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Attractants and repellents control demethylation of methylated chemotaxis proteins in *Escherichia coli*

(methylation/protein modification/sensory transduction)

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ABSTRACT **A group of methylated proteins, the methyl-accepting chemotaxis proteins (MCP), has been shown to play a central role in bacterial chemotaxis. Both methylation and demethylation of MCP occur continuously in the absence of added stimuli; these two processes are in balance such that a basal level of methylation is maintained. Attractants cause the methylation level to increase to a new value, whereas repellents bring about a decrease in level. Therefore, attractants and repellents must somehow perturb the balance between methylation and demethylation of MCP. In this report the effect of attractants on demethylation of MCP was monitored in two ways: (*i*) by following the disappearance of [*methyl*-^{3}H]MCP and (*ii*) by measuring formation of [^{3}H]methanol, the product of MCP demethylation. Both methods showed that addition of attractants causes a transient inhibition of MCP demethylation. Repellent addition has previously been shown to stimulate MCP demethylation. It is therefore concluded that control of *demethylation* plays a crucial role in changing the level of methylation of MCP in response to attractants and repellents.**

Throughout biology, reversible covalent modifications of regulatory proteins are known to occur (1), and the extent of modification is influenced by stimuli of various sorts (for example, sensory stimuli, hormones, neurotransmitters). How is the extent of modification controlled? In each case there is an enzyme that puts on the modifying group and another that takes it off. Perhaps the most studied case is protein phosphorylation and its modification enzymes, the protein kinases and protein phosphatases (1).

One simple system in which covalent modification of proteins plays an important regulatory role is bacterial chemotaxis (ref. 2 and other reviews cited there). Bacteria are able to detect chemical gradients in their environment by means of specific chemoreceptors (3), and consequently they alter their swimming pattern (4–7) through controlling the direction of rotation of their flagella (8–10). A protein methylation reaction that utilizes methyl groups of L-methionine has been shown to play a crucial role in chemotaxis by bacteria (2, 11–16). Methyl groups of L-methionine are transferred, via S-adenosylmethionine and a methyltransferase enzyme (15), to carboxyl groups of glutamic acid residues (17, 18) of several cytoplasmic membrane proteins, the methyl-accepting chemotaxis proteins (MCP) (11–13, 16). A methylesterase that removes methyl groups from MCP has also been identified (19), and the product of MCP demethylation has been shown to be methanol (19, 20).

In the absence of added stimuli, cells maintain a basal level of methylation of MCP (11, 14). This level is altered by attractants and repellents (11–14, 16). An increase in the concentration of attractants or a decrease in the concentration of repellents leads to an increase in the level of methylation, while a decrease in the concentration of attractants or an increase in the concentration of repellents causes a decrease in the methylation level (2, 11–14, 16). These changes in the level of methylation of MCP are involved in terminating the response to the stimuli (14, 21–23), the process of sensory adaptation.

Little is known at the molecular level about the way in which changes in binding of stimuli to chemoreceptors lead to changes in the level of methylation of MCP. In this report we show that one effect of addition of attractants to *Escherichia coli* is a transient, essentially total inhibition of demethylation of MCP. We also discuss previously published results which show that addition of repellents causes an increase in MCP demethylation (14, 20). Together, these data show that control of MCP demethylation plays a major role in bringing about changes in the level of methylation of MCP in response to stimuli and hence in controlling the behavioral response. Whether chemical stimuli also alter the methylation process has not yet been studied.

MATERIALS AND METHODS

Chemicals. L-Arginine, L-histidine, L-leucine, L-lysine, L-methionine, and L-threonine were obtained from Calbiochem. α-Aminoisobutyric acid, α-methyl-DL-aspartic acid, and α-methyl-DL-glutamic acid were obtained from Sigma and DL-serinol was purchased from Nutritional Biochemicals. L-[*methyl*-^{3}H]Methionine was purchased from New England Nuclear at approximately 15 Ci/mmol (1 Ci = 3.7×10^{10} becquerels) and diluted 1:2 to 1:5 with nonradioactive L-methionine prior to use. For experiments in which [^{3}H]methanol formation was measured, L-[*methyl*-^{3}H]methionine was taken to dryness under vacuum to remove volatile ^{3}H-labeled contaminants. [^{14}C]Methanol and [^{3}H]methanol were purchased from New England Nuclear.

