Activation of Methylesterase CheB: Evidence of a Dual Role for the Regulatory Domain†

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ABSTRACT: The response regulator CheB functions within the bacterial chemotaxis system together with the methyltransferase CheR to control the level of chemoreceptor methylation, influencing the signaling activities of the receptors. CheB catalyzes demethylation of specific methylglutamate residues introduced into the chemoreceptors by CheR. CheB has a two-domain architecture consisting of an N-terminal regulatory domain joined by a linker to a C-terminal effector domain. In the unphosphorylated state of the response regulator, the regulatory domain inhibits the methylesterase activity of the effector domain. Upon phosphorylation of a specific aspartate residue within the regulatory domain, the C-terminal methylesterase activity is stimulated, resulting in the subsequent demethylation of the chemoreceptors. We have investigated the mechanism of regulation of CheB activity by the N-terminal regulatory domain. First, we have found that phosphorylation of the N-terminal domain not only relieves inhibition of the C-terminal methylesterase activity but also provides an enhancement of this activity above that seen for the C-terminal effector domain alone. Second, we have identified mutations in CheB that show an enhancement of methylesterase activity in the absence of phosphorylation. Most of these single-site mutations are localized in the linker region joining the regulatory and effector domains. On the basis of these observations, we propose a model for activation of CheB in which phosphorylation of the regulatory domain results in a reorganization of the domain interface, allowing exposure of the active site to the receptor substrate and simultaneously stimulating methylesterase activity.

Response regulators comprise a very large family of proteins that are involved in a wide variety of phosphotransfer-dependent "two-component" signal transduction pathways found both in prokaryotes and eukaryotes (*1*, *2*). The methylesterase CheB is one of two response regulators that functions within the bacterial chemotaxis system (*3*, *4*). CheB is involved in the reversible covalent modification of the cytoplasmic domains of transmembrane chemoreceptors. CheB acts together with the methyltransferase CheR to control the level of receptor methylation, influencing the signaling activities of the receptors, and contributing to adaptation. CheB catalyzes deamidation of specific glutamine residues in the receptors (*5*) and demethylation of specific methylglutamate residues introduced into the chemoreceptors by CheR (*6*).

As for most members of the response regulator family, CheB has a two-domain architecture consisting of an N-terminal regulatory domain joined by a linker region to a C-terminal effector domain. In the unphosphorylated, inactive state of the response regulator, the N-terminal regulatory domain inhibits the methylesterase activity of the C-terminal effector domain (*7*, *8*). Upon phosphorylation of a specific aspartate residue, Asp56, within the N-terminal domain, the C-terminal methylesterase activity is stimulated, resulting in the subsequent demethylation of the chemoreceptors (*9*, *10*).

It has been proposed that activation of response regulators via phosphorylation involves communication between phosphorylated regulatory domains and downstream effector domains. In support of this idea, previous studies of CheB indicated a role for interdomain interactions in regulation of methylesterase activity. Removal of the N-terminal regulatory domain by either genetic manipulation (*7*, *8*, *10*) or proteolysis (*7*) results in unregulated activation of the C-terminal methylesterase catalytic domain. Thus, the N-terminal domain acts to inhibit C-terminal methylesterase activity. However, in some response regulator proteins, the regulatory domains play a stimulatory role. For instance, phosphorylation has been shown to facilitate dimerization in some response regulators, and this dimerization event is required for effector activation (*11*). Thus, both inhibitory and stimulatory roles of the regulatory domain have been identified in different response regulator proteins.

We have investigated the mechanism of regulation of methylesterase CheB activity by the N-terminal domain. In a screen to identify a surface of the C-terminal domain involved in inhibitory interactions with the N-terminal domain, we have identified mutations that result in enhanced methylesterase activity in the absence of phosphorylation of

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the N-terminal domain. Most of these single-site mutations are localized in the linker region joining the N-terminal regulatory and C-terminal effector domains. We have also found that phosphorylation of CheB not only relieves inhibition of the C-terminal methylesterase activity but also provides an enhancement of activity above that seen for the C-terminal domain alone. These observations and the recently determined crystal structure of CheB (*12*) enable us to propose a model for phosphorylation-dependent CheB activation. There are at least two effects of phosphorylation of the regulatory domain that contribute to enhancement of methylesterase activity. One effect is relief of inhibition that may be achieved through disruption of the interdomain interface and subsequent repositioning of the domains to expose the active site. The other is stimulation of methylesterase activity that could presumably be achieved either by facilitating interaction with the receptor substrate through a surface of the phosphorylated regulatory domain, or alternatively, by enhancing catalysis through direct or allosteric interactions.

MATERIALS AND METHODS

Materials. Ammonium sulfate from ICN and Tris from United States Biochemical were of ultrapure grade. *S*-Adenosyl L-methionine was obtained from Sigma. IPTG[1] was purchased from Boehringer Mannheim. *S*-Adenosyl [^{3}H]L-methionine (10.8 Ci/mmol) was purchased from DuPont-New England Nuclear. [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham. Ecoscint A scintillation fluid was from National Diagnostics. Low-range molecular weight standards, electrophoresis reagents, and Bradford protein assay solution were from Bio-Rad. SDS−polyacrylamide gels (15%) were run under reducing conditions using a Mini-PROTEAN II system (Bio-Rad) and were stained with Coomassie Blue. Nytran nylon membranes were from Schleicher and Schuell. ECL chemiluminescence kit was from Amersham. FPLC (5 mL HiTrap-SP) cation-exchange columns were from Pharmacia LKB. Sequenase DNA polymerase was purchased from United States Biochemical Corp. All other materials were of reagent grade from standard commercial sources. Liquid scintillation counting was performed with a Beckman scintillation spectrometer (model LS650), and densitometry was performed using a Bio-Rad densitometer (GS-670) with Molecular Analyst software (Bio-Rad). Ultraviolet and visible spectroscopy was done on a Beckman spectrophotometer (model DU 650).

Strains and Plasmids. Escherichia coli strain HCB437 [Δ(*tsr*)7021 Δ(*trg*)100 *zbd*::Tn5 Δ(*cheA-cheZ*)2209 *met*F159-(Am)] (*13*) containing plasmid pME98 (*7*) was used for expression of *Salmonella typhimurium* aspartate receptor Tar. *E. coli* HB101 (*14*) was used as a host strain for expression of *S. typhimurium* methyltransferase CheR from plasmid pME43 (*15*) and for expression of intact *S. typhimurium* CheB from plasmid pME30 (*16*), a derivative of pUC12 with an *Eco*RI−*Hin*dIII cassette containing the *cheB* gene. *E. coli* DH5α (*17*) was used as a host strain for expression of the catalytic domain of *S. typhimurium* CheB (residues 147−349), CheBc, from plasmid pCP1 (*18*) and for expression of all CheB mutant proteins encoded by pUC12-derived vectors. *S. typhimurium* PS430 (*cheB*111 *recA*$^-$ *srl-202*::Tn10) (*19*) was used as a CheB-deficient host for assaying restoration of swarming ability by controlled expression of CheBc, CheB D56N, and mutant CheB proteins as described below. *E. coli* DH5α cells were used for construction of these plasmids. Final plasmid constructs isolated from DH5α were electroporated into a restriction-deficient *S. typhimurium* strain LB5000 (*metA metB trpB val leu lac str hsdLT hsdSA hsdSB*) (*20*), and modified plasmid DNA was then isolated and introduced into PS430 by electroporation. All plasmid-containing strains were grown at 37 °C in LB media containing 40 μg/mL ampicillin. For growth of PS430 or HCB437, 10 μg/mL tetracycline or 25 μg/mL kanamycin, respectively, were included in the media.

