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N-Terminal Half of CheB Is Involved in Methylesterase Response to Negative Chemotactic Stimuli in *Escherichia coli*

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The chemotactic receptor-transducer proteins of *Escherichia coli* are responsible for directing the swimming behavior of cells by signaling for either straight swimming or tumbling in response to chemostimuli. The signaling states of these proteins are affected not only by the concentrations of various stimuli but also by the extent to which they have been methylated at specific glutamyl residues. The activities of a chemotaxis-specific methyltransferase (CheR) and a chemotaxis-specific methylesterase (CheB) are regulated in response to chemotactic stimuli to enable sensory adaptation to unchanging levels of stimuli by appropriately shifting the signaling states of the transducer proteins. For CheB this regulation involves a feedback loop that requires some of the components making up the chemotactic signal transduction machinery of the cell. This feedback loop causes the methylesterase activity of CheB to decrease transiently in response to attractant stimuli and to increase transiently in response to negative stimuli (repellent addition or attractant removal). In this report we demonstrate that the methylesterase response to negative stimuli involves the N-terminal half of the CheB protein, whereas the response to positive stimuli does not require this segment of the protein. Both aspects of the methylesterase response require CheA. In addition, we demonstrate that mutant forms of CheB lacking methylesterase activity can adversely affect the swimming behavior and chemotactic ability of cells and can markedly diminish modulation of the wild-type methylesterase activity in response to negative stimuli. The significance of these results is discussed in relation to the recent demonstration of phosphoryl transfer from CheA to CheB (J. F. Hess, K. Oosawa, N. Kaplan, and M. I. Simon, Cell 53:79–87, 1988) and the discovery of sequence homology between the N-terminal half of CheB and CheY (A. Stock, D. E. Koshland, Jr., and J. Stock, Proc. Natl. Acad. Sci. USA 82:7989–7993, 1985).

Considerable progress has been made recently toward identifying and characterizing the cellular components and processes that enable an *Escherichia coli* cell to control its swimming behavior in response to chemical stimuli and thereby enable chemotaxis. A normally motile cell alternates frequently between two distinct swimming behaviors: smooth swimming and tumbling. By regulating the relative time spent performing these two behaviors in response to various attractants and repellents, an *E. coli* cell accomplishes chemotaxis (1, 2, 24, 25, 50). In addition to being able to detect and respond rapidly to chemostimuli, *E. coli* cells are capable of shutting off these responses when the levels of such stimuli remain constant; i.e., the cells can adapt to the stimuli (3, 4, 25).

Among the cellular components that enable the chemotactic response and adaptation are the transducer proteins. These transmembrane proteins function as receptors for many chemostimuli and therefore are responsible for binding to specific attractant and repellent molecules and for communicating (signaling) these binding events to the cellular machinery that determines the swimming behavior of the cell (6, 10, 11, 21, 22, 26, 39, 61). The signaling mode (8, 29, 32; P. Ames, J. Chen, C. Wolff, and J. S. Parkinson, Cold Spring Harbor Symp. Quant. Biol., in press) adopted by a specific transducer protein is determined, in part, by its interactions with its specific attractant and repellent molecules. For example, the transducer protein Tsr signals for smooth swimming when it has bound an attractant ligand (such as serine), but it signals for tumbly behavior when it has bound a repellent ligand (such as leucine).

The signaling mode of a transducer protein is affected not only by its interactions with attractants and repellents, but also by the methylation of several of its glutamic acid residues (12–14, 35, 36, 49). Methylation of the transducer proteins is catalyzed by a chemotaxis-specific methyltransferase (CheR) (49), which utilizes *S*-adenosylmethionine as the methyl donor and generates the corresponding methyl esters of specific glutamate residues of the transducers. A chemotaxis-specific methylesterase (CheB) catalyzes the hydrolysis of these methylesters, producing methanol and regenerating the glutamate carboxylate moieties (56). All of the transducer proteins are methylated to some extent, reflecting the relative activities of CheR and CheB.

Transducer methylation levels change in response to chemostimuli (19, 20, 42, 46). For example, an increase in an attractant concentration causes an increased level of methylation of its receptor (transducer) protein; an increased repellent concentration causes decreased methylation of its receptor (transducer) protein. These changes of transducer methylation levels are thought to play a key role in adaptation by resetting the signaling bias of the transducer proteins following a stimulus. Thus, an attractant stimulus initially causes smooth swimming, but as the receptor becomes more methylated, this signaling bias is negated. For a repellent stimulus the initial behavioral response is to tumble, but as the receptor protein becomes less methylated, the signaling bias returns to the normal level (12, 13, 35–37, 47).

The methylesterase activity of CheB is modulated in response to chemotactic stimuli: attractants (such as serine) cause a transient decrease in methylesterase activity, whereas repellents (such as leucine) cause a transient activity increase (17, 48, 59). These activity changes are thought to play an important role in adjusting the signaling status of the transducer proteins to enable adaptation to occur. We

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are interested in understanding the events and interactions that modulate the rate of transducer demethylation in response to chemotactic stimuli in *E. coli*. As a first step toward studying these interactions, we have investigated whether the N-terminal portion of CheB is involved in this modulation. Previous work with CheB from *Salmonella typhimurium* by Simms et al. (44) has demonstrated that the N-terminal two-fifths of this enzyme is not required for its catalytic (methylesterase) activity, but analysis of the amino acid sequence of this region of the protein suggests that it may play some role in regulating the methylesterase activity of CheB. For example, Simms et al. (43) have suggested that this region of CheB may contain a nucleotide binding domain. In addition, the N-terminal portion of CheB (the first 129 amino acids) shares regions of homology with the CheY protein (the putative tumble generator), perhaps indicating interaction with a common regulator (52). Hess and coworkers have recently provided direct evidence for such interactions by demonstrating phosphorylation of CheB and CheY by CheA (15, 16, 31). These phosphotransfer reactions may provide a mechanism for modulating CheB methylesterase activity in response to chemostimuli and for activating CheY as a tumble generator (15, 16, 31, 34, 45). The sequence homology between CheY and the N-terminal portion of CheB and analogies with other bacterial sensory systems (18a, 38, 52) suggest that the site of CheB phosphorylation lies in its N-terminal half.

In this communication we report our investigations of the in vivo modulation of methylesterase activity for various N-terminal CheB deletion mutants in *E. coli*. We also report the altered modulation of the wild-type methylesterase caused by expression of some catalytically inactive forms of CheB in merodiploids. Our results provide some insight into the functional role of the N-terminal region of CheB.

MATERIALS AND METHODS

Chemicals. L-[*methyl*-^{3}H]methionine (80 Ci/mmol) was obtained from Dupont, NEN Research Products. Ready-Solv scintillation cocktail was purchased from Beckman Instruments, Inc. Synthetic L-serine and L-leucine were used as stimuli in flow experiments and were purchased from K and K Laboratories Division, ICN Pharmaceuticals Inc. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Calbiochem-Behring. Succinic acid and Tris used for making flow media were obtained from Sigma Chemical Co. Glass-distilled water was used in preparing all buffers and flow media.