Preparation of Bacteria. The bacterial strains used here either have been described previously (16, 20, 22) or are described in the legend to Table 1. All these strains require L-histidine, L-leucine, and L-threonine for growth; all except OW1 and AW660 also require L-methionine. Bacteria were grown at 35°C in minimal medium (25) containing D-ribose (0.2–0.5%) as carbon and energy source and L-histidine, L-leucine, L-methionine, and L-threonine (1 mM each). Cells were harvested and washed as described (23).

Experimental Procedures. The washed cells were resuspended to 3×10^8 cells per ml in chemotaxis medium (10 mM potassium phosphate, pH 7.0/0.1 mM potassium ethylenediaminetetraacetate) containing sodium DL-lactate (10 mM) and

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Abbreviation: MCP, methyl-accepting chemotaxis proteins.

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Table 1. [^{3}H]Methanol formation from L-[*methyl*-^{3}H]methionine by *E. coli*

Strain*	Chemotaxis genotype	Chemotaxis defect	Methanol formed, ^{3}H cpm†
RP487	Wild type	None	491 (±93, n = 9)
OW1	Wild type	None	1001
AW670	*flaI*	Lacks motility and chemotaxis proteins	53 (±13, n = 5)
AW660	*tsr tar trg*	MCP I$^-$, MCP II$^-$, MCP III$^-$ ‡	45
RP4332	*cheX202*	MCP methyltransferase$^-$	74
RP4333	*cheX203*	MCP methyltransferase$^-$	79
AW677	*cheB287*§	MCP methylesterase$^-$	53
AW678	*cheB294*§	MCP methylesterase$^-$	63

Cells were incubated with L-[*methyl*-^{3}H]methionine (4 μM, 6 Ci/mmol) for 30 min and then treated with trichloroacetic acid and centrifuged. [^{3}H]Methanol in 3 ml of the trichloroacetic acid-soluble fraction was then determined. Recovery of authentic [^{3}H]methanol in the center well under these conditions was 29%.

* AW660 is in the same genetic background as OW1, whereas the other mutant strains listed are in the same genetic background as RP487.

† Radioactivities of samples were measured for 10 min each. Values presented are the average of all determinations. For RP487 and AW670, the standard deviation and number of determinations are shown. All other values are the average of either two or three determinations; the deviation of experimental values from the average was never more than 20%.

‡ MCP I, MCP II, and MCP III are the three known types of MCP polypeptides (12, 13, 16).

§ Strains AW677 and AW678 were constructed by transferring the *cheB* mutation from strain e21e1 (11) [which is the same as *cheB287* (24)] or *cheB294* (24), respectively, into strain RP487 by phage P1 transduction, using *eda* as the selected marker.

chloramphenicol (50 μg/ml). After 5-min incubation in this medium at 30°C with rotary shaking, L-[*methyl*-^{3}H]methionine was added. At various times, samples of cells were treated with trichloroacetic acid (5%, final concentration), placed on ice for 15 min, and then centrifuged. The precipitates contain the [*methyl*-^{3}H]MCP, and the supernatant fractions contain the [^{3}H]methanol.

In some experiments, [*methyl*-^{3}H]MCP was measured. The trichloroacetic acid-insoluble precipitates from 3-ml samples were processed and subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis (11% polyacrylamide gels) and fluorography as described (23). The amount of radioactivity incorporated into MCP was determined by scanning the fluorograms with a Photomation scanning microdensitometer (Optronics International Inc., Chelmsford, MA).

