24 ml of buffer and centrifuged once more at low speed to remove any remaining whole cells or large debris. The protein concentration of these membranes was determined by a modified Lowry assay [13] using a standard of

bovine serum albumin whose concentration was determined by ultraviolet spectroscopy [A (10 mg/ml) at 279 nm = 6.7 [14]]. All samples for protein determination were first precipitated with 10% trichloroacetic acid. Membranes were stored at −20°C in small aliquots at protein concentrations of 25 to 35 mg/ml.

Preparation of soluble extracts of Salmonella containing *cheR* methyltransferase activity. Enzyme was prepared from wild-type ST1 cells, grown as described above for ST1038 cells but without supplemental thymine. Cells were sonicated as above in the standard buffer of 0.1 M $NaPO_4$, 1 mM EDTA, pH 7.0, and whole-cell debris, and membrane fractions were removed by centrifugation at 236,000g for 30 min at 4°C in a Ti45 rotor of a Beckman L5-75 ultracentrifuge. The soluble extract, at a protein concentration of about 15 to 20 mg/ml, was stored in small aliquots at −20°C. Under these conditions, methyltransferase activity was stable for months.

For experiments in which the level of methyltransferase was determined in various *che* and *fla* mutant strains of Salmonella, the protocol described above was followed but was scaled down so that only 1 liter of culture was necessary to obtain about 5 ml of soluble fraction. Sonication was performed for 2 min in 13-ml stainless steel cooling cells (Heat Systems), and membranes and cell debris were removed by centrifugation in a type 65 rotor at 264,000g for 30 minutes.

Quantitative assay of *cheR* methyltransferase by formation of alkali labile methanol. A modification of the assay protocol of Diliberto and Axelrod was employed [15]. Methylation was carried out at 30°C in incubation mixtures containing, in a total volume of 0.2 ml, 55 μM S-adenosyl-L-[^{3}H-methyl]methionine (80 cpm/pmole), ST1038 membranes containing unmethylated 60,000-dalton methyl acceptor proteins (1.4 mg protein), and various amounts of soluble fractions containing methyltransferase activity in a buffer of 0.1 M $NaPO_4$, 1 mM EDTA, pH 7.0. The reaction was terminated (generally after 30 min) by the addition of 2 ml 10% (wt/vol) trichloroacetic acid. Proteins were pelleted by centrifugation for 10 min at 4,000g at 4°C. The supernatant was removed by aspiration, and the pellet was resuspended once more in 2 ml 10% trichloroacetic acid and centrifuged as before. Control experiments demonstrated that no detectable loss of methyl groups in 60,000-dalton proteins occurred at this acid preparation step. For example, incubation of reaction mixtures at 30°C for 60 min in trichloroacetic acid before centrifugation did not affect the amount of methylation.

The trichloroacetic acid pellet containing [^{3}H-methylated]proteins was redissolved in 0.5 ml of 0.2 N NaOH, 0.4% sodium dodecyl sulfate, and 1% methanol. Samples were hydrolyzed 30 min at 30°C to remove methyl groups in base-sensitive ester linkages (for example, as in γ-glutamyl methyl esters in the 60,000-dalton membrane proteins [3, 4]. [^{3}H]methanol formed in this alkaline hydrolysis was extracted with 6.0 ml of a 3:1 v/v mixture of toluene/3-methyl-1-butanol [15]. Samples were vortexed in 15-ml Corex centrifuge tubes for 15 sec and centrifuged 5 min at 3,000g. Control experiments performed with [^{14}C]methanol indicated that 65.3% of the total methanol is partitioned into the organic phase at equilibrium under these conditions. This value is not affected by the amount of soluble or membrane protein in the trichloroacetic acid pellet and all results have been corrected for this factor. A portion of the organic phase (2.0 ml) was counted directly in 1.0 ml of Handifluor (Mallinckrodt).

Another 2.0-ml aliquot was evaporated to dryness at 50°C under an air stream. The difference in radioactivity of these two samples, corrected for the volumes counted, the efficiency of extraction, and a small amount (4%) of quenching by the toluene/3-methyl-1-butanol, was taken as the amount of methyl groups present in alkali-labile groups.