Mutagenesis of cheB. Using PCR oligonucleotide-directed mutagenesis with plasmid pME30 as template, a *Pst*I site was introduced into *cheB* through a silent mutation at a position encoding amino acid 129, at the end of the N-terminal domain of CheB as defined by homology to CheY, to yield plasmid pGA1. Asp56, the site of phosphorylation, was substituted with Asn (D56N) using oligonucleotide-directed mutagenesis of pGA1, yielding pAS4. A low-copy plasmid (pMLB1120.215) with a tightly regulated *lac* promoter derived from pBR322 and the M13 IG region of plasmid pZ150 (*21*) was used to construct plasmids that enabled IPTG-inducible expression of CheB. The *cheB D56N* and *cheBc* genes flanked by *Eco*RI and *Hin*dIII sites from plasmids pAS4 and pCP1, respectively, were inserted immediately downstream of the *lac* promoter in pMLB1120.215 to generate plasmids pAS5 and pAS6. The *Pst*I−*Hin*dIII fragment from pAS5, encoding the putative linker region and the effector domain of methylesterase CheB was then subjected to PCR amplification under conditions that allowed for a low level of random infidelity (*22*). Specifically, 100 ng of pAS5 template; 1 μM each 5′ and 3′ oligonucleotide primers overlapping the *Pst*I and *Hin*dIII sites, respectively; 200 μM each dGTP, dCTP, dTTP; 40 μM dATP; and 2.5 units of *Taq* DNA polymerase (Stratagene) were used for 25 cycles of PCR amplification in a 50 μL reaction mixture containing 20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, and 0.1 mg/mL nuclease-free bovine serum albumin, pH 8.8. The PCR mutagenized *Pst*I−*Hin*dIII fragments were then inserted into *Pst*I−*Hin*dIII-digested pAS5, replacing the wild-type effector domain with a mixture of randomly mutagenized fragments, and the resulting plasmids were electroporated into *S. typhimurium* LB5000. Transformants were plated on LB agar plates containing ampicillin. Cells were scraped from the plates, and plasmid DNA was isolated and then electroporated into *S. typhimurium* PS430. Transformants were plated onto LB agar plates containing antibiotics, and single colonies were used to screen for swarming ability as described below. Plasmids conferring enhanced swarming ability were isolated, and the *Eco*RI−*Hin*dIII fragments encoding CheB were inserted into high-copy pUC12 vectors. The mutagenized regions of the *cheB* genes, corresponding to the *Pst*I−*Hin*dIII fragments, were analyzed by dideoxynucleoside triphosphate sequencing.

[1] Abbreviations: IPTG, isopropyl-1-thio-β-D-galactopyranoside; SDS, sodium dodecyl sulfate; FPLC, fast protein liquid chromatography; PCR, polymerase chain reaction; EDTA, ethylenediamine/tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; βME, β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.

Swarm Assays for Bacterial Chemotaxis. Single colonies of *S. typhimurium* PS430 containing pMLB1120.215-derived plasmids were used to inoculate LB media containing 40 μg/mL ampicillin and 10 μg/mL tetracycline and were grown at 37 °C to stationary phase. Cultures were diluted 50-fold into LB media and inoculated into semisolid agar plates (1.3% tryptone, 0.7% NaCl, and 0.35−0.4% agar) containing 10 μg/mL tetracycline, 40 μg/mL ampicillin, and IPTG as indicated. Plates were incubated at 37 °C. Screening of mutants was performed in 100 × 15 mm plates using 0.4% agar in a grid of 24 inoculations per plate. Swarm diameters of potential mutants were scored relative to the sizes of PS430 containing plasmids pAS5 (CheB D56N) or pAS6 (CheBc) after a 12−16 h incubation. Swarm rate determinations were performed using 0.35% agar and a single inoculation into the center of a 60 × 15 mm plate. Rates were determined from a minimum of four swarm diameter measurements taken over a 10 h period beginning 10−12 h after inoculation.

Methylesterase Assay. *E. coli* membranes containing *S. typhimurium* aspartate receptors (Tar) with ^{3}H-labeled methyl groups were prepared by incubating Tar-containing membranes with methyltransferase CheR and *S*-adenosyl [^{3}H]L-methionine as a methyl group donor according to Simms et al. (*7*). Esterase activity was assayed by incubating the membrane-bound [*methyl*-^{3}H]Tar with methylesterase CheB (5 μM) or CheBc (0.5 μM) in the indicated buffer with 1 mM EDTA and 2.8 mM βME. To assay the activity of CheB upon phosphorylation, CheB (0.5 μM) was preincubated in the presence of 50 mM phosphoramidate [synthesized as its ammonium salt by the method of Sheridan et al. (*23*)] and 25 mM $MgCl_2$, unless otherwise specified, in either 50 mM Tris-HCl, pH 8.0, or 50 mM phosphate buffer, pH 7.0, for 30 s prior to initiation of the reaction by addition of [*methyl*-^{3}H]Tar. At indicated times, 5 μL aliquots of the reaction were removed and quenched by addition of 30 μL of 5 N acetic acid and applied to filter paper (Whatman). Volatile counts of [^{3}H]methanol released in the reaction were then measured with a Beckman LS-650 liquid scintillation spectrometer after at least a 12 h incubation at 25 °C. The specific activity of the methylated Tar was used to determine esterase activity (mmol of [^{3}H]methanol released mol of methylesterase^{-1} s^{-1}).

To minimize difficulties associated with assaying the wide range of activities associated with different forms of CheB, the concentrations of unphosphorylated CheB, CheBc, and phosphorylated CheB in the individual methylesterase assays were adjusted so that the absolute quantity of [^{3}H]methanol released fell within a range of 200−2000 counts/min. At all concentrations of CheB used, the initial rates of [^{3}H]-methanol released were linear with respect to the concentration of CheB. The average background was between 80 and 100 counts/min.