In other experiments, formation of [^{3}H]methanol was monitored. An aliquot of the trichloroacetic acid-soluble fraction was placed in the outer compartment of a Conway microdiffusion cell (as described in ref. 26, but without use of K_2CO_3) containing 1 ml of distilled water in its center well. Volatile components were allowed to diffuse into the center well, away from nonvolatile components, which remained in the outer compartment. After 3 hr, at which time equilibrium had been reached, the center well contents were recovered and a 20-μl aliquot was subjected to gas chromatography to separate [^{3}H]methanol from other volatile ^{3}H-labeled products.

Gas chromatography was performed with a Varian Aerograph Autoprep model A-700 equipped with a 1-foot (30 cm) Teflon column [1/8-inch (0.3 cm) outside diameter]. The column packing was 5% silicone SE-30 on 100/120 mesh Chromosorb W-HP (Alltech Associates, Arlington Heights, IL). Helium was used as carrier gas at a flow rate of 32 ml/min, and the injector and detector chambers were at 200°C and 230°C, respectively. The column temperature was maintained at 32°C for 3 min after sample injection, and two 90-sec fractions were collected by bubbling the effluent into 5 ml of Aquasol in scintillation vials. Recovery of authentic [^{14}C]methanol when collected in this manner was 61%. The column was then heated to 165°C for 4 min, and the effluent was collected in one vial containing 5 ml of Aquasol. (For the experiment shown in Fig. 1, 10-sec fractions were collected for 7 min after sample injection.) Radioactivity in the effluent samples was then determined by liquid scintillation counting.

RESULTS

Assays for Demethylation of MCP. Demethylation of MCP in *E. coli* was monitored either by measuring disappearance of the substrate, [*methyl*-^{3}H]MCP, or by measuring formation of the product, [^{3}H]methanol.

Measurement of disappearance of [methyl-^{3}*H*]*MCP during "cold chase."* When L-methionine is present, there is continuous turnover of the methyl groups of MCP in the absence of changes in attractant or repellent concentrations (11, 14). In "cold chase" experiments, a large excess of nonradioactive ("cold") L-methionine is added to cells that have been incubating in the presence of L-[*methyl*-^{3}H]methionine (11, 20). This stops further incorporation of radioactive methyl groups so that the disappearance of radioactivity from MCP under these conditions is a direct measure of the demethylation aspect of methyl group turnover. Radioactivity in MCP was quantitated after gel electrophoresis.

Measurement of formation of [^{3}*H*]*methanol.* The product of demethylation of MCP is methanol (19, 20); hence, measurement of methanol formation due to turnover of methyl

