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Aberrant Regulation of Methylesterase Activity in *cheD* Chemotaxis Mutants of *Escherichia coli*

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The adaptation process in several *cheD* chemotaxis mutants, which carry defects in *tsr*, the serine transducer gene, was examined. *cheD* mutants are smooth swimming and generally nonchemotactic; the defect is dominant to the wild-type *tsr* gene (J. S. Parkinson, J. Bacteriol. 142:953–961, 1980). All classes of methyl-accepting chemotaxis proteins synthesized in unstimulated *cheD* strains are overmethylated relative to the wild type. We found that the steady-state rate of demethylation in *cheD* mutants was low; this may explain their overmethylated phenotype. In addition, all *cheD* mutants showed diminished responsiveness of methylesterase activity to attractant and repellent stimuli transduced by either the Tsr or Tar protein, and they did not adapt. These results suggest that the dominant nature of the *cheD* mutations is manifested as a general defect in the regulation of demethylation. Some of these altered properties of methylesterase activity in *cheD* mutants were exhibited in wild-type cells that were treated with saturating concentrations of serine. The mutant Tsr protein thus seems to be locked into a signaling mode that suppresses tumbling and inhibits methylesterase activity in a global fashion. We found that the Tar and mutant Tsr proteins synthesized in *cheD* strains were methylated and deamidated at the correct sites and that the mutations were not located in the methylated peptides. Thus, the signaling properties of the transducers may be controlled at sites distinct from the methyl-accepting sites.

Bacteria detect chemical changes in their environment via specific receptors. This sensory information is transduced to the flagellar apparatus to produce a behavioral response. The response to attractant stimuli consists of a suppression of tumbling which promotes the net movement of cells in a favorable direction. Tumbling frequency increases in response to repellents. The initiation of responses is rapid and is followed by a period of adaptation after which the cells return to their prestimulus swimming pattern of alternating runs and tumbles (2, 3, 25, 30).

In *Escherichia coli*, a family of four known receptor proteins denoted the methyl-accepting chemotaxis proteins (MCPs) plays an essential role in the sensory transduction process (5, 22, 31, 33). Each class of protein mediates behavioral responses to certain subsets of attractant and repellent molecules. Mutants with defects in the *tsr* gene lack responsiveness to the attractant serine and to repellents such as leucine and weak acids. Responses to the attractants aspartate and maltose and the repellents Ni(II) and Co(II) are abolished in *tar* mutants (32, 33). Mutants in *trg* are unresponsive to the attractants ribose and galactose (15, 22). The products of the *tsr* and *tar* genes (MCPI and MCPII, respectively) are about an order of magnitude more abundant than the *trg* and *tap* gene products (5, 15).

The MCPs appear to be either primary or secondary sensory receptors and seem to be involved in generating the signal that controls the direction of flagellar rotation. In addition, behavioral adaptation of cells to stimuli correlates with changes in the level of methylation of the MCP class that mediates the response. Each MCP migrates on polyacrylamide gels as a set of multiple bands. The faster-migrating MCPs are more highly methylated and increase in abundance during adaptation to a positive stimulus (attractant increase or repellent decrease). Conversely, adaptation to a negative stimulus correlates with a decrease in the highly methylated forms and an increase in the slower-migrating, less-methylated bands (6, 8, 9, 11, 13). Thus, the level of methylation seems to represent a record of the previous environment of the cell, which the bacterium uses to compare with its current environment.

Methylation results in formation of the γ-methyl ester of glutamic acid residues on MCPs (21, 41). At least five sites on the Tsr protein and four sites on the Tar protein are available for methylation in chemotactically wild-type cells (18, 19). In addition, an enzymatic deamidation of glutamine to glutamic acid residues that accept methyl groups occurs at two sites on the Tsr and Tar proteins (17, 18). The methylation sites are located on two different tryptic peptides in the Tar and Tsr proteins (designated peptides K1 and R1) (18, 19). Peptide K1 contains lysine, methionine, the two deamidation sites, and after deamidation at least three specific glutamic acid residues that accept methyl groups (17, 19).

Methylation is catalyzed by a chemotaxis-specific methyltransferase encoded by the *cheR* gene (10, 35). Hydrolysis of the methyl esters produces methanol and the free γ-carboxyl of glutamic acid (36, 39) and is catalyzed by a methylesterase, the product of the *cheB* gene (14, 36). The *cheB* gene product also seems to catalyze deamidation (18, 28, 29). In the absence of stimuli, methyl groups on MCPs turn over with an average half-life of 20 min in a balance between the activities of methylesterase and methyltransferase (23, 40). These activities constitute the adaptation system of the cell and are precisely regulated by an unknown mechanism. By using methanol production as an assay for methylesterase activity, it has been possible to show that in wild-type cells an attractant stimulus transiently inhibits methylesterase activity (19a, 40). Similarly, a negative stimulus results in a brief increase in methylesterase activity (13, 39; Kehry et al., submitted for publication).

In the present work, we report studies on a class of dominant mutations found in the *tsr* gene, denoted *cheD* mutants. These mutants were isolated and characterized by

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TABLE 1. *E. coli* strains[a]

Strain	Chemotaxis genotype	Source
FD487	Wild type	This work
RP3841	Δ*tar*	J. S. Parkinson
RP5884	Δ*tsr*	J. S. Parkinson
RP2867	Δ(*tap-cheB*)	J. S. Parkinson
RP4791	*cheD191*	J. S. Parkinson (27)
RP4792	*cheD192*	J. S. Parkinson (27)
RP4793	*cheD193*	J. S. Parkinson (27)
159 *flaI*	*flaI*	M. Simon

[a] The original strain RP487 was obtained from J. S. Parkinson and made a λ *ind*⁻ lysogen. RP487 was previously designated RP477 *metF* (23).

