

Posttranslational processing of methyl-accepting chemotaxis proteins in *Escherichia coli*

(sensory transduction/membrane proteins/*cheR* and *cheB* mutants)

DAVID SHERRIS AND JOHN S. PARKINSON

Department of Biology, University of Utah, Salt Lake City, Utah 84112

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ABSTRACT Methyl-accepting chemotaxis proteins (MCPs) of *Escherichia coli* undergo changes in methylation state in response to chemical stimuli. The addition of methyl groups to MCP is dependent on *cheR* function; their removal is dependent on *cheB* function. This MCP methylation system is instrumental in establishing the unstimulated swimming pattern of *E. coli* and in enabling the cell to carry out sensory adaptation after a chemotactic response. We employed electrophoresis in sodium dodecyl sulfate-containing polyacrylamide gels to analyze MCP molecules synthesized in *cheR* deletion mutants lacking MCP-specific methyltransferase activity. MCP made under these conditions proved to be completely devoid of methyl groups. In the absence of *cheB* function as well, this unmethylated MCP is made in a form, designated 2*, that exhibits several properties characteristic of methylated MCP. In the presence of *cheB* function, MCP 2* is processed to a form, designated 1*, that no longer resembles methylated MCP. The rate of this conversion process is modulated by chemotactic stimuli. Both MCP 1* and MCP 2* are capable of initiating changes in flagellar rotation in response to stimuli, and, in the presence of *cheR* function, both forms can accept methyl groups. We suggest that MCP 2* is a normal intermediate in MCP synthesis in which one or more of the methyl-accepting glutamic acid residues carry a methyl-ester-like modification, which, like glutamic acid methyl esters, can be removed by *cheB* function. This *cheB*-dependent processing event does not appear to be reversible, but nevertheless it may play an important role in modulating the signaling behavior of newly synthesized MCP molecules.

Methyl-accepting chemotaxis proteins (MCPs) of *Escherichia coli* play an essential role in initiating flagellar responses to changing attractant or repellent levels ("excitation"), and in terminating those responses in static chemical environments ("adaptation") (1). Although the nature of these events is still poorly understood, these signaler proteins appear to hold the key to an eventual molecular description of the sensory transduction machinery responsible for chemotactic behavior in *E. coli*.

E. coli possesses at least three different membrane signalers, each of which is responsible for processing sensory inputs from a different subset of chemoreceptor types. The two major signalers, MCP I and MCP II, are products of the *tsr* and *tar* genes, respectively (2, 3). The *tsr* product processes type I stimuli, which include the attractant serine and several repellents. The *tar* product handles type II stimuli, which include the attractants aspartic acid and maltose as well as several repellents. In response to favorable stimuli, such as increasing levels of attractant, these signalers elicit counterclockwise flagellar rotation, which produces smooth swimming behavior. Unfavorable stimuli, such as repellent increases, produce clockwise rotation, which causes abrupt tumbles or changes in swimming direction (4).

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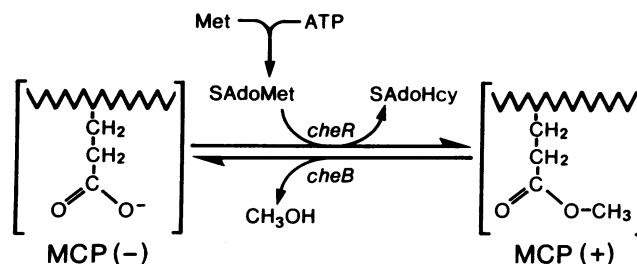


FIG. 1. Summary of MCP methylation and demethylation reactions. Unmethylated MCP [MCP(-)] is converted to methylated MCP [MCP(+)] by addition of methyl groups to several glutamic acid residues. This reaction is dependent on *cheR* function and utilizes *S*-adenosylmethionine (SAdoMet), derived from methionine (Met) and adenosine triphosphate (ATP) as the methyl donor, forming *S*-adenosylhomocysteine (SAdoHcy). Demethylation is dependent on *cheB* function and generates methanol.