Protein Expression and Purification. CheBc protein was expressed and purified according to West et al. (*24*). Both wild-type and mutant CheB proteins were expressed and purified by a modification of the procedure of Simms et al. (*7*). All steps were carried out on ice or at 4 °C unless otherwise noted. Cells expressing wild-type or mutant CheB proteins were grown to early stationary phase at 37 or 30 °C, respectively. Cells were harvested by centrifugation at 6000*g* for 20 min, washed once in 0.1 M potassium phosphate, 1 mM EDTA, 2.8 mM βME, and 0.5 mM PMSF, pH 7.0, and then resuspended in the same buffer using 3 mL of buffer/gram wet wt of cells. Cells were disrupted by sonication using a Heat Systems Ultrasonic Processor. Unbroken cells were removed by centrifugation for 10 min at 12000*g*. The supernatant was clarified by ultracentrifugation at 100000*g* for 1 h. The soluble portion was fractionated via precipitation by addition of 29.6 g of ammonium sulfate/100 mL of solution with gentle stirring for 30−60 min. The precipitate was collected by centrifugation at 27000*g* for 15 min, resuspended in a minimal volume of phosphate buffer (0.1 M potassium phosphate, 1 mM EDTA, 2.8 mM βME, and 0.5 mM PMSF, pH 7.0), and dialyzed against 2 × 4 L of phosphate buffer overnight. The dialyzed ammonium sulfate fraction was diluted 5-fold with Tris buffer (20 mM Tris-HCl, 1 mM EDTA, 2.8 mM βME, and 0.5 mM PMSF, pH 8.6) and applied to an 18 × 2.5 cm Q Sepharose Fast Flow (Pharmacia) column equilibrated in Tris buffer. The flow-through containing CheB was collected and protein was precipitated by the addition of 40.0 g of solid ammonium sulfate/100 mL of solution. The precipitate was collected as before, resuspended and dialyzed overnight against 2 × 4 L of 0.1 M potassium phosphate, 1 mM EDTA, 2.8 mM βME, and 0.5 mM PMSF, pH 6.0. The sample was diluted with 1 mM EDTA, 2.8 mM βME, and 0.5 mM PMSF to a final concentration of 20 mM potassium phosphate prior to loading onto a 5 mL HiTrap SP cation-exchange column equilibrated in 20 mM potassium phosphate, 1 mM EDTA, 2.8 mM βME, and 0.5 mM PMSF, pH 6.0. The column was washed with the same buffer and protein was eluted with a 200 mL linear gradient of 0 to 200 mM sodium chloride in 20 mM potassium phosphate, pH 6.0. Fractions containing CheB were pooled and precipitated with 40.0 g of solid ammonium sulfate/100 mL, collected, resuspended, and dialyzed as before against 2 × 4 L of 0.1 M potassium phosphate, 1 mM EDTA, 2.8 mM βME, and 0.5 mM PMSF, pH 6.0. The sample was concentrated using an Amicon Centriprep 10 filtration device and then applied to a 40 × 2.5 cm Bio-Gel P-60 (Bio-Rad) gel filtration column equilibrated in 80 mM potassium phosphate, 1 mM EDTA, 2.8 mM βME, 0.5 mM PMSF, and 100 mM sodium chloride, pH 6.0. Purity was assessed by SDS−polyacrylamide gel electrophoresis. CheB proteins were precipitated with ammonium sulfate [50% (w/v) saturation] and the precipitates were aliquoted and stored at 4 °C. CheB, stored in ammonium sulfate, was prepared for analysis by overnight dialysis against 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 2.8 mM βME, and 0.5 mM PMSF, pH 8.0. Concentrations of purified proteins were estimated by measuring the absorbance at 280 nm (wild-type CheB and mutants (except R255W), ϵ_{280} = 10 900 M^{-1}cm^{-1}, R255W, ϵ_{280} = 16 600 M^{-1}cm^{-1}, CheBc, ϵ_{280} = 9600 M^{-1}cm^{-1}). Extinction coefficients were estimated based on the tryptophan and tyrosine content of the proteins (*25*). Coomassie Blue staining of the purified proteins analyzed by SDS−PAGE correlated directly with the UV absorbance quantitation.

Immunoblot Analysis of CheB. Western blot analyses of the esterase protein were performed using an ECL chemiluminescence kit (Amersham). Proteins were transferred from SDS−polyacrylamide gels to Nytran nylon membranes using a Bio-Rad Trans-Blot apparatus with recommended

transfer buffer. CheB protein was detected using rabbit anti-CheBc antisera and horseradish peroxidase (HRP)-labeled anti-rabbit antibody (ECL kit) according to the manufacturer's instructions. The developed immunoblots were quantitated by densitometry.

RESULTS

Isolation of Mutations within the Linker and Catalytic Domain That Relieve Inhibition by the Regulatory Domain in the Absence of Phosphorylation. Previous studies have shown that limited proteolysis of intact CheB generates a form of the protein consisting of the C-terminal catalytic domain, CheBc (residues 147−349), which has approximately 10-fold higher methylesterase activity than the unphosphorylated intact protein (*7*). These results established an inhibitory role for the N-terminal domain of CheB. Phosphorylation of CheB by the histidine protein kinase CheA in the presence of ATP and Mg^{2+} has been shown to result in an increase in methylesterase activity equivalent to the removal of the N-terminal domain (*9*, *10*, *26*), suggesting that phosphorylation of CheB results in relief of inhibition of methylesterase activity by the N-terminal domain. In this study, we set out to examine the inhibitory role of the N-terminal domain in the regulation of methylesterase activity of CheB. Our approach was to attempt to identify a putative surface on the C-terminal domain that might be involved in the inhibitory interactions with the N-terminal domain. To define this surface region, we sought to identify mutations outside of the regulatory domain that relieve the inhibitory effect of the regulatory domain in the absence of phosphorylation.

Cells exhibiting a null phenotype for the *cheB* gene lack chemotactic ability (*27*). These strains are unable to form expanding rings or swarms in semisolid agar plates. While complete restoration of chemotaxis in these strains can only be achieved by expression of appropriate levels of wild-type CheB, partial restoration of swarming behavior is observed when active and unregulated esterase is provided by expression of the C-terminal domain of CheB, CheBc, at low levels (*10*). On the basis of the assumption that alterations in CheB that resulted in activation of esterase activity in the absence of phosphorylation would restore swarming behavior similar to that observed with CheBc, we developed a screen to select for such *cheB* mutations. Site-directed mutagenesis of the *cheB* gene was carried out to generate an asparagine substitution at Asp56, the site of phosphorylation of CheB, producing a mutant protein, CheB D56N, that cannot be phosphorylated. *cheB D56N* and *cheBc* were inserted downstream of a *lac* promoter to generate plasmids pGA1 and pGA2, respectively, to enable controlled expression of both gene products by IPTG. The plasmids were introduced into an *S. typhimurium* CheB-deficient cell line, PS430, and the rates of swarming of these cells were measured as described in the Materials and Methods. Swarm rates were compared for different levels of expression of CheB protein by varying the concentration of IPTG in the semisolid agar plates. At low concentrations of IPTG, cells expressing CheBc, an unregulated and active methylesterase, showed higher rates of swarming than cells expressing CheB D56N, an unregulated inhibited methylesterase (Figure 1). At higher concentrations of IPTG, a reversal of this effect of IPTG-induced expression of the active and inactive methylesterases

