Sensory transduction in *Escherichia coli*: Role of a protein methylation reaction in sensory adaptation

(bacterial chemotaxis/protein modification/electrophoresis)

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Communicated by Arthur Kelman, September 6, 1977

ABSTRACT The behavioral response of *Escherichia coli* to the addition of a stimulatory compound is transient; thus the organism undergoes sensory adaptation. When the compound is removed, *E. coli* undergoes the inverse process, called de-adaptation, and very rapidly regains its sensitivity to the stimulus. In this communication we demonstrate that the previously reported methylation of several cytoplasmic membrane proteins is correlated with, and very likely controls, the state of adaptation of the cell.

In the absence of an added stimulus these proteins are methylated to a basal level. When the bacteria are stimulated by the addition of an attractant, the extent of methylation increases over a period of several minutes to a new level, which is maintained as long as the attractant is present. The magnitude of the increase in methylation is a function of the size of the stimulus and is directly proportional to the duration of the behavioral response. Upon removal of the attractant the level of methylation very rapidly falls to the basal value. Previously we have shown that adaptation requires methionine, but maintenance of the adapted state and de-adaptation do not [Springer, M. S., Goy, M. F. & Adler, J. (1975) *Proc. Natl. Acad. Sci. USA* 74, 183-187]; here we demonstrate that methylation requires methionine but maintenance of an attractant-induced level of methylation and the demethylation that occurs following removal of the attractant do not. These results strongly indicate a role for protein methylation in sensory adaptation.

Bacterial chemotaxis is a behavioral response to variations in the chemical composition of the environment. As in a eukaryotic receptor cell, stimuli are detected by interaction with specific receptor sites. Subsequently, the information obtained from these interactions is converted from one form to another until it ultimately leads to a change in the output of the cell. This conversion process is known as sensory transduction.

In the bacterium *Escherichia coli* the sensory transduction process controls the swimming behavior of the cell. Unstimulated bacteria swim in smooth lines interrupted at random intervals by a tumbling motion that abruptly alters the direction of travel (1, 2); smooth swimming is accomplished by means of counterclockwise rotation of the flagella, while tumbling is due to clockwise rotation (3-5). When presented with a stimulus, specifically a change in chemical concentration, the cells respond by altering the frequency at which they tumble (2, 6, 7). The addition of attractants leads to the suppression of tumbling (2, 7), while addition of repellents has the opposite effect, namely an enhancement of tumbling (6). However, these responses are transient: the tumbling frequency eventually returns to the pre-stimulus level even though there is no further change in the concentration of the stimulatory chemical (2, 6). This decline in response, known as sensory adaptation, is characteristic of the transduction process in many sensory systems (8).

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The length of time that the cell responds, called the adaptation time, has been shown for attractants to be directly proportional to the change in the fraction of receptor binding sites occupied as a result of the stimulus (9). Thus, the response reflects the magnitude of the concentration change.

In the adapted state cells have fully lost their sensitivity to the continued presence of the stimulus, and they will maintain this insensitivity as long as the stimulatory chemical remains in the environment. However, even though adapted cells have stopped responding to the stimulus, some component of the chemotaxis machinery still registers the fact that the stimulus is present. This is simply demonstrated by removing the chemical: the removal of an attractant from cells that have already responded and adapted to it is followed by a second behavioral response, in this case an increase in tumbling frequency (2).

Furthermore, adapted cells sense exactly how much of the chemical is present. The magnitude of the response following a change in attractant concentration is dependent on the initial concentration to which the cells have already adapted. For example, the length of response to sufficient attractant to give 50 mM final concentration becomes progressively shorter if the cells have been previously adapted to 0, 0.5, or 5 mM of the same compound (9, 10). Therefore, in response to the addition of an attractant some part of the sensory transduction machinery of the cell must undergo a long-term change that reflects the magnitude of the stimulus. This change is maintained even after the behavioral response is ended, and lasts as long as the attractant is present.