The *tsr* and *tar* gene products can be reversibly methylated by the chemotaxis machinery as summarized in Fig. 1. Methyl groups are attached to specific glutamic acid residues in these MCPs to form γ -carboxyl methyl esters, which upon hydrolysis yield methanol and an unmodified glutamic acid residue (5-7). The *in vivo* addition and removal of MCP methyl groups are controlled by the *cheR*[†] and *cheB* gene products, which are thought to be an MCP-specific methyltransferase and methyl-esterase, respectively (9, 10). Mutants defective in *cheR* function have much reduced levels of methylated MCP (11) and exhibit an extreme counterclockwise bias in flagellar rotation (12). Conversely, *cheB* mutants have very high levels of methylated MCP (13) and exhibit a strong clockwise bias in flagellar rotation (14). Thus the signaling properties of MCPs appear to be influenced by their methylation state.

Chemotactic stimuli elicit changes in MCP methylation state (15). For example, stimuli that trigger counterclockwise flagellar responses also cause an increase in the steady-state level of MCP methylation. These changes in methylation state appear to be associated with the adaptation phase of the chemotactic response (15, 16). They somehow cancel or counteract the initial excitatory signal elicited by the stimulus and return the cell to its prestimulus swimming pattern. Mutants such as those with *cheR* or *cheB* mutations, which are unable to alter their MCP methylation levels, are capable of initiating flagellar responses to stimuli but exhibit defects in sensory adaptation (refs. 11, 12, 17; unpublished data).

In this report we describe the properties of MCP molecules

Abbreviation: MCP, methyl-accepting chemotaxis protein.

[†]The *cheR* gene of *E. coli* was formerly designated *cheX*. Workers in the chemotaxis field have agreed to this nomenclature change in order to standardize gene notations in *E. coli* and *Salmonella typhimurium* (8).

Table 1. Bacterial and phage strains

Strain	Chemotaxis functions [†]									MCP pattern [‡]
	<i>cheA</i>	<i>cheW</i>	<i>tar</i>	?	<i>cheR</i>	<i>cheB</i>	<i>cheY</i>	<i>cheZ</i>	<i>tsr</i>	
RP1267	+	+	+	+	+	+	+	+	+	Wild type
RP1269 ($\Delta 2206$)	+	+	+	-	-	-	-	-	+	2*
RP1271 ($\Delta 2234$)	+	+	-	-	-	-	+	+	+	2*
RP1272 ($\Delta 2216$)	-	-	-	-	-	+	+	+	+	1*
RP1273 ($\Delta 2241$)	+	+	+	-	-	-	+	+	+	2*
RP1274 ($\Delta 2231$)	-	-	-	-	-	+	+	+	+	1*
RP1275 ($\Delta 2203$)	+	-	-	-	-	-	-	+	+	2*
$\lambda che22\Delta 25-5$	-	-	+	+	-	-	-	-	-	λtar
$\lambda che22\Delta 11-37$	-	-	-	+	+	-	-	-	-	$\lambda cheR$
$\lambda che22\Delta 25-37$	-	-	+	+	+	-	-	-	-	$\lambda tar cheR$
$\lambda che22\Delta 16-3\Delta 28$	-	-	-	-	-	+	-	-	-	$\lambda cheB$
$\lambda fla91$	-	-	-	-	-	-	-	-	+	λtsr

[†] The genes listed constitute two adjacent operons in the *E. coli* chromosome and are shown in their correct map order. The ? indicates a newly discovered gene that makes a 65,000-dalton product (M. K. Slocum, personal communication). Its role in chemotaxis is not yet clear.

[‡] See text and Fig. 2.

synthesized in strains from which the *cheR* locus has been deleted. Although these mutants proved to have no detectable MCP methylation activity, they were nevertheless able to synthesize MCP molecules with many of the biochemical and functional properties of methylated MCP. We do not know the chemical nature of the methyl-ester-like moiety in these molecules, but its removal can be effected by a *cheB*-dependent processing event. The possible role of these events in chemotaxis is discussed.

MATERIALS AND METHODS

Bacterial Strains. *E. coli* K-12 derivatives used in this work are listed in Table 1. RP1267 [$F^- thi thr leu his eda uvrA rpsL zja::Tn10 (\lambda ind)$] is a *met*⁺ *uvrA* derivative of strain RP437 (18) which is wild type for chemotaxis. Deletions of the *cheR* locus were transferred to RP1267 by cotransduction with the *eda* locus to generate the other bacterial strains in Table 1. The isolation of mutants with these *che* deletions will be reported elsewhere.

