Methylation of Chemotaxis-Specific Proteins in *Escherichia coli* Cells Permeable to *S*-Adenosylmethionine†

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ABSTRACT: Using a modification of the EGTA treatment of Oishi and Smith [Oishi, M., & Smith, C. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3569], *Escherichia coli* cells have been made permeable to *S*-adenosylmethionine and other related molecules in order to facilitate the study of methylation in chemotaxis. The permeable cells are nonmotile but respond to chemotactic stimuli by reversible methylation of their methyl-accepting chemotactic proteins (MCP I and MCP II) in a manner similar to that of untreated, motile cells. Addition of *S*-adenosyl-L-[*methyl*-^{3}H]methionine to the permeable cells specifically labels two proteins, MCP I and MCP II. Methylation of these MCP's is dependent on the presence of wild-type gene products of *flaI*, *flaA*, *cheB*, *cheX*, *tsr*, and *tar*. The extent of methylation of the MCP's is affected by the presence of attractants or repellents: addition of attractant increases the steady-state level of methylation; addition of repellent causes rapid demethylation to a new steady-state level. Methylation is inhibited by the addition of the transmethylase inhibitors A9145C and Sinefungin, which are *S*-adenosylmethionine analogues, and by *S*-adenosylhomocysteine.

Bacterial chemotaxis is a complex phenomenon in which bacterial cells can detect changes in concentration of specific chemicals, behaviorally respond to these changes, and then adapt to the new concentration of stimuli. The behavioral adaptation necessary for chemotaxis is associated with reversible methylation of at least three chemotaxis-specific membrane proteins, called methyl-accepting chemotaxis proteins (MCP's)[1] [for a review, see Springer et al. (1979)]. The steady-state level of methylation of the MCP's reflects the concentration of attractant or repellent. After a change in the concentration of either an attractant or repellent, a new steady-state level is reached at approximately the same time at which the cells are adapting behaviorally. This new level of methylation is maintained for as long as the stimulus is present.

Methylation of the MCP's in *Escherichia coli* has been detected by incubating cells with methionine in which the methyl group is radiolabeled. The methionine is converted in vivo to the methyl donor *S*-adenosylmethionine (*S*-AdoMet), which retains the radioactive methyl group. Methylation has been shown to occur at one or more sites on the MCP (Chelsky & Dahlquist, 1980b), at least one of which is a glutamic acid residue (van der Werf & Koshland, 1977; Kleene et al., 1977). The gene products involved in the methylation and demethylation of the MCP's in both *E. coli* (Parkinson, 1977) and *Salmonella typhimurium* (DeFranco et al., 1979) have been identified. These include the chemotaxis-specific methyltransferase (*E. coli cheX*; *S. typhimurium cheR*), methylesterase (*E. coli cheB*; *S. typhimurium cheX*), and at least two other gene products (*E. coli cheZ* and *cheY*; *S. typhimurium cheT* and *cheQ*) whose functions are unknown but are hypothesized to be regulatory (Parkinson, 1977).

How the level of methylation is regulated by the concentration of stimuli and how this affects adaptation are not known. In order to study the mechanism of control of the level of methylation, it appeared advantageous to develop a procedure by which cells could be made permeable to exogenous *S*-AdoMet. Using a procedure of Oishi & Smith (1978) which makes *E. coli* permeable to ATP and other nucleotides, we have been able to specifically label MCP I and MCP II of *E. coli* through a methylation reaction which closely resembles the reaction in untreated cells. The major advantage of this procedure over the previously described methionine labeling procedure is that there is a higher efficiency of incorporation of radiolabel into the MCP's and that greater than 90% of the radioactivity incorporated into macromolecules is found in the MCP's, whereas, when radioactive methionine is used, other proteins and lipids are labeled. Other advantages are that there is greater control of the internal concentrations of *S*-AdoMet when exogenous *S*-AdoMet is added and that the cells are now permeable to other molecules, such as the methyltransferase inhibitors *S*-adenosylhomocysteine (*S*-AdoHcy), Sinefungin, and A9145C.

Materials and Methods

Chemicals. A9145C and Sinefungin were the gift of R. T. Borchardt. Synthetic L-serine was obtained from ICN Pharmaceuticals. The *S*-AdoMet from Sigma Chemical Co. was purified to remove contaminating *S*-AdoHcy on an AG 1-X8 column described by Shapiro & Ehninger (1966). Adenosyl-L-[*methyl*-^{3}H]methionine was obtained from New England Nuclear with a specific activity of 5–15 Ci/mmol; [^{35}S]methionine was obtained from Amersham at ~1000 Ci/mmol.