J. S. Parkinson and co-workers (6a, 26, 27) and are generally nonchemotactic. Our studies and others (6a, 27) suggest that the altered Tsr protein in *cheD* strains is locked into the signaling mode and has globally activated the adaptation system. Cells are smooth swimming and seem incapable of adapting behaviorally (27). We measured the rate at which methyl groups turn over and stimulus-induced changes in methylesterase activity in cells carrying three *cheD* alleles. These studies suggest that abnormal regulation of MCP-specific demethylation may be responsible for the aberrant behavioral phenotype of *cheD* mutants. Possible mechanisms by which MCP signaling controls adaptation are discussed.

MATERIALS AND METHODS

Strains. The$_{S1}$ *E. coli* strains used are listed in Table 1. All strains were gifts from J. S. Parkinson or M. I. Simon.

Chemicals. L-[*methyl*-^{3}H]methionine (80 Ci/mmol) and [^{14}C]methanol (3.3 mCi/mmol) were obtained from New England Nuclear Corp. and diluted before use as described previously (19a). Synthetic L-serine and L-aspartate used as attractants were from ICN Pharmaceuticals, K and K Laboratories Division. Succinic acid was purchased from Sigma Chemical Co. All solvents for high-performance liquid chromatography were glass distilled and obtained from Burdick and Jackson, Inc. Trifluoroacetic acid (sequanal grade) was purchased from Pierce Chemical Co. in sealed 1-g ampoules and stored at 4°C. All buffers and flow media were prepared from glass-distilled water.

Growth of bacteria and measurement of methanol production. Bacteria were grown in minimal medium exactly as described elsewhere (19a). Dilute overnight cultures of *cheD* strains were grown by using nonchemotactic colonies from fresh tryptone swarm plates. Only smoothly swimming isolates were used in experiments. [^{3}H]methanol was measured by vapor-phase transfer of medium directly in scintillation vials as described previously (19a, 38; D. Chelsky et al., Anal. Biochem., in press).

Continuous-flow assay. The filter assembly (Gelman acrodisc, 0.2 μm) used in all flow experiments was described previously (19a). Briefly, the outlet was channeled to a fraction collector, and the filter inlet was connected to peristaltic pump tubing. For chase turnover experiments, between 1.2×10^9 and 1.8×10^9 washed cells were incubated with 40 μCi of L-[*methyl*-^{3}H]methionine (not dried) at 30°C for 45 min. The chase was initiated by pumping the cells onto the filter and maintaining them at 23°C by a continuous flow of medium (0.38 ml/min) that contained 0.1 mM methionine. Fractions of 0.8 min were collected and 0.15-ml aliquots of each fraction were placed in 0.5-ml microcentrifuge tubes for vapor-phase transfer. A stimulus consisted of quickly switching the inlet tubing to medium lacking or containing attractants. Preparation of all media (see below) has been described in detail previously (19a).

The number of MCP-methyl groups turning over per cell was measured by a combined steady-state turnover and chase turnover experiment. The rate of [^{3}H]methanol production was measured in the steady state, and the average half-life of methyl groups was measured from a subsequent chase. Washed cells (1.8×10^9) were resuspended in 2.0 ml of steady-state medium (10 mM Tris-hydrochloride [pH 7.4], 50 mM sodium succinate [pH 7.4], 0.25 μM methionine, 2.0 μCi of L-[*methyl*-^{3}H]methionine per ml, 5 μl of stock [^{14}C]methanol per 9 ml of medium), equilibrated, and maintained on the filter in the same medium. After 20 min on the filter, the medium was changed to chase medium [10 mM Tris-hydrochloride [pH 7.4], 50 mM sodium succinate [pH 7.4], 0.1 mM methionine, 5 μl of stock [^{14}C]methanol per 9 ml of medium] for an additional 40 min. Aliquots of medium that flowed over the cells were subjected to vapor-phase transfer, and the volatile ^{3}H and ^{14}C counts per minute were measured (Beckman LS 7000 apparatus with automatic quench compensation) (19a). Aliquots (10 μl) were also counted directly for calculation of the specific activity of [*methyl*-^{3}H]methionine. Efficiency of counting ^{3}H was taken as 37%. The ^{14}C in the medium was measured from an aliquot of medium that was removed prior to the addition of ^{3}H. Medium containing only ^{14}C was used to measure the transfer efficiency.

The number of methyl groups turning over per cell was calculated for each strain and for the strain carrying the deletion Δ(*tap-cheR-cheB*) from the above information as follows:

$$\frac{\text{molecules of methanol}}{\text{cell}} = \frac{(^3\text{H})(\text{eff})(\text{vol})\,[\text{met}]\,(t_{1/2})(6.02 \times 10^{11})}{(0.8)(0.15)(0.69)(\text{cell})(\text{cpm})} \quad (1)$$

where ^{3}H is the ^{3}H counts per minute of methanol transferred, eff is the transfer efficiency, vol is fraction volume in milliliters, [met] is the methionine concentration in picomoles per milliliter, $t_{1/2}/0.69$ is the average rate constant of demethylation in minutes obtained from the chase, cell refers to the number of cells on the filter, cpm is the ^{3}H counts per minute per milliliter of medium, and each fraction is 0.8 min with 0.15 ml subjected to vapor-phase transfer per fraction. The number of MCP-specific methyl groups turning over per cell was obtained by subtracting the background in the Δ(*tap-cheR-cheB*) deletion from each strain:

$$\frac{\text{MCP methyl groups}}{\text{cell}} = \frac{\text{methanol}}{\text{cell}} - \frac{\text{methanol}}{\text{cell}} \text{ for } \Delta(\textit{tap-cheR-cheB}) \quad (2)$$

Radioactive labeling of cells and polyacrylamide gel electrophoresis. Procedures for labeling and electrophoresis were exactly as described previously (18, 19a). Films were scanned with a densitometer (Quick Scan, Jr.; Helena Laboratories). Peaks corresponding to Tar and Tsr proteins were integrated with a planimeter. The half-life of methyl groups was obtained from a graph of peak area for methylated MCP versus time after the chase.