TABLE I. Methanol Release Assay for Alkali-Labile Methyl Groups: Specificity for Reaction Catalyzed by *cheR* Methyltransferase

Source of methyltransferase (soluble fraction)	Source of methyl acceptor (membrane fraction)	Methanol-formed (pmoles)[a]	Radioactivity incorporated into 60,000-dalton membranes components[b]
ST1 (wild-type)	ST1038 (*cheR*$^-$)	115.3	++++[d]
ST1038 (*cheR*$^-$)	ST1038	22.6	–
None	ST1038	11.7	–
ST1	None	3.6	–
ST1038	None	4.1	–
ST1	SL1507 (Δ*flaAmotC*)	24.0	–[c]

[a]Standard assay (90-min incubation) performed as described in Methods with 0.24 mg of soluble protein.
[b]Analyzed by dodecylsulfate polyacrylamide gel electrophoresis as described in Methods.
[c]Membranes from this strain, supplied by Dr. B. A. D. Stocker, Stanford University, lack the 60,000-dalton methylated membrane protein [S. Clarke, K. T. Sparrow, and D. E. Koshland, unpublished experiments].
[d]Nomenclature: ++++, 100–75%, +++, 75–50%; ++, 50–10%; +, 10–1%; –, less than 1% (not detectable).

Specific incorporation into the 60,000-dalton membrane proteins associated with chemotaxis was obtained by subtracting control values obtained when membranes or soluble fractions were incubated alone with S-adenosyl-L-[^{3}H-methyl] methionine, or when *cheR*$^-$ and *fla*$^-$ soluble fractions were incubated with *fla*$^-$ membranes (see Results below).

Qualitative assay of *cheR*-methyltransferase-dependent methylation of 60,000-dalton membrane proteins by sodium dodecylsulfate polyacrylamide gel electrophoresis. ST1038 membranes (1.2 mg), S-adenosyl-L-[^{3}H]methionine (20 μM, 2,000 cpm/pmole), and a soluble extract of cells (0.4 mg protein) were mixed in a total volume of 120 μl in a buffer of 0.1 M $NaPO_4$, 1 mM EDTA, pH 7.0 and incubated 90 min at 30°C. Membranes were collected by centrifugation at 8,000g for 60 min in an SE12 rotor of a Sorvall RC-5-centrifuge, resuspended in 100 μl H_2O, and solubilized in 100 μl dodecyl sulfate buffer. Dodecyl sulfate polyacrylamide gel electrophoresis (12.5% acrylamide in separating gel) was performed as described by Ames [16]. Gels were prepared for fluorography as described by Bonner and Laskey [17]. The relative amount of radioactivity in the 60,000-dalton region of radioautographs was determined by densitometry.

RESULTS

Specificity of the Quantitative Methanol Release Assay for the *cheR* Methyltransferase

The assay described here takes advantage of the base-lability of groups such as the γ-glutamyl methyl ester found in the 60,000-dalton membrane chemotactic proteins [3, 4]. Methyl groups incorporated into trichloroacetic acid-precipitable linkages from S-adenosyl-L-[methyl-^{3}H]methionine are hydrolyzed in NaOH and the methanol produced is extracted into solvent and counted (see Methods). The data in Table I show the specificity of this assay, the reaction catalyzed by the *cheR* methyltransferase in crude soluble extracts of Salmonella. When wild-type extract was incubated with a *cheR*$^-$ membrane fraction (methyl group acceptor), 115.3 pmoles of methanol were formed. However, only 22.6 pmoles of

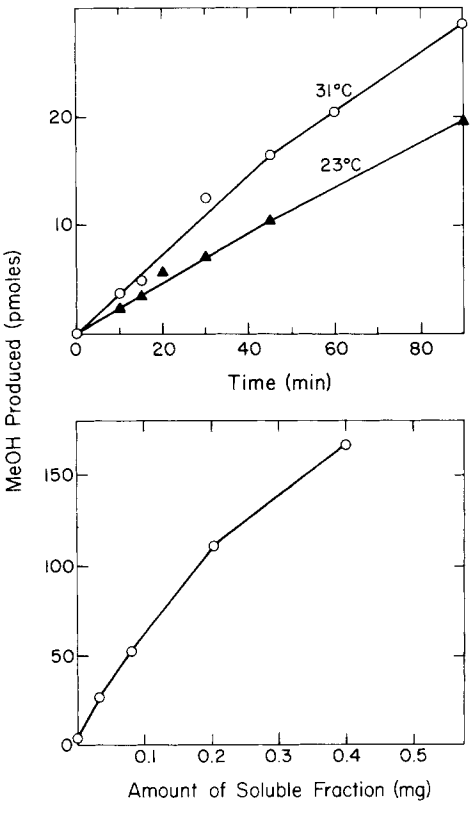

Fig. 1. Methanol-forming assay for *cheR* methyltransferase. Top, linearity of amount of methanol produced as a function of incubation time of crude soluble extract of Salmonella (0.1 mg protein) with S-adenosyl[methyl-^{3}H]-L-methionine and a membrane fraction as described in Methods. Bottom, dependence of methanol formation on the amount of soluble extract. Incubation time was 60 minutes.