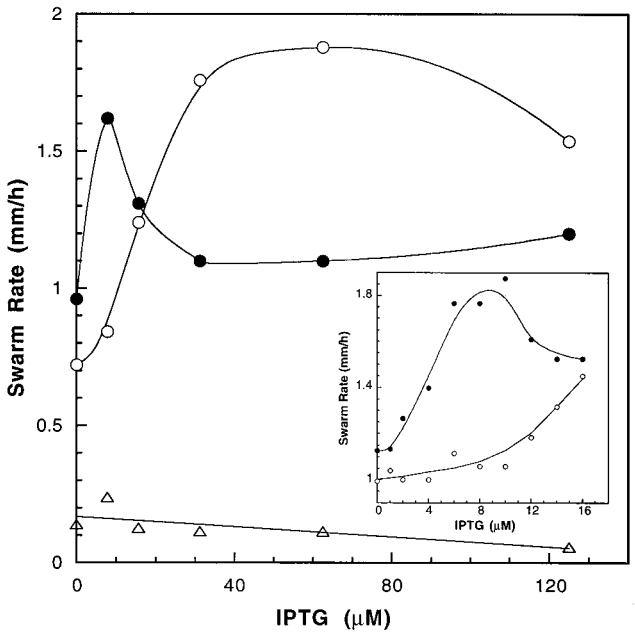

FIGURE 1: Partial restoration of swarming ability by expression of CheB D56N and CheBc in CheB-deficient cells. Swarming rates of *S. typhimurium* PS430 containing plasmid-encoded, IPTG-inducible genes for CheB D56N (○), CheBc (●), or no CheB protein (△) were determined in the presence of the indicated concentrations of IPTG as described in the Materials and Methods. The inset shows results of an independent experiment in which a lower range of IPTG concentrations was used.

was observed. The altered swarming was specific to cells containing plasmids expressing the methylesterase, as cells containing a control plasmid without a gene encoding CheB did not show any change in chemotactic ability. The greatest enhancement in swarming rates of cells expressing the active methylesterase CheBc relative to those expressing the inhibited methylesterase CheB D56N was observed at an IPTG concentration of 8 μM. This concentration of IPTG was used in subsequent swarm assays to select for activating mutants of CheB D56N.

A pool of plasmid-encoded mutant *cheB* genes was then constructed as described in the Materials and Methods. The genes encoded proteins with a D56N substitution in the N-terminal domain and randomized substitutions in the linker and C-terminal regions. PS430 cells containing these plasmid-encoded *cheB* mutant genes were subjected to an initial screen in which swarm diameters of these cells were scored relative to those of cells expressing either CheB D56N or CheBc after 12−16 h growth in semisolid agar plates. In the initial screen of approximately 1200 mutants, 42% exhibited swarm diameters similar to those of PS430 expressing CheB D56N, while 55% had significantly smaller swarm diameters. These two populations presumably reflect *cheB* with no mutations (or silent mutations) and *cheB* mutations encoding nonfunctional protein, respectively. The remainder of the mutants exhibited swarm diameters intermediate in size to those of cells expressing CheB D56N or CheBc in the initial screen. Of these, only about one-third of the isolates exhibited swarm rates significantly greater than that of cells expressing CheB D56N in a subsequent more rigorous analysis of swarm rates. Plasmids were isolated from these cells and the genes encoding CheB were sequenced. To verify that the mutations identified in *cheB*

Table 1: Chemotactic Ability of *S. typhimurium* PS430 Cells Expressing CheB Mutant Proteins Relative to Cells Expressing CheB D56N

	relative chemotactic ability (mutant CheB/CheB D56N)[a]	
mutant protein	trial I[b]	trial II[c]
R132P[d]	1.7	1.4
R134P	1.8	1.7
A137T	1.1	1.1
H138R	1.3	1.4
H138Q	1.2	1.2
L147S	1.7	1.6
L153P	1.2	1.1
R255W	1.4	1.1

[a] Values are expressed as the relative swarming rates of cells expressing mutant CheB and CheB D56N proteins. Swarm rate determinations were performed by single inoculations into semi-solid agar plates (1.3% tryptone, 0.7% NaCl, 0.35% agar) containing 40 μg/mL ampicillin, 10 μg/mL tetracycline, and 8 μM IPTG. [b] Swarm rates of the mutants isolated from the initial screen. [c] *Pst*I−*Hin*dIII fragment encoding the linker and catalytic domain of CheB from the sequenced genes in trial I were reinserted into *Pst*I−*Hin*dIII-digested pAS5. The reconstructed plasmids were electroporated into *S. typhimurium* PS430 and the rates of swarming of the transformants were determined. [d] This mutation was isolated three times.

were in fact responsible for the altered phenotype, the *Pst*I−*Hin*dIII fragment encoding the linker and catalytic domain of CheB from the sequenced genes were isolated and ligated to *Pst*I−*Hin*dIII-digested pAS5, replacing the corresponding portion of the *cheB* gene. The reconstructed plasmids were then electroporated into *S. typhimurium* PS430 and the transformants were screened for swarming ability.

A total of 10 isolates that exhibited enhanced swarming relative to cells expressing CheB D56N were identified, and were shown to have phenotypes linked to mutations in the C-terminal region of CheB (Table 1). Within the 10 independently selected isolates, one of the mutations, R132P, occurred three times, resulting in a final collection of eight different mutations. Mapping of the sites of the mutations onto the three-dimensional structure of CheB indicated that the mutations are not clustered onto a specific surface of the C-terminal domain (Figure 2). All but one of the mutations are located in the linker that connects the N- and C-terminal domains and none of the mutations are in, or near, the methylesterase active site.

Expression and Purification of Mutant Proteins. For isolation of proteins for subsequent biochemical analyses, we reconstructed the mutant *cheB* genes in high-copy expression plasmids to enable production of CheB proteins containing the mutated CheB C-terminal region and a wild-type N-terminal domain competent for phosphorylation at Asp56. The *Pst*I−*Hin*dIII fragments encoding the linker and catalytic domain of each of the mutant proteins were inserted into the wild-type *cheB* gene contained in constitutive expression plasmids derived from pUC12. Four of the eight mutant proteins, CheB A137T, H138R, L153P, and R255W, yielded stable, purified products, and these proteins were subjected to further characterization as reported below. Three mutant proteins, CheB R132P, R134P, and L147S, were unusually susceptible to proteolysis and showed evidence of degradation when analyzed by SDS−PAGE at all stages of purification. One mutant protein, CheB H138Q, was not purified because the position of this mutation was