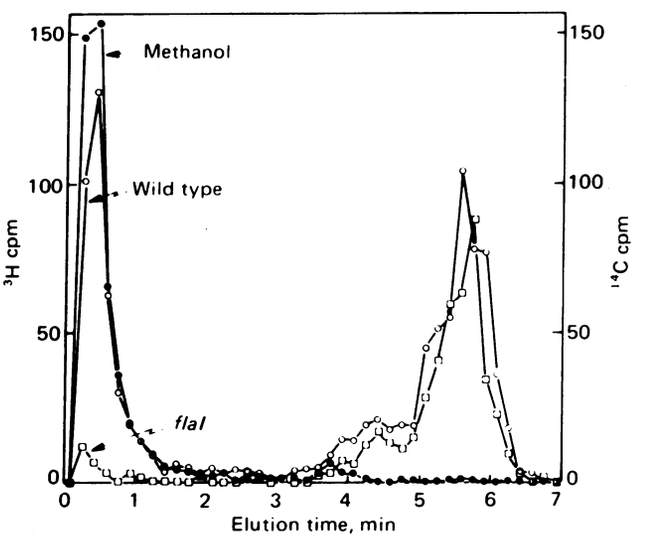

FIG. 1. Gas chromatography of [^{14}C]methanol and the volatile ^{3}H-labeled products formed by cells incubated with L-[*methyl*-^{3}H]methionine. [^{14}C]Methanol was mixed with the volatile ^{3}H-labeled product (from the center well of the Conway cell) formed by the chemotactically wild-type strain RP487, and a 20-μl aliquot was subjected to gas chromatography. In a separate experiment, the volatile ^{3}H-labeled products formed by the nonchemotactic strain AW670 (a *flaI* mutant, which lacks flagella and chemotaxis proteins) were subjected to gas chromatography under identical conditions. Radioactivities of samples were measured for 30 min each. ●, [^{14}C]-Methanol; ○, wild type; □, *flaI*. Recovery of [^{14}C]methanol during gas chromatography was 84%, while recovery of total ^{3}H-labeled products was 86% for wild type and 85% for *flaI*.

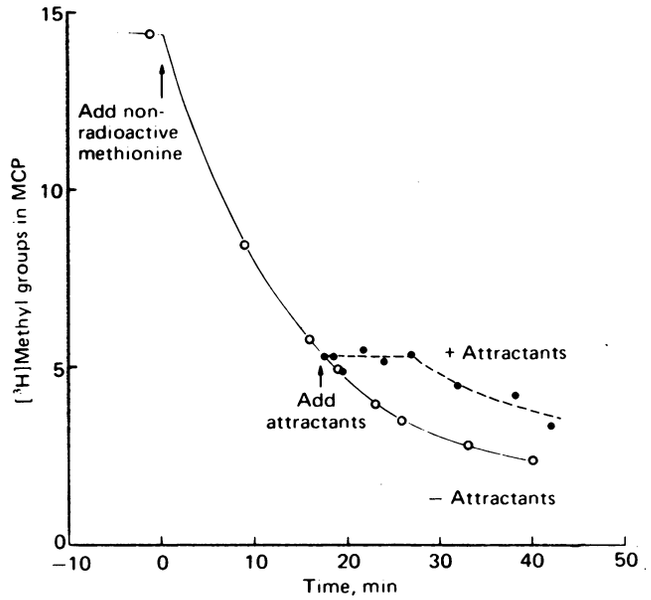

FIG. 2. Effect of attractants on "cold chase" of [*methyl*-^{3}H]MCP. Wild-type cells (RP487) were incubated with L-[*methyl*-^{3}H]methionine (1 μM, 4 Ci/mmol) for 60 min to label MCP to a plateau level. A 1000-fold excess of nonradioactive L-methionine (1 mM) was then added (0 min) to initiate chase of ^{3}H-labeled methyl groups. After 17 min of chase, attractants (50 mM α-aminoisobutyrate plus 5 mM α-methyl-DL-aspartate) were added to one portion of cells (+ attractants, ●– –●) and water was added to another portion as a control (– attractants, O—O). At various times during the experiment, cells were treated with trichloroacetic acid and their MCP [^{3}H]methylation levels were determined after gel electrophoresis. Each point is the average of duplicate determinations. A second experiment (data not shown) gave the same result, except that the period of inhibition of demethylation was 8 min instead of the 10 min shown here.

groups of MCP should provide an assay for the demethylation process. For these experiments, cells were incubated with L-[*methyl*-^{3}H]methionine and production of [^{3}H]methanol was then determined. This particular assay has not been used previously for studies of bacterial chemotaxis[§]; therefore, it is important to first establish that measurement of methanol formation under these conditions provides a valid assay for the demethylation of MCP.

Fig. 1 shows the elution patterns of radioactivity from the gas chromatograph for authentic [^{14}C]methanol and for the volatile ^{3}H-labeled products formed by cells incubated with L-[*methyl*-^{3}H]methionine. These cells form a volatile ^{3}H-labeled product that comigrates with authentic [^{14}C]methanol. The involvement of chemotaxis in formation of this product is demonstrated by comparing the amount formed by chemotactically wild-type cells with the amount formed by various nonchemotactic mutants. As shown in Fig. 1 and Table 1, the *flaI* mutant, which lacks all known chemotaxis-related proteins as well as flagella (28), forms only about 10% of the amount of methanol that is formed by the chemotactically wild-type strain. More specifically, the *tsr tar trg* strain, which lacks functional MCP polypeptides (12, 13, 16), is as deficient in methanol production as the *flaI* strain (Table 1). Furthermore, *cheX* mutants, which are defective in the MCP methyltransferase (refs. 15, 29; unpublished results), and *cheB* mutants, which are missing MCP methylesterase activity (19, 30), also show low levels of [^{3}H]methanol formation relative to wild-type strains (Table 1). These results demonstrate that at least 90% of the methanol formed by chemotactically wild-type bacteria results from turnover of the methyl groups of MCP.