Bacterial strains. The isogenic *E. coli* strains RP487 (a chemotactically wild-type strain), RP4972 (a Δ*cheB* mutant), and RP1788 (a Δ*cheA* mutant) were kindly provided by J. S. Parkinson. RP487 and RP4972 were made *recA* by P1 transduction with P1 *vir* bacteriophage grown on a *recA srl*::Tn*10* host and were renamed D213 (RP487 *recA*) and D263 (RP4972 *recA*), respectively. These strains require histidine, threonine, leucine, methionine, and thiamine for growth.

Media. AB minimal medium was prepared as described by Clark and Maaloe (9) and was supplemented with 5 μg of thiamine per ml, 1% glycerol, and the required amino acids at 0.1 mg/ml. λ tryptone broth contained 1% tryptone (Difco Laboratories) and 0.5% NaCl. Swarm plates contained λ tryptone broth and 0.35% agar. Flow medium contained 50 mM succinic acid and 10 mM Tris adjusted to pH 7.4 with NaOH. Flow chase medium contained flow medium and 100 μM cold (unlabeled) methionine.

Growth of bacteria. Cells for use in flow assays, for observation of swimming behavior, or for immunoblotting were obtained as follows. Single colonies from fresh LB plates were used to inoculate λ tryptone swarm plates (0.35% agar) containing the desired concentration of IPTG; both types of plates contained ampicillin at 100 μg/ml when appropriate for maintenance of plasmids. After 8 to 10 h of growth at 30°C, swarm colonies were used to inoculate overnight cultures in AB minimal medium supplemented with histidine, threonine, leucine, methionine, thiamine, and 1% glycerol plus the same concentrations of ampicillin and IPTG (when appropriate) as used in the swarm plates. These overnight cultures were grown at 30°C for 8 to 12 h and then used to inoculate 25 ml of λ tryptone broth containing 1% glycerol and appropriate concentrations of IPTG and ampicillin. These cultures were grown at 30°C for approximately 2 h to a cell density of 1×10^8 to 2×10^8 cells per ml, at which time the cells were pelleted by centrifugation, washed twice in 10 mM Tris hydrochloride (pH 7.4), and suspended in flow medium at a density of 1×10^9 to 2×10^9 cells per ml (for flow experiments) or 1×10^8 to 2×10^8 cells per ml (for polyacrylamide gels or observation of swimming behavior). For each of the *cheB* plasmids we determined the concentration of IPTG that gave the maximal swarm size when the plasmid was inserted in the Δ*cheB* host D263. These levels of IPTG were added to the AB minimal and λ tryptone growth media and differed from one plasmid to another, depending on the strength of the promoter and the extent of the deletion within the *cheB* gene.

Flow-chase assays of methylesterase activity. The response of methylesterase activity to chemotactic stimuli was determined by using the procedures and apparatus described previously by Kehry et al. (17). This involved a 30- to 40-min labeling of cells (prepared as described above) with L-[*methyl*-^{3}H]methionine (30 μCi/10^9 cells) to enable formation of [^{3}H]methylester groups on the transducer proteins. These cells were pelleted briefly in a microcentrifuge, suspended in flow-chase medium, and pumped onto a Gelman filter (pore size, 0.2 μm). A continuous flow of chase medium was maintained over the cells, and flowthrough fractions were collected at 1-min intervals. Cells were subjected to stimuli (attractant or repellent addition or removal) by changing the positions of three-way inlet valves to direct the flow of the desired solution over the cells. The flow rate was approximately 0.42 ml/min, and the $t_{1/2}$ of mixing was 20 to 30 s.

The volatile ^{3}H in flowthrough fractions was determined by subjecting each fraction to vapor phase transfer in capped scintillation vials as described by Kehry et al. (17). Each fraction contained 0.42 ml of flowthrough, and each scintillation vial contained 2.5 ml of scintillation fluid. After vapor phase transfer had proceeded for approximately 16 h at room temperature, each sample was counted for 5 min in a Beckman scintillation counter by using an appropriate ^{3}H window.

Plasmid constructions. The expression vectors pHS1400 and pCR43 were constructed in this laboratory by D. C. Muchmore and C. B. Russell, respectively. pTTQ18 was purchased from Amersham Corp. Each of these plasmids carries an IPTG-inducible promoter and the *lacI*^{q} gene to enable regulated expression of genes placed downstream of the *lacUV5* promoter (for pCR43) or *tac* promoter (for pHS1400 and pTTQ18). To clone the *cheB* gene of *E. coli*, we first isolated a 2.3-kilobase-pair *Cla*I-*Sal*I fragment from the transducing phage λche22 (obtained from J. S. Parkinson). This fragment contained the tail end (280 base pairs) of *tap*, all of the *cheR* and *cheB* genes, and approximately the

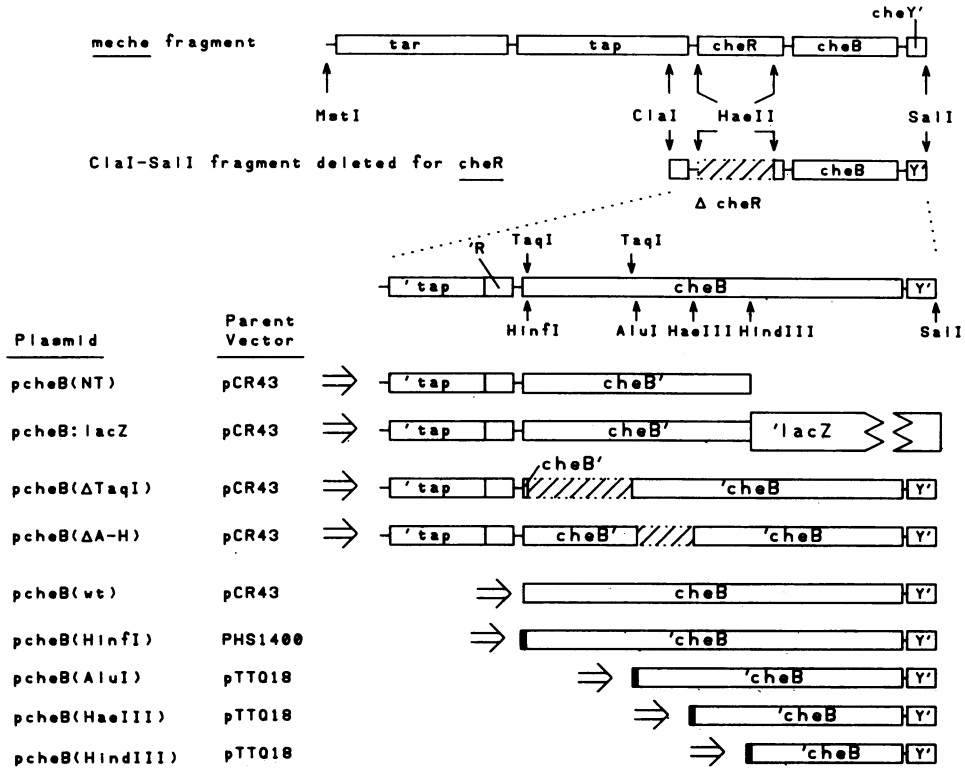

FIG. 1. Summary of plasmid constructions expressing *cheB* deletion mutants. For each plasmid the expression vector used is indicated and the chemotaxis-related DNA inserted downstream of the promoter (⇒) in the vector is depicted. The restriction sites used to generate these plasmids (as described in Materials and Methods) are indicated. Internal deletions (i.e., within the *cheB* gene) are shown (▨). The small segments of the T4 lysozyme gene or the pTTQ18 multiple-cloning site that were fused to segments of the *cheB* gene (see Materials and Methods) in some of the constructions are depicted (■).

first 100 base pairs of *cheY*. We then deleted an 800-base-pair *Hae*II segment from this fragment to remove virtually all of the *cheR* gene (Fig. 1).