Strains. RP477*met*$^-$ (Kort et al., 1975) was obtained from J. Adler but is Thr$^+$ in our hands. The λ *E. coli* hybrid λ*fla3*Δ*14* and the *E. coli* strains 159λ, 159λ*flaI*$^-$, MS5234 (*tsr*$^-$), and MS5235 (*tar*$^-$) were from M. Simon. *E. coli* strains RP4230 (*cheY*$^-$), RP4208 (*cheZ*$^-$), RP4209 (*cheB*$^-$), RP4227 (*cheA*$^-$), RP4228 (*cheX*$^-$), and RP4252 (*flaA*$^-$) were from J. S. Parkinson.

EGTA Treatment. Bacteria were made permeable to *S*-AdoMet by a procedure adapted from Oishi & Smith (1978). *E. coli* were grown in λ tryptone (10 g of tryptone plus 5 g of NaCl) plus 1% glycerol to (2–5) × 10^8 cells/mL. They were washed twice in cold EGTA buffer (10 mM Tris-HCl, pH 7.7,

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[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; MCP, methyl-accepting chemotaxis protein; *S*-AdoMet, *S*-adenosyl-L-methionine; *S*-AdoHcy, *S*-adenosylhomocysteine; *S*-Ado[^{3}H]Met, adenosyl-L-[*methyl*-^{3}H]methionine; NaDodSO$_4$, sodium dodecyl sulfate.

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10 mM $MgCl_2$, 50 mM NaCl, and 1 mM EGTA) and resuspended in 1/20 to 1/50 their original volume in cold 2 M sucrose in EGTA buffer. After 10 min on ice, 20–40 volumes of cold RS buffer (10 mM Tris-HCl, pH 7.7, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM $CaCl_2$) was added, and the cells were centrifuged. They were resuspended to $\sim 1 \times 10^9$ cells/mL in cold RS buffer and could be kept on ice until used, usually within 1 h.

Radiolabeling of MCP's. EGTA-treated cells were warmed to room temperature, and 1 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (*S*-Ado[^{3}H]Met; 2×10^{-7} M final concentration) per mL was added, unless otherwise stated. Cells were incubated at room temperature under conditions described in the text. One-milliliter samples were taken, and formalin was added to a final concentration of 5% to stop further methylation. The samples were centrifuged, resuspended in the suitable dissociation buffer for the electrophoresis procedure employed, and boiled for 2 min.

Electrophoresis. Slab gel electrophoresis was performed on 7.5% $NaDodSO_4$–polyacrylamide gels as described by Laemmli (1970). Following electrophoresis, slab gels were treated for fluorography (Laskey & Mills, 1975). Radioactive proteins were visualized by exposing Kodak XR-5 X-ray film to the gel.

For quantitation of the extent of methylation of the MCP's, 3–5 μL of dansylated bovine serum albumin (Talbot & Yphantis, 1971) was added to each 50-μL sample in Tris-acetate–$NaDodSO_4$ dissociation buffer (Bio-Rad Laboratories, 1974). The dansylated bovine serum albumin could be visualized by UV light as an aid in determining the location of the MCP's. The samples were subjected to electrophoresis on 7.5% Biophore tube gels preequilibrated with Tris-acetate–$NaDodSO_4$ gel buffer (Bio-Rad Laboratories, 1974). The gels were cut into 1-mm slices from which protein was eluted by shaking overnight at room temperature in 0.35 mL of elution buffer containing 5 mM $NaHCO_3$, 0.05% $NaDodSO_4$, and 0.5 M urea. In general, only the eluate from the 10–20 slices including and below the dansylated BSA standard was counted since the MCP's migrate to that region of the gel. The eluate was counted in 3 mL of scintillation fluid for aqueous samples.

Phage-Directed Chemotaxis Proteins. Chemotaxis-specific proteins were labeled with [^{35}S]methionine following the procedure of Silverman et al. (1976) as modified by Chelsky & Dahlquist (1980a). Ultraviolet-irradiated 159λ was infected wtih λ*fla3Δ14* which carries the genes for *cheB*, *-M*, *-X*, *-Y*, and *-Z*. The bacteria were incubated with [^{35}S]methionine, harvested, and prepared for electrophoresis. Under the conditions of this procedure the predominant proteins labeled are chemotaxis specific.

S-AdoMet Uptake. The amount of *S*-AdoMet taken up by the cells was estimated by using a filter binding assay. Aliquots of 200 μL of EGTA-treated cells which had been incubated with *S*-Ado[^{3}H]Met were applied to a Millipore HA filter with a 0.45-μm pore size and washed with 2–5 mL of RS buffer. The amount of radioactivity bound to the filter was determined.