However, when the attractant is removed this change in the transduction machinery must be reversed, because sensitivity to the chemical is restored. This phenomenon is the inverse of adaptation and we refer to it as de-adaptation. In *E. coli*, characteristic asymmetry exists in the properties of the sensory transduction machinery: adaptation to attractants occurs slowly, often requiring many minutes for completion, whereas de-adaptation is very rapid. Within a second or two after removal of the attractant the cells have fully regained their sensitivity to a subsequent addition of the same stimulus (10).

What are the biological mechanisms that underlie these phenomena? Several years ago it was discovered that L-methionine is absolutely required for chemotaxis in *E. coli* (11). Subsequently it was shown that sensory adaptation involves a methionine-dependent process, but that maintenance of the adapted state and de-adaptation are both methionine-independent (12). Thus, methionine appears to play a role in the biochemistry of the sensory adaptation process. Previously we

Abbreviations: AIBu, α -aminoisobutyrate; MCP, methyl-accepting chemotaxis proteins.

[†] The first two authors have contributed equally to this work and the order, therefore, was arbitrarily chosen.

had discovered that methionine serves as the methyl donor for a protein-methylation reaction that is involved in the chemotactic response (13). In this paper we demonstrate that the methylation reaction appears to regulate the state of adaptation of the cell.

MATERIALS AND METHODS

Chemicals. L-Threonine, L-leucine, L-histidine, L-methionine, L-aspartic acid, and α -aminoisobutyric acid (AiBu) were obtained from Calbiochem (A grade). L-[methyl- ^3H]Methionine was purchased from Amersham-Searle and New England Nuclear at approximately 15 Ci/mmol and diluted 1:6 with unlabeled methionine before use. All other chemicals were reagent grade.

Bacteria. All experiments were performed with *E. coli* strain RP477 *metF* (13). This strain is chemotactically wild type and requires threonine, leucine, and histidine, as well as being *metF*.

Preparation of Samples. Cells were grown, washed, and resuspended as described previously (14). Radioactive methionine was added ($1.33 \mu\text{M}$ per 3×10^8 bacteria/ml per 20 min.), and cells were subjected to the addition or removal of chemical stimuli, chloramphenicol, and/or methionine, as indicated in the figures. At various times 5-ml samples were taken, fixed with formalin, and prepared for electrophoresis as described previously (14).

Electrophoresis, Fluorography, and Quantitation. Slab gel electrophoresis was performed on 12% acrylamide gels (24×0.15 cm) as described by Laemmli (15). Gels were prepared for fluorography (16) and exposed to film for 2–3 days at -70° . The region of interest in the developed film was scanned with a Joyce-Loebl scanning microdensitometer and the area under the curve was measured. This area is proportional to the amount of radioactivity present. The “level of methylation” of each sample is the amount of radioactivity in the methyl-accepting chemotaxis proteins of that sample divided by the amount of protein that was applied to the gel, as determined by the method of Lowry *et al.* (17). [In some experiments (Figs. 2 and 4 and the additivity experiments described in *Results*), samples were prepared and electrophoresed by a different, but equivalent, procedure (13)].

The methyl-accepting chemotaxis proteins studied here have been shown to be the products of two genes, called *tsr* and *tar* (14, 18). Each of these genes codes for several methylated protein bands of very similar molecular weights (14, 18). Because we could not achieve sufficient resolution to present quantitative data for each protein band, we have summed the radioactivity of the entire set and report data for the aggregate. Thus, it is important to bear in mind that although the bulk of the methylation shows the characteristics described below, nevertheless a minor component, represented by one or more bands, may differ significantly in its properties.

