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Sensory transduction in *Escherichia coli*: Regulation of the demethylation rate by the CheA protein

(bacterial chemotaxis/methylated protein/stimulation/feedback)

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ABSTRACT **The reversible methylation of three membrane proteins plays an essential role in bacterial chemotaxis. Chemotactic stimuli bring about changes in the levels of methylation of these proteins, at least in part, by regulation of the demethylation reaction. Addition of attractants causes an increase in the methylation level and a transient, but essentially complete, inhibition in the rate of the demethylation reaction, while addition of repellents results in a decrease in level and a transient increase (of at least 25- to 30-fold) in rate. We have now found that the increase, but not the decrease, in rate requires the presence of the *cheA* gene product, a protein that is distinct from the demethylase. The demethylation reaction is therefore regulated by two distinct mechanisms—one, which involves the CheA protein, that mediates the increase in rate and a second, which does not involve the CheA protein, that mediates the decrease in rate. Several pieces of evidence already in the literature imply that the CheA protein functions downstream of the methylation system at the flagellar end of the chemotactic machinery. These data, in conjunction with the newer results, suggest that the CheA protein helps to regulate the demethylation reaction through a feedback mechanism.**

Escherichia coli and other enteric bacteria respond to changes in the concentrations of certain chemicals in their environment by altering their pattern of swimming, a phenomenon known as chemotaxis. In isotropic media, the bacterial flagella alternately rotate in the counterclockwise and clockwise directions to produce periods of smooth translational motion or episodes of tumbling, respectively (1–3). When the cells are exposed to a positive stimulus (addition of attractant or removal of repellent), the flagella rotate exclusively in the counterclockwise direction; when they are exposed to a negative stimulus (addition of a repellent or removal of an attractant), the flagella rotate in the clockwise direction (2). These responses are transient and the cells eventually adapt (4, 5).

The reversible methylation of three membrane proteins, methyl-accepting chemotaxis proteins (MCPs) I, II, and III, plays a central role in these responses (see ref. 6 for a review). In the absence of a stimulus, the MCPs are methylated to a basal level. When a chemical attractant is added, there is a slow increase in that level until a new steady state is attained, a condition that is maintained for as long as the attractant is present. However, if the attractant is removed, there is a rapid loss of methyl groups until the original basal level is reached. Responses to repellents are the inverse of those to attractants; addition of a repellent causes a rapid decrease in the level of methylation while removal of the repellent causes a slow increase in level. These changes in methylation appear to regulate the adaptation process. The MCPs also act as transducers and define three separate but complementary pathways through which information flows in the chemotactic machinery (7–9). Information about certain stimuli is transmitted through MCPI while information concerning other stimuli is transmitted through MCPII or MCPIII. Thus, any single stimulus affects the equilibrium level of methylation of only one of the three MCPs. The methylation of all three MCPs is carried out by the product of the *cheR* gene (10), while methyl groups are removed from these proteins by the product of the *cheB* gene (11, 12). Under normal circumstances, both enzymes are active and the methyl groups on the MCPs undergo continuous turnover (13, 14). Although we do not understand how the levels of methylation of the proteins are controlled, changes in those levels are accompanied by changes in the rate of the demethylation process (14, 15). Positive stimuli greatly diminish the rate of demethylation, while negative stimuli increase the rate at least 30-fold. These large variations in the rate of the demethylation reaction are transient and persist only while the level of methylation is changing. The rate returns to prestimulus values when the change in methylation is complete.

How is the demethylation reaction regulated? In this communication, we report that the product(s) of the *cheA* gene is necessary for the increase in the rate of the demethylation reaction that follows a negative stimulus but is not required for the decrease in rate that follows a positive stimulus. The *cheA* gene is one of a number of genes whose products are known to be required for chemotaxis but for which, until now, no function had been identified.

MATERIALS AND METHODS

Chemicals. L-[*methyl*-^{3}H]Methionine (15 Ci/mmol; 1 Ci = 37 GBq) was obtained from New England Nuclear or Amersham.