Trypsin digestion and peptide analysis. Preparative labeling, digestion with trypsin, and high-performance liquid chromatography were as described previously (18, 19). The aqueous buffer was 0.1% trifluoroacetic acid. A linear gradient of 0.1% trifluoroacetic acid in 90% acetonitrile was applied over the course of 85 min as described before (19, 20).

RESULTS

Phenotpye of *cheD* mutants. The *cheD* mutants used in these studies were isolated, characterized, and mapped by Parkinson and co-workers (6a, 26, 27). Mutant strains exhibit a general defect in chemotaxis; the cells are unable to tumble and consequently do not respond behaviorally to chemotactic stimuli (27). All of these mutations map to the *tsr* structural gene and are dominant to wild type (6a). The three *cheD* mutants we used in this work differed in the extent of their chemotaxis defects, ranging from severely (strain RP4793) to mildly (strain RP4791) nonchemotactic. RP4791 cells actually form very small swarms on semisolid agar (27).

Examination of the methylation level of MCPs synthesized in *cheD* strains illustrated two important features of the mutant phenotype. Methyl groups on MCPs were labeled by incubating cells in L-[*methyl*-^{3}H]methionine in the absence of protein synthesis, and the proteins were subjected to electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS). The distribution of methylated forms of MCPs synthesized in wild-type cells (Fig. 1, lanes 2 and 9) was very different from the distribution found in unstimulated *cheD* mutants (Fig. 1, lanes 3 to 5). The most-methylated (fastest-migrating) forms of both the Tsr and Tar proteins predominated in *cheD* mutants in contrast to a relatively uniform distribution in wild-type cells (Fig. 1; compare lanes 2 and 3). Thus, all MCP classes were overmethylated in *cheD* mutants. Similar results have been obtained by Callahan and Parkinson (6a) by labeling mutant Tsr proteins with [^{35}S]methionine after infection of UV-irradiated host strains with λ phage derivatives carrying *cheD* mutations.

Addition of attractants to wild-type cells results in a redistribution of MCP bands to the faster-migrating, more-methylated forms (6, 8, 9, 11). However, *cheD* strains exhibited little or no change in the methylation pattern of their [^{3}H]methyl-labeled MCPs after attractant stimulation (Fig. 1; compare lanes 3 and 8). The least severe *cheD* mutants (lanes 5 and 6 or 4 and 7) showed a greater change in methylation level in response to attractants; the Tar protein also seemed to exhibit greater attractant-stimulated methylation changes in *cheD* strains than did the Tsr protein. This suggests that the adaptation system in the severe *cheD* mutants does not respond to environmental stimuli in the normal fashion.

Steady-state methyl turnover rate in *cheD* mutants is low. In steady-state or adapted conditions (no stimulus), methyl groups on MCPs in wild-type cells turn over in a precise balance between methylation and demethylation (23, 40). The fact that MCPs produced in *cheD* mutants are overmethylated implies that in these cells, relative to wild-type cells, the rate of methylation exceeds the rate of demethylation. One possible explanation is that methyltransferase is more active in wild-type cells. Alternatively, methylesterase activity might be depressed.

We assayed methylesterase activity in cells whose methyl groups were labeled with [*methyl*-^{3}H]methionine by monitoring the production of [^{3}H]methanol (see above) the product

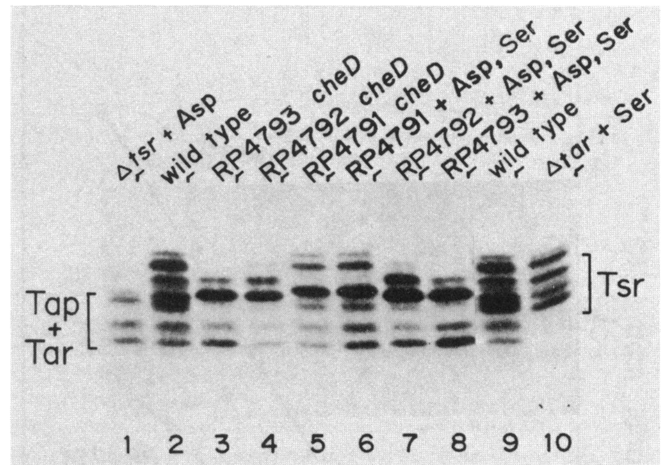

FIG. 1. MCPs synthesized in *cheD* mutants were overmethylated. Methyl groups on MCPs were specifically labeled with L-[*methyl*-^{3}H]methionine as described previously (18). Positions of the methylated Tsr, Tap, and Tar bands are indicated. The most methylated bands of Tsr protein overlapped with methylated forms of the Tap protein. Lanes: 1, RP5884 (Δ*tsr*) plus 1 mM aspartate; 2 and 9, FD487 (wild type), unstimulated; 3 and 8, RP4793 (*cheD193*); 4 and 7, RP4792 (*cheD192*); 5 and 6, RP4791 (*cheD191*); 10, RP3841 (Δ*tar*) plus 50 μM serine. The cells in lanes 6 to 8 were stimulated with 1 mM aspartate and 50 μM serine. The mutation in the *tsr* gene in RP4791 results in a slower mobility for the mutant Tsr protein relative to wild type (lanes 5 and 6) (6a).