Results

EGTA Treatment. When chemotactic *E. coli* strain RP477*met*$^-$ was treated with EGTA as described under Materials and Methods, 90–99% of the cells were nonmotile and nonviable in either RS buffer or rich broth. This lack of motility persisted for the length of the labeling procedure (up to 2 h). Protein synthesis in the EGTA-treated cells as determined by Cl_3AcOH-precipitable radioactivity was negligible in RS buffer supplemented with [^{35}S]methionine.

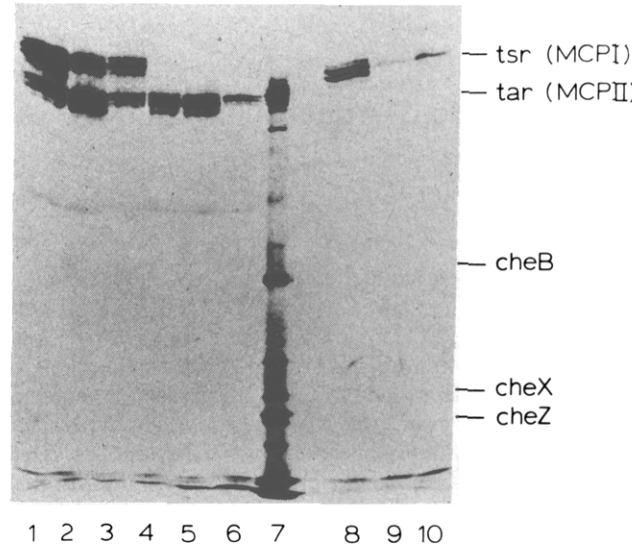

FIGURE 1: $NaDodSO_4$–polyacrylamide electrophoresis of *S*-Ado-[^{3}H]Met-labeled EGTA-treated cells. Cells were treated with EGTA as described under Materials and Methods and incubated in the presence of 2 μCi of *S*-Ado[^{3}H]Met (4×10^{-7} M final concentration) per 1×10^9 cells for 40 min at room temperature. Aliquots of 5×10^8 cells were applied to a 7.5% slab gel, and the proteins were separated by electrophoresis. The gel was treated for fluorography, and the radioactivity was visualized as described. Lanes 1–3 are RP477*met*$^-$ labeled in the presence of L-serine (1×10^{-3} M, lane 1), L-aspartate (4×10^{-4} M, lane 2), or buffer alone (lane 3); lanes 4–6 are MS5234 (*tsr*$^-$) labeled in the presence of L-serine (lane 4), L-aspartate (lane 5), or buffer alone (lane 6); lanes 8–10 are MS5235 (*tar*$^-$) labeled in a similar manner. Lane 7 is an aliquot of 159λ infected with λ*fla3Δ14* to produce [^{35}S]Met phage-directed chemotaxis proteins as molecular weight standards.

Methylation of MCP's by Exogenously Added S-AdoMet. Addition of *S*-Ado[^{3}H]Met at 1 μCi/mL (2×10^{-7} M final concentration) to chemotactic *E. coli* strains, which had been EGTA-treated, resulted in the incorporation of radioactivity into two species of proteins as shown in Figure 1. Aliquots of EGTA-treated RP477*met*$^-$ labeled in the presence of L-serine (lane 1), L-aspartate (lane 2), or buffer alone (lane 3) were applied to an $NaDodSO_4$–polyacrylamide slab gel. Lane 7 contains a sample of [^{35}S]methionine-labeled λ*fla3Δ14*-directed chemotaxis proteins used as molecular weight standards, as indicated. The two major proteins labeled by the addition of exogenous *S*-Ado[^{3}H]Met appear to be MCP I and MCP II because they have approximate molecular weights of 60 000–70 000 and the protein of lower molecular weight is similar in size and multiple banding pattern to the bacteriophage-directed ^{35}S-labeled MCP II (compare lanes 1 and 7). The larger protein is similar to ^{35}S-labeled MCP I (data not shown).

Further evidence that these labeled proteins are MCP I and MCP II is shown in Figure 1 by the requirement for wild-type *tar* and *tsr* for incorporation of radioactivity into the MCP's. Silverman & Simon (1977) have shown that *tsr* and *tar* are the structural genes for MCP I and MCP II, respectively. Samples of EGTA-treated MS5234 (*tsr*$^-$, lanes 4–6) and EGTA-treated MS5235 (*tar*$^-$, lanes 8–10) were labeled with *S*-Ado[^{3}H]Met. As shown in Figure 1, *tsr*$^+$ is required for the presence of labeled MCP I (lanes 8–10) and *tar*$^+$ is required for the presence of labeled MCP II (lanes 4–6).