Phage Strains. $\lambda fla91$, which carries the *tsr* locus, was obtained from M. Silverman. Other transducing phages used in this work were derivatives of $\lambda che22$ (S. Houts, personal communication) and are listed in Table 1. These phage strains were constructed by crosses between deleted $\lambda che22$ strains.

Radiolabeling and Polyacrylamide Gel Electrophoresis of MCP. MCP molecules synthesized in UV-programmed cells by λ transducing phages were labeled with [³⁵S]sulfate essentially as described (19). Samples were analyzed in discontinuous polyacrylamide gels containing 11% acrylamide and 0.11% bisacrylamide as described by Boyd and Simon (20).

Stimulus-Induced Changes in MCP Banding Patterns. After 90-min incubation with λ transducing phages, UV-programmed cells containing labeled MCP molecules were subjected to attractant or repellent compounds for a period of 30 min. The attractants aspartic acid and serine were added to a final concentration of 10 mM; the repellents leucine and acetate were added to a final concentration of 20 mM each. Chloramphenicol was also added at 90 min to a final concentration of 200 μ g/ml to inhibit any further protein synthesis.

Base Hydrolysis of MCP. Labeled MCP in NaDodSO₄ sample buffer was treated with 0.5 M NaOH for 30 min at 30°C. The samples were then neutralized by addition of HCl and analyzed by NaDodSO₄/polyacrylamide gel electrophoresis.

RESULTS

NaDodSO₄/Polyacrylamide Gel Electrophoretic Analysis of MCP Methylation States. Methylated forms of the *tar* and *tsr* gene products can be observed by using a [³H]methyl label, but this method permits detection of only those MCP molecules that contain at least one exchangeable methyl group. In order to observe both methylated and unmethylated forms of these proteins, we infected heavily UV-irradiated cells with λtar or λtsr transducing phage and labeled the MCPs with [³⁵S]sulfate. The backbone-labeled MCP molecules were then analyzed by electrophoresis in NaDodSO₄-containing polyacrylamide gels and subsequent autoradiography. Several recent studies have demonstrated that this gel technique is able to resolve MCP molecules with different methylation states (20–23). Under these conditions, unmethylated molecules migrate slower than methylated ones, but the basis for this effect is not known. It may be that the formation of glutamic acid methyl ester groups, which eliminate negative charges on MCP, enables the protein to bind significantly more NaDodSO₄ molecules. Alternatively, MCP molecules in this detergent may retain some secondary structure that influences their electrophoretic mobility. In any event, NaDodSO₄/polyacrylamide gel electrophoresis appears to be a reliable method for assessing the methylation states of MCP molecules. Moreover, the MCP methylation system, including its responsiveness to stimuli, appears to behave normally in UV-programmed cells (20–22).

The NaDodSO₄/polyacrylamide gel electrophoresis profiles of MCPI (*tsr* product) and MCPII (*tar* product) synthesized in wild-type hosts are shown in Fig. 2, lanes a and b and f and g. Although the apparent *M_r* of MCPI is slightly greater than that of MCPII, in most respects there is little difference in their behavior and all of the properties to be discussed below apply to both gene products. Under the conditions used here (long gels, low crosslinking) each MCP can form as many as eight different bands. For simplicity we have grouped these bands into sets that we designate MCPI-1, †-2, -3, and -4 and MCPII-1, -2, and -3, beginning with the slowest forms of each MCP. The faster migrating bands represent methylated forms of MCP. The relative proportion of MCP in these fast forms can be increased by subjecting UV-programmed cells to attractant stim-

† MCPI-1 is not observed after 90-min radiolabeling time using [³⁵S]sulfate; however it can be seen by using a shorter labeling time, 15 min, with [³⁵S]methionine. MCPI-1 is located at the same position as MCPI-1* and is also a doublet.

