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Biochemistry

Methylation of a membrane protein involved in bacterial chemotaxis*

(*Escherichia coli*/methionine/flagella/electrophoresis)

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ABSTRACT **A protein methylation reaction involved in chemotaxis of *Escherichia coli* has been identified. The involvement of this reaction in chemotaxis is indicated by four lines of evidence. (*a*) The methylation reaction is altered in several classes of generally nonchemotactic mutants and is coreverted with the chemotaxis defects. (*b*) The methylation level of the protein is affected by chemotactic stimuli. (*c*) The transferred methyl group is derived from methionine and is labile, in accord with the known fact that chemotaxis requires a continuous supply of methionine. (*d*) Methylation is abnormal in various mutants having defective or missing flagella.**

Bacteria such as *Escherichia coli* and *Salmonella typhimurium* swim in smooth lines, interrupted frequently by brief periods of tumbling that produce random directional changes (1). Chemotaxis results from modification of the frequency of this tumbling in response to spatial (1) or temporal (2–4) chemical gradients: there is a decrease in frequency when the cell encounters an increasing attractant or decreasing repellent concentration, and a less pronounced increase in frequency in response to the opposite changes in concentration. The chemical stimuli are detected by chemoreceptors (5–8). The receptors produce a signal ultimately affecting the direction of rotation of the flagella (9), which can be either clockwise or counterclockwise (10, 11). It is the direction of flagellar rotation which determines whether the cell swims smoothly or tumbles (9).

Control over tumbling frequency in *E. coli* involves the products of at least four genes, *cheA, B, C,* and *D* (12, 13). Mutations in these genes can produce a generally nonchemotactic phenotype (14) characterized by abnormal regulation of the tumbling frequency (13, 14). In all four genes mutants have been isolated that have lost the ability to tumble, either spontaneously or in response to stimuli (13, 14). In *cheB* additional mutants have been found which tumble incessantly (13); these *cheB* mutants show partially or completely impaired chemotaxis (13).

A useful probe for biochemical analysis of the signalling process and the tumble-regulating mechanism was provided by the discovery that L-methionine is required for chemotaxis in *E. coli* (15). After the removal of methionine, methionine auxotrophs lose the ability to tumble and do not respond to either attractants or repellents (8, 15–19), much like mutants defective in the *che* genes. These studies have been extended to indicate that *S*-adenosylmethionine, a metabolite of methionine, is required in chemotaxis, probably as a methyl donor (20, 21, 32).

Using radioactive L-methionine, we have identified a protein in the envelope of *E. coli* that contains a labile methyl group donated by methionine. This methylation is shown to be involved in chemotaxis by the use of generally nonchemotactic (*che*) mutants and chemotactic stimuli. The identification of this methyl-accepting chemotaxis protein (MCP) opens the way for a biochemical investigation of the mechanisms of information processing in bacterial chemotaxis.

MATERIALS AND METHODS

Chemicals. L-[*methyl*-^{3}H]Methionine, 2–11 Ci/mmol, and L-[*methyl*-^{14}C]methionine, 56 mCi/mmol, were obtained from Amersham/Searle Corp; L-[*1*-^{14}C]methionine, 8 mCi/mmol, was obtained from Calbiochem. These isotopes were used at or near their purchased specific activities. Amino acids (A grade) and chloramphenicol (B grade) were purchased from Calbiochem. Trypsin and pepsin (Sigma Chemical Co.) were used at 100 and 60 units/ml, respectively. All other chemicals were reagent grade.

Bacteria. Chemotaxis mutants and their parents are described in the legend to Table 1. Mutants *flaA* 64.1, *flaB* Mu1557, *flaC* 80.12, *flaI* 101.6, and *hag* 726 were isolated by Silverman and Simon (22, 23) from MS1350.