Level of Methylation in Response to Chemotactic Stimuli. Figure 1 also demonstrates qualitatively that the amount of radioactivity incorporated into the MCP's is affected by the presence or absence of attractants. The samples in lanes 1,

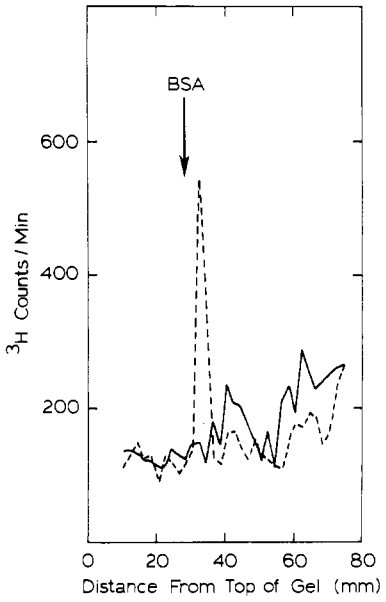

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of EGTA-treated *flaI*$^+$ and *flaI*$^-$ strains labeled in the presence of *S*-Ado[^{3}H]Met. Aliquots of EGTA-treated 159λ (*flaI*$^+$) and 159λ*flaI*$^-$ were incubated in the presence of 1 μCi of *S*-Ado[^{3}H]Met (2×10^{-7} M final concentration) for 40 min at room temperature. Aliquots of 1×10^9 cells were applied to 7.5% tube gels. The gels were sliced into 2-mm segments, and the radioactivity in each slice was determined as described under Materials and Methods. (---) 159λ; (—) 159λ*flaI*$^-$.

4, and 8 were labeled in the presence of L-serine, lanes 2, 5, and 9 were labeled in the presence of L-aspartate, and lanes 3, 6, and 10 were labeled in buffer alone. L-Serine caused an increase in the amount of radioactivity in MCP I (lanes 1 and 8) and L-aspartate caused an increase in that in MCP II (lanes 2 and 5) as compared to buffer alone. The increase in methylation of MCP II in the presence of L-serine is probably due to contamination of the L-serine with other compounds which act at MCP II since synthetic L-serine does not cause this increase.

The specific, differential labeling of the MCP's in response to the presence or absence of attractant and the fact that there is negligible protein synthesis occurring during labeling with *S*-AdoMet indicate that the incorporation of radioactivity is due to methylation alone. In order to quantitate the extent of methylation of the MCP's, aliquots of EGTA-treated cells were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis in the tube gels, sliced, and eluted, as described under Materials and Methods. Under these conditions, MCP I and MCP II run as a single broad band, as shown in Figure 2. Profiles of this type can be used to determine the extent of methylation under a variety of conditions. For example, aliquots of an EGTA-treated wild-type chemotactic strain (159λ) or of the nonchemotactic isogenic strain (159λ*flaI*$^-$) were analyzed as described above. The strain 159λ*flaI*$^-$ lacks the positive regulatory function of *flaI*$^+$ necessary for the expression of *tsr* and *tar* gene products. The elution profiles of the radioactivity on the tube gels are compared in Figure 2. The *flaI*$^+$ cells show labeling of the MCP peak while the *flaI*$^-$ cells do not. The radioactivity incorporated into the MCP peak varied in wild-type cells from ~1000 cpm for 159λ(*met*$^+$) to 10000 cpm in RP477*met*$^-$.

In EGTA-treated RP477*met*$^-$, a steady-state level of methylation is reached 15 min after the addition of *S*-Ado-[^{3}H]Met to unstimulated cells and after 30 min in serine-stimulated cells (data not shown). This steady-state level remains constant for at least 90 min. There is an approximate

Table I: Level of Methylation in the MCP Peak after Addition of Attractants Serine or Aspartate

addition to the cells	cpm in the MCP peak	ratio of stimulated to unstimulated[a]
L-serine (9.5×10^{-4} M)	4610	2.1
L-aspartate (3.9×10^{-4} M)	3550	1.6
L-serine and L-aspartate	4950	2.3
RS buffer	2160	1.0

[a] This ratio is calculated by dividing the amount of radioactivity incorporated (cpm) in each sample by the amount in the RS buffer control.