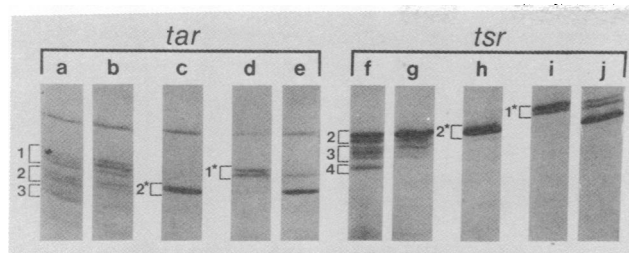


FIG. 2. NaDodSO₄/polyacrylamide gel electrophoretic analysis of *tar* (MCPII) and *tsr* (MCPI) gene products synthesized in *cheR* deletion mutants. MCP molecules were labeled by infecting UV-irradiated host strains with λ che22 Δ 25-5 (lanes a-e) or λ fla91 (lanes f-j). Only the region containing MCP is shown. The uppermost band in a-e is not a product of the *tsr* locus, but rather appears to be the product of a previously undiscovered chemotaxis gene adjacent to the *tar* locus (M. K. Slocum, personal communication). Lanes a and f: host strain RP1267 (*cheR*⁺ *cheB*⁺), attractant stimulated pattern (aspartate, a; serine, f); lanes b and g: host strain RP1267 (*cheR*⁺ *cheB*⁺), unstimulated pattern; lanes c and h: host strain RP1273 (*cheR* Δ *cheB* Δ), unstimulated (stimulated patterns were identical to these); lanes d and i: host strain RP1272 (*cheR* Δ *cheB*⁺), unstimulated (stimulated patterns were essentially identical to these); lanes e and j: host strain RP1273 (*cheR* Δ *cheB* Δ) coinfecting with λ tar or λ tsr and λ che22 Δ 16-3 Δ 28 to furnish *cheB* function, unstimulated.

uli, which increase MCP methylation state (compare lanes a and b or f and g in Fig. 2).

MCP Patterns in *cheR* Mutants. The NaDodSO₄/polyacrylamide gel electrophoresis profiles of MCP molecules synthesized in deletion mutants lacking *cheR* function are shown in Fig. 2, lanes c and d and h and i. Two different patterns, which we designate 1* and 2*, were observed. Mutants with the 2* pattern exhibited a single MCP band at the same position as one of the 2 bands for MCPI and MCPII from wild-type hosts. Strains with the 1* pattern exhibited two bands at the 1 position for MCPI and MCPII. The genotypes of the deletion mutants used in these experiments and their MCP patterns are summarized in Table 1. Hosts lacking both the *cheR* and *cheB* functions gave the 2* pattern, whereas hosts that lacked *cheR* function but retained *cheB* function gave the 1* pattern. The presence or absence of other *che* functions had no effect on MCP banding patterns in these *cheR* deletion strains. Thus the presence of *cheB* activity appears to be responsible for producing the 1* rather than the 2* pattern. We confirmed this point by using a λ cheB transducing phage to restore *cheB* function to host strains that had the 2* pattern. The resulting MCP profiles exhibited both the 1* and 2* bands (Fig. 2, lanes e and j). Our failure to obtain complete conversion to the 1* pattern is probably due to an inability of the superinfected cells to synthesize adequate amounts of *cheB* product.

MCP 2* is a Precursor to MCP 1*. A pulse-chase experiment was performed to test the possibility that MCP 2* and MCP 1* shared a precursor-product relationship. Type 2* hosts were infected with λ tar or λ tsr after UV irradiation and allowed to accumulate labeled MCP 2*. We then removed the [³⁵S]sulfate label, infected the cells with λ cheB phage, and added unlabeled sulfur-containing amino acids. At various times during the chase period, samples were extracted and analyzed by NaDodSO₄/polyacrylamide gel electrophoresis. Densitometer tracings of the resultant autoradiograms were used to determine the relative amounts of the 1* and 2* forms. We observed a slow decline in the amount of 2* form and a concomitant increase in the amount of 1* form under these conditions (Fig. 3), implying that MCP 2* is a precursor to MCP 1*. We also found that the rate of 2* to 1* conversion could be enhanced by subjecting the cells to repellent stimuli during the