of the demethylation reaction (19a, 38; Chelsky et al., in press). After a slight lag after the addition of L-[*methyl*-^{3}H]methionine, the rate of [^{3}H]methanol production in wild-type cells was linear (Fig. 2). The rate of [^{3}H]methanol production by a *flaI* mutant, which lacks flagella and all chemotaxis proteins, was approximately fivefold lower than the rate in wild-type cells. The high rate of methanol production measured by this assay was chemotaxis specific and required the presence of functional methyltransferase (*cheR*), methylesterase (*cheB*), and MCP (*tsr* and *tar*) genes (Fig. 2) (19a, 40). The rate of methanol produced in the most severe *cheD* allele, RP4793, was identical to the low rate in the nonmotile *flaI* mutant. The remaining two *cheD* mutants produced [^{3}H]methanol at a rate intermediate to *flaI* and wild-type cells (Fig. 2). This decreased methylesterase activity in *cheD* strains thus could account for the observed overmethylation of MCPs relative to wild-type cells.

***cheD* mutants show diminished responsiveness of methylesterase activity to stimuli.** We have previously described a flow assay which measured stimulus-induced changes in methylesterase activity in intact cells (19a). Briefly, cells were labeled with L-[*methyl*-^{3}H]methionine, washed free of radioactivity, and maintained on a disposable filter assembly by a continuous flow of medium containing a chase of nonradioactive methionine. Medium that was pumped over the cells during the chase was collected (0.8-min fractions) and assayed for [^{3}H]methanol. Cells were given positive or negative stimuli by switching the pump inlet tubing to medium that was identical except for the presence or absence of attractant (i.e., serine or asparate) (see above and reference 19a). In the absence of attractant stimulation, we observed a multiple exponential decay in the rate of [^{3}H]methanol produced by wild-type cells. The weighted average half-life of methyl groups and the average rate constant of demethylation were obtained from the unstimulated chase experiments (19a).

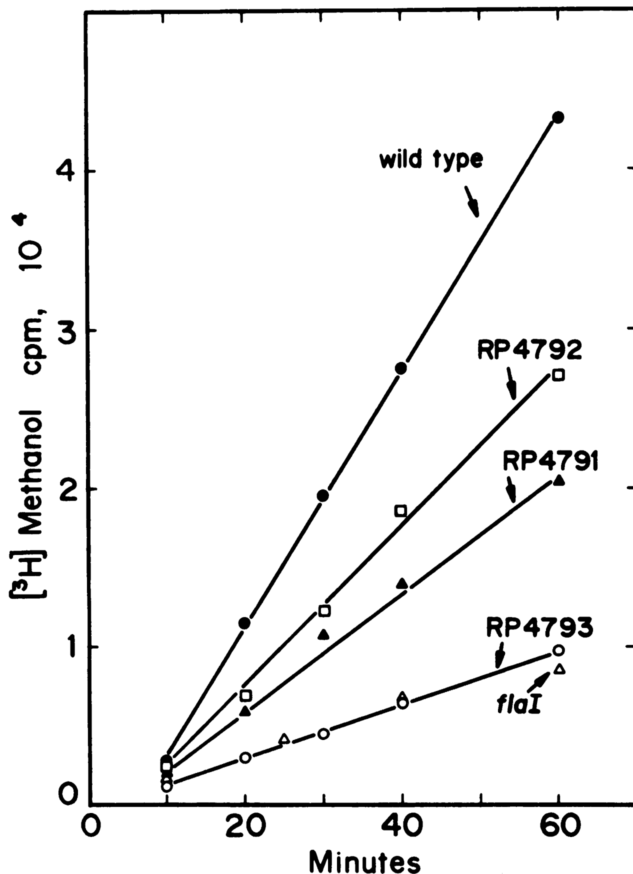

FIG. 2. Steady-state rate of [^{3}H]methanol production was low in *cheD* mutants. *E. coli* strains were grown and labeled with L-[*methyl*-^{3}H]methionine as described previously (19a). Aliquots of cells were removed at various times after the addition of radioactivity and treated with trichloroacetic acid. Volatile ^{3}H in the supernatant fraction was measured by vapor-phase transfer (19a, 38; Chelsky et al., in press). Transfer efficiency of a parallel [^{14}C]methanol sample was 51%. Symbols: ●, FD487 (wild type); ■, RP4792 (*cheD192*); ▲, RP4791 (*cheD191*); ○, RP4793 (*cheD193*); △, 159 *flaI*. Nonspecific background (counts per minute) was subtracted from each sample as follows: FD487, 700; RP4792, 630; RP4791, 550; RP4793, 640; 159 *flaI*, 660.

Wild-type cells responded to attractant stimulation (35 μM serine and 1 mM aspartate; Fig. 3A and 4A, respectively) by transiently decreasing the rate at which [^{3}H]methanol was produced. The decrease was followed by a recovery period of about 10 min. Although we are as yet unable to directly observe the detailed timecourse of methylation, we assumed that during the time that methylesterase activity was depressed a net increase in the level of MCP methylation occurred which corresponded to behavioral adaptation.