Fig. 1 shows that other volatile ^{3}H-labeled products, in addition to [^{3}H]methanol, are produced by cells incubated with L-[*methyl*-^{3}H]methionine. Formation of this material does not appear to be related to chemotaxis: the *flaI* mutant produces nearly as much as the wild-type strain (Fig. 1), and even in the wild type formation of this material is unaffected by chemical stimuli (data not shown).

Effect of Attractants on Demethylation of MCP. The two assays described above were used to study the effect of addition of attractants on demethylation of MCP.

Results obtained with the "cold chase" assay. Demethylation of MCP occurs continuously in the absence of any added chemical stimuli, as shown by "cold chase" experiments (11, 20; – attractants in Fig. 2). The nonmetabolizable attractants (31) α-aminoisobutyrate and α-methylaspartate (added together for maximal effect) caused a rapid and severe inhibition of the demethylation process (+ attractants in Fig. 2). This inhibition was transient; after 8–10 min, demethylation resumed even though the attractants were still present.

Results obtained with the methanol formation assay. In the

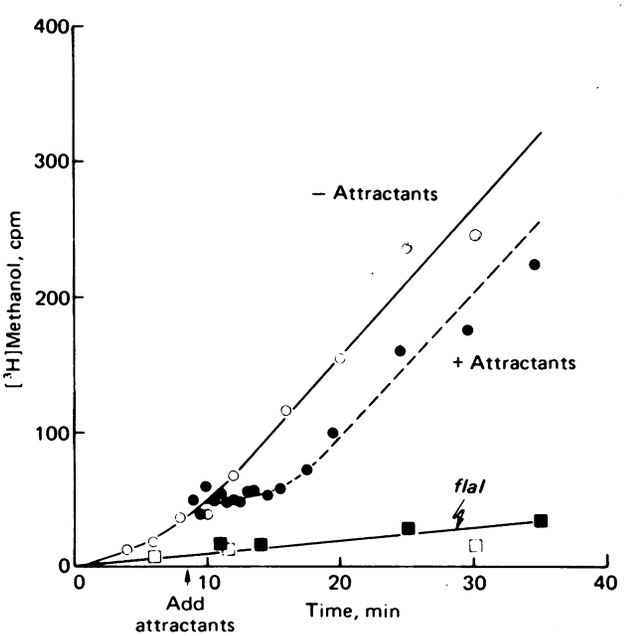

FIG. 3. Effect of attractants on [^{3}H]methanol formation. Wild-type cells (RP487) and a *flaI* mutant (AW670) were incubated with L-[*methyl*-^{3}H]methionine (4 μM, 3 Ci/mmol). After 8½ min (arrow), attractants (50 mM α-aminoisobutyrate plus 5 mM α-methyl-D-L-aspartate) were added to one portion of each strain (+ attractants: ●– –● for wild type, ■—■ for *flaI*) and water was added to a second portion as a control (– attractants: O—O for wild type, □—□ for *flaI*). At various times during the experiment, cells were treated with trichloroacetic acid and centrifuged. [^{3}H]Methanol in 2 ml of the trichloroacetic acid-soluble fraction was measured. Recovery of authentic [^{3}H]methanol in the center well under these conditions was 24%. The results shown are from one experiment; these results have been confirmed in seven similar experiments. We wish to emphasize that the rate of methanol formation in attractant-stimulated cells after the transient inhibition was the same as that in cells not treated with attractant. In the five experiments with sufficient data for analysis, the two rates varied by 14% (data from this figure), 0%, 0.4%, 1.0%, and 1.6%.