Bacterial Strains. The *cheA* strains were gifts of J. S. Parkinson. Alleles *129*, *137* (class 2), and *145* (class 1) (16) were used in an RP421 background (an explanation of the classes will be given in *Results*). The nonpolar deletion mutant RP1788 [Δ(*cheA*)*m102-11*] lacks 94% of the 5′ end of the gene and is functionally a class 3 mutant (J. S. Parkinson, personal communication). cheA101 is allele *101* (16) in an RP487 background and is a class 1 mutant. The wild-type RP487 has been described (14).

Growth and Preparation of Cultures. Cells were grown, harvested, and washed as described (17).

Labeling of Cells. All experiments were carried out at 30°C with rotary shaking. Cells were suspended at a concentration of 1×10^9/ml in 10 mM potassium phosphate, pH 7/10 mM sodium D,L-lactate/10 μM EDTA containing chloramphenicol at 50 μg/ml. After 5 min of incubation L-[*methyl*-^{3}H]methionine (2.3 Ci/mmol; 9 μM) was added and the cells were incubated for another 75 min to allow incorporation of [^{3}H]methyl groups to reach equilibrium. At various times after the appropriate manipulations, 1-ml aliquots were removed and the reactions were stopped by the addition of

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Abbreviation: MCP, methyl-accepting chemotaxis protein.

trichloracetic acid (5% final concentration). For experiments using the type II repellent Co^{2+}, the type II attractant D,L-2-methylaspartate was added after 50 min of incubation with [^{3}H]methionine and the incubation was allowed to continue for the remainder of the 75 min. The attractant was added to allow a larger fractional demethylation on addition of the repellent.

Measurement of Methylation Levels. The levels were measured by HPLC and Conway analysis as described (17). This procedure does not distinguish between the different MCPs and, therefore, the results are totals for all three proteins. $NaDodSO_4$ gel electrophoresis was carried out as described (17).

RESULTS

The loss of methyl groups that occurs on addition of a repellent can be followed, as shown in Fig. 1*a*, by $NaDodSO_4$ gel electrophoresis of cells in which [^{3}H]methyl groups have been incorporated into the MCPs. Each of the MCPs runs as a series of bands, a complexity that arises from the fact that each protein can be methylated at more than one site, and the more heavily methylated a particular molecule is the more rapidly it migrates (18–21). Thus, changes in the relative intensities of the different bands, as well as the overall density, reflect whether there has been a net gain or loss of methyl groups. The pattern obtained for a wild-type strain that had been stimulated with and adapted to a type II attractant (one using MCPII as the transducer) is shown in lane 1 of Fig. 1*a*. The attractant was used to make the effects of the subsequent addition of repellent more apparent. The major effect of the repellent is the large loss of density from bands 7 and 8, the two most rapidly migrating species of MCPII (bands 9 and 10 are due to MCPIII, which is not affected by this repellent). The demethylation is very rapid and is largely complete in less than 1 min. In contrast, the repellent-stimulated loss of methyl groups from a *cheA* deletion mutant (Fig. 1*b*) is slow. Little change can be seen in the electrophoretic pattern prior to 5 min and 10–20 min is required for demethylation to reach completion. Similar results have been obtained on dilution of an attractant.