Removal of attractant (Fig. 3A and 4A, second arrows) resulted in a peak of increased methylesterase activity which correlated with the adaptation of cells to the negative stimulus. Neither of these changes in the rate of [^{3}H]methanol production was observed when a strain lacking the *cheR* and *cheB* gene products was given identical stimuli (Fig. 3A).

All *cheD* mutants showed diminished responsiveness of methylesterase activity to attractant stimuli. A positive serine stimulus resulted in a slight inhibition of [^{3}H]methanol production that seemed to persist until the stimulus was removed (Fig. 3); the *cheD* cells did not adapt. Removal of serine (negative stimulus) elicited only a small peak of [^{3}H]methanol in the least-severe *cheD* mutant (Fig. 3B). The rate of [^{3}H]methanol production in the other *cheD* alleles merely returned to the unstimulated rate and exhibited no peak in [^{3}H]methanol production (Fig. 3C and D). Thus, the magnitude of the responses to serine stimuli (transduced by Tsr) decreased with increasing severity of the *cheD* defect (Fig. 3B to D).

Responsiveness of methylesterase activity to aspartate, which is mediated by Tar, also was abolished in *cheD* mutants (Fig. 4B and C). As with a positive serine stimulus, aspartate addition resulted in a slight inhibition in the rate of [^{3}H]methanol production that returned to the prestimulus rate when the stimulus was removed. These experiments were consistent with previous results showing that *cheD* defects in the *tsr* gene are dominant over the remaining MCP classes (6a, 26). In addition, our data suggest that the dominance is manifested as a general defect in regulation of demethylation during chemotactic responses.

Half-life of methyl groups in *cheD* mutants is long. The average half-life of methyl groups that turned over on MCPs was measured by two different methods. In the first type of experiment, methyl groups were radioactively labeled with L-[*methyl*-^{3}H]methionine and then chased with an excess of nonradioactive methionine. At various times after the start of the chase, aliquots of cells were removed and solubilized for SDS-polyacrylamide gel electrophoresis. Radioactive methyl groups remaining in MCP bands were quantitated by densitometry of the resulting fluorographs. These data for wild-type cells and two *cheD* mutants are summarized in Table 2. Methyl groups on Tsr and Tar proteins in unstimulated wild-type cells turned over with a half-life of 26 and 23 min, respectively. The half-life of methyl groups in the *cheD* mutant RP4792 was similar to that in wild-type cells (Table 2). This is consistent with the similar time courses of methanol production observed in RP4792 and wild-type cells (Fig. 1). In contrast, the half-life of methyl groups on mutant Tsr protein in the *cheD193* strain (RP4793) was very long (85 min). The half-life of methyl groups on Tar protein from RP4793 also was long relative to that from the wild type (35 min). However, in this *cheD193* strain, the methyl groups on Tar turned over considerably faster than did those on the Tsr protein.

A second measurement of half-life was made by using a modification of the continuous-flow experiments as described in the text. Labeled cells were maintained on the filter in the presence of L-[*methyl*-^{3}H]methionine as in a steady-state turnover experiment. From data obtained from this portion of the flow experiment, we calculated the rate in molecules of methanol per cell per minute at which methyl groups turned over (Table 3; see above). Radioactive methyl groups were then chased by changing the medium flowing over the cells to medium containing an excess of nonradioactive methionine. The weighted average half-life of basal methyl groups was obtained from this chase. The information in the two parts of this experiment was combined to give an estimate of the number of methyl groups on MCPs that were turning over in the absence of any attractant or repellent stimulation (see above).

The data based on methanol production (Table 3) showed a good correlation with the half-lives measured from loss of methyl groups on MCPs (Table 2). In wild-type cells, the weighted average half-life of methyl groups was 18 to 20 min; methyl group half-lives in the *cheD* mutants RP4792 and RP4793 were longer (26 and 35 min, respectively; Table 3). Methyl groups therefore turned over slower in *cheD* mutants; this reflected their low methylesterase activity. We