The N-terminal deletion mutants were constructed by removing the indicated restriction fragments (*Taq*I, *Alu*I, *Hae*III, etc.) (Fig. 1). On the basis of the published sequence of *cheB* (30), these deletions should maintain the correct reading frame through the remainder of the *cheB* gene. We confirmed that the *cheB* gene we isolated (see above) has an identical DNA sequence to that reported by Mutoh and Simon (30). In addition, we have sequenced across the junctions of all the deletion constructions reported in this study. The wild-type CheB protein is composed of 349 amino acid residues. The CheB(*Hin*fI) construction removes the first 11 amino acids of CheB (replacing them with the first 8 amino acids of T4 lysozyme) and was formed by ligating the *Hin*fI site of *cheB* to the *Sna*BI site of T4 lysozyme. For the CheB(*Alu*I) and CheB(*Hae*III) constructions, the blunt end of the appropriate DNA fragment was ligated to the *Sma*I site of the pTTQ18 multiple-cloning site. This added 7 amino acids (Met-Asn-Ser-Ser-Ser-Val-Pro) encoded by the plasmid multiple-cloning site to the N terminus of these shortened forms of CheB. The p*cheB*(*Hae*III) construction encodes a form of CheB that lacks amino acids 1 to 150. p*cheB*(*Alu*I) encodes a CheB variant that lacks amino acids 1 to 98. The protein encoded by p*cheB*(*Hin*dIII) lacks residues 1 to 206. CheB(Δ*Taq*I) and CheB(ΔA-H) lack amino acid residues 10 to 68 and 99 to 151, respectively. The *cheB-lacZ* fusion has the *Cla*I-*Hin*dIII *cheB* coding sequences (through codon 205) joined to codon 6 of *lacZ* by a *Hin*dIII linker that maintains the correct reading frame.

Immunoblotting. Sodium dodecyl sulfate-polyacrylamide gels were run by the method of Laemmli (23) with solubilized whole-cell extracts obtained by boiling cells in sodium dodecyl sulfate loading buffer. Proteins were transferred to nitrocellulose in a commercial (CBS Scientific) blotting apparatus with Towbin buffer plus 0.01% sodium dodecyl sulfate and a current of 150 mA for 10 h. Western blots were performed with rabbit antiserum raised against purified *E. coli* CheB, prepared essentially as described by Simms et al. (44). The specificity of the antiserum was improved either by adding sodium dodecyl sulfate-solubilized whole-cell extract of a *flbB-flaH* deletion strain during the first antibody hybridization step or by affinity purifying anti-CheB immunoglobulin Gs on a CheB-Sepharose 4B column. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G was used as the second antibody, and blots were visualized by using the NBT/X-phos color reaction as specified by the supplier (Promega). Prior to hybridization with the CheB antibodies, blocking was performed for 60 min in Tris-buffered saline (TBS) containing 1% nonfat dry milk and 0.1% Tween 20. Two 10-min washes of the blot in TBS and TBS–0.05% Tween 20 followed each antibody hybridization step.

RESULTS

N-terminal deletion mutations of CheB. By using various convenient restriction sites in the *cheB* gene, we constructed several deletion mutations that eliminate portions of the N-terminal coding sequences without disrupting the reading frame of the remaining coding sequences of *cheB*. The extents of these deletions are shown in Fig. 1. We trans-

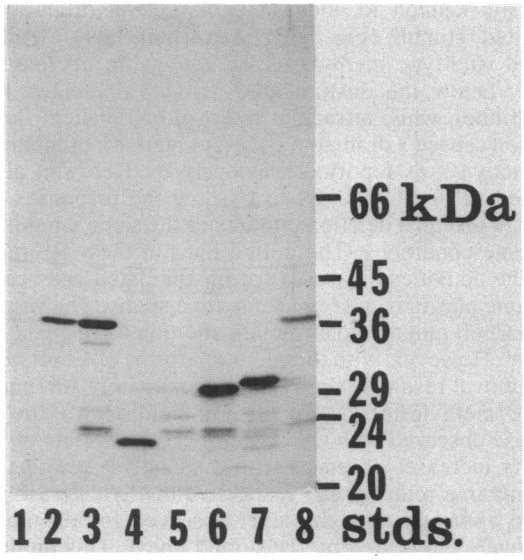

FIG. 2. Western blot of wild-type and N-terminal-deleted forms of CheB. Cells were grown, solubilized, and run on a 10% polyacrylamide gel under denaturing conditions as described in Materials and Methods. The separated proteins were electroblotted onto nitrocellulose and hybridized to anti-CheB antibodies as described. Lanes: 1, strain D263 (Δ*cheB*); 2, strain D213 (wild type); 3, strain D263 containing plasmid p*cheB*(*Hin*fI); 4, strain D263 containing p*cheB*(*Hae*III); 5, strain D263 containing p*cheB*(*Alu*I); lane 6, strain D263 containing p*cheB*(Δ*Taq*I); 7, strain D263 containing p*cheB* (ΔA-H); 8, strain D263 containing p*cheB*(wt). In each case the cells were grown in the presence of a concentration of IPTG that gave maximal complementation (with respect to swarming) in the Δ*cheB* strain D263. Sizes and mobilities of molecular weight standards (stds.) are indicated on the right. Abbreviation: kDa, kilodaltons.

formed the *recA* Δ*cheB* host D263 with plasmids (Fig. 1) expressing these mutant forms of CheB to determine whether the N-terminal deleted proteins could (i) maintain methylesterase activity and functionally complement the Δ*cheB* host to enable chemotaxis and (ii) maintain the interactions with additional chemotaxis components that enable modulation of the methylesterase activity in response to transducer-mediated stimuli.

Western blots (using CheB antiserum) of whole-cell extracts of strain D263 containing these plasmids indicate that each of the deletion constructions directs synthesis of a shortened form of CheB having approximately the predicted size (Fig. 2). These shortened forms of CheB appear to be somewhat more susceptible to proteolysis than is the full-length protein; however, for all but one of the constructions [CheB(*Alu*I)], the major CheB species present appeared to be the unproteolyzed version. With CheB(*Alu*I) over half of the protein appears to have been proteolyzed to a smaller form. We want to emphasize that the cells used to prepare extracts for Western blotting were treated in exactly the same way as the cells used for the flow experiments described below. This treatment involves several washes in Tris buffer (requiring approximately 30 min) followed by incubation in Tris-buffered succinate for 30 to 40 min. The presence of the various N-terminal-deleted versions of CheB after this treatment indicates that these proteins are relatively stable and that in each case the observed methylesterase activity is in fact associated with the intended shortened form of CheB.