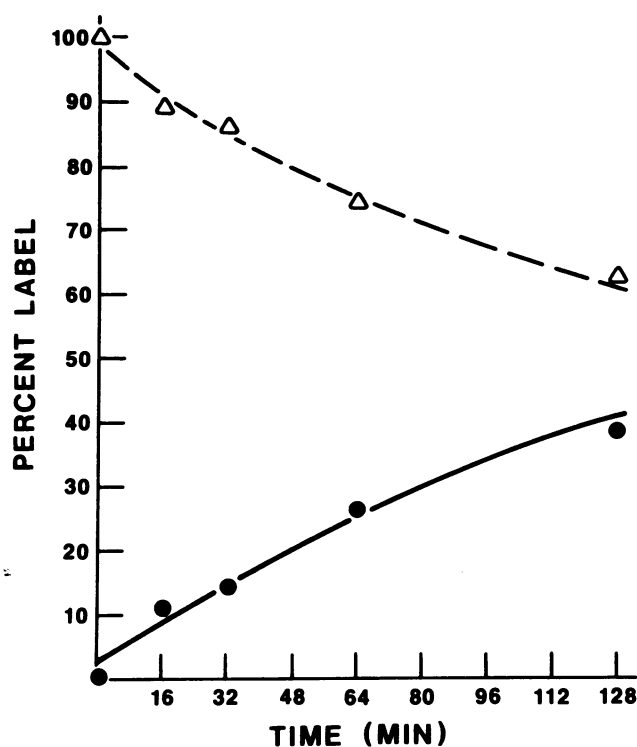


FIG. 3. Results of pulse-chase analysis of MCP 2* to MCP 1* conversion. Strain RP1273 (*cheR* Δ *cheB* Δ) was infected with λ fla91 and allowed to accumulate labeled MCP 2* for 90 min under UV-programming conditions. At this point the cells were superinfected with λ che22 Δ 16-3 Δ 28 (*cheB*⁺) at a multiplicity of 10 and the medium was replaced by one containing unlabeled sulfate and 1 mM each cysteine and methionine. Incubation was continued and samples were removed at the time points indicated and analyzed by NaDodSO₄/polyacrylamide gel electrophoresis. Densitometer tracings of the 2* and 1* peaks were made from autoradiograms and the area under each was used to calculate the amount of material in each MCP form. Δ , MCP 2*; \bullet , MCP 1*.

chase period, whereas attractant stimuli seemed to inhibit conversion of 2* to 1* (data not shown). The *cheB*-dependent methylesterase activity is known to be modulated by stimuli in the same manner.

The two 1* bands generated by the *cheB*-dependent conversion of MCP 2* to 1* did not appear simultaneously: the faster 1* band invariably appeared before the slower one (data not shown). This finding indicates that the conversion of MCP 2* to 1* occurs in two sequential steps. The first processing step (conversion of 2* to the faster 1* band) is clearly dependent on *cheB* function. The second step (conversion of fast 1* to slow 1*) need not be dependent on *cheB* activity, although it seems likely that it is. If some other function proves to be responsible for this second processing step, it clearly cannot act in the absence of *cheB* function.

Properties of MCP 1* and MCP 2*. Both the 1* and 2* forms of MCP can be observed in *cheR* deletion mutants, which should not have any MCP-specific methyltransferase activity. In fact, we were unable to achieve any detectable incorporation of [³H]methyl label from methionine into the 1* or 2* forms, whereas MCP molecules synthesized in *cheR*⁺ hosts were readily labeled under the same conditions (data not shown). The possibility remained, however, that MCP 1* or 2* could carry a methyl or some other alkyl group modification, provided that the modifying group was derived from some donor other than S-adenosylmethionine. To exclude this possibility, we subjected MCP 1* and MCP 2* to base hydrolysis before analysis

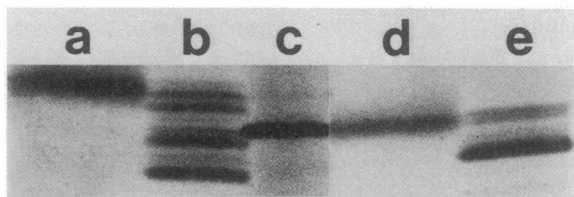


FIG. 4. NaDodSO₄/polyacrylamide gel electrophoretic analysis of base-hydrolyzed MCP molecules. Results shown are for MCP II (*tar* product) labeled by UV programming. Lanes a and b: host strain RP1267 (*cheR*⁺ *cheB*⁺), aspartate stimulated; lane a, base hydrolyzed; lane b, untreated; lanes c, d, and e: host strain RP1273 (*cheR*^Δ *cheB*^Δ); lane c, untreated; lane d, treated with NaOH; lane e, infected with *λche22Δ25-37* (*cheR*⁺) to furnish *cheR* function, untreated.