[§] It should be pointed out that this assay measures methanol formed *in vivo* from turnover of methyl groups of methylated proteins in the presence of methionine, in contrast to a previous assay (20), which measured methanol formed from MCP in bacteria first washed free of radioactive methionine. Both of these assays are distinct from a measure of methylated proteins by alkaline hydrolysis to produce methanol as in ref. 27.

wild-type strain, continuous demethylation of MCP in the absence of added stimuli was also seen with the methanol formation assay (− attractants in Fig. 3). When the attractants α-aminoisobutyrate and α-methylaspartate were added together to these cells, there was an immediate decrease in the rate of methanol formation (+ attractants in Fig. 3) to the rate seen in *flaI* cells (*flaI* in Fig. 3). This indicates that MCP demethylation was completely or nearly completely inhibited. This inhibition was transient; after 8–10 min, the attractant-stimulated cells formed methanol at the same rate as the unstimulated cells.

Several lines of evidence support the idea that this inhibition of demethylation is related to chemotaxis. (*i*) Attractants produce the inhibition (Figs. 2 and 3), whereas chemicals that are neither attractants (31) nor repellents (32) (50 mM serinol plus 10 mM α-methylglutamate or 10 mM lysine plus 10 mM arginine) fail to inhibit demethylation (only tested in the methanol formation assay, data not shown). (*ii*) In both of the demethylation assays, the inhibition of MCP demethylation by α-aminoisobutyrate and α-methylaspartate shows the same specificity with respect to *tsr* and *tar* mutants as does the chemotactic response (data not shown). The *tsr* mutants are attracted to α-methylaspartate but not to α-aminoisobutyrate (12, 31); α-methylaspartate causes an inhibition of demethylation of MCP in a *tsr* mutant, whereas α-aminoisobutyrate is ineffective. Similarly, the *tar* mutants are unable to respond chemotactically to α-methylaspartate but are attracted to α-aminoisobutyrate (12, 31), and α-methylaspartate does not cause inhibition of demethylation in a *tar* mutant whereas α-aminoisobutyrate does.

DISCUSSION

The basal level of methylation of MCP (methyl-accepting chemotaxis proteins) observed in the absence of stimuli represents a steady state in which the methylation and demethylation processes occur at the same rate (11, 14). Attractants and repellents in some way perturb this balance and thereby cause the level of methylation of MCP to change (2, 11–14, 16). Addition of attractants brings about an increase in the level of methylation and addition of repellents a decrease in the methylation level (11–14, 16). The new level again represents a steady state, with methylation and demethylation once more in balance. This new level of methylation is maintained as long as the attractants or repellents remain in the cells' environment (14). Removal of the stimuli causes a return to the unstimulated basal level of methylation (2, 14).

How is the level of methylation altered by chemical stimuli? Addition of attractants could increase the level of methylation by stimulating methylation, inhibiting demethylation, or both. Similarly, addition of repellents might stimulate demethylation or inhibit methylation or both.

With regard to repellents, previously published results show that they stimulate demethylation of MCP. (*i*) The decrease in level of methylation of MCP that occurs in response to repellents is rapid, with the new steady-state level being reached within 20 sec (14). If repellents only inhibited methylation, and demethylation continued at the unstimulated rate (as measured by "cold chase"; see ref. 11 or Fig. 2), more than 10 min would be required to reach the level of methylation actually achieved (14) in repellent-stimulated cells. This suggests that repellents activate demethylation at least 30-fold above the unstimulated rate. (*ii*) A rapid demethylation of MCP in response to addition of repellents is seen even in cells depleted of L-methionine (20). Because methylation does not occur when cells are depleted of L-methionine (14), the effects of repellents on the level of MCP methylation in these cells must be exerted on the demethylation step.