Sample Preparation. Cells were grown in tryptone broth (1% Difco Bactotryptone, 0.5% sodium chloride) at 35° to approximately 3×10^8 bacteria per ml, collected by centrifugation at 22°, washed three times in chemotaxis medium (10 mM potassium phosphate pH 7.0, 0.1 mM ethylenediaminetetraacetate, pH 7.0), and put on ice. Methylations were carried out at 30° in chemotaxis medium plus 10 mM D,L-sodium lactate as an energy source, and 200 μg/ml of chloramphenicol. Cells were added to this medium at 30° 5 min before addition of radioactive methionine. L-[*methyl*-^{3}H]Methionine was added to cells (4×10^8 bacteria per ml) to a concentration of 1 μM (except where noted). Ten-milliliter samples were taken at times indicated, formalin was added to 2.5%, since it could be shown to stop methylation and demethylation reactions, and the samples were put on ice. Similarly, L-[*methyl*-^{14}C]methionine was added to a control culture (1.5×10^9 bacteria per ml) to yield 4 μM, incubated for 20 min, brought to 2.5% formalin, and chilled. All incubations were carried out with rotary shaking. Twenty-milliliter aliquots of the ^{14}C-treated cells were added to each of the ^{3}H-labeled samples to allow correction for the variable loss in each sample in subsequent steps (the normalization control described in the legend to Fig. 2).

From this point on, samples were kept at 2°. Treated cells were collected by centrifugation and resuspended in 1.5 ml of 10 mM sodium phosphate, pH 7.2. Cell envelopes were prepared by sonication and centrifugation according to the method of Ames (24), except that the final centrifugation was at 300,000 × *g* (R_{max}) for 25 min, and sodium phosphate buffer was used throughout. Envelope pellets were resuspended in 0.1 ml of boiling medium containing 3% sodium dodecyl sulfate (NaDodSO$_4$), 1% mercaptoethanol, 3% urea, 10% glycerol, 10 mM sodium phosphate buffer pH 7.2,

Abbreviations: MCP, methyl-accepting chemotaxis protein; NaDodSO$_4$, sodium dodecyl sulfate.

* An account of this work was presented at the Cold Spring Harbor Conference on Cell Motility, September 9–14, proceedings in press.

Table 1. Methylation of MCP in chemotaxis mutants and their revertants*

Strain designation†	Genotype‡	Phenotype		% of wild-type methylation
		Chemotaxis defect§	Tumbling property	
e12i1	*cheA* amber	General	Smooth	52
e14f1	*cheA* amber	General	Smooth	56
e10g1	*cheA*	General	Smooth	62
A593	*cheA*	General	Smooth	103
e16b1	*cheA*	General	Smooth	159
e14i1	*cheA*	General	Smooth	254
e12i1 Che$^+$	*cheA* revertant	None	Normal	99
e10g1 Che$^+$	*cheA* revertant	None	Normal	107
B590	*cheB*	General	Smooth	0
e10d3	*cheB*	General	Smooth	6¶
e17p1	*cheB*	General	Smooth	6¶
e14n1	*cheB*	General	Smooth	178¶
e14h3	*cheB*	General	Smooth	273
e17p1 Che$^+$	*cheB* partial revertant	Partial	Low frequency	62
e17p1 Che$^+$	*cheB* revertant	None	Normal	94
e25p3 Che$^+$	*cheB* revertant	None	Normal	276
e21e1	*cheB*	General	Tumbler	1
e20t2	*cheB* amber	General	Tumbler	6
AW628	*cheB*	General	Tumbler	57
e26e2	*cheB* amber	General	Tumbler	120
C497	*cheC*	General	Smooth	126
AW518	*cheD*	Serine	Normal	16
$S2_3$	*cheD*	Serine	Normal	91
3.4	*cheD*	General	Smooth	1

* Methylated samples were prepared as described in *Materials and Methods,* and methylated MCP was quantitated as described in the legend to Fig. 2. Labeling was allowed to proceed for 20 min. In all cases the parent and the mutant were analyzed on the same day using aliquots from the same ^{14}C-labeled culture as the normalization control.