RESULTS

Previous publications have described the methylation of proteins in the cytoplasmic membrane of *E. coli* that are involved in chemotaxis (13, 14, 18). These proteins, collectively called MCP for methyl-accepting chemotaxis proteins, can be divided into two functional units, MCP I and MCP II, which are the products of different genes (14, 18). Early work indicated that the level of methylation of these proteins changes when chemotactic stimuli are presented (13). Some stimuli alter the level of methylation of MCP I, while other stimuli alter the level of methylation of MCP II (14, 18). We have initiated a detailed

FIG. 1. Effect of attractants on the methylation of MCP. Tritiated methionine was added at time zero. After 40 min of incubation the culture was divided in two. One group of cells (O) was stimulated by addition of the attractant AiBu (50 mM final concentration), while the other group of cells (●) received no addition. The concentration of AiBu was subsequently lowered by diluting the adapted cells 100-fold into medium containing no AiBu (Δ). A 100-fold dilution of the adapted cells into medium containing AiBu had no effect on the level of methylation (data not shown). Results are the average of two experiments. For ease of comparison, the data in all figures have been normalized so that the basal level of methylation is the same.

investigation of the methylation properties of these proteins with the goal of defining their role in the chemotactic response. Although most of the stimuli used in this work affect MCP I, the properties of MCP II appear to be entirely similar (data not shown).

Effects of Attractants on the Methylation of MCP. Fig. 1 illustrates the methylation properties of MCP. Upon the addition of labeled methionine, radioactive methyl groups were incorporated into MCP until a plateau was reached, indicating that these cells maintain a basal level of methylation in the absence of added stimuli. When 50 mM of the attractant AiBu was given, the level of methylation rose, with a half-time of 3 min, until a new plateau was established. This is a long-term effect, because the new level of methylation was maintained without decrement for more than 50 min.[§] In contrast, the behavioral response to this concentration of AiBu lasted for only about 5 min. However, the increase in methylation is not irreversible: removal of the attractant led to a very rapid demethylation to the basal level (Fig. 1). Within 15 sec, the earliest point at which a measurement could be obtained, the effect of the attractant had been completely reversed.

For a given attractant the magnitude of the increase in methylation is related to the size of the stimulus and is directly proportional to the duration of the behavioral response to the same stimulus. This is seen in Fig. 2, which shows both properties plotted against attractant concentration. The two functions can be superimposed, strongly suggesting a relationship between them.

Examination of the kinetics of the reaction shows that the half-time of methylation apparently reflects the concentration of the attractant. Data for the time course of methylation in

[§] This appears to contradict previously published data which indicate that, following addition of an attractant, a transient rise and fall in the level of methylation occurs (13); however, in the earlier experiment the possibility that the decline in the level of methylation was due to metabolism of the attractant was not ruled out. In Fig. 2 we use a non-metabolizable attractant to demonstrate that in the absence of metabolism there is a sustained change in the level of methylation of MCP after an attractant stimulus. This has been confirmed with a variety of attractants, including metabolizable attractants at concentrations so high that metabolism cannot significantly affect the concentration of the stimulus (data not shown).

FIG. 2. Increase in methylation of MCP and duration of the behavioral response as functions of AiBu concentration. In these experiments the cells were stimulated by raising the concentration of AiBu from zero to the value listed on the abscissa. The behavioral responses (O) were determined by the moving boundary method of M. S. Springer (unpublished). To determine the methylation levels, cells were incubated for 30 min with tritiated methionine, then the culture was split into aliquots, each of which received a different concentration of AiBu or no addition. After 30 min of additional incubation the reactions were terminated and the methylation levels (●) were measured. Each value is the average of five determinations (the standard deviation is about 10%) and is plotted as the percent increase of methylation relative to the unstimulated level. Day-to-day variations in the length of the behavioral response have been observed (12). Therefore, it is not surprising that the percent change of methylation in some of the other experiments is greater than expected from the data presented here. The solid line is derived from the law of mass action, assuming a dissociation constant K_D of 5 mM. The fact that the experimental points fall on this line is consistent with the idea that both adaptation time and extent of methylation depend on the change in the degree of binding of AiBu to a chemoreceptor with this K_D .

response to the addition of 5 and 100 mM AiBu are presented in Fig. 3. The ratio of the half-times of these reactions was 0.6. This is close to the value 0.55 obtained from the ratio of the adaptation times to the same stimuli (Fig. 2). Thus, the half-time of the reaction, like the magnitude of the increase in methylation, appears to be related to the duration of the behavioral response.