Table II: Level of Methylation in *che*$^-$ Mutants in the Presence or Absence of L-Serine

strain	phenotype	% methylation[a] +serine	% methylation[a] −serine	ratio of stimulated to unstimulated[b]
RP477*met*$^-$	*che*$^+$	257	100	2.6
RP4227	*cheA*$^-$	47	30	1.5
RP4209	*cheB*$^-$	7	4	1.9
RP4228	*cheX*$^-$	<1	<1	
RP4230	*cheY*$^-$	<1	<1	
RP4208	*cheZ*$^-$	56	25	2.2
RP4252	*flaA*$^-$	<1	<1	

[a] Percent methylation was calculated by dividing the cpm in the MCP peak of the mutants by the cpm in the MCP peak of the wild-type strain in the absence of L-serine (−serine). [b] This ratio was calculated by dividing the percent methylation in the +serine MCP peak by the percent methylation in the −serine MCP peak for each strain.

twofold increase in state level of methylation in attractant-stimulated cells compared to unstimulated cells. Table I presents the results of a typical experiment in which EGTA-treated RP477*met*$^-$ was incubated in the presence of 2×10^{-7} M *S*-AdoMet and the indicated concentration of attractant until a steady-state level of methylation was reached (40 min). The ratios of methylation levels in stimulated cells compared to those in unstimulated cells are 2.1 for L-serine, 1.7 for L-aspartate, and 2.3 for L-serine plus L-aspartate. In 11 experiments of this kind, using L-serine as the stimulus, the average ratio of radioactivity in the MCP peak of stimulated cells to unstimulated cells was 2.1 ± 0.2.

The increase in extent of methylation in the L-serine stimulated cells was not due to an increased permeability of the cells to *S*-AdoMet in the presence of serine, as determined by a filter binding assay (see Materials and Methods). There was no significant difference in the amount of *S*-Ado[^{3}H]Met bound to filters in the presence or absence of serine either by a chemotactically wild-type strain RP477*met*$^-$ which had been EGTA-treated or by EGTA-treated RP4252 (*flaA*$^-$) which is unable to methylate its MCP's.

The extent of methylation in various chemotactic mutants was determined. Aliquots of these mutants which had been EGTA-treated and incubated with *S*-Ado[^{3}H]Met (2×10^{-7} M final concentration) in the presence or absence of L-serine until a steady-state level of methylation was reached (40 min) were analyzed. The results are presented in Table II, the gene products of *cheB*, *cheX*, *cheY*, and *flaA* are necessary for incorporation of radioactive methyl groups to occur to even 10% of wild type. The level of methylation in the *cheA*$^-$ and *cheZ*$^-$ mutants also appears to be reduced; however, this may reflect differences in labeling efficiency rather than an effect of the specific chemotaxis mutation. Methylation in *cheW*$^-$

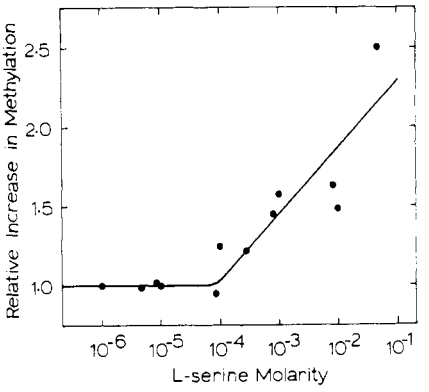

FIGURE 3: Level of methylation of the MCP peak as a function of L-serine concentration. EGTA-treated RP477*met*$^-$ was incubated in the presence of 1 μCi of *S*-Ado[^{3}H]Met (2×10^{-7} M final concentration) per 1×10^9 cells and the indicated concentrations of L-serine at room temperature for 40 min. Aliquots of 1×10^9 cells were analyzed by tube gel electrophoresis and the amount of radioactivity incorporated into the MCP peak was determined, as described under Materials and Methods. The relative increase in methylation was determined by dividing the cpm in the MCP peaks incubated in the presence of L-serine by the cpm in the MCP peak of an equivalent unstimulated sample.

mutants is allele dependent and has not been included in Table II.

The increase in level of methylation in EGTA-treated cells can be shown to be dependent on the concentration of attractant above a threshold concentration present during incubation of *S*-Ado[^{3}H]Met. EGTA-treated RP77*met*$^-$ was incubated in the presence of *S*-Ado[^{3}H]Met and concentrations of L-serine varying from 10^{-6} to 2×10^{-1} M. The samples were applied to $NaDodSO_4$–polyacrylamide tube gels, and the radioactivity in the MCP peak was determined as described under Materials and Methods. Figure 3 is representative data from two such experiments expressed as the percent increase in radioactive methyl groups incorporated in the MCP peak as compared to an unstimulated control. The data demonstrate that, at L-serine concentrations above $\sim 10^{-4}$ M, the level of methylation increases as the concentration of attractant increases.