(i) Complementation ability. Virtually all of the CheB N-terminal deletion mutants restored chemotactic (swarming) ability to the nonchemotactic Δ*cheB* host and changed the unstimulated swimming behavior of this host from very tumbly to random (alternately smooth swimming and tumbling) (Table 1). In addition, all but one of these mutant forms of CheB maintained methylesterase activity (Table 1; Fig. 3 and 4). The p*cheB*(*Hin*dIII) construction that eliminated the N-terminal 60% of CheB did eliminate methylesterase activity and was not able to confer swarming ability to the Δ*cheB* host. It is remarkable that as much as 45% of CheB can be removed from its N terminus without destroying the ability of the residual protein to properly fold into a reasonably stable protein with a functional methylesterase active site. Simms et al. (44) have reported a similar ability for the C-terminal 60% of CheB from *S. typhimurium*.

(ii) Response of methylesterase activity to chemotactic stimuli. Using the in vivo flow-chase assay of CheB developed by Kehry et al. (17), we determined whether our N-terminal deletion mutants could respond to chemotactic stimuli in the same manner as does the intact, wild-type methylesterase. We found that these mutant forms of CheB responded normally to positive chemotactic stimuli (attractant addition) but exhibited a markedly diminished response to negative chemotactic stimuli (attractant removal or repellent addition). Plasmid-encoded wild-type CheB responded in char-

TABLE 1. Properties of various N-terminal-deleted forms of CheB

Plasmid	Methylesterase properties[a]				% Complementation (swarming of Δ*cheB* host)[b]
	Activity	Modulation			
		+Att	−Att	+Rep	
p*cheB*(wt)	+	Strong (0.6)	Strong (6.0)	Strong (3.5)	100
p*cheB*(*Hin*fI)	+	Strong (0.7)	Weak (1.5)	Weak (1.5)	80
p*cheB*(*Alu*I)	+	Strong (0.6)	Weak (1.8)	Weak (1.3)	50
p*cheB*(*Hae*III)	+	Strong (0.7)	Weak (1.6)	Weak (1.3)	35
p*cheB*(*Hin*dIII)	−	None (1.0)	None (1.0)	None (1.0)	<10
p*cheB*(Δ*Taq*I)	+	Strong (0.7)	Weak (1.4)	Weak (1.4)	80
p*cheB*(ΔA-H)	+	Strong (0.5)	Weak (1.5)	Weak (1.3)	50

[a] The presence (+) or absence (−) of methylesterase activity and the ability of the enzyme to respond to chemotactic stimuli were determined in flow-chase assays (described in Materials and Methods). The extent of this response is described qualitatively (strong, weak, or no response) for each stimulus. The numbers in parentheses indicate the ratio of the observed activity following a stimulus to the activity of an identical unstimulated control sample at the same time point. For attractant removal (−Att; 50 μM L-serine) and repellent addition (+Rep; 20 mM L-leucine) the ratio was determined at the point of highest activity following stimulation. For attractant addition (+Att; 50 μM L-serine) the ratio was determined 8 min after stimulation.

[b] The percentages indicate the swarm size of Δ*cheB* host D263 transformed with the listed plasmids relative to the swarm size of a wild-type chemotaxis strain (D213) under the same conditions (expressed as percentage of wild-type swarm). The λ tryptone swarm plates used for these determinations were supplemented with appropriate concentrations of IPTG to give the maximal swarm size for each version of CheB.

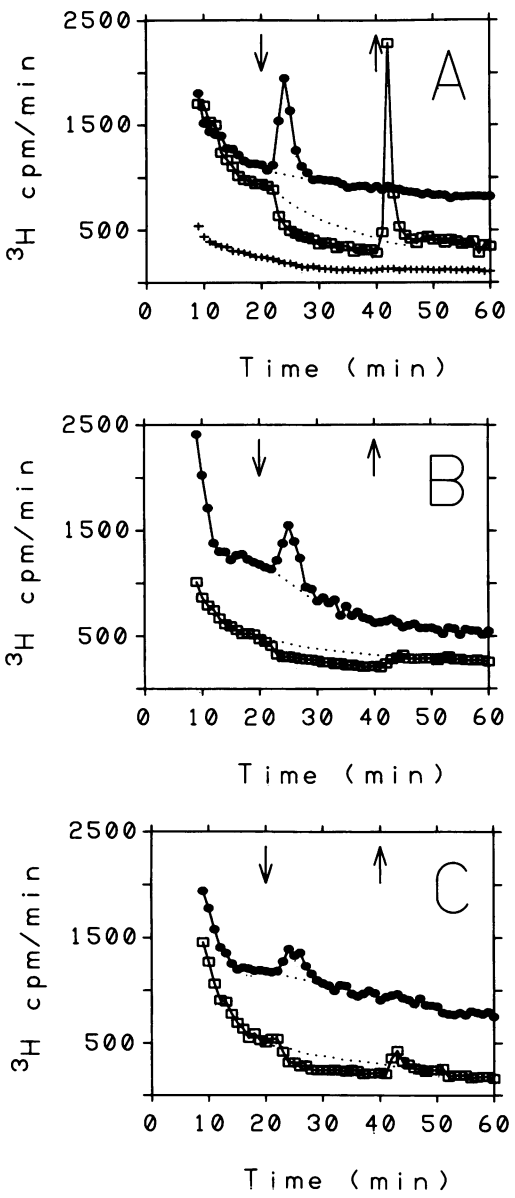

FIG. 3. Results of flow-chase experiments measuring the ability of CheB(wt) and N-terminal-deleted forms of CheB to respond to attractant and repellent stimuli. Each panel shows plots of methylesterase activity (volatile ^{3}H counts generated per minute) as a function of time following initiation of the cold chase (as described in Materials and Methods) and the response of this activity to serine and leucine stimuli. Symbols: □, the flow medium was switched from neutral medium (no amino acid additions) to the same medium containing 50 μM L-serine at t = 20 min (↓), and this serine solution was allowed to flow over the cells until t = 40 min (↑), when the medium flowing over the cells was switched back to neutral (no-serine) medium; ●, the flow medium was switched from neutral medium to the same medium containing 20 mM L-leucine at t = 20 min (↓), and this leucine solution was allowed to flow over the cells until t = 40 min (↑), when neutral medium (no leucine) was used again. In each panel the plots for the leucine stimulus experiments have been shifted upward by 500 cpm/min so that they do not obscure the plots of the serine stimulus experiments. For each stimulation experiment, a no-stimulus control was run with the same cell-plasmid combinations. This involved monitoring [^{3}H]methanol production for cells that were not subjected to any attractant or repellent stimuli over the same time course as used for the stimulation experiments. These data are shown (· · · ·) for the interval between 20 and 48 min for the serine stimulation experiments and between 20 and 30 min for the leucine stimulation experiments. (A) Results for host strain D263 transformed with plasmid p*cheB*(wt) (□, ●) and for the Δ*cheB* host in the absence of any plasmid (++++) subjected to a leucine stimulus as described above. The latter results demonstrate that the low level of non-CheB-dependent volatile ^{3}H counts does not respond to repellent stimulation; similar results were obtained with the Δ*cheB* strain with serine as a stimulus. (B) Results for host strain D263 transformed with plasmid p*cheB*(*Hin*fI). (C) Results for D263 transformed with p*cheB*(*Alu*I). Note that for wild-type CheB and for each mutant version of CheB, the methylesterase activity dips below that for the unstimulated control following serine addition.