methanol were formed when a *cheR*$^-$-soluble extract was used in place of wild-type extract. Furthermore, only 24 pmoles of methanol were formed when a wild-type soluble extract was incubated with membranes derived from an *fla*$^-$ strain, which lacks the 60,000-dalton acceptor proteins. Thus, of the total 115.3 pmoles of methanol formed, approximately 90 pmoles are derived from 60,000-dalton membrane proteins methylated by the *cheR* methyltransferase. The methanol produced in the absence of membrane acceptor proteins (no membranes, *fla*$^-$ membranes) or with *cheR*$^-$ methyltransferase appears to be from reactions not related to chemotaxis; these methyl groups are not incorporated into 60,000-dalton proteins as evidenced from dodecyl sulfate gel electrophoretic analysis of the reaction mixture (see Methods). In the experiments presented here, incorporation of methyl groups when the membrane fraction was incubated alone with S-adenosyl-L-[^{3}H-methyl] methionine has been subtracted out as a blank value less than 5% of catalyzed amount.

Figure 1 shows that the incorporation of methyl groups into 60,000-dalton proteins is linear with the time of incubation of membranes and soluble extract at both 23°C and 31°C, and that the incorporation is dependent upon the amount of soluble extract used as

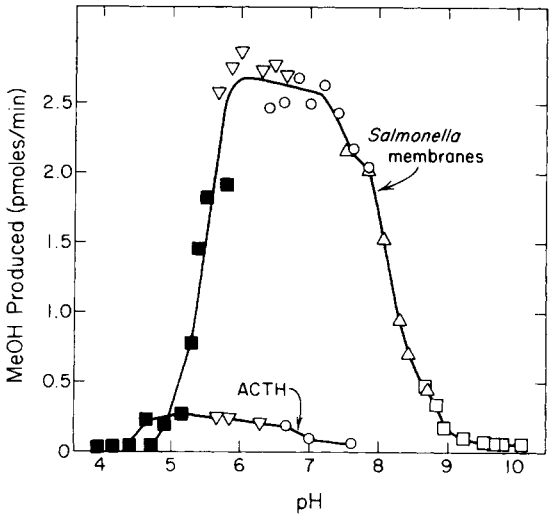

Fig. 2. pH dependence of carboxyl methyltransferase activity in crude extracts of S thyphimurium. Assays were performed as described in Methods by diluting equal parts of a mixture of an ST1038 membrane fraction and an ST1 soluble fraction in 10 mM $NaPO_4$, 0.2 M NaCl, pH 7.0 with S-adenosyl-L-[methyl-^{3}H]methionine in 0.1 M of buffer at the indicated pH. The final concentration of soluble protein was 2.2 mg/ml, and reactions were performed for 30 minutes. The activity shown with ACTH as a substrate is not due to the *cheR* methyltransferase because extracts of *cheR*$^-$ strains catalyzed this methylation reaction at the same rate (Table II). Buffers used included the sodium salt of citric acid (■–■–■), maleic acid (▽–▽–▽), phosphoric acid (○–○–○), glycylglycine (△–△–△), and glycine (□–□–□).

a source of methyltransferase activity. Michaelis-Menten kinetics were obtained for the activity as a function of S-adenosyl-L-methionine and membrane concentration. The K_m for S-adenosyl-L-methionine in this reaction was determined to be 12 μM; the effective K_m for the *cheR*$^-$-derived membrane substrate was found to be approximately 1.6 mg membrane protein/ml.