Time Course of Methylation and Demethylation. In order to determine the time course of demethylation after the addition of repellents, EGTA-treated RP477*met*$^-$ was incubated with *S*-ado[^{3}H]Met until a steady-state level of methylation had been reached. The extent of methylation was determined and then the repellents sodium acetate and cobalt chloride were added to concentrations of 0.1 M and 0.5 mM, respectively. Duplicate samples were taken at times after this addition and analyzed on $NaDodSO_4$–polyacrylamide tube gels. The data, shown in Figure 4, demonstrate that within 2 min after the addition of the repellent, a new steady-state level of methylation of almost half the original level had been reached and maintained. A similar time course of decrease in the level of methylation has been shown to occur when cells lebeled in the presence of attractant were diluted 10-fold into buffer (data not shown).

If attractant was added to cells which had reached a steady-state level of methylation, it was expected that the level of methylation would increase in response to the attractant. Attempts to demonstrate this increase in EGTA-treated RP477*met* incubated with 10^{-7} M *S*-Ado[^{3}H]Met were unsuccessful. It seemed possible that at such a low external concentration of *S*-AdoMet, the rate of increase in methylation level might be limited by the internal concentration of *S*-AdoMet. In order to circumvent this problem, nonradioactive

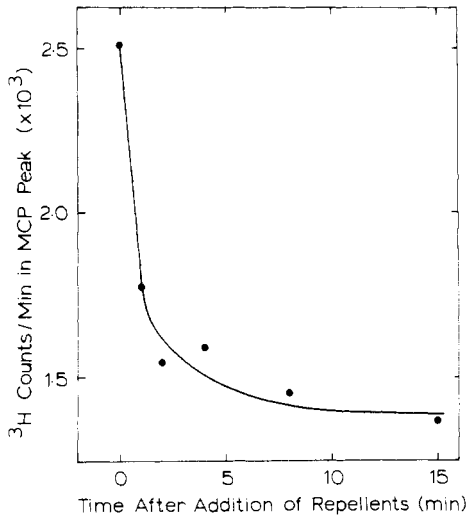

FIGURE 4: Time course of the decrease in methylation of the MCP's following the addition of repellents. EGTA-treated RP477*met*$^-$ was incubated in the presence of 1 μCi of *S*-Ado[^{3}H]Met (2×10^{-7} M final concentration) per 1×10^9 cells for 40 min. Aliquots of 1 mL (1×10^9 cells) were taken in triplicate and analyzed on tube gels to establish the steady-state level of methylation. 0.5 mM $CoCl_2$ and 0.1 M sodium acetate were added to the cells, and 1-mL samples were analyzed on tube gels in duplicate at the times indicated. The radioactivity in the MCP peak was determined, as described under Materials and Methods.

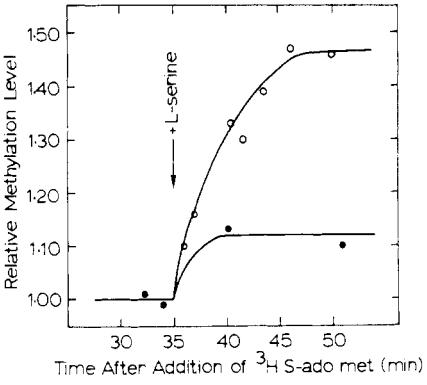

FIGURE 5: Time course of increase in methylation level following the addition of L-serine. EGTA-treated RP477*met*$^-$ at 1×10^9 cells/mL was incubated in the presence of 2 μCi of *S*-Ado[^{3}H]Met/mL (4×10^{-7} M final concentration) and 1×10^{-5} M unlabeled *S*-AdoMet until a steady-state level of methylation had been reached. Samples of 1 mL were taken at the times indicated. At 35 min, the culture was divided and part was treated with 1×10^{-3} M L-serine; the rest was treated with an equivalent volume of RS buffer. Samples of 1 mL were taken at the times indicated. The samples were analyzed on tube gels to determine the amount of radioactivity in the MCP peak. The relative methylation level was calculated by dividing the cpm in the MCP peak by the average cpm in the MCP peak before the culture was divided. (○) L-Serine; (●) buffer.