Effects of Repellents on the Methylation of MCP. Because attractants and repellents produce opposite behavioral effects, the methylation response to a repellent might be anticipated as a decrease in methylation from the basal level, rather than the increase observed for attractants. Fig. 4 shows this to be so. Here again, a long-term change in methylation was observed. The new level of methylation was maintained for more than 30 min, while the behavioral response lasted for only about 60 sec.

Additivity of Stimuli. In bacteria, responses to stimuli detected by different types of chemoreceptors are additive, permitting a single integrated response when several stimuli are encountered simultaneously (6, 10, 19). Accordingly, we tested the effect on the stimulation of methylation by AiBu alone, α -methylaspartate alone, and AiBu and α -methylaspartate given simultaneously. The length of the behavioral response to simultaneous addition of these compounds is the sum of the responses to the compounds presented individually (10). Using concentrations that saturate the chemoreceptors, we found that the final level of methylation, in arbitrary units, was 6.8 ± 0.8 , 8.7 ± 0.7 , 11.1 ± 0.7 , and 13.0 ± 0.7 for unstimulated, α -methylaspartate-stimulated, AiBu-stimulated, and AiBu plus α -methylaspartate-stimulated cells, respectively (each value

FIG. 3. Kinetics of the attractant-stimulated increase in methylation of MCP. Tritiated methionine was added and the cells were incubated for 50 min. The culture was then divided into three parts. One group of cells was stimulated by addition of 5 mM AiBu (●), the second was stimulated by addition of 100 mM AiBu (O), and the third served as the unstimulated control. Zero time refers to the time at which the attractant was added. Results are the average of two experiments. Data are plotted as the percent increase of methylation compared to the unstimulated level. We find half-times for the reactions of 2.0 min (5 mM) and 3.5 min (100 mM).

is the mean $\pm$ the standard deviation, $n = 6$), indicating additivity of the methylation responses.

Repellent and attractant stimuli are also integrated by bacteria (6, 10, 19). However, these stimuli have opposing polarities: the response to an attractant can be progressively reduced if greater and greater repellent stimuli are presented simultaneously with the attractant (6, 10, 19). When the repellent stimulus described in Fig. 4 is given simultaneously with a saturating concentration of the attractant AiBu, the resulting methylation level is intermediate between the levels observed if either stimulus is presented alone: the levels for repellent alone, attractant alone, attractant plus repellent, and unstimulated cells are, in arbitrary units, 3.7 ± 0.3 , 10.1 ± 1.0 , 6.0 ± 0.3 , and 6.8 ± 0.4 , respectively (each value is the mean $\pm$ the standard deviation, $n = 6$). This indicates that attractants and repellents produce an integrated effect on the extent of methylation of MCP.

FIG. 4. Effect of the addition of repellents on the methylation of MCP. Tritiated methionine was added and the cells were incubated for 30 min. The culture was then split into two parts, one of which received no addition (●) and one of which was stimulated (O) by addition of a mixture of repellents (0.3 mM indole + 17 mM L-leucine + 17 mM sodium acetate). Zero time refers to the time at which the stimulus was added. The first sample was obtained from the stimulated culture 20 sec after this addition. We have not studied the effect of removal of repellent on methylation.

DISCUSSION

When a chemotactic stimulus is presented to a bacterial cell, a change in swimming behavior is immediately observed. However, as the cell adapts to the stimulus the swimming behavior returns to normal. Therefore, some component of the sensory transduction machinery must deliver a transient signal to the motor apparatus of the cell, in order to regulate the behavioral response.

In 1975 we discovered a protein methylation reaction that is involved in chemotaxis, and we demonstrated that chemotactic stimuli could affect the level of methylation of the protein substrate, called MCP (13). Methylation of MCP, therefore, is a possible candidate for this type of signal. However, Figs. 1 and 4 demonstrate that there is a long-term change in the level of methylation of MCP after a stimulus, in contrast to the short duration of the behavioral response. Thus, the level of methylation *per se* cannot be the transient signal that controls the length of the response. Instead, we would like to propose that the level of methylation of MCP plays a role in the *generation* of this signal, by regulating the process of sensory adaptation. The following lines of evidence indicate a role for the methylation of MCP in sensory adaptation.