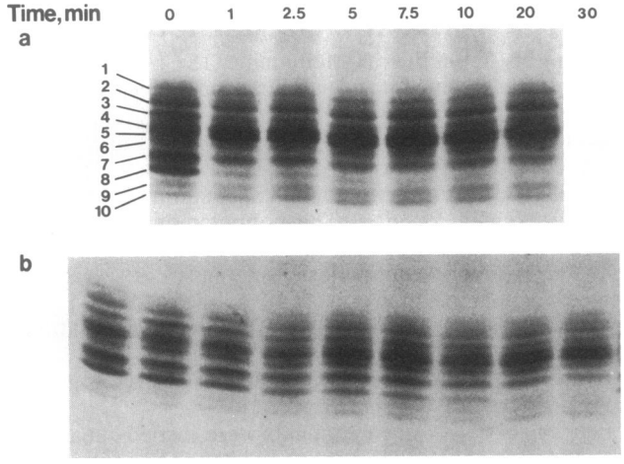

FIG. 1. Effect of repellent stimulation on electrophoretic patterns of the MCPs. Cells were incubated with [^{3}H]methionine for 50 min, at which time the type II attractant DL-2-methylaspartate (1 μM) was added, and incubation was continued for the remainder of the 75 min. At this time (indicated as 0 in the figure), cells were stimulated by addition of the type II repellent $CoSO_4$ (0.5 mM), and aliquots were removed at appropriate intervals. Samples containing equal quantities of protein were run on 7% $NaDodSO_4$/polyacrylamide gels. (*a*) Wild type (RP487). (*b*) *cheA* deletion mutant (RP1788), except for the extreme left lane, which repeats RP487 without repellent.

It is clear from these data that the mutant does not demethylate at a normal rate in response to the repellent. There are several possible explanations for this result: (*i*) the mutant may be unable to increase the rate of demethylation when challenged with the repellent; (*ii*) there may be a normal increase in rate, but the basal rate itself may be very low; (*iii*) some combination of *i* and *ii*. These possibilities can be distinguished by kinetic measurements of basal and stimulated rates of demethylation.

This analysis should take into consideration the unusual nature of the *cheA* gene (16, 22). The gene has two translation initiation sites that are in phase and, therefore, codes for two polypeptides. The larger one has a molecular weight of 76,000 and is found in the cytoplasmic membrane, while the smaller one has a molecular weight of 66,000 and is found in the cytoplasm. Genetic analysis has revealed the existence of two complementation classes within the gene. Members of class 1 complement with members of class 2 but not with each other and vice versa. There is a third class of mutants that fail to complement at all. The simplest explanation for these results is that the *cheA* gene products serve two functions represented by the class 1 and class 2 mutants. The class 3 *cheA* strains evidently have defects that cause the loss of both functions. It is not known whether the two functions are distributed between the two proteins or even whether the smaller protein is needed at all (16). In any case, it is important to compare the properties of mutants in the two groups. The deletion mutant used in Fig. 1 belongs to the third group.

Unstimulated Rates of Demethylation. Demethylation rates can be obtained, under steady-state conditions, from label–chase experiments. In the absence of a stimulus, the half-time for loss of label was 20 min for the wild type and 40 min for both *cheA101* (class 1) and *cheA129* (class 2) (Fig. 2*a*). An identical experiment was carried out with cells that had been exposed and then adapted to a mixture of type I and type II attractants (Fig. 2*b*). In all strains, these rate constants are the same as those measured in the absence of attractant. Taken together, the data show that the unstimulated or adapted rates of demethylation for the *cheA* mutants are about half those of the wild type. While these differences are significant, they are not sufficient to account for the low rates of demethylation exhibited by the deletion mutant in response to the addition of a repellent (Fig. 1).

Repellent-Stimulated Rates of Demethylation. The responses of the wild type to type I and type II repellents are shown in Fig. 3. Both repellents resulted in a net loss of about 30% of the labeled methyl groups. Moreover, the demethylation was very rapid, reaching completion in less than 0.5 min. The rapid kinetics of the loss requires an increase of at least 25-fold in the rate of demethylation as compared with the rates observed in the unstimulated state. If this were not the case and the net loss of methyl groups resulted simply from inhibition of the methylation process, the decrease in level would have required 10 min to reach completion (calculated from Fig. 2).

In contrast, the class 1 *cheA* mutant (*101*) demethylated slowly in response to either repellent (Fig. 4*a*). The actual kinetics of the loss are those that would be predicted if the methylation reaction were inhibited and demethylation continued at the rates observed in the unstimulated or adapted states. Results with a second class 1 mutant (*145*) and the class 3 deletion mutant were similar (data not shown). It should also be pointed out that the absolute magnitude of the loss of methyl groups is somewhat smaller for the mutants than the wild type.