Establishment of Optimal Conditions for In Vitro Methylation: Characteristics of the *cheR* Methyltransferase

Methyltransferase activity from crude extracts using a membrane fraction as the acceptor species was found to be maximal between pH 5.7 and 7.4, with sharply declining activities at higher or lower values (Fig. 2). Figure 3 shows the effect of adding excess salt to the standard assay mixture. In all cases there was decreased activity. The ionic strength of 0.1 M $NaPO_4$ appears to be optimal for this methylation system. Both the substrate and the enzyme appear to lose activity in solutions of low ionic strength. For example, if membranes are washed with 0.01 M $NaPO_4$, 1 mM EDTA, pH 7.0, the efficiency of methylation is 20–30% of that of membranes washed in 0.1 M $NaPO_4$, 1 mM EDTA, pH 7.0 buffer. This inhibition is not reversed with added salt. Additionally, when crude soluble extracts are dialyzed in 0.01 M $NaPO_4$, 1 mM EDTA, a similar loss of activity occurs. In this case, gel filtration experiments with Sephadex G-100 similar to those reported in Figure 4 have shown that the residual activity elutes in the void volume of the column. These results suggest that the methyltransferase aggregates to a less active or stable form

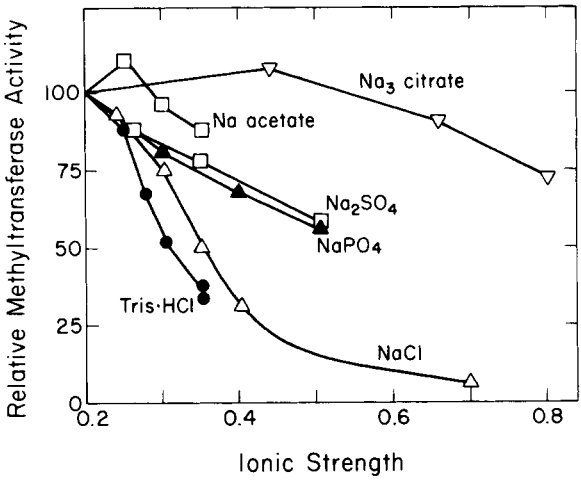

Fig. 3. Dependence of methyltransferase activity upon ionic conditions in the assay medium. Crude soluble extracts of Salmonella were assayed as described in Methods in the presence of additional salts at pH 7.0. Methyltransferase activity (pmoles MeOH produced/min/mg soluble protein) relative to that demonstrated under standard assay conditions (ionic strength, 210 mM) is shown as a function of the total ionic strength.

in low salt. Finally, the amount of activity that can be extracted from cells by sonication is also dependent upon the ionic strength of the buffer. Maximal soluble activity is obtained at concentrations of $NaPO_4$ above 0.1 M. For these reasons, all of the experiments reported here were performed at pH 7.0 in a buffer of 0.1 M $NaPO_4$, 1 mM EDTA.

The hydrodynamic properties of this enzyme in crude extracts have been investigated by gel filtration chromatography and sucrose gradient sedimentation. Using reference marker proteins with known coefficients, a diffusion coefficient of 7.6×10^{-7} cm^2/sec and a sedimentation coefficient of 3.5 S were determined for the *cheR* methyltransferase (Fig. 4). Assuming a partial specific volume of 0.73 cm^3/gm, the molecular weight calculated from the Svedberg equation is 41,000 and the frictional ratio (f/f_0) is 1.23. Assuming a hydration of 0.3 gm H_2O/gm protein, the axial ratio of an equivalent prolate ellipsoid is 2.5:1 [18].

The *cheR* Methyltransferase is Specific for 60,000-Dalton Substrate Proteins in the Bacterial Membrane

To test the specificity of the methyltransferase it was tested on a variety of membrane- and non-membrane-bound proteins. The experiments reported in Table II show that crude extracts of wild-type Salmonella do catalyze the transfer of methyl groups to exogenous substrates. Significantly, however, Salmonella extracts prepared from *cheR*$^-$ cells also catalyzed these carboxyl methyl group transfers. Thus, the *cheR* carboxyl methyltransferase appears to have a narrow specificity for methylation of proteins in the bacterial membrane, and the methylation observed of ACTH and other proteins (Table II) must be catalyzed by other enzyme(s) of unknown function in the bacterial cell.

Evidence obtained so far strongly suggests that the only proteins in the bacterial membrane or soluble fraction methylated by the *cheR* methyltransferase is the class of 60,000-dalton membrane proteins. The data in Table I showed that similar background