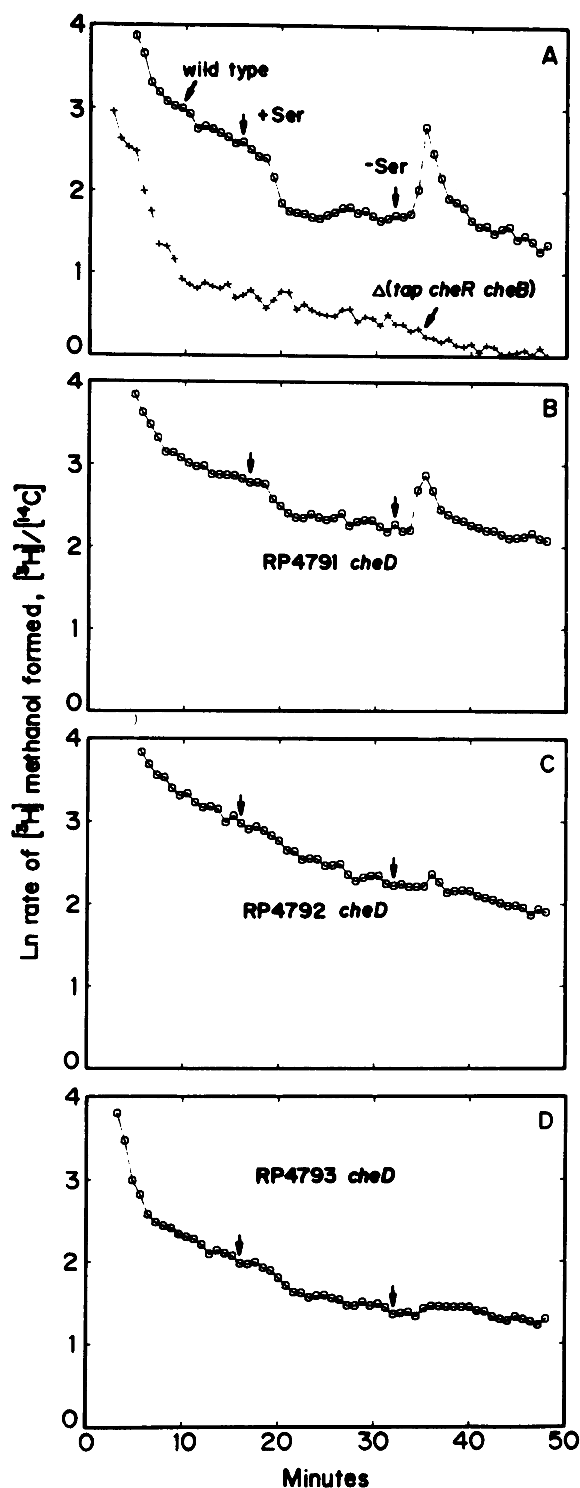

FIG. 3. Methylesterase activity in *cheD* mutants did not respond normally to stimuli through the Tsr transducer. Cells (2×10^9) were labeled for 40 min as described for flow-chase experiments (see text and reference 19a). At 0 min, medium containing L-[*methyl*-^{3}H]methionine was removed and replaced with medium containing a 300-fold excess of nonradioactive methionine, and cells were pumped onto the filter. The flow rate was 0.38 ml/min, and each fraction was 0.8 min. An internal standard of [^{14}C]methanol was added to the medium. Each data point was expressed as the natural logarithm of the volatile ^{3}H/^{14}C counts per minute transferred in a 150-μl portion of the fraction. A stimulus of 35 μM serine was applied at the first arrow (16 min) and was removed at the second arrow (32 min) for each curve. Media used for each strain were identical. (A) Symbols: O, FD487 (wild type); +, RP2867 Δ(*tap-cheR-cheB*). (B) RP4791 *cheD191*. (C) RP4792 *cheD192*. (D) RP4793 *cheD193*.

calculated the number of methyl groups turning over in *cheD* cells and found that up to twice as many methyl groups were turning over in the most severe *cheD* mutant (RP4793) as compared with wild-type cells. This provides additional support for the proposal that the low methylesterase activity in *cheD* cells plays an important role in maintaining overmethylation of MCPs.

The differences in the exchange times (Tables 2 and 3) reflected the fact that the flow method is sensitive to methyl groups which turn over most rapidly. The more direct assay performed with protein gels is sensitive to all the different methyl group turnover rates. The flow assay generally gives shorter half times than does the gel method. Both methods demonstrate the lowered rate of methyl turnover in *cheD* mutants.

Methyl-accepting sites seem normal in *cheD* mutants. One possible explanation at the structural level for the decreased activity of methylesterase in *cheD* cells is that the locations of *cheD* mutations may be adjacent to or in the methyl-accepting sites or deamidation sites in the mutant Tsr protein. We therefore compared [^{3}H]methyl-labeled tryptic peptides from Tar and Tsr (the two fastest-migrating bands of each MCP [Fig. 1]) synthesized in mutant and wild-type cells. As expected, peptide maps of methylated peptides derived from Tar protein synthesized in wild-type, *tsr*, and *cheD* strains were identical (data not shown). Methylated peptides derived from Tsr protein produced in wild-type, *tar* cells, and all three *cheD* mutants also eluted at identical positions (data not shown). Therefore, it is likely that the *cheD* mutations were not located in the methyl-accepting tryptic peptides of the Tsr protein. In addition, the deamidations seemed to be occurring normally to produce methyl-accepting glutamic acid residues.

Only one difference was observed in the methylated peptides from Tsr protein synthesized in *cheD* and wild-type cells. In protein from *cheD* mutants, the trimethylated form of tryptic peptide K1 showed relatively less incorporation of [^{3}H]methyl groups than did the same peptide derived from Tsr synthesized in wild-type cells (data not shown). This difference presumably reflected the lower rate of turnover of methyl groups in *cheD* strains. We predict that the difference in incorporation would not be observed if the cell labeling period were extended longer than the 60 to 70 min routinely used for peptide map preparation.

Effects of high serine concentrations in wild-type cells. In agreement with other studies (1, 4), we observed an unusual effect of high serine concentrations (1 to 10 mM serine) on bacterial behavior. Addition of 1 mM serine to wild-type cells completely suppressed their tumbling behavior. A 1 mM serine stimulus in steady-state flow experiments also inhibited methylesterase activity such that no recovery to the prestimulus rate occurred. This indicated that the cells were unable to adapt. Adler has noted (1) that serine inhibits chemotaxis in a general fashion. These observations suggested that if, as discussed below, the Tsr protein in *cheD* mutants is locked in a signaling mode, we might expect wild-type cells that have been treated with high serine concentrations to phenotypically resemble *cheD* mutants.