The class 2 mutant (*129*) also demethylates slowly in response to type I and type II repellents (Fig. 4*b*). However, with this strain, the kinetics cannot be accounted for without an increase in the demethylation rate of 2- to 3-fold. If there

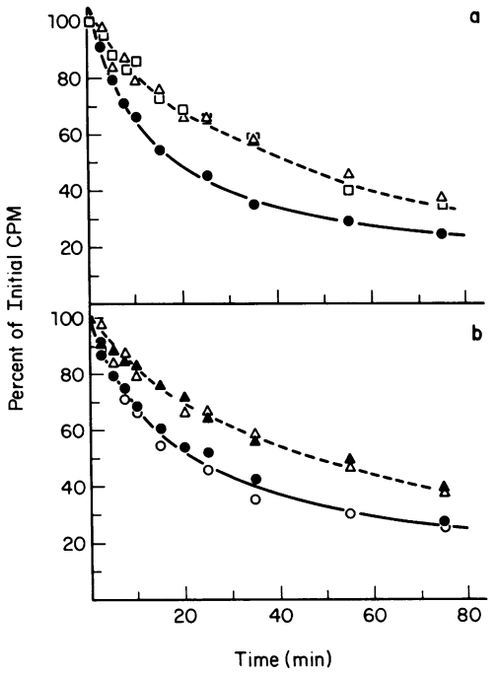

FIG. 2. Unstimulated rates of MCP demethylation. Cells were incubated with [^{3}H]methionine for 75 min and then a 100-fold excess of unlabeled methionine was added. At the indicated times, aliquots were removed, the reactions were stopped by addition of trichloroacetic acid, and levels of methylation were determined by HPLC and Conway analysis. There was a delay of 5 min between the addition of unlabeled methionine and the first measurable loss of label so that 5 min have been subtracted from all time points. (*a*) Rates in the absence of added attractant. ●, Wild type; △, *cheA101*; □, *cheA129*. (*b*) Rates after adaptation to an attractant. A mixture of the type I attractant α-aminoisobutyrate (50 mM) and the type II attractant DL-2-methylaspartate (0.3 mM) was added 50 min after addition of [^{3}H]methionine, and cells were then incubated in this mixture for the remainder of the 75 min. ● and ○, Wild type with and without attractant; ▲ and △, *cheA101* with and without attractant. The no-attractant data are repeated from *a*.

were no increase in rate, 15 min would have been required for the demethylation instead of the observed 5–7 min. Results with a second class 2 mutant (*137*) also indicate that an increase of about 2-fold takes place. These increases in rate, however, are small compared with the greater than 25-fold increase that occurs in the wild type.

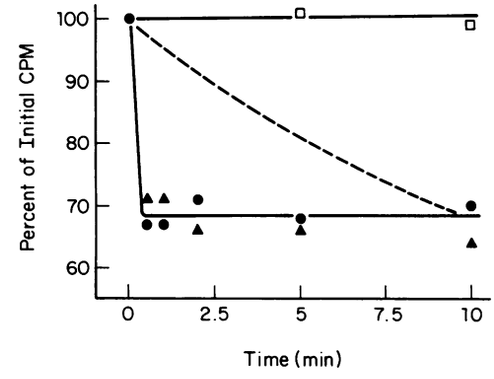

FIG. 3. Response of the wild type to addition of repellents. After incubation for 75 min, cells were stimulated by addition of a mixture of the type I repellents sodium acetate (17 mM), L-leucine (17 mM), and indole (0.3 mM) (●) or by addition of the type II repellent $CoSO_4$ (0.5 mM) (▲). □, Buffer was used as a control. ---, Kinetics expected if the methylation reaction is inhibited and no increase in demethylation rate takes place (calculated from Fig. 2).