† AW518, $S2_3$, *cheA* 593, *cheB* 590, and *cheC* 497 were isolated from AW405 (6, 12,[a]). AW620, AW627, AW628, and 3.4 were isolated by R. W. Reader from AW574 (19), a *gal*$^+$ *sup*$^+$ derivative (21) of AW405. These strains were made Met$^-$ by P1 cotransduction with *rif* of the *metF* mutation in a *rif* derivative of 2-46 (17). Other generally nonchemotactic mutants used were isolated from RP461 and RP470 (derivatives of AW574) by Parkinson (13, 31) and transferred into RP477 *metF* by P1 cotransduction with *eda*. RP477 is a *galE eda* derivative of AW574 (31). Chemotaxis revertants were selected on semisolid tryptone plates (14). All strains were made *metF* to facilitate incorporation of methionine, but this was later found unnecessary. Each mutant is compared to its isogenic wild-type strain. Mutants are listed in increasing order of methylation of MCP.

‡ Mutations are missense, unless otherwise indicated.

§ As determined on a semisolid tryptone plate (14). Constantly tumbling *cheB* strains often have some residual taxis in temporal stimulation (13).

¶ To evaluate reproducibility, strains were tested on four different days. The results were: e17p1, 4, 5, 2, and 11%; and e14n1, 126, 156, 134, and 297%. Five isolates of e10d3 were tested on the same day, giving 3, 5, 6, 7, and 9%. The table reports the means.

and 20 μg/ml of bromphenol blue. Samples were then boiled for 1 min to solubilize membrane proteins, including MCP.

After boiling, the protein in each sample was precipitated and extracted twice with 2 ml of 100% ethanol by mixing on a Vortex mixer and centrifugation. This removed a highly radioactive component with high electrophoretic mobility, probably lipid, which partially obscured the peak of interest in 13-cm gels. The extracted samples were again boiled for 1 min in 0.1 ml of boiling medium and applied to the tops of $NaDodSO_4$-polyacrylamide gels.

Electrophoresis. $NaDodSO_4$-polyacrylamide gel electrophoresis was performed according to Medappa *et al.* (25). Twenty-centimeter gels, of 10% acrylamide, were fractionated into 1-mm sections by a Gilson (Middleton, Wisc.) Aliquogel Fractionator. Thirteen-centimeter gels, of 6.5% acrylamide, were manually sliced into 5-mm sections. Gel sections were dissolved in 0.1 or 0.2 M sodium hydroxide, and radioactivity was determined in a liquid scintillation spectrometer. Analysis of the data is described in the legend of Fig. 2.

RESULTS

Molecular weight, chemical properties, and localization of MCP

Fig. 1 shows the electrophoretic pattern of solubilized envelopes isolated from a wild-type strain after labeling *in vivo* with L-[*methyl*-^3H]methionine. The peak with relative mobility 0.4 is analyzed in this paper and is given the name MCP for methyl-accepting chemotaxis protein. It has an apparent molecular weight of 62,000. The molecule is present in excess of 700 copies per cell. This is only an estimate, since the calculation assumes that all MCP molecules are unmethylated at the start of the experiment, that only one methyl group is transferred to each MCP molecule, and that there is no dilution of the specific activity of the methionine after it is taken up by the cells.

The protein nature of MCP was demonstrated by incubating an envelope preparation with proteolytic enzymes (see *Materials and Methods*). Pepsin reduced the radioactivity recovered in MCP to 6% and trypsin to 8% of that before treatment (data of Steven J. Kleene).