acteristic fashion to addition and removal of transducer-mediated stimuli (Fig. 3A). Attractant (serine) addition caused wild-type methylesterase activity to decrease transiently below the unstimulated activity (indicated by the dotted line), while attractant removal or repellent (leucine) addition caused a dramatic, transient increase of methylesterase activity, as reported previously by Kehry et al. (17). Figures 3B and C and 4A to C show the responses of the CheB N-terminal deletion mutants to the same stimuli under the same conditions. The dotted lines in these figures indicate the activities measured for unstimulated cells carrying the same plasmids over the same time spans. The responses of the CheB mutants to attractant addition were qualitatively normal. However, with these mutant forms of CheB, attractant removal resulted in only an approximately 50% increase of the methylesterase activity, in contrast to the 600% increase observed with wild-type CheB. The relatively small activity increases observed with the CheB N-terminal deletion mutants returned the respective methylesterase activities to approximately the level observed for unstimulated controls (as indicated by the dotted lines). This diminished response was not enhanced by using stronger stimuli (larger concentration jumps), although an extensive study of this was not attempted. The diminished response to attractant removal obtained with the N-terminal deletion mutants causes an easily recognized qualitative difference in comparing the flow plots of the mutants with that obtained with wild-type CheB. In response to attractant removal, the mutant versions of CheB are incapable of increasing their methylesterase activities significantly above that of the respective unstimulated controls. This essentially eliminates the activity spike that is the major feature of the response of the wild-type protein. Therefore, although the mutant versions of CheB are capable of shifting between some basal activity level and an inhibited activity level, they are not able to achieve or maintain the high activity level that is transiently reached by the wild-type protein.

The N-terminal deletion mutants of CheB also exhibited diminished responses to repellent (leucine) addition (Fig. 3B and C and 4A to C), as compared with the wild-type response (Fig. 3A). This loss of sensitivity was relatively small for the CheB(*Hin*fI) protein, but was quite noticeable for the other deletions. For example, a typical leucine response was a 3.5-fold activity increase for wild-type CheB, but increases of only 30 to 40% were observed for most of the deletion mutants. The responsiveness of the wild-type and mutant versions of CheB are summarized in Table 1. Leucine removal was expected to cause a transient decrease of methylesterase activity similar to that observed for serine addition (18). However, this response is not readily visible for either the wild-type or mutant versions of CheB in the experiments presented in Fig. 3 and 4. This appears to have

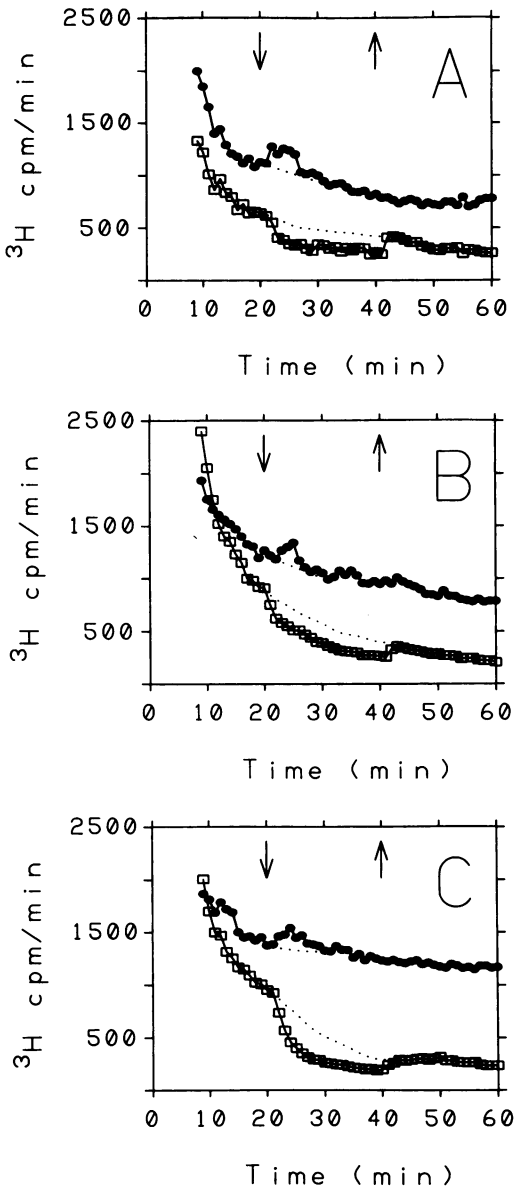

FIG. 4. Results of flow-chase experiments demonstrating the diminished ability of N-terminal-deleted forms of CheB to respond to negative chemotactic stimuli. The three panels show plots of methylesterase activity (expressed as volatile ^{3}H counts generated per minute) as a function of time following initiation of the cold chase (as described in Materials and Methods) and the response of this activity to serine and leucine stimuli. Symbols: □, the flow medium was switched from neutral medium (no amino acid additions) to the same medium containing 50 μM L-serine at t = 20 min (↓), and this attractant level was maintained until t = 40 min (↑), when the medium flowing over the cells was switched back to neutral (no-serine) medium; ●, the flow medium was switched from neutral medium to medium containing 20 mM L-leucine at t = 20 min (↓), and this leucine solution was allowed to flow over the cells until t = 40 min (↑), when neutral (no-leucine) medium was used again. As in Fig. 3, the plots for the leucine stimulus experiments have been shifted upward by 500 cpm/min so that they do not obscure the plots for the serine stimulus experiments. (A) Results for strain D263 carrying the plasmid p*cheB*(*Hae*III); (B) results for strain D263 carrying p*cheB*(Δ*Taq*I); (C) results for strain D263 carrying plasmid p*cheB*(ΔA-H). For each panel, the results of a no-stimulus control are shown (· · · ·) for the interval following stimulation.

resulted from the diminished sensitivity of the flow assay to small changes of methylesterase activity at the time of leucine removal. In these experiments, leucine removal occurred 40 min after initiation of the cold chase. By this time many of the radioactive methyl groups had been replaced by cold methyl groups, and it therefore became very difficult to detect small activity decreases with this system. By prelabeling cells in the presence of leucine and then subjecting them to the removal stimulus at t = 20 min, we were able to observe the expected activity decrease in the more sensitive portion of the chase (data not shown).