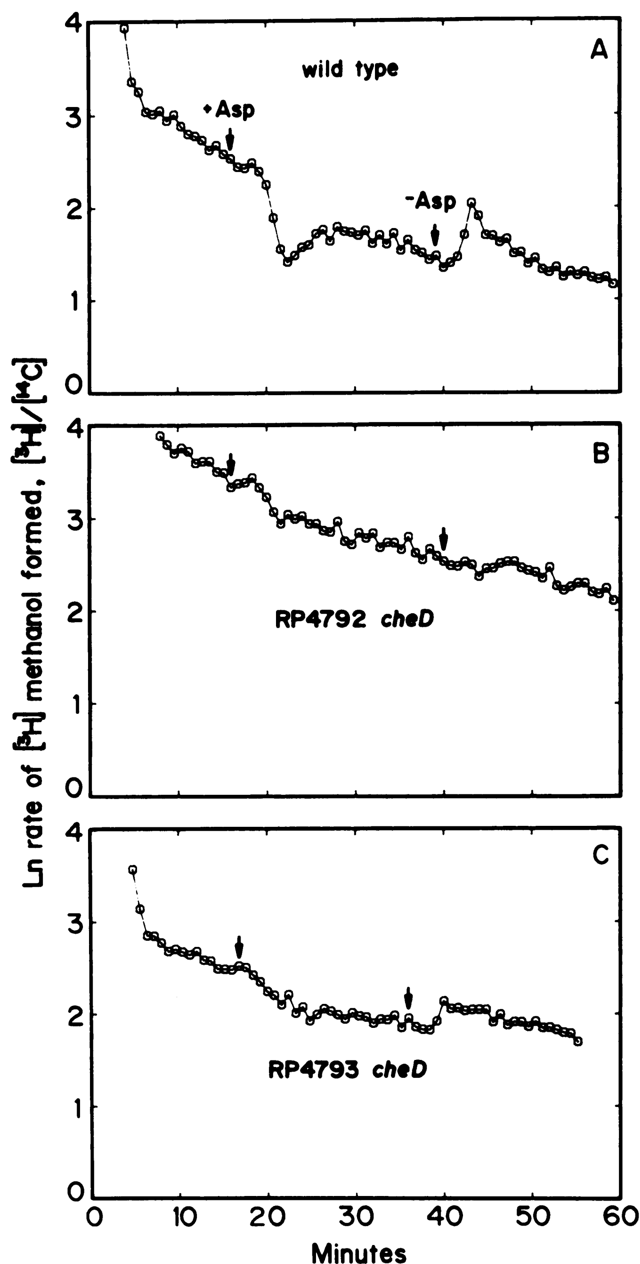

FIG. 4. Aberrant regulation of methylesterase activity in *cheD* mutants in response to stimuli through the Tar transducer. Cells were labeled and chased on the filter apparatus as described in the legend to Fig. 3. A stimulus of 1 mM aspartate was applied at the first arrow and was removed at the second arrow along each curve. (A) FD487, 1.8×10^9 cells (wild type). (B) RP4792, 2×10^9 cells (*cheD192*). (C) RP4793, 1.2×10^9 cells (*cheD193*). The curve in B is shifted upward to an apparently higher rate of [^{3}H]methanol production relative to those in A and C because of the larger number of cells used.

This idea was tested by performing chase turnover experiments similar to those shown in Fig. 4 in the absence or continuous presence of 1 mM serine in the medium. Wild-type cells and *tsr* deletion mutants were given a stimulus of 1 mM aspartate (Fig. 4) with or without 1 mM serine present. High serine concentrations decreased the initial rate at which wild-type cells produced [^{3}H]methanol in the chase and virtually eliminated the exponential decay of [^{3}H]methanol production observed in an unstimulated chase; the slope was almost zero. This implies that the half-life of methyl groups was very long in the presence of 1 mM serine. However, the responses of methylesterase activity to the positive and negative aspartate stimuli were similar to those observed in wild-type cells in the absence of high serine (data not shown). The peak of [^{3}H]methanol produced by removal of aspartate actually was larger in the presence of 1 mM serine; this increase presumably resulted from the longer half-life of methyl groups during the chase. Moreover, none of these effects of high serine concentrations were observed in the *tsr* deletion mutant; decreases in methyl turnover rate and increased half-life of methyl groups required the normal chemoreception of serine.

TABLE 2. Half-lives of basal methyl groups[a]

Strain and genotype	$t_{1/2}$ (min)		
	Tsr	Tar	Total[b]
FD487 (wild type)	26	23	24
RP4792 *cheD192*	20	22	20
RP4793 *cheD193*	85	35	69

[a] Methyl groups on MCPs were radioactively labeled and quantitated from SDS-polyacrylamide gels as described previously (18, 19a). Half-lives were obtained from a plot of total peak area in methylated MCP versus time after the chase. All labeling and chases were done in the absence of stimulation. Time points of 0, 6, 12, 20, 30, 45, and 60 min were taken.

[b] Bands corresponding to the Tsr and Tar proteins were quantitated separately. Total MCP is the sum over all MCP bands that were labeled (Tsr, Tap, and Tar proteins).

DISCUSSION

cheD mutations produce signaling defects in the Tsr protein. The phenotype of *cheD* mutants seems to result from an altered form of the *tsr* gene product (6a, 27). Previous studies (6a, 27) and the results presented here support the idea that the altered Tsr protein is locked into the signaling mode induced by a positive stimulus and is unresponsive to the actions of the adaptation system. Parkinson has shown that *cheD* mutants regain chemotactic functions by acquiring

TABLE 3. Half-lives and numbers of basal methyl groups[a]

Experiment	Strain and genotype	$t_{1/2}$ (min)	No. of MCP-methyl groups[b]
1	FD487 (wild type)	20	1,000
	RP4792 *cheD192*	26	1,700
2	FD487 (wild type)	18	3,300
	RP4793 *cheD193*	35	6,700

[a] Cells were labeled with [*methyl*-^{3}H]methionine, and the rate of methanol production was measured in a steady-state turnover experiment. Half-life was obtained by subsequently chasing the cells with nonradioactive methionine in the flow apparatus. Experiments were done in the absence of stimulation as described in the text.