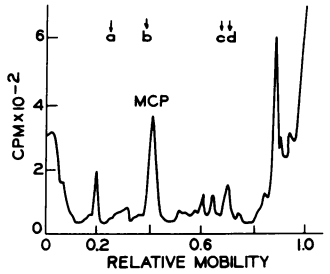

FIG. 1. Electrophoretic pattern of envelopes isolated after treatment with L-[*methyl*-^{3}H]methionine. Envelopes were prepared from RP477 *metF* as described in *Materials and Methods*, except that formalin was not used to stop reactions and the samples were not extracted with ethanol. The sample was electrophoresed in a 20-cm, 10% acrylamide gel, and radioactivity in 1-mm sections was determined. Mobilities are relative to bromphenol blue. The peak with a relative mobility of 0.88 was not consistently found. The molecular weight standards used, indicated by arrows, were (a) phosphorylase A, (b) bovine serum albumin, (c) carboxypeptidase, and (d) chymotrypsinogen of molecular weights 94,000, 68,000, 34,600, and 24,700, respectively. They were prepared by acetylation with [^{14}C]acetic anhydride (a gift of R. Z. Montelaro and R. R. Rueckert) and were applied to the gel along with the labeled envelopes.

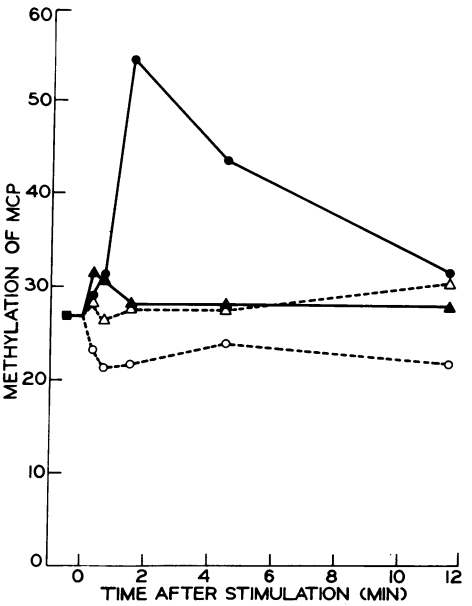

FIG. 2. Effect of temporal attractant stimulation on the methylation of MCP. Methylation was carried out as described in *Materials and Methods*. At 22 min after addition of methionine, L-serine (final concentration 0.5 mM) or an equal volume of water was added to the cells (time zero). Samples were prepared as described in *Materials and Methods*. Methylation of MCP was quantitated by integrating the radioactivity counted in the [^{3}H]MCP peak (the test sample) and the [^{14}C]MCP peak (the normalization control) from the same gel, dividing the ^{3}H value by the OD_{590} of the cells in the test sample to normalize for the slightly different number of cells in each sample, and then dividing by the ^{14}C value to normalize for loss of material during the sample preparation. Since each sample originally contained an identical amount of ^{14}C-labeled MCP, sample loss should be accurately reflected by variations in the ^{14}C value. The resulting number is also a function of the specific activity of the labeled methionine used in each experiment. At 22 min after methionine addition, the methylation of MCP in Figs. 2 and 3 is comparable when the specific activities are taken into account. Curves are shown for the chemotactically wild-type strain 2-46 after addition of L-serine (●) or water (○), and for $S2_3$, an isogenic mutant[a] defective in serine taxis, after addition of L-serine (▲) or water (△). Curves were normalized so that the −0.5 min points (■) were equal; this adjustment was less than 15%.

S-Adenosylmethionine has been implicated in chemotaxis (see Introduction), which suggests a transfer of the methyl group of methionine. MCP receives at least the methyl group, since it is labeled by L-[*methyl*-^{3}H]methionine. It does not, however, incorporate the entire methionine molecule, since cells incubated with L-[*1*-^{14}C]methionine fail to incorporate label into MCP.