We have previously reported that the *cheA* deletion mutation in strain RP1788 eliminates modulation of wild-type CheB in response to both positive and negative stimuli (R. C. Stewart, C. B. Russell, A. Roth, and F. W. Dahlquist, Cold Spring Harbor Symp. Quant. Biol., in press). To determine whether this deletion also affected the residual level of modulation observed with the various N-terminal deletion mutants of CheB, we placed the appropriate plasmids expressing these forms of CheB into a Δ*cheB* Δ*cheA* host. This strain was constructed by moving the *cheA* deletion segment of strain RP1788 into the Δ*cheB* strain RP4972. Wild-type CheB fails to respond to addition or removal of serine or leucine stimuli in this strain, and each of the various N-terminal deletion mutants shows a similar lack of response in this strain (data not shown). These results suggest that CheA is required for both the positive and negative aspects of modulation of the methylesterase activity of CheB.

Effects of catalytically inactive CheB on regulation of the wild-type methylesterase. We have also studied several *cheB* mutations that eliminate methylesterase activity. These include three point (missense) mutations that map in the C-terminal half of CheB and a CheB-LacZ fusion protein that deletes the C-terminal half of CheB. Details of the construction of this fusion and of the expression vectors used to enable regulated production of the *cheB* mutants are found in Materials and Methods. We believe that these three point mutants and the fusion protein completely lack methylesterase activity for several reasons. First, using a P_{lacUV5} expression vector, we expressed these proteins in a wild-type host at about 10 times the level of the wild-type enzyme (see Materials and Methods) without affecting transducer methylation levels (data not shown). Second, flow-chase experiments performed with these plasmid-encoded mutants expressed in a Δ*cheB* host indicated no methylesterase activity in vivo, even at high levels of expression. Finally, the CheB-LacZ fusion deletes the region of the protein that contains at least part of the methylesterase active site (43, 44).

Despite this lack of methylesterase activity, these mutant forms of CheB were found to affect the swimming behavior and swarming ability of host cells when expressed at high levels (about 10 times the level of wild-type CheB in a wild-type host, as estimated from Western blots). For example, the swarming ability of Δ*cheB* hosts improved modestly to 15 to 25% of wild-type ability when *cheB627*, *cheB633*, or *cheB-lacZ* was expressed at high levels. This apparently resulted from the ability of these overexpressed catalytically inactive proteins to diminish the extremely tumbly bias of the Δ*cheB* host. (The limited ability of cells to swarm in the absence of methylation-mediated adaptation has been discussed by Block et al. [3] and by Stock et al. [53–55]). Effects on swimming behavior were also observed when these mutant proteins were overexpressed in wild-type host cells (that had normal levels of functional, wild-type CheB): the swarming ability of the wild-type host was diminished to

TABLE 2. Properties of various inactive forms of CheB and their effects on the properties of wild-type CheB

Plasmid	Expression level[a]	Δ*cheB* host[b]		Wild-type host[b]				
		Esterase activity	% Swarming ability	Esterase activity	Modulation			% Swarming ability
					+Att	−Att	+Rep	
p*cheB-lacZ*	10×	−	20–25	+	Strong (0.6)	Weak (2.0)	Weak (1.3)	50–60
p*cheB*(NT)	10×	−	10–15	+	Strong (0.6)	Strong (6.0)	Strong (4.0)	90–100
p*cheB*627	10×	−	20	+	Strong (0.6)	Weak (2.0)	Weak (2.0)	15
p*cheB*631	10×	−	<10	+	Strong (0.6)	Weak (1.7)	ND[c]	<10
p*cheB*633	10×	−	15	+	Strong (0.6)	Weak (1.9)	ND	30

[a] Expression of the various *cheB* alleles and the *cheB-lacZ* fusion was regulated by the concentration of IPTG in the growth medium (0 IPTG for 1× expression; 1 mM IPTG for 10× expression relative to the wild-type level of CheB as detailed in Materials and Methods).

[b] Methylesterase activity and swarming ability (relative to wild-type strain D213) associated with the various forms of CheB were measured by using Δ*cheB* host D263 transformed with the indicated plasmids. The wild-type chemotaxis strain D213 was transformed with the same plasmids to enable determination of their effects on modulation of wild-type CheB and on the chemotactic ability of this host. Swarming abilities of these cell-plasmid combinations are indicated as the percentage of the swarm size of strain D213 under identical conditions. The presence (+) or absence (−) of methylesterase activity as detected in flow assays is indicated. Responses of methylesterase activity to attractant addition (+Att), attractant removal (−Att), and repellent addition (+Rep) as determined in flow assays are indicated qualitatively as strong or weak responses and quantitatively (numbers in parentheses). The method of calculating these numbers and the stimulus concentrations used for these experiments are detailed in Table 1, footnote *a*.

[c] ND, Not done.

varying extents under these conditions (Table 2), although the growth rate was not affected (data not shown). This apparently resulted from a diminished ability to tumble under these circumstances, as indicated by the smooth swimming behavior of unstimulated cells (data not shown).

In merodiploids generated by placing plasmid vectors expressing inactive versions of CheB in a wild-type host cell, the level of methylesterase activity of the wild-type host was not noticeably affected by the presence of excess inactive CheB mutant proteins, but the ability of the wild-type enzyme to respond to transducer-mediated chemotactic stimuli was markedly altered (Fig. 5; Table 2). The response to attractant addition was essentially normal in these merodiploids, but the response of the wild-type enzyme to attractant removal or repellent addition was diminished by the presence of excess CheB627, CheB631, CheB633, or CheB-LacZ. These altered responses resemble those observed with the CheB N-terminal deletion mutants expressed in the absence of the wild-type allele (Fig. 3 and 4).

Although the CheB-LacZ fusion protein clearly affected modulation of the wild-type methylesterase, high levels of expression of the N-terminal half of CheB alone [using p*cheB*(NT)] had no noticeable effect on modulation of wild-type CheB and only a very minor influence on swimming and swarming ability of host cells (Table 2). This probably resulted from instability of this CheB fragment (as deduced from Western blots; data not shown). Fusion of this segment to a stable protein (LacZ) apparently enhances its stability, thereby enabling it to influence modulation of the wild-type methylesterase and swimming behavior.

DISCUSSION

The extent of methylation of the transducer proteins is an important factor in determining their signaling state (tumbly versus smooth) and therefore affects the regulation of swimming behavior in *E. coli* and *S. typhimurium* (12, 13, 35–37, 47). One important determinant of transducer methylation is the activity of CheB, the transducer methylesterase (56, 64). The activity of this enzyme is regulated in response to transducer-mediated stimuli by some unknown mechanism (17, 18, 48, 59). The work presented here provides some insight into this regulation and suggests some possible regulatory mechanisms.