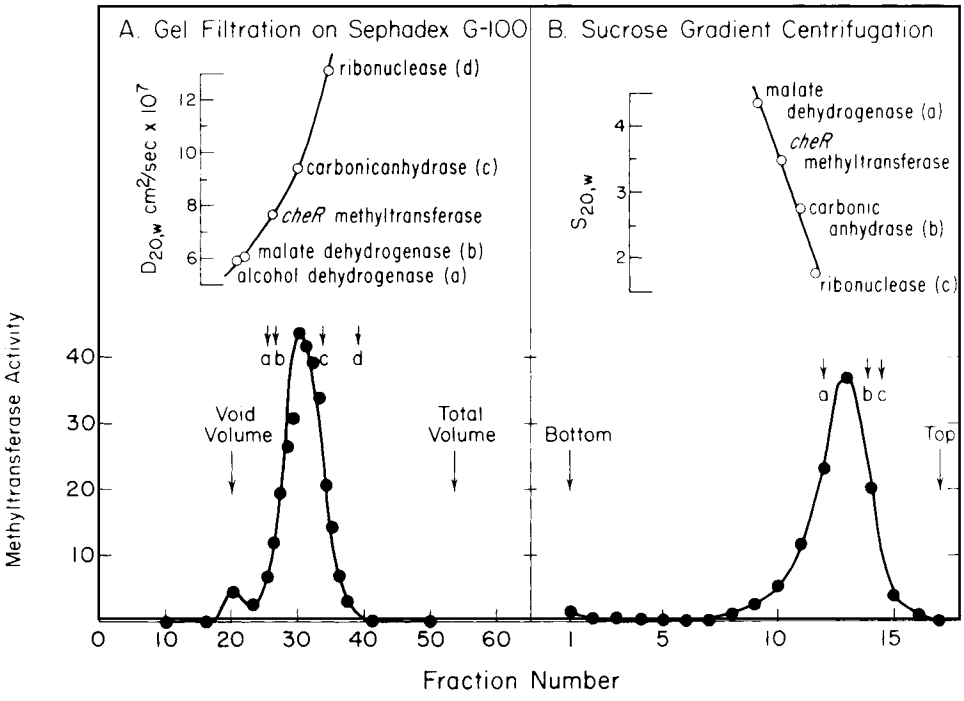

Fig. 4. Determination of hydrodynamic properties of the *cheR* methyltransferase for native molecular weight estimation. Left: Determination of $D_{20,w}$ by gel filtration chromatography. A mixture of crude extract (10 mg protein) and marker proteins were applied to a 1.0 cm × 40-cm column of Sephadex G100 in 0.1 M $NaPO_4$, 1 mM EDTA, pH 7.0. Fractions were collected and assayed for enzymatic activities. The apparent $D_{20,w}$ of the methyltransferase (7.6×10^{-7} cm²/sec) was interpolated from a plot of the peak positions of the marker enzymes (arrows). Right: Determination of $s_{20,w}$ by sucrose gradient centrifugation. Crude extract (5 mg protein) was applied to the top of a 5-ml 5–20% sucrose gradient in 0.1 M $NaPO_4$, 1 mM EDTA, pH 7.0 with marker enzymes. The gradient was centrifuged in an SW50.1 rotor at 4°C at 50,000 rpm for 14 hours. Fractions were collected from the bottom of the tube and assayed for the enzyme activities shown. The sedimentation coefficient of the methyltransferase (3.5 S) was interpolated from a plot of the peak positions of the marker enzymes. Values of $s_{20,w}$ and $D_{20,w}$ ($\times 10^7$ cm²/sec) used, respectively, for these calculations include liver alcohol dehydrogenase, 5.96 S; malate dehydrogenase, 4.32 S, 6.1; carbonic anhydrase 2.75 S, 9.4; and ribonuclease 1.9 S, 13.1.

TABLE II. Methyl-Acceptor Specificity of the *cheR* Methyltransferase

Methyl-accepting[a] substrate	Methanol formed after[b] alkaline hydrolysis (pmoles) with soluble fraction	
	from wild-type cells	from *cheR*⁻ cells
None	5.7	6.3
Salmonella *cheR*⁻ membrane fraction	208.5	13.7
Lysozyme	7.6	7.1
Ribonuclease	9.9	9.0
Ovalbumin	10.2	8.4
ACTH	54.6	56.9

[a]Amount of protein is 2.2 mg.
[b]Enzyme fraction (0.5 mg protein) from wild-type cells (ST1) or a mutant strain (ST1038) lacking *cheR* methyltransferase activity were incubated 30 min with the acceptor species shown as described in Methods but in a buffer of 0.1 M Na citrate, pH 5.6.