S-AdoMet was increased 100-fold to a final concentration of 10^{-5} M, while the specific activity was only decreased 50-fold. The data in Figure 5 were obtained by quantitating radioactivity in the MCP peak in EGTA-treated RP477*met*$^-$, labeled in the presence of 10^{-5} M nonradioactive *S*-AdoMet and 2×10^{-7} M *S*-Ado[^{3}H]Met. These cells were incubated in buffer alone plus *S*-AdoMet, and samples were taken for analysis at the times indicated. After steady-state methylation level had been reached (35 min), the incubation mixture was divided into two aliquots, one of which was treated with L-serine and the other of which was treated with an equal amount of buffer as a control. Samples were withdrawn at the times indicated. At this higher concentration of *S*-AdoMet, an increase in the level of methylation occurred within 7–8 min in the presence

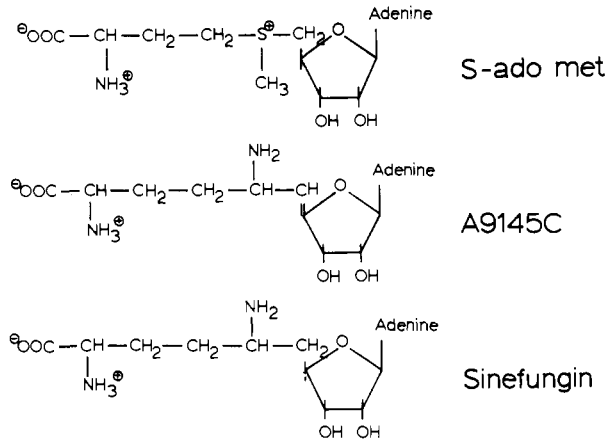

FIGURE 6: Structures of *S*-AdoMet and its analogues, A9145C and Sinefungin.

of L-serine. The small increase in the level of methylation in the samples in buffer alone is reproducible and is probably due to increased aeration during mixing.

Effect of Methyltransferase Inhibitors on the Level of Methylation. *S*-AdoHcy (Borchardt, 1977) and the two *S*-AdoMet analogues, Sinefungin and A9145C (Pugh et al., 1978), have been shown to be reversible inhibitors of many methyltransferases. The structures of these compounds are shown in Figure 6. In order to determine if these molecules were effective inhibitors of the chemotaxis methyltransferase (*E. coli cheX*), EGTA-treated RP477*met*$^-$ was incubated in the presence of *S*-Ado[^{3}H]Met (2×10^{-7} M) and varying concentrations of each of these inhibitors. The radioactivity in the MCP peak was determined for each concentration and expressed as percent inhibition by using the radioactivity in the MCP peak of a noninhibited sample as the 0% inhibited value.

As shown in Figure 7, 50% inhibition of methylation occurs at 10^{-8} M Sinefungin, 10^{-9} M A9145C, and 10^{-5} M *S*-AdoHcy. This inhibition does not appear to be due to a decreased permeability of the *S*-Ado[^{3}H]Met in the presence of these inhibitors. Addition of Sinefungin or A9145C after a steady-state level of methylation has been reached does not inhibit demethylation due to the addition of repellents, nor do these molecules act as repellents by causing a rapid decrease in the level of methylation. Further, methylation due to the addition of L-serine as described above is inhibited after a 5-min preincubation with A9145C at 10^{-6} M (data not shown).

Discussion

Methylation of the MCP's is the only assayable enzymatic event associated with the chemotactic response and, therefore, is an important tool in understanding the mechanism of chemotaxis. In order to study the role of methylation and its regulation, it was advantageous to develop a procedure to permeabilize *E. coli* to exogenous *S*-AdoMet, the methyl donor. Using an EGTA–sucrose treatment, we have been able to permeabilize cells under conditions in which addition of radioactive methyl-labeled *S*-AdoMet to these treated cells results in the incorporation of radioactivity into only two protein species. These were identified as methylated MCP I and MCP II based on these criteria: (1) approximate molecular weights on $NaDodSO_4$–polyacrylamide gel electrophoresis are 60 000–70 000; (2) the multiple banding patterns are similar to those of known MCP's on $NaDodSO_4$–polyacrylamide gels; (3) the occurrence of the labeled proteins requires the presence of the wild-type gene products of *flaI*, *cheB*, *cheX*, *tsr*, and *tar*; (4) the level of methylation of the