First, as described in the introduction, sensory adaptation involves a long-term change in some property of the transduction machinery. This property regulates the cell's sensitivity to the addition of a new stimulus, by reflecting the presence (and the magnitude) of stimuli already in the environment. The characteristics of the methylation reaction are compatible with such a role: the addition of attractant produces an increase in methylation that lasts as long as the attractant remains in the environment, but no longer (Fig. 1). This long-term change is a measure of the amount of the attractant that is present (Fig. 2).

Second, the length of the behavioral response appears to be related to the time required for the methylation system to reach the new level of methylation following a stimulus (see analysis of Fig. 3 in *Results*).

Third, the reaction exhibits the asymmetry of the adaptation and de-adaptation process. Behaviorally, adaptation to an attractant stimulus occurs relatively slowly compared to the de-adaptation that follows removal of the same stimulus (10). The methylation reaction shows strikingly similar asymmetric kinetics (Figs. 1 and 3): methylation following addition of 50 mM AiBu occurs very much slower than the demethylation that follows removal of the same stimulus.

Fourth, the methylation reaction, like the behavioral response, shows additivity if several stimuli are presented simultaneously. Furthermore, attractants and repellents have opposing effects on methylation, just as they do on behavior.

All of the evidence presented thus far is circumstantial in nature, and shows only a correlation between methylation of MCP and the process of adaptation. However, it is possible to demonstrate the relationship between these two phenomena more directly, by blocking methylation. If the methylation reaction actually regulates adaptation, such a blockade should prevent the adaptation process from occurring.

We have blocked methylation in two ways, and in both cases we have found that, as expected, adaptation was prevented. Our first method was to deprive cells of methionine, the methyl donor in the reaction. When cells were starved for methionine the methylation reaction was indeed blocked: Fig. 5A shows that when an attractant stimulus was given to such cells there was no increase in methylation, even if the cells were incubated with the attractant for a time longer than it would have taken for them to adapt if methionine had been available. However,

as soon as methionine was added a typical attractant-induced methylation increase was seen. When methionine-starved cells were tested for the ability to adapt, it was found that adaptation could not occur until methionine was added to the culture (12). Thus, adaptation is very likely dependent on methylation. Furthermore, it was demonstrated that once adaptation has taken place in the presence of methionine, the removal of methionine did not cause cells to de-adapt (12). This indicates that maintenance of the adapted state is methionine-independent. Fig. 5B demonstrates that maintenance of an attractant-induced increase of methylation also does not require methionine.[†] Similarly, both de-adaptation (12) and demethylation (Fig. 5B) following removal of the attractant are methionine-independent.

Our second method to block methylation involved the use of a mutant that is genetically defective in the ability to methylate MCP. A *cheX* strain that has the methyl-accepting polypeptides present in the cytoplasmic membrane but cannot carry out methylation (refs. 14 and 18; M. L. Toews and S. J. Kleene, unpublished observations) was tested for ability to adapt to stimuli. We found that such a mutant responded to the addition of attractants or repellents, but that the response was incessant; i.e., the mutant could not adapt to the stimulus (M. F. Goy, M. S. Springer, and J. Adler, unpublished observations). Similar results have been obtained by J. S. Parkinson (unpublished observations). Thus, by this method as well, adaptation is found to be dependent on the ability to methylate MCP.

The results with the *cheX* mutant indicate further that the initiation of the chemotactic response (which we call "excitation") is not dependent on the methylation of MCP. However, other mutants (*tar*, *tsr*), which fail to synthesize the MCP polypeptides, cannot even initiate responses to stimuli (14, 18). Therefore, MCP plays a central role in the chemotactic response, not only in adaptation but also in excitation. It must be that some property of MCP other than methylation, for instance a possible second covalent modification, is involved in excitation.