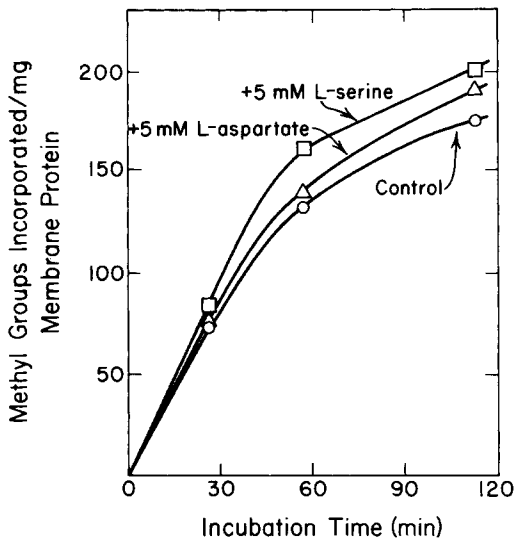

Fig. 5. Effect of amino acid chemotaxis attractants on the time course of methylation of ST1038 membranes. Membranes (1.38 mg protein) were mixed in a standard assay with a wild-type soluble fraction (0.55 mg protein) for the times indicated. Alkali-labile methyl groups were determined against a blank of membranes incubated without the soluble fraction.

levels of carboxyl methylation were obtained in methanol-forming assays where *fla*$^-$ membranes (which lack the 60,000-dalton membrane proteins) were incubated with wild-type soluble extracts. Further in vitro experiments where [^{3}H]methylated polypeptides of the membrane and soluble fraction were examined by dodecyl sulfate gel electrophoresis (see Methods) indicated that *cheR*$^-$ and wild-type soluble extracts only differed in the labeling of 60,000-dalton membrane proteins. Furthermore, there was no difference in the labeling of *fla*$^-$ membranes when incubated with *cheR*$^-$ or wild-type soluble fractions. Finally, the only difference in the in vivo labeling pattern of wild-type and *cheR*$^-$ cells is in the 60,000-dalton region [10].

Regulation of the *cheR* Methyltransferase Reaction by Chemoeffectors

The primary chemoreceptors appear to be closely linked to the membrane-bound methyl-accepting class of 60,000-dalton proteins. In vitro studies of L-aspartate and L-serine binding to membrane fractions have suggested that the chemoreceptors for these substances are identical to the two major methylated proteins [7]. In any event, changes in the concentration of a variety of chemoeffectors in vivo are paralleled by changes in the methylation level of specific membrane proteins [8, 19–21]. Because the regulation of the methylation level of these proteins is an essential feature of sensory adaptation, and because it is presently not known at what level this regulation is achieved, we investigated the effect of chemoeffectors on the in vitro methylation assay developed here. Figure 5 shows the time course of methylation of membranes derived from *cheR*$^-$ cells catalyzed by wild-type soluble extract in the presence and absence of L-serine and L-aspartate, two of the best chemotactic attractants of S typhimurium. Small but reproducible increases in the rate of methylation occur for both of these compounds. Figure 6 shows data for various concentrations of these attractants. Maximal stimulation of about 1.3-fold has been observed. Data is also presented in Figure 6 for 4 repellents. At appropriate concentrations these substances inhibit the methyltransferase reaction by as much as 30%.

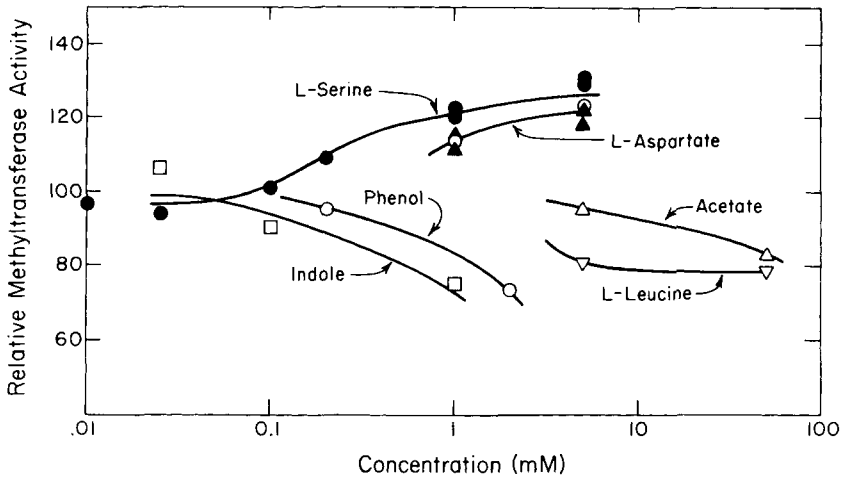

Fig. 6. Effect of attractants (L-serine, L-aspartate) and repellents (phenol, indole, acetate, L-leucine) on the initial rate of in vitro methylation of ST1038 membranes. Standard assays were performed in the presence of the indicated concentration of chemoeffector. A crude extract of wild-type cells (0.46 mg) was used as a source of methyltransferase; the incubations were carried out for 30 minutes.