In the experiments reported here, we have studied the effects of attractants on MCP demethylation. Demethylation of MCP was monitored by two independent assays, one that measures disappearance of the substrate, [*methyl*-^{3}H]MCP, and another that measures formation of [^{3}H]methanol, the product of [*methyl*-^{3}H]MCP demethylation. In both assays, addition of attractants was found to cause a transient severe inhibition of demethylation of MCP.

It is not known whether the stimulation of demethylation caused by repellents and the inhibition of demethylation caused by attractants are due to changes in the activity of the demethylating enzyme or are due to changes in the susceptibility of the MCP substrate sites for demethylation. Whether changes in the rate of *methylation* of MCP also occur in response to stimuli has not yet been determined.

It is interesting that, after the period of essentially complete inhibition of demethylation of MCP, demethylation resumes at the same rate as in unstimulated cells (Fig. 3), even though the level of methylated MCP is now nearly twice as high in stimulated cells (14). One explanation for this is that the increase in methylated MCP, the substrate for demethylation, is just balanced by a partial but permanent inhibition of the demethylating enzyme; thus the rate of demethylation would be the same as that found prior to stimulation. A second possibility is that the demethylating enzyme is saturated with substrate (methylated MCP) even in the unstimulated state, and it is therefore operating at its maximum velocity. [The rapid demethylation of MCP that occurs in response to addition of repellents (14) would seem to argue against this idea of demethylation occurring at maximum velocity in the unstimulated state. However, the demethylation that occurs in response to repellents does not require methionine (20), whereas turnover demethylation does (14); perhaps two different demethylation processes are involved.]

What role does the inhibition of demethylation play in chemotaxis? The behavioral response of cells to chemical stimuli is transient: after some time, the cells stop responding even though the stimuli remain present (5, 6, 33, 34), a phenomenon called sensory adaptation. Increases and decreases in the level of methylation of MCP have been shown to be involved in adaptation to addition of attractants and repellents, respectively (14, 21–23). In fact, the time required for behavioral adaptation to a stimulus has been shown to approximately equal the time required for the new level of methylation of MCP to be reached (14). For the attractant combination used in the experiments reported here, the duration of the inhibition of demethylation (8–10 min, Figs. 2 and 3) is approximately equal to the adaptation time for the behavioral response (10–12 min, determined as in ref. 5), and hence approximately equal to the time required for the new level of methylation to be established. It therefore seems likely that the transient inhibition of demethylation upon addition of attractants brings about the increase in level of methylation and is centrally involved in controlling behavioral adaptation in these cells.

Furthermore, there is an apparent relationship between MCP demethylation activity and the swimming behavior of cells. (*i*) Demethylation is inhibited during periods of smooth swimming (after attractant addition, ref. 5) and stimulated during periods of tumbling (after repellent addition, ref. 6). (*ii*) Demethylation activity is the same in unstimulated cells as in cells adapted to attractants; the tumbling frequency is also the same in these two conditions (5, 33, 34). (*iii*) In the absence of added stimuli, demethylation of MCP does not occur in cells depleted of L-methionine (14), and it has been known for some time that these cells exhibit smooth swimming (35–37). Thus, smooth swimming occurs when the rate of demethylation is low, and

tumbling occurs when the rate of demethylation is high. One possible explanation for this is that methanol formation from demethylation of MCP is the signal for tumble generation. A second possibility is that control of tumbling frequency and control of demethylation activity are both mediated by the same regulator.

The methyltransferase enzymes involved in protein-carboxyl methylation in mammals have been identified and characterized (38, 39). A protein methylesterase has recently been discovered in mammalian tissues as well (40). It will be interesting to learn if control of demethylation is of major importance in regulating cellular responses in eukaryotic systems such as these. However, in all higher organisms studied to date the methylated proteins are highly unstable (38, 39) and difficult to analyze directly. Thus, an *in vivo* assay that uses methanol formation as a measure of demethylation, such as described in this paper and refs. 27 and 41, may prove particularly useful.

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