In order to localize MCP in the envelope of *E. coli*, cells were labeled for 20 min with radioactive methionine, and cytoplasmic and outer membranes were separated by the method of Osborn *et al.* (26). The isolated cytoplasmic membrane contained 81% of the methylated MCP of the unfractionated envelope, while the isolated outer membrane contained 17%, indicating an association of MCP with the cytoplasmic membrane. Purity of the separated membranes was evaluated as follows: the cytoplasmic membrane fraction (density = 1.183) contained nine times as much NADH oxidase (26) as the outer membrane fraction (density = 1.230), and less than one-eighth as much 2-keto-3-deoxyoctanoate (26) (an indicator of lipopolysaccharide).

The cytoplasmic fraction of *E. coli* [the supernatant after 25 min of centrifugation at 300,000 × *g* (R_{max}) to remove cell envelopes] contains methylated material that coelectrophoreses exactly with methylated MCP from isolated envelopes. If this material is MCP, there is about 1.3 times as much of it in the cytoplasmic fraction as there is in the membrane fraction. Centrifugation at the same speed for five times as long reduces the amount found in the cytoplasm to about half that found in the envelopes. We therefore consider it most likely that this cytoplasmic material represents MCP contained in contaminating vesicles, too small to sediment rapidly. In agreement with this hypothesis, it was found that the cytoplasmic fraction contains NADH oxidase activity (26), and that the activity decreases 4-fold with the longer centrifugation. All further experiments reported in this paper are concerned only with the envelope fraction.

Methylation of MCP in nonchemotactic mutants

Since both methionine and the products of the *che* genes play a role in the regulation of tumbling frequency, we investigated the methylation of MCP in generally nonchemotactic (*che*) mutants. Table 1 shows the amount of label incorporated into MCP in a number of *che* mutants compared to otherwise isogenic wild-type strains after 20 min of exposure to radioactive methionine. Data are also given for revertants selected for chemotaxis.

As can be seen, there is a broad range of values for incorporation of label into MCP. A number of mutants representing several *che* genes show significant abnormalities, ranging from total absence of incorporation to severalfold above wild type. We do not know whether the observed defects represent a change in the amount of MCP, a change in the rate of methylation of MCP, or a change in the recovery of methylated MCP with the envelope fraction. It is important to notice that when affected mutants are reverted to normal chemotaxis behavior, normal or greater-than-normal incorporation is also restored, indicating that the defective incorporation really is a consequence of the *che* mutations and not due to a second mutation. A partial revertant shows a partial restoration of incorporation. However, based on these data there is no obvious correlation between the

[a] E. N. Kort, R. W. Reader, and J. Adler, in preparation.

amount of methylation observed and chemotaxis genotype or phenotype; in particular, the amount of label found in MCP bears no consistent relationship to the tumbling frequencies of the mutant strains.

Effect of chemotactic stimuli on the amount of methyl incorporation into MCP

As seen in Fig. 2, a rapid increase in the concentration of an attractant, L-serine, was accompanied by a 2-fold increase in the amount of label found in wild-type MCP, followed by a gradual decline to the baseline level. A parallel water addition had little or no effect. An identical stimulus was given to a strain isogenic to the wild-type except for a *cheD* mutation which renders the mutant virtually unresponsive to this concentration of L-serine in spatial gradients (6, [a]). After the stimulus, this mutant showed little or no change in incorporation of label (Fig. 2). Both strains are fully chemotactic in spatial gradients of L-aspartate, an attractant detected by a different chemoreceptor from the one for serine (6). Both responded equally well to a temporal gradient of L-aspartate (final concentration 1.4 mM) by an approximately 2-fold increase in the amount of label found in MCP (data not shown).

Addition of the attractant DL-α-methylaspartate, a nonoxidizable analog of aspartate (6), also increased the methylation of MCP, 1.3-fold at 1.4 mM. Thus the effects of attractants on incorporation are not due simply to oxidation of the attractant.