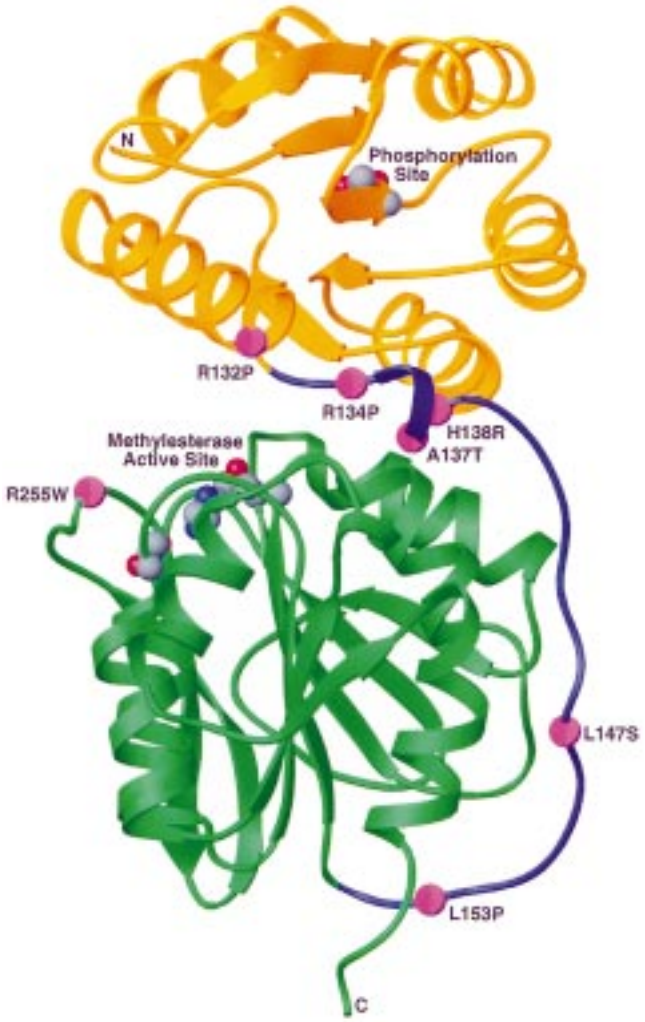

FIGURE 2: Ribbon representation of the X-ray crystal structure of CheB (*12*) showing locations of activating mutations. The N-terminal regulatory domain is colored gold, the C-terminal catalytic domain is shown in green, and the linker region is colored blue. The N- and C-termini of the protein are labeled by N and C, respectively. The positions of the activating mutations are shown as magenta spheres. The site of phosphorylation (Asp-56) in the N-terminal domain and the methylesterase active site (Ser-164, His-190, and Asp-286) in the C-terminal domain are shown as Corey−Pauling−Koltun (CPK) models with carbon atoms colored gray, oxygen colored red, and nitrogen colored dark blue. The figure was prepared with the program RIBBONS (*38*).

redundant in primary sequence location to the mutation in CheB H138R.

Limited proteolysis of CheB both in vitro and in vivo produces a number of fragments of CheB, including a relatively stable 21 kDa form, CheBc, which has significantly higher methylesterase activity than the intact protein (*7*). To analyze the susceptibility of the mutant CheB proteins to proteolytic degradation in vivo relative to wild-type CheB, lysates of cells constitutively expressing mutant and wild-type CheB from high-copy plasmids were analyzed by SDS−PAGE followed by immunoblotting using rabbit polyclonal antibodies raised against CheBc. There was no significant difference in the quantity of CheBc fragments in lysates of cells expressing mutant CheB protein relative to cells expressing wild-type CheB (data not shown). However, the extent of CheB proteolysis in lysates of cells expressing low levels of mutant and wild-type CheB proteins, under condi-

Table 2: Methylesterase Activity of Wild-type and Mutant CheB Proteins in the Presence and Absence of Phosphoramidate

CheB protein	methylesterase activity[a]	
	−phosphoramidate/Mg^{2+} [b]	+phosphoramidate/Mg^{2+} [c]
CheB (wt)	0.09 ± 0.007	6.3 ± 0.69
CheB A137T	0.26 ± 0.010	5.8 ± 0.46
CheB H138R	0.24 ± 0.010	7.3 ± 0.92
CheB L153P	0.21 ± 0.007	6.8 ± 0.56
CheB R255W	0.34 ± 0.007	6.1 ± 0.21
CheB D56N	0.07 ± 0.000	0.05 ± 0.0

[a] Methylesterase activities are expressed relative to that of CheBc in 50 mM potassium phosphate, pH 7.0. A relative activity of CheBc of 1.0 corresponded to an average methylesterase activity of 7.2 ± 0.3 mmol of [^{3}H]methanol released mol of $CheBc^{-1}$ sec^{-1}. Values are represented as the mean ± SD of at least two independent experiments. [b] Methylesterase activity of wild-type and mutant CheB proteins was determined by incubating membranes containing [*methyl*-^{3}H]Tar with methylesterase in a reaction buffer of 50 mM potassium phosphate, 1 mM EDTA, and 2.8 mM βME, pH 7.0. [c] Phosphorylation of CheB was carried out by preincubation of CheB with 25 mM $MgCl_2$ and 50 mM phosphoramidate for 30 s in phosphate buffer without EDTA.

tions comparable to those used in the swarm assay, could not be estimated, since the concentration of fragments at this level of expression of CheB fell below the detection limit of the chemiluminescent immunoblotting method.

Methylesterase Activity of Mutant Proteins in the Presence and Absence of Phosphoramidate. An in vitro assay that measures the rate of demethylation of the aspartate receptor, Tar, was used to determine the methylesterase activities of wild-type and mutant CheB proteins (*7*). Esterase activity was assayed by incubating membranes containing methylated Tar with methylesterase CheB in a reaction buffer of 50 mM potassium phosphate, pH 7.0 with 1 mM EDTA and 2.8 mM βME at 25 °C. Under these conditions, each of the four mutant CheB proteins showed a 2−3-fold higher methylesterase activity than wild-type CheB (Table 2). Absolute rates of demethylation vary from assay to assay due to a number of factors including preparation-dependent variation in the methylated receptor substrate and differences in the extent of equilibration in the vapor diffusion assay. For this reason, all assays included a control reaction using CheBc, and methylesterase activities were always normalized to this value. CheBc rather than CheB was chosen as a standard to avoid the complication of enhanced activity of CheB introduced by the presence of small quantities of highly active proteolytic fragments. Rates of demethylation for CheBc were typically in the range 6.1−8.7 mmol of methanol released mol of $CheBc^{-1}$ sec^{-1}.

To determine if the methylesterase activity of CheB was entirely due to small quantities of highly active CheBc contaminant, it was necessary to estimate the quantity of CheBc in samples of intact CheB. To determine the percentage of CheBc in samples of CheB, aliquots of purified CheB from 1 to 1500 ng were subjected to SDS−PAGE, transferred to nylon membranes and immunoblotted with antibodies against CheBc. The detection limit for CheBc in the chemiluminescent immunoblotting procedure was ∼5 ng of protein. Quantitation through densitometric scanning of the developed immunoblots showed the sum of proteolytic fragments to constitute less than 1% of the total CheB protein. The methylesterase activity of CheB relative to CheBc is 0.09 (Table 2). In order for CheBc contamination to account for this activity, samples of intact CheB would have to contain CheBc at a level approximately 9% that of the total amount of protein. These data imply that intact CheB must have intrinsic methylesterase activity.

To determine whether the increased methylesterase activity of the CheB mutant proteins was due to increased amounts of CheBc contamination in purified preparations, the purified proteins were examined for evidence of proteolytic degradation. Samples of the four purified mutant CheB proteins were analyzed by SDS−PAGE, transferred to nylon membranes and immunoblotted using antibodies raised against CheBc as described above. Densitometric analysis of the immunoblots of three of the four mutants characterized, A137T, L153P, and R255W revealed no detectable degradation products. H138R contained degradation products constituting approximately 8% of the total CheB protein. For all the methylesterase activity of CheB H138R to be a result of degradation products, the sample would have to contain CheBc fragments at a level approximately equivalent to 25% of the total protein, thus the enhanced methylesterase activity cannot be entirely attributed to the presence of CheBc in the sample of mutant CheB H138R.

Initial investigations to probe the activities of phosphorylated mutant CheB proteins were performed using phosphoramidate as a phosphodonor (*28*). The CheB proteins were preincubated with 5 mM phosphoramidate and 5 mM $MgCl_2$, in 50 mM phosphate buffer, pH 7.0, for 30 s at 25 °C prior to initiation of the demethylation reaction by the addition of membranes containing [*methyl*-^{3}H]Tar. When assayed in the presence of phosphoramidate, wild-type and mutant CheB proteins all showed similar increases in methylesterase activity relative to that of unphosphorylated CheB (data not shown). Interestingly, in the presence of 5 mM phosphoramidate and 5 mM $MgCl_2$, CheB showed 2-fold higher methylesterase activity than CheBc.