How, then, might MCP function in bacterial chemotaxis? When the cell is presented with an attractant stimulus, the receptors immediately signal MCP to initiate the chemotactic response. Simultaneously, the receptors indicate that an increase in methylation of MCP is required, and the methylating system is activated. As long as there is a difference between the required level of methylation and the actual level of methylation the cell responds. However, the addition of methyl groups to MCP tends to counteract the effect of excitation. Thus, when the actual level of methylation reaches the required level the response terminates and the cell is adapted. Nevertheless, the cell detects the continued presence of the attractant, because the new level of methylation reflects the concentration of the attractant. Thus, the transduction machinery has undergone a long-term change following the stimulus, even though the behavioral response is transient. Conversely, when the attractant is removed, methylation returns to the basal level and the cell

[†] The results shown here appear to contradict previously published data, which indicate that when cells are washed free of methionine there is slow spontaneous demethylation of MCP (13). However, in the earlier experiment chloramphenicol was present during the starvation period, thus blocking incorporation (by protein synthesis) of nonradioactive methionine liberated through proteolysis. This nonradioactive methionine was able to serve as a donor in the methylation reaction, replacing radioactive methyl groups with nonradioactive ones and producing an apparent demethylation (13). Here we wash away the chloramphenicol and supply the other amino acids required for protein synthesis (threonine, leucine, and histidine), thereby eliminating this artifact.

FIG. 5. The role of methionine in controlling the level of methylation of MCP. (A) Cells were given tritiated methionine and incubated for 40 min. They were then washed by centrifugation at 30° to remove methionine and chloramphenicol,[†] and resuspended to approximately the same cell density as before the wash. Zero time in the figure refers to the time of this resuspension. AiBu (to 50 mM) and tritiated methionine were added at the times indicated. (Chloramphenicol was added with the methionine to block protein synthesis.) Results are the average of two experiments. (B) Cells were given tritiated methionine and incubated for 30 min. The culture was then split and half was stimulated with 50 mM AiBu while half remained unstimulated. After 20 min of further incubation both stimulated and unstimulated cells were washed free of methionine and chloramphenicol by Millipore filtration at 30°, and resuspended to approximately the same cell density as before the wash. AiBu was always included for the stimulated cells. Zero time in the figure refers to the time of resuspension. O, Washed stimulated cells; Δ, washed stimulated cells diluted 100-fold into medium containing no AiBu; ●, the mean of three samples of washed unstimulated cells. As a control to show that these cells have been washed free of methionine, a fraction of the washed AiBu-stimulated culture was given a large dose of a second attractant (100 mM aspartate), whose effects on methylation can be easily seen when methionine is present; no effect was observed, indicating that these cells are truly depleted of methionine (data not shown).

is ready to respond again (i.e., it has de-adapted). In this scheme chemotaxis is regulated by a rapid process, excitation, and a slower process, adaptation. The interaction between these two processes is capable of generating a transient signal that governs the response to the stimulus. This mechanism has features in common with other two-process models that have been proposed to explain sensory transduction mechanisms involving sensory adaptation (2, 10, 20).

In conclusion, considerable progress has been made in defining the function of MCP in bacterial chemotaxis: it has been shown that MCP plays an essential role in both excitation (14, 18) and adaptation, the two fundamental processes that control the chemotactic response. Furthermore, methylation of MCP is very likely responsible for regulating sensory adaptation. However, we know nothing yet of how the chemoreceptors influence the level of methylation of MCP, or of how this level, in combination with the excitation process, affects the direction of rotation of the flagella. The mechanism of these linkages remains to be discovered.

We are grateful to D. J. Zagrodnik and L. de la Hueraga for expert technical assistance. This work was supported by U.S. Public Health Service Grant A108746 from the National Institute of Allergy and Infectious Diseases, National Science Foundation Grant PCM75-21007, and a grant from the Graduate School of the University of Wisconsin-Madison. M.F.G. was a National Science Foundation Predoctoral Fellow and in addition received support from National Institutes of Health Training Grant 5-T01-GM00398-15 and the Graduate School of the University of Wisconsin-Madison. M.S.S. held a postdoctoral fellowship from the National Institutes of Health.

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