The most obvious result indicated by this work is that the N-terminal half of CheB is unnecessary for methylesterase

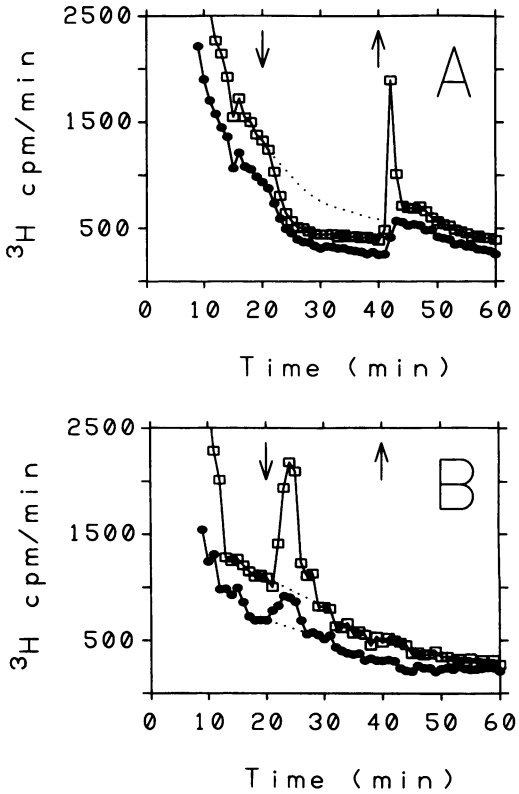

FIG. 5. Results of flow-chase experiments demonstrating the effect of excess CheB-LacZ on modulation of CheB(wt) in merodiploids. The plots indicate the observed methylesterase activity as a function of time for wild-type chemotactic host strain D213 transformed with plasmid p*cheB-lacZ*. Symbols: □, cell-plasmid combinations grown in the absence of IPTG; ●, cell-plasmid combinations grown in the presence of 1 mM IPTG [which results in approximately 10 times more CheB-LacZ than CheB(wt)]. (A) Responses to addition and removal of 50 μM L-serine at t = 20 min and t = 40 min, respectively. (B) Activity changes caused by addition (t = 20 min) and removal (t = 40 min) of 20 mM L-leucine. The methylesterase activity observed with unstimulated cell-plasmid combinations is shown (· · · ·).

catalytic activity and is not required for folding of the C-terminal half into a stable, active protein. This result confirms the earlier work of Simms et al. (44), who found that a proteolytic fragment representing the C-terminal three-fifths of the *S. typhimurium* methylesterase maintains catalytic activity. In fact, Simms et al. (44) determined that this truncated form of CheB is 15-fold more active than the intact 37-kilodalton CheB protein in an in vitro assay. Although our results do not indicate any significantly greater activity for the *E. coli* CheB N-terminal deletion mutants than for the wild-type protein, our in vivo flow assay (chosen to enable observation of CheB interactions with regulatory elements) may be less sensitive to such changes than is the in vitro assay used by Simms et al. (44). For example, factors such as the in vivo stability of the modified enzyme and the level of transducer protein methylation achieved during the preflow labeling period can affect the observed absolute rate of methanol production in the flow assay. It should also be noted that none of our intragenic *cheB* deletions encode a shortened protein that corresponds exactly to the proteolytic fragment studied by Simms et al. (44), although two of our deletions encode smaller CheB fragments. It is conceivable that the exact site of proteolytic cleavage (or the extent of the deletion) is important for this enhancement of the methylesterase activity.

Since the N-terminal half of CheB is unnecessary for methylesterase catalytic activity, we have attempted to determine what its functional role might be. Our results indicate that although the N-terminal region of CheB is not necessary for some aspects of modulation of methylesterase activity in response to chemotactic stimuli, it is required to achieve the complete extent of regulation observed with the wild-type enzyme. The activities of our N-terminal deletion mutants are modulated in the same qualitative manner as that of the wild-type protein: increased attractant concentration results in transiently diminished activity, whereas attractant removal or repellent addition results in activity increases. However, these mutant forms of CheB do not respond nearly as dramatically to negative chemotactic stimuli (attractant removal and repellent addition) as does the native enzyme. On the basis of these results, the N-terminal region of CheB appears to be involved in the ability of the enzyme to respond to negative chemotactic stimuli, but it is not required for a response to positive chemotactic stimuli.

A wide variety of N-terminal deletions (ranging from removal of the first 11 amino acids to removal of the first 150 amino acids) affects CheB modulation in essentially the same manner: by diminishing responsiveness to negative chemotactic stimuli. We find it intriguing that the same altered modulation is observed with the wild-type enzyme when it functions in the presence of excess inactive CheB (point mutants or CheB-LacZ fusion). One possible explanation for this similarity is that the N-terminal region of these inactive mutant proteins or of the fusion protein competes with the wild-type protein for some regulatory factor. By soaking up such a regulator, these inactive forms of CheB could cause the wild-type enzyme to respond in the same altered manner as do the various N-terminal deletion mutants of CheB, which are incapable of interacting with or responding to this hypothetical regulatory factor.

Although it is possible that this factor is actually a stimulus-induced transducer conformation, several observations lead us to believe that components in addition to the transducers and CheB are involved in modulating CheB activity. First, Kehry et al. (18) demonstrated that methylesterase activity responds to some signal that reflects integrated input from the different classes of transducer proteins. Second, an in vivo-reconstituted methylation system constructed by expressing *cheR*, *cheB*, *tar*, and *tsr* in a Δ(*flbB-flaH*) host (which is deleted for or expresses none of the known flagellar and chemotaxis components) does not exhibit modulation of methylesterase activity in response to chemotactic stimuli (C. B. Russell, R. C. Stewart, and F. W. Dahlquist, submitted for publication). Finally, we have found that deletion of *cheA* or *cheW* (putative chemotaxis signaling components [15, 16, 31, 33, 34, 40, 51, 62, 63]) abolishes or greatly diminishes the modulation of CheB methylesterase activity (Stewart et al., in press). In addition, Springer and Zanolari (48) have demonstrated impaired methylesterase response (to negative stimuli) in some *cheA* point mutants. It therefore appears that some of the components of the chemotactic signal transduction pathway that directs flagellar rotation (62) also participate in the modulation of CheB methylesterase activity (Stewart et al., in press).

It is interesting that the altered methylesterase responsiveness to negative stimuli we observed with our N-terminal CheB deletion mutants is similar to that reported by Springer and Zanolari (48) for *cheA* point mutants and similar to that observed in this laboratory for several *cheA* point mutants. Taken together, these results suggest that two distinct factors may modulate methylesterase activity: one that diminishes CheB activity in response to attractant stimuli and one that enhances CheB activity in response to negative stimuli. This methylesterase activator could interact with the N-terminal region of CheB, and its generation could be CheA dependent. However, we want to emphasize that our results do not necessarily provide evidence for direct interaction of an activity modulator with the N-terminal half of CheB. For instance, in a situation involving sequential activation and deactivation steps such as we generate with negative stimuli in flow experiments,

$$\text{CheB}^{\text{less active}} \xrightarrow{k_a} \text{CheB}^{\text{more active}} \xrightarrow{k_d} \text{CheB}^{\text{less active}}$$

the height of the observed activity spike depends on the level of accumulation of $\text{CheB}^{\text{more active}}$ and the rate at which it interacts with methylated transducer proteins. The level of $\text{CheB}^{\text{more active}}$ can be diminished by slowing k_a (the rate of CheB activation) or by speeding up k_d (the rate of CheB deactivation) in response to activity modulators without eliminating the ability of the enzyme to interact directly with a modulator. Alternatively, any factor that slows the interaction between $\text{CheB}^{\text{more active}}$ and the transducers could diminish the extent of modulation. It is not yet clear whether the activation and deactivation result from reversals of a common event or interaction. However, the recent results of Hess et al. (15, 16) suggest that phosphorylation of CheB by CheA may be responsible for activation of methylesterase activity. Because the CheB-phosphoryl linkage is relatively unstable to hydrolysis, this activation would be transient; deactivation would result from loss of the phosphoryl group. If our CheB N-terminal deletion mutants are incapable or less capable of being phosphorylated, we would expect them to exhibit diminished activation. Alternatively, these deletions could decrease the stability of the CheB-phosphoryl linkage and thereby diminish the lifetime of the activated form of CheB.