To determine whether attractants increase the rate of the methyltransferase reaction by exposing new methyl group acceptor sites, the experiment in Figure 7 was performed. If the presence of serine only increased the rate of reaction, one would expect that the maximal level of methylation would be the same at long incubation times. This was not the result obtained here. A possible explanation of these results is that serine changes the conformation of the 60,000-dalton polypeptides to change the relative rates of methylation and demethylation.

Presence of Methyltransferase in the *che* and *fla* Mutant Strains

Table III shows the result of experiments where the level of *cheR* methyltransferase in various strains was determined with two different assays. A semiquantitative gel electrophoresis assay for radioactivity in 60,000-dalton membrane proteins was used to determine whether the absolute levels of activity in the quantitative methanol formation assay were due to *cheR* methyltransferase activity or were due to other methylation reactions.

All of the classes of generally non-chemotactic mutants that contained a functional *cheR* gene showed specific methyltransferase activity. This result is in agreement with that of Springer and Koshland [10], who employed a different type of in vitro assay for methyltransferase activity. Mutants in the structural gene for flagellin (Hl^-) also demonstrated *cheR* methyltransferase activity. These cells have normal basal body structures [22].

On the other hand, generally no *cheR* methyltransferase activity could be demonstrated in flagellar mutants, which either do not produce basal body structures (*flaC*$^-$, *flaK*$^-$) or produce altered basal body structures (*flaFV*$^-$, *flaFVIII*$^-$, *flaL*$^-$) [23]. These results show that the synthesis of the *cheR* methyltransferase is co-regulated with flagellin synthesis.

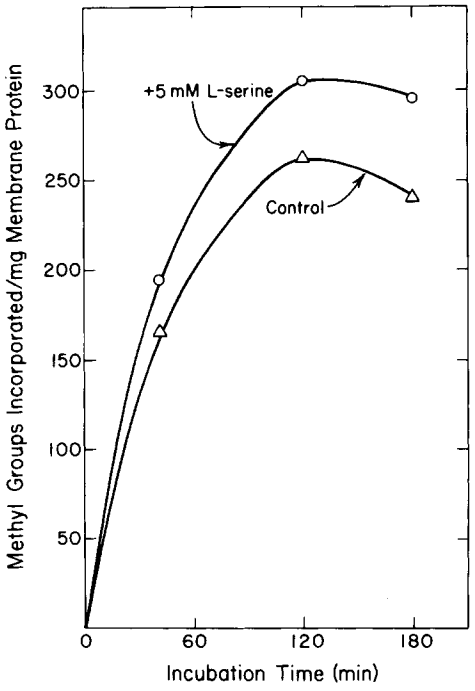

Fig. 7. Determination of the total number of sites available for methylation in ST1038 membranes in the presence and absence of 5 mM L-serine. Membranes (1.38 mg protein) were incubated with a soluble fraction (1.66 mg protein) as usual. The values for the pmoles of methyl groups incorporated have been corrected for controls where the soluble fraction alone or membranes alone were incubated for the indicated times.

DISCUSSION

A New Class of Protein Carboxyl Methyltransferases

The enzyme described here has properties which distinguish it from eukaryotic enzymes [24–27] which catalyze the transfer of methyl groups from S-adenosyl-L-methionine to alkali-sensitive linkages on proteins. In contrast to the purified carboxyl methyltransferase from animal cells, the *cheR* methyltransferase has a very narrow substrate specificity. Methyl transfer has only been detected to the 60,000-dalton bacterial membrane proteins: ACTH, ovalbumin, and ribonuclease, which are excellent substrates for the enzyme from bovine pituitary [24], calf spleen [25], red blood cells [26], and calf thymus [27] are not methylated by bacterial *cheR* methyltransferase (although they are methylated by one or more other Salmonella enzymes (see below). The native molecular weight of the *cheR* methyltransferase, 41,000, is slightly larger than that of the calf thymus [27] or the ox brain enzyme (35,000 daltons) [28] and markedly larger than that of the erythrocyte enzyme (25,000 daltons) [26]. The K_m for S-adenosyl methionine for the *cheR* methyltransferase is also roughly an order of magnitude higher than measured for the mammalian carboxyl methyltransferases.