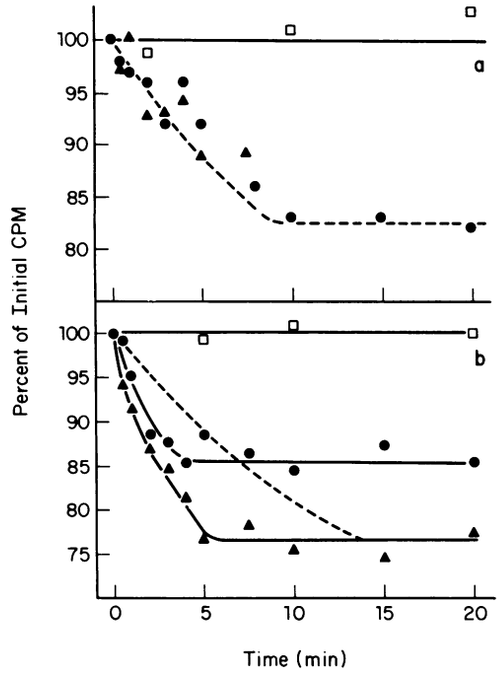

FIG. 4. Response of *cheA* mutants to the addition of repellents. The protocol used is the same as for Fig. 3. Responses of *cheA101* (class 1) (*a*) and *cheA129* (class 2) (*b*) to the type I repellent mixture (●), to the type II repellent (▲), or to buffer (□) as a control are shown. ---, Kinetics expected if the methylation reaction is inhibited and no increase in the demethylation rate takes place (calculated from Fig. 2).

Attractant-Inhibited Rates of Demethylation. Is a functional *cheA* gene also required for the attractant-induced inhibition of the demethylation rate? This question can be an-

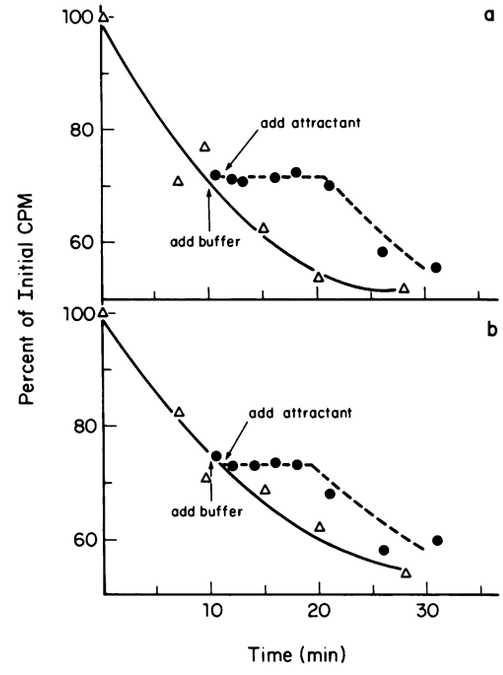

FIG. 5. Attractant-stimulated inhibition of demethylation. Cells were incubated with [^{3}H]methionine for 75 min, and then [^{3}H]methionine was removed by filtration. Cells were resuspended and the chase was initiated (0 time) by addition of unlabeled methionine (10 μM). Ten minutes later, a mixture of the attractants α-aminoisobutyrate (50 mM) and DL-2-methylaspartate (0.3 mM) was added to one portion of cells (●) and buffer was added to another as a control (△). (*a*) Wild type. (*b*) *cheA101* (class 1).