The level of phosphorylation of CheB in the presence of phosphoramidate represents a balance between two rapid reactions, phosphoryl transfer and dephosphorylation. To achieve a steady-state maximal level of phospho-CheB we attempted to optimize the conditions for CheB phosphorylation as measured by enhancement of methylesterase activity. Methylesterase activity of wild-type CheB was assayed at different concentrations of $MgCl_2$ and phosphoramidate. Maximum methylesterase activity of wild-type CheB was observed in the presence of phosphoramidate at concentrations above 20 mM and $MgCl_2$ at concentrations between 20 and 70 mM (data not shown). In the presence of 50 mM phosphoramidate and 25 mM $MgCl_2$, both the mutant and wild-type CheB proteins had similar methylesterase activities, approximately 6-fold higher than that of CheBc (Table 2).

Phosphorylated CheB has Higher Methylesterase Activity than CheBc. Phosphorylation of CheB is known to result in increased methylesterase activity (*9*, *10*, *28*−*30*) but an activity greater than that of CheBc has not previously been reported. Therefore, we decided to further characterize the reaction. Phosphorylation of intact CheB by incubation in the presence of 50 mM phosphoramidate and 25 mM $MgCl_2$ resulted in a 70-fold increase in methylesterase activity over CheB and a 6-fold increase in activity over CheBc in 50 mM potassium phosphate, pH 7.0. Examination of the methylesterase activity of both CheB and CheBc at pH 6.0 in 50 mM MES and at pH 8.0 in 50 mM Tris-HCl produced somewhat different activities, but under all conditions, the

Table 3: Methylesterase Activity of CheB, CheBc and CheB in the Presence of Phosphoramidate (NH_2PO_3) at pH 6.0, 7.0, and 8.0

	methylesterase activity[a]		
	50 mM MES, pH 6.0	50 mM potassium phosphate, pH 7.0	50 mM Tris-HCl, pH 8.0
CheB	0.75 ± 0.035	0.09 ± 0.007	0.09 ± 0.001
CheBc	2.35 ± 0.460	1.00	0.55 ± 0.092
CheB + NH_2PO_3 + Mg^{2+} [b]	3.75 ± 0.035	7.00 ± 0.640	4.40 ± 0.420
CheB + Mg^{2+} [c]		0.24	
CheBc + Mg^{2+} [c]		0.44	

[a] Methylesterase activities are expressed relative to that of CheBc in 50 mM potassium phosphate, pH 7.0. Values are expressed as the mean ± SD of at least two independent experiments. CheB and CheBc were assayed for methylesterase activity as described in the Materials and Methods. [b] Phosphorylation of CheB was carried out by preincubation of CheB with 25 mM $MgCl_2$ and 50 mM phosphoramidate for 30 s in the indicated buffers without EDTA. [c] CheB and CheBc were assayed in the presence of 25 mM $MgCl_2$.

presence of phosphoramidate and Mg^{2+} produced activities greater than those of CheBc. CheB and CheBc showed higher activity at pH 6.0 than at pH 7.0 or at pH 8.0, consistent with previous results indicating the optimum pH for methylesterase activity at approximately pH 6.0 (*8*, *31*). CheB phosphorylated in the presence of phosphoramidate showed highest activity in 50 mM phosphate buffer at pH 7.0 (Table 3).

Phosphorylation of response regulators by small molecule phosphodonors such as phosphoramidate requires Mg^{2+} (*28*), and we did not observe any activation of methylesterase activity in the presence of phosphoramidate alone without $MgCl_2$. To examine the role of Mg^{2+} in the activation of methylesterase activity, CheB was incubated with 25 mM $MgCl_2$ alone resulting in a 2-fold increase in activity of CheB. This is consistent with the activation of methylesterase activity of intact CheB by Mg^{2+} previously reported (*30*). This effect was specific to intact CheB, as CheBc in the presence of 25 mM $MgCl_2$ showed a 3-fold reduction in methylesterase activity (Table 3). The activation of methylesterase activity in the presence of both phosphoramidate and Mg^{2+} was specific to intact CheB. Incubation of CheBc with phosphoramidate and $MgCl_2$ resulted in a 3-fold reduction in methylesterase activity, indicating that the stimulatory effect of phosphoramidate observed with intact (full-length) CheB required the involvement of the N-terminal regulatory domain of CheB (Figure 3). There was no activation of methylesterase activity of CheB D56N in the presence of phosphoramidate and Mg^{2+}, indicating that Asp-56 is essential for activation (Table 2). These data suggest that the enhancement of methylesterase activity observed in the presence of phosphoramidate and Mg^{2+} results from phosphorylation of the regulatory domain at Asp-56 and cannot be accounted for by nonspecific effects of either phosphoramidate or Mg^{2+} on other regions of the protein.

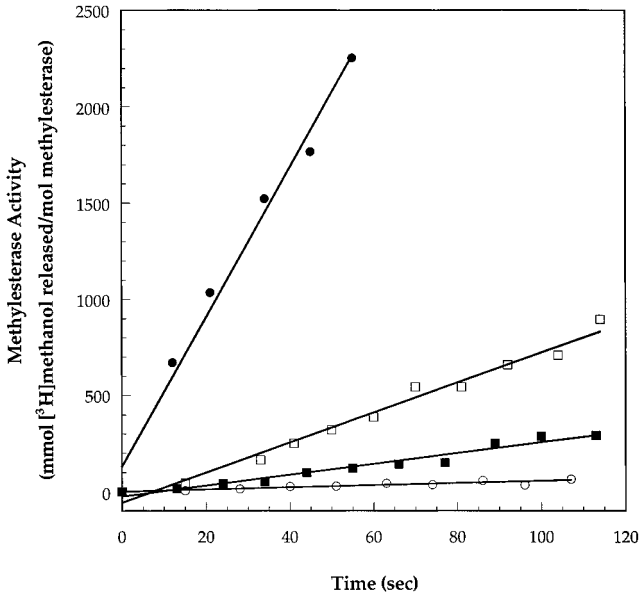

FIGURE 3: Methylesterase activity of CheB and CheBc in the presence and absence of phosphoramidate. Methylesterase activity was determined as described in the Materials and Methods by incubating membranes containing [*methyl*-^{3}H]Tar with methylesterase in a reaction buffer of 50 mM potassium phosphate, 1 mM EDTA, and 2.8 mM βME, pH 7.0. Assays in the presence of phosphoramidate were carried out by preincubation of methylesterase with 25 mM $MgCl_2$ and 50 mM phosphoramidate in phosphate buffer without EDTA for 30 s prior to initiation of the reaction by addition of Tar-containing membranes. Assays were performed using 5 μM CheB (○), 0.5 μM CheBc (□), 0.5 μM CheB + phosphoramidate (●), and 0.5 μM CheBc + phosphoramidate (■).