by NaDodSO₄/polyacrylamide gel electrophoresis. In wild-type cells methylated MCP molecules are readily demethylated by such treatments due to the unstable nature of the glutamic acid methyl ester linkage. Chemically demethylated MCP bands at the MCP 1* position as shown in Fig. 4. Neither the 1* (not shown) nor the 2* (Fig. 4, lanes c and d) form of MCP was affected by base hydrolysis, indicating that neither form contains an alkyl ester modification.

Both MCP 1* and MCP 2* are capable of accepting methyl groups in the presence of *cheR* function. To prove this, we infected type 1* and 2* hosts with *λcheR* transducing phage to furnish MCP methyltransferase activity. Under these conditions both the 1* and 2* forms of MCP generated additional faster-migrating bands characteristic of methylated MCP. An example of methylated MCP 2* is shown in Fig. 4, lane e. Two lines of evidence indicated that these new bands did in fact carry methyl ester groups. First, the relative amount of MCP in the fast bands was enhanced by attractant stimuli. Second, base hydrolysis caused this material to migrate once again at the 1* or 2* position.

DISCUSSION

The *tar* and *tsr* gene products, the major MCPs of *E. coli*, exhibit complex banding patterns when analyzed by NaDodSO₄/polyacrylamide gel electrophoresis. Recent studies have demonstrated that much of this complexity is due to the existence of multiple MCP methylation states, which for unknown reasons influence the migration rates of MCP molecules in NaDodSO₄/polyacrylamide gel electrophoresis (20–23). However, methylation is not the only factor involved in this phenomenon (24). In the present study we examined the properties of MCP molecules synthesized in deletion mutants lacking the *cheR*-dependent methyltransferase activity and found that the MCP in such strains is completely devoid of methyl groups, but nevertheless can exist in two different forms that are resolved by NaDodSO₄/polyacrylamide gel electrophoresis. Strains having deletions of both the *cheR* and *cheB* functions synthesize MCP 2*, which migrates somewhat faster than MCP 1*, which is found in mutants lacking only *cheR* activity. Our findings are summarized by the scheme shown in Fig. 5.

We suggest that MCP 1* and MCP 2* are part of the normal biosynthetic pathway for MCP. Both the *tar* and *tsr* products are initially synthesized in the 2* form. In *cheB*⁺ strains, MCP 2* is then converted to MCP 1* by a *cheB*-dependent process that appears to involve two sequential steps. Ordinarily, this conversion may take place during MCP synthesis, but it is clear from pulse-chase experiments that MCP 2* can be processed to MCP 1* after translation of the MCP molecule has been completed. There is no evidence as yet to suggest that this pro-

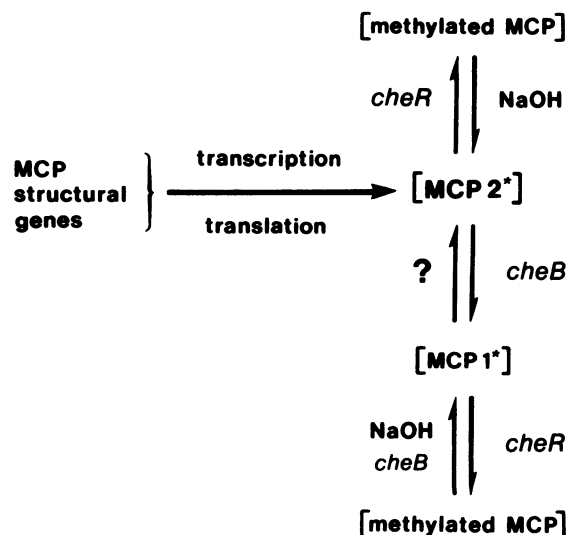


FIG. 5. Summary of posttranslational processing of MCP by the *cheB* and *cheR* functions. See text for details.

cessing event is reversible, so MCP 1* probably represents the "mature" (but unmethylated) form of MCP. However, in the presence of *cheR* function, both MCP 1* and MCP 2* are capable of accepting methyl groups, so the 2* → 1* conversion is not an obligatory step in the methylation process.