[b] The number of methyl groups turning over per cell was obtained from the half-life and the rate of [^{3}H]methanol production (molecules of methanol per cell per minute) calculated as described in the text. In each experiment, the same measurements were made on a strain carrying the deletion Δ(*tap-cheR-cheB*). Molecules of methanol produced per cell in this strain were subtracted as background from the wild type and from other mutants to give the above numbers. Absolute numbers of methyl groups in wild-type cells varied with the experiment, probably because of differences in cell motility; the average of 17 experiments was 2,050 methyl groups per cell (range of 850 to 3,700).

a second, null mutation in the *tsr* gene (27). Thus, the dominant nature of the *cheD* phenotype is exerted by the aberrant activity of the mutant *tsr* product.

Our measurements of methylesterase activity and the regulation of demethylation in *cheD* strains suggest that the mutant Tsr protein is actively signaling. In wild-type cells immediately after an attractant stimulus, the rate of methyl group hydrolysis decreases four- to fivefold (19a, 40), tumbling is suppressed, and the level of methylation on the appropriate MCP increases (13, 22, 23, 32–34). This reponse is exactly what we observed in unstimulated *cheD* mutants: a low rate of methyl turnover, the absence of tumbling behavior, and overmethylation of MCPs. It appears that *cheD* cells have activated their adaptation system, but accumulation of methyl groups is unable to cause adaptation. Callahan and Parkinson (6a) have reached similar conclusions from their analyses of the behavior and methylation levels in numerous *cheD* alleles. We concluded that *cheD* strains may be useful as a model for investigating the biochemical properties of continuously signaling sensory transducer proteins.

In addition, we showed by peptide mapping that the altered Tsr protein seemed to be deamidated and accepted methyl groups at the appropriate sites. These results were in agreement with fine-structure deletion mapping, which placed these three *cheD* mutations at sites distinct from the methyl-accepting regions, and the observation that deamidated forms of the altered Tsr protein are synthesized in a *cheR* background (6a). These results do not exclude the possibility that the *cheD* mutations may actually be adjacent to the methylation sites in the three-dimensional structure of the Tsr protein and may include potential binding sites for methylesterase or methyltransferase. However, methylation of the mutant protein does occur and apparently does not result in the same structural changes that in the wild-type gene product inhibit the production of an excitatory signal. This suggests that in the structure of an MCP molecule the signaling properties may be controlled at sites that are distinct from the sites at which methylation and demethylation occur.

We observed that high serine concentrations produce inhibitory effects on chemotactic responses of wild-type cells. These effects may be similar to some of the aberrations in the chemotactic ability of unstimulated *cheD* strains. Serine suppresses tumbling in wild-type cells (4), partially inhibits responses to stimuli transduced by other MCPs (1), and in the present study decreased the rate of methyl turnover while increasing the half-life of methyl groups (see above). Chemoreception of serine by the Tsr protein was required to produce these effects because they did not occur in *tsr* deletion mutants. However, normal transient responses in methylesterase activity resulted from application of Tar stimuli to wild-type cells in the continuous presence of saturating serine. In *cheD* mutants these stimulus-induced changes in methylesterase activity were diminished or absent, and no recovery was observed. Thus, saturating the Tsr protein in wild-type cells with serine did not entirely mimic the dominant effects of a *cheD* mutation. It is possible that the ability of the signaling properties of the wild-type Tsr protein to change as a result of increased methylation levels may account for this difference.

Implications for regulation of demethylation in wild-type bacteria. If we consider the *cheD* transducer as a model for a continuously signaling Tsr protein, what can the study of *cheD* mutants tell us about the mechanism by which MCP signaling controls adaptation? We cannot support or refute any models for regulating the methylation process because we are not yet able to assay methyltransferase activity with sufficient temporal resolution. The observation that the continuous signaling of Tsr decreases methylesterase activity and responsiveness to stimuli transduced through all MCP classes suggests two possible mechanisms for regulation of demethylation (37).

In one case, the dominant effect would be mediated by a direct communication between transducers of different classes. Tsr and Tar molecules would be asociated in a complex (7). Regulation of demethylation occurs directly by a change in reactivity to esterase that is induced in the MCP as a result of chemoreception. Stimulation of one MCP class is communicated to the other MCP class within the complex and accounts for the nonspecific methylation. If this model were correct, it is difficult to understand why methylation is apparently specific to the MCP class transducing the stimulus in wild-type cells (34). In addition, coordinate synthesis of the mutant Tsr protein and wild-type Tar protein is not required to produce the dominant overmethylated *cheD* phenotype (Parkinson, personal communication). This suggests that unless complexes of Tsr and Tar molecules dissociate and reassociate in the inner membrane, the complexes proposed in this model may not be required to produce the dominant effect of *cheD* mutations.

Another mechanism for regulating demethylation is that the signaling of one MCP class produces a general or global effect on the adaptation system (i.e., demethylation). In this case, signaling of the Tsr transducer regulates demethylation throughout the cell via some intermediate that fluctuates according to the adapted state of the cell (40). *cheD* cells seem to produce something that suppresses tumbling. An entity has been postulated to regulate tumbling frequency and is designated "tumble regulator" or "response regulator" (16, 24, 25). This tumble regulator or its predecessor in the flow of information may be the same intermediate that regulates demethylation in a global fashion. This model accounts for the dominant overmethylated phenotype and the lack of responsiveness observed in *cheD* mutants. Specificity of methylation in wild-type cells would be maintained by MCP-specific regulation of methylation in a manner similar to that proposed by Stock and Koshland (37) and Goldbeter and Koshland (12).

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