DISCUSSION

The N-Terminal Domain Plays a Dual Role in Regulation of Methylesterase Activity. While activation of methylesterase activity by phosphorylation of CheB has been observed previously (*9*, *10*, *28*, *30*), a level of activity greater than that of CheBc has not been reported. We have found that CheB, in the presence of phosphoramidate at pH 7.0, has 6- and 60-fold higher methylesterase activity than CheBc and CheB, respectively. Our results indicate that phosphorylation of CheB enhances methylesterase activity to a greater extent than is achieved by simply relieving inhibition by removal of the regulatory domain. Because of the rapid autodephosphorylation of phospho-CheB, it is not possible to maintain CheB in a stoichiometrically phosphorylated state, and the methylesterase activity observed in assays of CheB in the presence of phosphorylating agents will vary with the steady-state level of phospho-CheB attained under the particular conditions of the assay. When CheB is phosphorylated in the presence of the histidine protein kinase CheA and ATP, only a fraction of CheB exists in the phosphorylated state at any given time (*9*, *10*, *32*). Thus, it seems likely that the greater activation of CheB observed in this study relative to that of earlier studies (*9*, *10*, *30*) indicates that a higher steady-state level of phospho-CheB can be generated by using phosphoramidate rather than CheA and ATP as the phosphodonor. CheB has previously been shown to be activated in the presence of phosphoramidate and Mg^{2+} (*28*). We have optimized conditions for stimulation of methylesterase activity of CheB in the presence of phosphoramidate and Mg^{2+} and have found a requirement for significantly higher concentrations of both phosphoramidate and Mg^{2+} than those used previously.

The amount of phosphorylated CheB that exists at any given time is dependent on both the rate of phosphotransfer and the rate of dephosphorylation of phosphorylated CheB.

Phospho-CheB has been shown to have a very short half-life of ~2 s at 25 °C and a first-order rate constant for its dephosphorylation of 21 min^{-1} (*30*). The rate of phosphotransfer from phosphoramidate to CheB has been estimated to be 1.8 min^{-1} in the presence of 18 mM potassium phosphoramidate and 16 mM $MgCl_2$ in 100 mM Tris-HCl, pH 7.9 (*28*). Using these rates, it can be calculated that under steady-state phosphorylation conditions less than 10% of CheB exists in the phosphorylated state. If our observed activation of methylesterase is corrected for the level of CheB phosphorylation, phospho-CheB would be approximately 600-fold more active than unphosphorylated CheB and 60-fold more active than CheBc. It should be noted, however, that these values are only rough estimations. The conditions used for the determination of the rate of phosphotransfer from phosphoramidate to CheB are not identical to those used in methylesterase assays. In optimizing conditions for phosphorylation of CheB by phosphoramidate, we observed maximum methylesterase activity at phosphoramidate concentrations above 20 mM and at $MgCl_2$ concentrations between 20 and 70 mM. Extrapolating to 18 mM phosphoramidate and 16 mM $MgCl_2$ in our analyses of the dependence of methylesterase activity on phosphoramidate and $MgCl_2$, we obtain activities approximately 90% of the maximum, suggesting that the rate of phosphotransfer determined at these concentrations, slightly underestimates the phosphotransfer rate that exists in our methylesterase assays. Further uncertainty in the estimate results from the use of different buffer conditions in the methylesterase assays and in determination of the rates of phosphotransfer and CheB dephosphorylation. Nonetheless, these calculations emphasize that the 6-fold higher activity of phosphorylated CheB relative to CheBc observed in our study is a minimum value for the effect of phosphorylation on the activation of CheB.

Mutations in the Linker Region Relieve Inhibition of the Regulatory Domain in the Absence of Phosphorylation. Our screen for activating mutations of CheB was designed to identify residues outside of the N-terminal domain involved in inhibitory interactions with the regulatory domain. These mutations are distinct from those previously selected within the regulatory domain, which exhibited enhanced methylesterase activity as a result of altered kinetics of phosphorylation (*30*). The recently determined crystal structure of CheB provides insight into the mechanism of inhibition by the regulatory domain and provides a basis for interpreting the relief of inhibition by the mutations selected in this study. In the crystal structure of unphosphorylated CheB, the regulatory domain is positioned against the active site of the catalytic domain with a relatively long, 24 residue linker region connecting the two domains (Figure 2). Simple docking analysis indicates that a glutamate residue of a coiled-coil region designed to represent the receptor methylation region cannot approach closer than 7 Å to the active-site serine (Ser-164) of CheB (*12*). Thus, the regulatory domain exerts its inhibitory effect by occluding the active site. Relief of inhibition likely involves a repositioning of the regulatory domain, creating a more open form of CheB that allows access of substrate to the active site.

The mutations isolated in this study, unexpectedly, did not cluster in a single surface at the domain interface. All but one of the mutations are localized in the linker. Five of these mutations were substitutions to proline, one of which, R132P, was isolated three times through independent rounds of selection. At these positions within the linker, proline residues cannot be accommodated without disrupting the backbone conformation. Indeed, two of the proline-substituted proteins that we attempted to isolate, R132P and R134P, were very unstable and degraded during purification. Analysis of the degradation products of partially purified preparations of one of the unstable mutants, R132P, showed CheBc-like activity, suggesting that the enhanced esterase activity may reflect increased proteolytic susceptibility. While all of the mutations were isolated from a screen designed to select for enhanced methylesterase activity, this was directly confirmed for only the four mutant proteins that were purified. These were found to exhibit a modest increase in activity over wild-type CheB.

The linker that connects the two domains of CheB contains one helical turn that forms part of the domain interface and then continues in an extended conformation running along a surface of the C-terminal domain. Mutations in the linker, at sites near or within the interface (R132P, R134P, A137T, and H138R), could potentially directly disrupt the interface, promoting a more open conformation of CheB. Mutations also occur distal to the interdomain region at sites that are located at the interface of the linker and the C-terminal domain (L147S and L153P). Three of the residues in the linker (His-138, Leu-147, and Leu-153) have side chains that are directed inward, fitting into well-defined hydrophobic patches on the surface of the C-terminal domain. It is likely that these interactions of the linker, as well as interactions at the domain interface, contribute to stabilizing the closed, inhibited conformation of CheB. Our data suggest that disruption of these interactions allows CheB to adopt a more active conformation.

One of the isolated mutations, R255W, lies outside the linker. Arg-255 is located in a type III′ β-turn connecting β5 and helix αC of the catalytic domain. Arg-255 is completely accessible to solvent and is on the outer edge of the substrate-binding cleft of the protein. The location of this residue suggests that it might be part of a recognition surface involved in binding to the substrate Tar. Alternatively, the Trp substitution might act allosterically to influence either the catalytic site or the domain interface.

Unphosphorylated CheB Has a Basal Level of Methylesterase Activity. Methylesterase activity of samples of purified wild-type CheB show some variation due to the presence of minor amounts of CheBc. Further purification of an aliquot of purified wild-type CheB, by the application of additional cation exchange and gel filtration steps immediately prior to use, yielded a highly purified preparation. This sample showed a moderate decrease in methylesterase activity as a result of a decrease in CheBc contamination (data not shown). Analysis of samples of CheB by immunoblotting revealed the presence of small amounts (<1%) of CheBc. However, methylesterase activity of unphosphorylated CheB could not be entirely accounted for by the estimated amount of CheBc present. Additional evidence that unphosphorylated CheB has intrinsic methylesterase activity comes from the observation that the methylesterase activity of CheB is decreased in the presence of the histidine protein kinase CheA. CheA binds to CheB, presumably via the N-terminal domain of CheB, with a dissociation constant

of 3.2 μM (*33*). Other studies have shown that CheA, in the absence of Mg^{2+} and ATP, inhibits the methylesterase activity of intact CheB but not CheBc (*9*, *10*). CheA would not inhibit the methylesterase activity of CheB if all of the activity was contributed by the contaminating CheBc. This supports the conclusion that pure CheB, in the absence of any CheBc, has methylesterase activity.