Time course of methylation and demethylation

Fig. 3 shows a time course of the methylation and demethylation of MCP in wild-type *E. coli*. The incorporation rate is rapid for 10 min, and continues at a reduced rate for at least 75 min more. Addition of a 70-fold excess of unlabeled L-methionine stops apparent radioactive methylation and gives an apparent demethylation or exchange half-time of 17 min. If, however, the methionine is washed away by centrifugation, leading to conditions of methionine starvation, the turnover half-time is somewhat longer, 48 min.

We do not know what fraction of the MCP was still methylated at the start of this experiment. Possibly it is a large fraction, considering the rather long turnover time for demethylation under conditions of methionine starvation. Therefore, the observed methylation may represent a combination of two processes, *de novo*, methylation and exchange. Indeed, a logarithmic plot of the methylation data is biphasic, indicating contributions from at least two processes occurring at different rates.

It has been shown that removal of methionine leads to loss of chemotaxis within a few minutes, and that methionine rapidly restores chemotaxis to methionine-depleted cells (16, 18, 19). Thus, if the methylation reaction plays a role in chemotaxis, it should be rapid, and the donated methyl group should be removed with time. The observed time courses in general fulfill these requirements.

Relationship between MCP and flagella

Since the mechanism that regulates tumbling frequency must ultimately communicate with the flagella, it is possible that some or all of the tumble-regulating machinery may be a part of the flagellum itself. An *E. coli* flagellum consists of a basal body (a rod and four rings) inserted into the cell envelope, a short (45 nm long) hook just outside the cell, and a several micrometer-long filament attached to the hook (27). It is a multicomponent organelle whose synthesis and assembly are under the control of many genes, easily complex

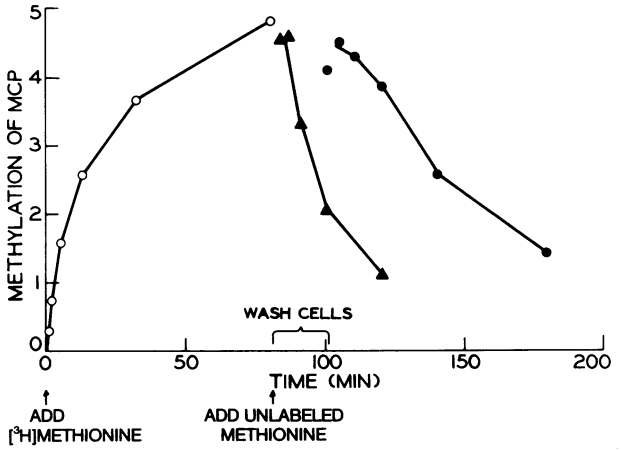

FIG. 3. Time course of methylation and demethylation of MCP in RP477 *metF*. Methylation was carried out as described in *Materials and Methods*, except that the L-[*methyl*-^{3}H]methionine concentration was 7.2 μM to allow for the long time course. After 80 min, a fraction of the cells was washed once in chemotaxis medium plus chloramphenicol and lactate at 22° to remove methionine, and resuspended in the same at 30°; the remainder of the cells were brought to 0.5 mM methionine with unlabeled L-methionine. Samples were prepared as described in *Materials and Methods*. Methylation of MCP, the ordinate, is defined in the legend to Fig. 2. Methylation (○), demethylation after washing (●), and apparent demethylation after chase with unlabeled methionine (▲) are shown.

enough to contain elements of the chemotaxis machinery. If MCP is associated with the flagellum, it is most likely to be found in the basal body, since MCP has been shown to be associated with the cytoplasmic membrane.

With this in mind, a number of *fla* mutants, strains that lack flagella (22, 23), were tested for capacity to methylate MCP, by the procedure described in Table 1. Four *fla* mutants showed, after 20 min, less than 4% of the methylation measured for their wild-type parent. This indicates that the methylation system is part of the flagellar structure, requires such a structure for incorporation into the cell envelope, or is under coordinate control with flagellar synthesis.