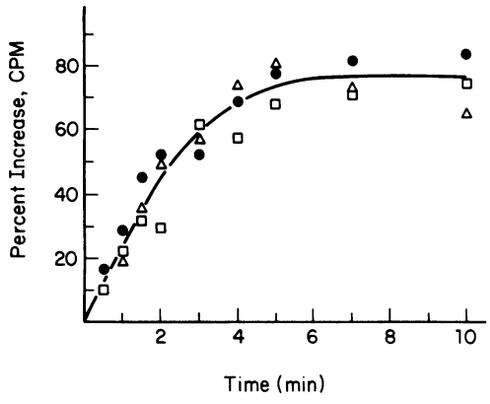

FIG. 6. Attractant-stimulated increase in methylation levels. Cells were incubated with [^{3}H]methionine for 75 min, and then (0 on the graph) they were stimulated by the addition of α-aminoisobutyrate (50 mM) plus DL-2-methylaspartate (0.3 mM). ●, Wild type; □, *cheA101* (class 1); △, *cheA129* (class 2).

swered by addition of an attractant after a chase with unlabeled methionine has begun. When the wild type is challenged with a mixture of a type I and type II attractant, there is almost total cessation of the loss of label for a period of 8–10 min, after which chase resumes (Fig. 5). This implies that the demethylation reaction has been inhibited for that 8- to 10-min period. When the same experiment was repeated using a class 1 *cheA* mutant (*101*), a similar inhibition, again of about 8 min duration, was observed (Fig. 5). Depression of the demethylation rate also occurred in the class 2 mutant *cheA137* (data not shown). Thus, it appears that a functional *cheA* gene is not required for the inhibition.

Similarly, as shown in Fig. 6, mutations in the *cheA* gene appear to have little effect on either the extent or the kinetics of the increase in methylation that follows addition of an attractant. Although the data in Fig. 6 have been normalized to basal levels of methylation, these levels do not vary greatly between wild-type and cheA strains (two left lanes of Fig. 1*b*), so that the absolute magnitudes of the changes in methylation are similar as well.

DISCUSSION

The constant levels of methylation that *E. coli* maintains in the absence of stimuli, or after adaptation to stimuli, represent steady states in which the rates of methylation and demethylation are equal. For the level to change on stimulation, there must be at least a transient change in the ratio of the two rates. This is most strikingly illustrated by the rapid decrease in the level of methylation that follows a negative stimulus. The kinetics of the change, which is complete in less than 20–30 sec (ref. 14; this communication), requires that an increase of at least 25- to 30-fold occur in the rate of the demethylation reaction. We have now shown that this acceleration in rate requires the presence of a functional *cheA* gene. Strains in which this gene is mutated undergo net demethylation after addition of a repellent but do so very slowly with 5–12 min being required for completion of the biochemical response. In fact, the kinetics obtained with the class 1 *cheA* strains (and the deletion mutant) are those expected if the demethylation reaction continued at its basal rate and the methylation reaction became inhibited. Although we believe this to be the actual situation, the same kinetics could also be generated by any combination of changes in rates that left the net demethylation rate (demethylation rate minus methylation rate) equal to the original basal demethylation rate. For example, a doubling of the demethylation rate without effect on the methylation rate would produce the observed results. The unstimulated rates of demethylation are also lower, by about 50%, in the *cheA* mutants than the wild type. Thus, the *cheA* product(s) plays a role in establishing the basal rates of demethylation as well as mediating the transient increase in those rates which follows a negative stimulus.

Just as the rate of demethylation is greatly increased by addition of a repellent to a wild-type strain, that rate is decreased by addition of an attractant. Surprisingly, this regulation does not involve the *cheA* product(s) since *cheA* mutations of either complementation group are without effect. These results indicate that different mechanisms mediate the stimulated increases and decreases in the rate of the demethylation reaction.