Nature of the Difference Between MCP 1* and MCP 2*. Although MCP 2* bands at about the same position in NaDodSO₄/polyacrylamide gel electrophoresis as singly methylated MCP from wild-type cells, two lines of evidence demonstrate that the MCP 2* migration pattern is not caused by methylation. First, methyl label could not be incorporated into MCP 2* in the absence of *cheR* activity. Second, unlike the pattern of methylated MCP molecules, the migration pattern of MCP 2* could not be altered by base hydrolysis. In most respects, however, MCP 2* seems to mimic methylated MCP. For example, the conversion of MCP 2* to MCP 1* is dependent on *cheB* function, as is the removal of methyl groups from MCP. These facts suggest that MCP 2* may contain a chemical moiety that influences the biochemical and physical properties of MCP in much the same manner as do glutamic acid methyl ester groups.

Rollins and Dahlquist (25) have used our *che* deletion mutants to examine MCP 1* and MCP 2* by isoelectric focusing. They find that the MCP 1* forms are more acidic than MCP 2* by one or two charge differences, indicating that the *cheB*-dependent conversion of MCP 2* to MCP 1* entails either the removal of positive charges or the addition of negative charges. Because this processing event is so similar to the *cheB*-dependent removal of glutamic acid methyl ester groups on MCP, it seems likely that one or more of the methyl-accepting glutamic acid residues of MCP could be involved. One possibility is that MCP 2* contains glutamine rather than glutamic acid at these sites. This would not only account for the similar behavior of MCP 2* and methylated MCP in NaDodSO₄/polyacrylamide gel electrophoresis and isoelectric focusing but could also explain why *cheB* function is able to catalyze both demethylation and the MCP 2* to MCP 1* conversion, because glutaminases often have methyltransferase activity as well (26).

Functional Properties of MCP 1* and MCP 2*. Mutants containing either the 1* or 2* forms of MCP are able to initiate changes in their pattern of flagellar rotation in response to chemotactic stimuli (unpublished data). Because type 1* and 2* strains have no methyltransferase activity, they are unable

to alter MCP methylation state and are therefore unable to carry out sensory adaptation. However, in the presence of functional *cheR* product, both MCP 1* and MCP 2* are capable of accepting methyl groups. The extent of methylation under these conditions is subject to control by chemotactic stimuli and can lead to sensory adaptation. Both excitation and adaptation are mediated by MCP signalers in wild-type cells and presumably by MCP 1* or MCP 2* in mutant strains. Because stimuli have no effect on the profile of either the 1* or 2* forms in the absence of methylation, excitation need not be associated with a change in electrophoretic behavior of the MCP molecules involved in the signaling event.

Role of MCP 2* in Chemotaxis. Although MCP 2* is not greatly altered in its signaling properties, it is perhaps significant that both the *tar* and *tsr* products are initially synthesized in the MCP 2* form, which is subsequently converted to the 1* form by a *cheB*-dependent process. It may be that MCP molecules must be synthesized in the 2* form in order to assume their proper native conformation or to permit their insertion into the cytoplasmic membrane. However, the similarity in behavior of MCP 2* and methylated MCP suggests that MCP 2* could have an even more important role in chemotaxis. Mutants that produce MCP in the 1* form exhibit an extreme counterclockwise bias in flagellar rotation, whereas mutants containing MCP 2* display a clockwise bias (unpublished data). This difference in behavior is presumably due to the methylation-like signaling properties of MCP 2*. In wild-type strains methylated MCP seems to be associated with clockwise flagellar rotation, because adaptation to stimuli that inhibit clockwise rotation is accompanied by an increase in methylation levels. Clockwise flagellar rotation is essential for chemotactic behavior because it enables *E. coli* to change swimming direction. Perhaps by synthesizing MCP in the 2* form initially, the cell ensures that it will have an adequate frequency of spontaneous turning movements as it swims.

It is now apparent that neither methylation-demethylation of MCP nor the *cheB*-dependent processing events described in this report are required to initiate flagellar responses to stimuli. Although the molecular nature of the excitatory forms of MCP remains very much a mystery, further study of these interesting membrane proteins and their posttranslational processing systems should eventually enable us to understand these signaling events.

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