It was, therefore, of interest to determine whether flagellin, the protein monomer of the bacterial flagellar filament, is MCP, since flagellin has an apparent molecular weight near 60,000 (close to that of MCP) as analyzed by the electrophoretic system used here (28), and has further been shown to be methylated (29, and references cited there). Four lines of evidence demonstrate that MCP is not flagellin: (*a*) as mentioned earlier, MCP is associated with the cytoplasmic membrane, and thus is not likely to be found in the flagellar filament; (*b*) sheared flagella (30) obtained from cells treated with L-[*methyl*-^{3}H]methionine are not labeled (which shows that the flagellin in filaments cannot be methylated under these conditions); (*c*) when electrophoresed on the same gel, [^{3}H]MCP has a slightly greater mobility than [^{14}C]flagellin isolated from the same strain, indicating a lower molecular weight by approximately 2000; and (*d*) a mutant that does not make flagellar filaments due to failure to make flagellin made 270% as much methylated MCP as an isogenic wild-type strain after 20 min of labeling.

DISCUSSION

In this report we describe the methylation of a protein (MCP) involved in chemotaxis and in the methionine requirement for chemotaxis. The supporting evidence follows:

(*a*) Certain nonchemotactic mutants fail to carry out this

methylation, and others are partially defective. Chemotactic revertants have lost the defects.

(*b*) Addition of attractants affects the methylation of MCP, while a mutant defective in chemotaxis toward a particular chemical is also defective in the methylation caused by addition of that chemical.

(*c*) The source of the methyl group in this reaction is methionine, and the donated methyl group is removed as the methionine is depleted. This is in agreement with the observation that methionine must be continuously present for chemotaxis to occur (15, 16).

(*d*) Mutations in a number of flagellar genes result in defective methylation of MCP. This suggests a role for the methylation reaction in chemotaxis or motility.

Although we are convinced for these reasons that the methylation reaction functions in chemotaxis, we do not know its exact role. This is emphasized by several results that are still unexplained:

(*a*) There is no apparent relationship between the measured methylation of MCP and tumbling frequency or chemotactic ability, except that many strains defective in chemotaxis show little or no methylation.

(*b*) Starvation for methionine leads to a suppression of tumbling (18, 19) and also to demethylation of MCP. Therefore, by analogy, the addition of attractant, which also suppresses tumbling (2, 4), might be expected to lead to demethylation of MCP. This is contrary to the observations reported in Fig. 2. However, though not understood at this time, this finding provides important information about the role of methionine in the mechanism of bacterial chemotaxis.

(*c*) The time course of methylation of MCP, though rapid, is not parallel to the time course of recovery of chemotaxis after the addition of methionine to methionine-starved cells. Fully chemotactic cells are observed within 5 min (18, 19), but the plateau of incorporation is not achieved until after 85 min. (Perhaps a full level of methylation is actually reached within a few minutes, since the protein may already be extensively methylated at the start of the experiment.)

There are many possible explanations for each of these points taken individually; however, taken *in toto* they suggest that the level of methylation of MCP may not directly regulate tumbling frequency or control chemotaxis.

The incorporation of methyl groups into MCP is influenced by a large number of genes that are involved in chemotaxis, motility, and flagellar synthesis. This is not surprising if the methylation of MCP plays a role in the tumble-regulating mechanism. Since tumbling involves a change in direction of rotation of the flagellum (9), there might well be close physical interactions between the tumble-generating mechanism and the flagellum, and these interactions could be influenced by defective flagellar or chemotaxis gene products. In addition, three or more genes could be required for the methylating system, coding for MCP, a methylase, and a demethylase.

Perhaps the most important unanswered question raised by this work is how the methylation of MCP functions in chemotaxis. The discovery of this reaction should allow the identification of any other components involved in methylation and demethylation, should lead to an understanding of the properties of the mutants and the effects of chemotactic stimuli, and may provide an *in vitro* assay for the study of chemotaxis. This should ultimately lead to a better understanding of the information linkage between the chemoreceptors and the flagella.

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