How and where in the chemotactic machinery does the *cheA* product(s) function? The *cheA* product(s) is not the demethylase (which is encoded by the *cheB* gene) and therefore most likely exerts its regulatory influence on the demethylation reaction in an indirect manner. This latter point is supported by three pieces of data that suggest that the CheA protein(s) functions at the downstream or flagellar end of the machinery. First, *cheA* appears to be required for clockwise rotation of the flagella as *cheA* mutants have a complete counterclockwise bias and fail to respond, or respond only briefly, to the strongest clockwise stimuli (addition of repellent or removal of attractant) (23). Second, the *cheA* gene is located in the *Mocha* operon (22). Two of the four members of that operon, *motA* and *motB*, are required for operation of the flagellar motor because in their absence the flagella are paralyzed (24). Since genes with related functions are often clustered, it is reasonable to assume that such a relationship may exist between the *mot* and *cheA* genes. The fourth member of the operon is *cheW*, a gene whose product is necessary for chemotaxis but for which no biochemical role is known. Third, reversion analyses suggest that the *cheA* product(s) may interact with flagellar components (25). Taken as a whole, these data and the results presented in this paper strongly imply that there is a feedback loop in the chemotactic machinery, in which the CheA protein(s) participates, that regulates the demethylation rate. Our model for the feedback loop in shown in Fig. 7.

In the absence of a stimulus, or after adaptation to a stimulus, clockwise and counterclockwise signals are generated somewhere in the chemotactic machinery and transmitted to the flagellar motor so that it rotates largely, but not exclusively, in the counterclockwise direction. The bias of the motor is established by the balance of the two signals. The clockwise signal is transmitted to the motor and is fed back to the demethylase (CheB protein) through the CheA protein(s). This feedback loop is one of the factors that sets the basal rate of demethylation. When the cell is presented with a negative stimulus, additional clockwise signal is generated by the transducing MCP and is relayed to the flagellar motor through the CheA protein. This signal shifts the bias of the motor and produces enhanced clockwise rotation. The signal is also fed back, again through the CheA protein, to the demethylase, greatly increasing its activity and thereby drastically shortening the time necessary for the methylation level

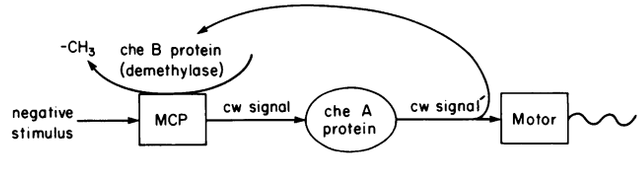

FIG. 7. Proposed feedback loop. A negative stimulus is transduced by the appropriate MCP so as to produce a clockwise (cw) signal. This signal is transmitted to the motor through the *cheA* protein and is also fed back to the methylation system, where it regulates the rate of the demethylation reaction.

to reach its new steady-state value. When the new level is attained the generation of the additional clockwise signal is terminated and adaptation is complete. Thus, the rotational bias of the motor and the demethylation rate (actually, the rate constant) are restored to their prestimulus values. In the model, the steady-state levels of methylation are determined by the conformation (i.e., exposure of the glutamic acid residues that can be methylated) of the MCPs and not by the rates of the methylation and demethylation reactions.* These rates, however, are responsible for the kinetics of the changes in levels.

This model, which accounts for most of the properties of the *cheA* mutants, has an additional implication that may be of considerable importance. The real key to understanding the function of the chemotactic machinery lies in uncovering the nature and identity of the signal or signals that control the rotational bias of the motor. The direct approach, establishment of an *in vitro* system that contains a rotating flagellum, is likely to be an extremely difficult undertaking. However, if the stimulated increase in the demethylation rate and the clockwise rotation of the motor are regulated by the same signal, then study of the demethylation reaction may be a less difficult route to that signal. Such a study should also yield information about the effect of positive (counterclockwise) stimuli on the machinery because these stimuli also result in the regulation of the demethylation reaction, possibly through a second feedback loop.

*This facet of the model is in agreement with the data in Figs. 1 and 2. These results show that, despite differences of a factor of 2 in the steady-state rates of demethylation between the wild-type and *cheA* mutants, the overall levels of methylation are similar (compare the densities and band patterns of the extreme left lanes of Fig. 1*b*). If the methylation levels were determined by the rates of the methylation and demethylation reactions, we would expect the methylation level to be higher in the mutants. Our data indicate that the methylation reaction is unaffected by defects in *cheA* (Fig. 6).

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