Positive and negative modulation of CheB methylesterase activity may result from different levels or mechanisms of control. Simms et al. (44) have proposed that methylesterase activity is modulated by both a local mechanism and a global mechanism. The local mechanism is postulated to result

from ligand-induced conformational changes of the transducer proteins that alter the accessibility of methyl groups to CheB (7, 44). Global regulation is postulated to involve some second messenger that also plays some role in determining the swimming behavior of cells. It is conceivable that we have eliminated or diminished the global modulation mechanism by deleting the N terminus of CheB or by overexpressing this region of CheB and that the remaining modulation represents the local form of regulation. However, we have demonstrated that in intact cells even the limited response to attractant addition or removal observed with the various N-terminal deletion mutants requires the presence of CheA. To us, this indicates that this aspect of methylesterase modulation requires interactions with the central chemotactic signaling machinery and does not result directly from interactions of CheB with the transducer proteins. Apparently, the interactions responsible for the decrease of methylesterase activity (in response to attractants) require that the C-terminal portion of CheB be intact and/or functional but do not require the presence of the N-terminal half of the protein. This would account for the inability of the CheB-LacZ fusion and the three CheB point mutants to affect this aspect of modulation of the wild-type enzyme in merodiploids and would explain the unaltered response of the CheB N-terminal deletion mutants to attractant addition.

An interesting alternative explanation for the effects of excess inactive CheB on modulation of wild-type methylesterase activity (in merodiploids) arises if we consider the possibility that CheB functions in some multimeric state (there is no compelling evidence supporting or disproving this possibility). If such a multimeric state were necessary to achieve the regulated conformation (but not for activity per se), inactive forms of CheB could poison such multimers, rendering them incapable of the full range of modulation. Furthermore, if the N-terminal portion of CheB were to participate in this multimeric interaction, we would expect deletion of the N terminus or the presence of the CheB-LacZ fusion to disrupt regulation.

It is not obvious how elevated levels of inactive CheB can detrimentally affect the swarming ability of chemotactically wild-type host cells. It is possible that in wild-type hosts the resulting altered modulation of the wild-type CheB is deleterious for chemotaxis; however, we also see effects on the swarming ability of Δ*cheB* hosts, and we have demonstrated that CheB N-terminal deletion mutants (in Δ*cheB* hosts) readily support chemotaxis even though their activities are modulated in the same altered manner. It is therefore unlikely that the diminished extent of CheB modulation caused by excess inactive CheB is responsible for the observed loss of chemotactic ability. It is also unlikely that this loss results from interference with the transducer methylation-demethylation reactions, because overexpression of CheB-LacZ or the three inactive CheB proteins does not noticeably alter the steady-state level of transducer methylation in wild-type host cells. We believe that the transducer-modulated signal(s) that communicates the presence of negative chemotactic stimuli to the components directing flagellar rotation (causing tumbling) also interacts with CheB. Possibly, this signal causes the methylesterase activity to increase. Excess CheB (regardless of its catalytic ability) would soak up this tumble messenger and prevent its interaction with other chemotaxis components. This would disrupt the chain of events that enables appropriate regulation of swimming behavior in response to chemotactic stimuli and would certainly diminish the chemotactic ability of the cell. The resulting swimming bias of a wild-type host cell would be excessively smooth. In the same manner, high levels of inactive CheB would diminish the extreme tumbly bias of a Δ*cheB* host cell by diminishing the availability of the tumble messenger. This could modestly improve the swarming ability of the Δ*cheB* host, as was observed.

With the recent demonstration of phosphorylation of CheB and CheY by autophosphorylated CheA (15, 16) and the elucidation of the probable role of CheY phosphorylation in causing tumbling (45), we can now speculate on how the interactions of CheB with CheA could account for many of the observations reported in this communication. In doing so, it is important to realize that the wild-type CheB protein has two distinct catalytic activities: CheB functions both as a transducer protein methylesterase and as a CheA phosphatase. These two enzymatic activities do not necessarily involve the same regions of the CheB protein molecule. Indeed, many of our results can be readily understood if we propose that the phosphorylation site and phosphatase activity of CheB involve the N-terminal portion of this protein, while the C-terminal region is involved in methylester hydrolysis. Under these conditions, overproduction of any version of CheB that maintains an intact N-terminal domain would greatly diminish the intracellular pool of phospho-CheA. We have evidence suggesting that CheB and CheY compete for a common component (Stewart et al., in press), which may be the phosphorylation site on CheA. Therefore, the presence of a greater than normal concentration of the phosphatase domain of CheB in a cell would diminish the ability of phospho-CheA to phosphorylate CheY in response to chemotactic stimuli. As a result, chemotactic ability would be impaired, and the ability of the cell to tumble would be greatly diminished. These are, in fact, the same effects observed when wild-type CheB, CheB-LacZ, or a CheB point mutant lacking methylesterase activity is overproduced in an otherwise wild-type cell. In addition, overproduction of these versions of CheB would be expected to hinder phosphorylation-dependent regulation of methylesterase activity either by depleting the available phospho-CheA pool or by competing with the wild-type protein for phosphorylation by CheA. Our data are consistent with this prediction in that overproduction of CheB-LacZ or the three CheB point mutants diminishes the activation response of the methylesterase (the putative phosphorylation-dependent response [15]) but does not affect the attractant-induced decrease in methylesterase activity. Furthermore, by deleting or altering the N-terminal segment of CheB, we may have eliminated or incapacitated the phosphorylation site(s) of CheB, thereby eliminating modulation of its methylesterase activity in response to negative stimuli, as we have reported here.

In the model outlined above, CheB phosphorylation in its N-terminal domain is the result of phosphoryl transfer from CheA, and this modification results in marked activation of the CheB methylesterase activity. This activation is transient because the CheB-phosphoryl linkage is unstable, either by virtue of its inherent hydrolytic instability or because the CheB protein catalyzes this hydrolysis. We are currently testing several aspects of this model by determining the site of CheB phosphorylation, the effect of this phosphorylation on CheB methylesterase activity, the susceptibility of various CheB deletion and fusion mutants to phosphorylation, and the in vivo phosphorylation levels of numerous CheB missense mutants that exhibit defective modulation in response to chemotactic stimuli. By doing so, we hope to better define and understand the interactions that enable CheB to modulate its methylesterase activity in

response to chemotactic stimuli and to understand the interplay of the phosphatase and methylesterase activities of CheB.

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