Model for Phosphorylation-Dependent Activation of CheB. The structure of unphosphorylated CheB provides a basis for understanding the inhibitory role of the N-terminal domain. Inhibition of methylesterase activity in intact, unphosphorylated CheB is due to partial occlusion of the active site by the N-terminal domain, thereby restricting access of the substrate chemoreceptors. The intrinsic methylesterase activity of CheB is postulated to result from an equilibrium between at least two conformations, a closed, less active conformation and an open, more active conformation. Phosphorylation is predicted to shift this equilibrium toward the open, more active conformation through a repositioning of the two domains. A similar mechanism has been proposed for the activation of another multidomain response regulator, NarL (*34*). In the crystal structure of NarL, the homologous N-terminal regulatory domain packs against the DNA-binding domain, blocking access of DNA to the recognition helix. Thus, stimulation of DNA binding upon phosphorylation of the regulatory domain is predicted to involve a repositioning of the N- and C-terminal domains.

Phosphorylation has been shown to promote oligomerization of certain response regulators (*11*). There is, however no evidence for dimerization of CheB upon phosphorylation. Unphosphorylated CheB and CheBc have been shown to behave as monomers during gel filtration (*7*). Due to the short half-life of phosphorylated CheB, it has not been possible to directly examine its oligomeric state.

The two domains of CheB form an interface (approximately 1000 Å^2) consisting of a small hydrophobic core stabilized by hydrogen bonds and salt bridges arrayed around the perimeter of the interface. Two phenylalanine residues are positioned at the center of the hydrophobic core of the interdomain interface: Phe-104 of the N-terminal domain and Phe-195 of the C-terminal domain. Phe-104 corresponds to a highly conserved aromatic residue in the response regulator family (*35*). The corresponding residue in CheY (Tyr-106) has been shown to exist in two distinct conformations. The outward, solvent-exposed conformation of Tyr-106 has been correlated with the inactive unphosphorylated state of CheY while the inward, solvent-excluded conformation has been correlated with the active phosphorylated state of CheY (*36*). These observations led to the proposal that the conformation of Tyr-106 is involved in CheY activation. In CheB, Phe-104 adopts an outward conformation like that described for CheY in its inactive state. If a similar type of switching mechanism occurred in CheB, then Phe-104 would adopt an inward orientation upon phosphorylation of CheB. This would likely contribute to disruption of the interdomain interface, allowing for opening of the cleft between the two domains and methylesterase activation.

On the basis of our observations and the recently reported X-ray crystal structure of intact unphosphorylated CheB, we propose a model for the phosphorylation-induced activation of methylesterase CheB (Figure 4). In the unphosphorylated state, we believe CheB exists in an equilibrium between a closed, less active conformation and an open, more active conformation. The small amount of protein existing in the active conformation would provide the low levels of methylesterase activity observed in unphosphorylated CheB. Phosphorylation-induced changes in the regulatory domain are postulated to shift the equilibrium toward the open, more active form of CheB. Specifically, phosphorylation at Asp-56 in the N-terminal domain is expected to cause a conformational change that is propagated to the interdomain interface, causing a disruption of the interface that allows separation of the domains and formation of a cleft wide enough to accommodate the coiled-coil of the substrate chemoreceptors.

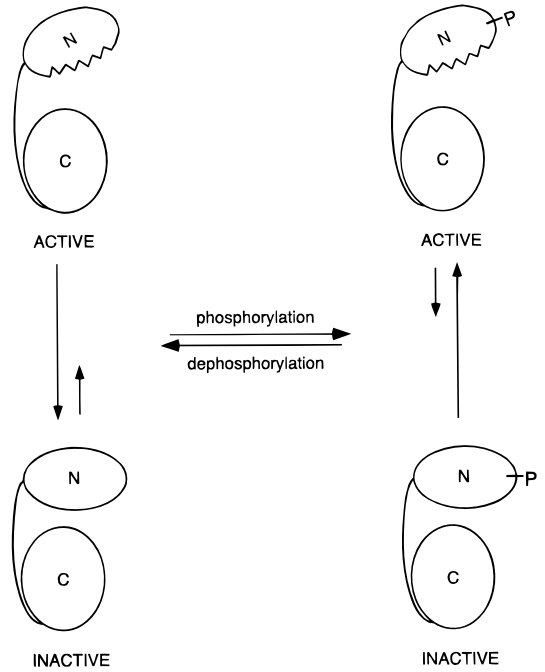

FIGURE 4: Model for phosphorylation-dependent activation of methylesterase CheB. We postulate that CheB can exist in at least two conformations: a closed, inactive form and an open, active form. Phosphorylation of the N-terminal domain of CheB presumably shifts the equilibrium between these two conformations, with the inactive form predominating for unphosphorylated CheB and the active form predominating for phosphorylated CheB. This is a minimal model with regard to the number of different states of CheB proposed. The extent of similarity between the unphosphorylated active form and the phosphorylated active form, both with respect to structure and methylesterase activity, remains an open question.

While the structure of CheB indicates a mechanism for relief of inhibition, it is less clear how phosphorylation of the regulatory domain acts to stimulate methylesterase activity. One possibility is that the domain rearrangement that is postulated to be necessary for relief of inhibition results in presentation of a recognition surface that facilitates interaction with the substrate receptors. The involvement of the N-terminal domain in substrate recognition has a precedence in receptor interactions proposed for the antagonistic enzyme, receptor methyltransferase CheR (*37*). Comparison of the structures of CheB and CheR reveal significant similarities with both proteins consisting of two domains, with active sites located in similar positions within the C-terminal domains near the domain−domain interfaces. In both enzymes, a cleft between the domains forms an opening for interaction with substrates, and in CheR, a region of the

N-terminal domain flanking this cleft contains a putative receptor interaction surface. But stimulation of methylesterase activity need not involve altered recognition of the receptor substrate. Alternatively, activation could be achieved by stimulation of catalysis. Alteration of the domain interface could influence the methylesterase catalytic triad either directly or through allosteric effects. By either mechanism, a single conformational change induced by phosphorylation may facilitate activation by both relief of inhibition and gain of stimulatory interactions.

One of the central questions in the field of two-component signal transduction focuses on how the conserved regulatory domain functions to regulate so many diverse effector domains. From numerous studies of many different response regulator proteins, an answer to this question is beginning to emerge. The regulatory domain can be considered to exist in two predominant conformations with phosphorylation serving to shift the equilibrium between the two states. Each conformation presents different molecular surfaces that promote distinct sets of protein−protein (or possibly protein−DNA) interactions. Thus response regulators can potentially be used to control any type of regulation that can be achieved through macromolecular interactions. The methylesterase CheB provides an illustrative example in which both inhibitory and stimulatory interactions appear to be involved.

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