TABLE III. ***cheR*** **Methyltransferase Activities in Non-Chemotactic (*che*$^-$) and Flagellar (*fla*) Strains of Salmonella typhimurium**

Strain[a]	Genotype	Methanol formation assay pmoles/min/mg protein	In vitro gel electrophoresis assay for methylation of 60,000-dalton membrane proteins[e]
ST1	wild-type	7.5	++++
ST23[b]	wild-type his$^-$ thy$^-$	7.2	++++
SJW797[c]	wild-type	8.6	++++
LT2[d]	wild-type	5.0	+++
ST1002	*cheP*$^-$	12.5	++++
ST1001	*cheQ*$^-$	13.1	++++
ST1038	*cheR*$^-$	0.8	–
ST108	*cheS*$^-$	6.5	++++
ST171	*cheT*$^-$	7.4	++++
ST155	*cheU*$^-$	7.9	++++
SL2516	*cheV*$^-$	2.4	+++
ST1024	*cheW*$^-$	12.8	++++
SL4041	*cheX*$^-$	3.8	+++
π434	*cheX*$^-$*cheQ*$^-$*cheT*$^-$	7.2	++++
π282	*cheQ*$^-$*cheT*$^-$	9.8	++++
SL4045	HI$^-$	3.7	+++
SL1507	Δ*fla-motC*	0.7	–
SL1509	*flaC*$^-$	0.9	–
SL4044	*flaK*$^-$	0.8	–
SL4052	*flaFV*$^-$	0.6	–
SL4053	*flaFVIII*$^-$	0.6	–
SJW801	*flaL*$^-$	0.6	+

[a]Strains SJW797, SJW801, SL4050, and SL4053 were obtained from Dr. T. Iino, University of Tokyo. Strains SL2516, SL4041, SL4045, SL1507, SL1509, and SL4044 were obtained from Dr. B. A. D. Stocker, Stanford University. All other strains are from this laboratory.
[b]Parent of ST strains.
[c]Parent of SJW801.
[d]Parent of SL strains.
[e]For meanings of ++++, etc, see footnote (d), Table I.

We have also demonstrated here the presence in Salmonella cytosolic extracts of protein methyltransferase activity with substrate specificity similar to that found in eukaryotic enzymes (Table II). A similar activity has been demonstrated in E coli [29].

Thus, at the present time, there appear to be two classes of protein carboxyl methyltransferases. The bacterial enzyme involved in the chemotactic response is highly specific for substrates in the bacterial membrane, while the mammalian enzyme and other bacterial enzymes appear to be relatively nonspecific in their methyl group acceptor specificity. Possibly these enzymes have specific substrates in vivo. If there are enzymes in mammalian tissues with specific substrate requirements, assays presently performed might not detect these activities. Thus, an entire class of substrate-specific protein carboxyl methyltransferases in mammalian cells may not have been identified for this reason.

Structure of the *cheR* Methyltransferase

The native molecular weight of the Salmonella enzyme has been determined to be 41,000 from its hydrodynamic properties. Since molecular cloning techniques have estab-

lished that the polypeptide molecular weight of the *cheR* gene product is 31,000 daltons [A. L. DeFranco and D. E. Koshland, Jr., unpublished], it is possible that another gene specifies a second subunit of approximately 10,000 daltons in the native enzyme.

Effects of Attractants and Repellents on In Vitro Methylation: Role of Methylation in Chemotaxis

Chemoeffectors have been shown to change the in vivo level of methylation of the 60,000-dalton membrane proteins in E coli [8] and in S typhimurium [19]. The in vitro data presented here indicate that at least part of this control is exercised at the methylation reaction. The changes in methylation detected here in the presence of chemoeffectors are in general qualitatively similar to but quantitatively less than those obtained in vivo or in the Escherichia in vitro system [12].

Regulation of the Synthesis of the *cheR* Methyltransferase

The expression of the *cheR* methyltransferase appears to be co-regulated with flagellin synthesis. Several *fla* mutants which result in the assembly of partial basal body structures failed to synthesize active methyltransferase (Table III). One of these mutants (*flaL*$^-$) appears to have a nearly complete basal structure [23] but still has little or no enzyme activity. Only in flagellin mutants themselves (H1$^-$) can methyltransferase activity be detected. Similar experiments have also indicated that this is the situation for a *cheX* methylesterase [11], the 60,000-dalton methylated proteins in the membrane [31], and the chemoreceptors for aspartate and serine [7].

This makes considerable evolutionary sense since there is little point in having a sensing system to direct motion if there is no motility apparatus to carry out the instructions.

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