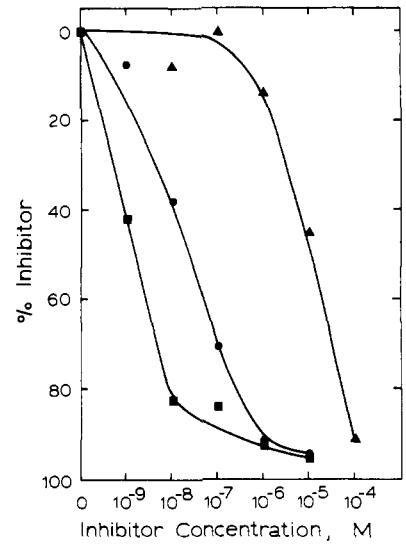

FIGURE 7: Inhibition of methylation of MCP's as a function of inhibitor concentration. EGTA-treated RP477*met*$^-$ was incubated in the presence of 1 μCi of *S*-Ado[^{3}H]Met (2×10^{-7} M final concentration) per 1×10^9 cells and the indicated concentration of inhibitor for 40 min at room temperature. Aliquots of 1×10^9 cells were analyzed on tube gels and the amount of radioactivity in the MCP peak was determined as described. The percent inhibition was calculated by dividing the cpm in the MCP peak with inhibitor present by the cpm in the MCP peak when no inhibitor was present. (■) A9145; (●) Sinefungin; (▲) *S*-AdoHcy.

bands responds to the presence or absence of chemotactic stimuli. A minor radioactive protein band of ~40 000–50 000 daltons has been detected on some of the gels. This appears to be a non-chemotaxis-specific protein, because it occurs in some *flaI*$^-$ preparations. Methylation of MCP III (Kondoh et al., 1979) does not occur at a detectable level in EGTA-treated cells.

The EGTA-treated cells, although nonmotile, appear to have an intact chemotaxis sensing and methylation mechanism. These treated cells respond to stimuli in ways that are similar to those shown for untreated motile cells using radioactive methyl-labeled methionine to label the MCP's. These responses include the following: (1) Approximately twice as much radioactivity is incorporated into the MCP's in the presence of high concentrations of the attractants L-serine or L-aspartate as compared to buffer alone. (2) The steady-state level of methylation increases as the concentration of the attractant L-serine increases. This effect has a threshold of 10^{-4} M serine. (3) The decrease in the level of methylation following addition of repellent is rapid, reaching a new steady-state level within 2 min.

The time course of methylation in EGTA-treated cells in 10^{-5} M *S*-AdoMet following the addition of L-serine is surprisingly slow, requiring 8–10 min to reach a new steady-state level. We (unpublished data) and other workers (Springer et al., 1979; Paoni & Koshland, 1979) have reported that the time required to reach the new level is within 5 min. It is possible that the slower time course with EGTA-treated cells may be due to limiting internal *S*-AdoMet concentration. This is further supported by the fact that this increase is not detected in EGTA-treated cells labeled in the presence of only 10^{-7} M *S*-AdoMet but requires 10^{-5} M *S*-AdoMet.

Because EGTA-treated cells are now permeable to molecules similar to *S*-AdoMet, we were able to determine the effect on methylation of the known methyltransferase inhibitors *S*-AdoHcy, Sinefungin, and A9145C. The latter two molecules are *S*-AdoMet analogues which have been shown to be potent reversible inhibitors of transmethylases (Pugh et al.,

1978). Inhibition of 50% of normal methylation occurs at 10^{-5} M *S*-AdoHcy, 10^{-8} M Sinefungin, and 10^{-9} M A9145C. These molecules do not cause a rapid demethylation in a manner similar to that of repellents. We have also shown that the inhibitor A9145C does not inhibit demethylation due to the addition of repellent but does inhibit methylation due to the addition of attractant. These inhibitors may offer a powerful tool for the study of the mechanism of regulation of methylation.

As described above, incorporation of radioactive methyl groups into the MCP's of EGTA-treated cells using exogenous *S*-Ado[^{3}H]Met closely resembles incorporation in untreated cells using radiolabeled methylmethionine. Recently, Paoni & Koshland (1979) published an EDTA–toluene treatment which permeabilizes *S. typhimurium* and *E. coli* to *S*-AdoMet and allows methylation of the MCP's. This methylation reaction also appears to be representative of the reaction in vivo and shows sensitivity to *S*-AdoHcy.

Permeabilization of chemotactic bacteria to *S*-AdoMet has several advantages over the use of methionine with untreated cells. The major advantage is that radioactivity is incorporated at high levels into only the two MCP's. This high degree of specificity allows the production of radiochemically pure methylated MCP's after $NaDodSO_4$–polyacrylamide gel electrophoresis without further purification. We have exploited this specificity to show by chemical cross-linking in EGTA-treated cells that MCP I and MCP II exist as functional tetramers (Chelsky & Dahlquist, 1980a) and that at least three methylation sites for both MCP I and MCP II may exist as indicated by tryptic peptide analysis (Chelsky & Dahlquist, 1980b).

Other advantages of this labeling procedure allow it to be used to study the mechanism of regulation of methylation. These advantages are that the cells are now permeable to methyltransferase inhibitors and that the size of the internal *S*-AdoMet pool may be more easily controlled.

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