Kinetic Basis for the Stimulatory Effect of Phosphorylation on the Methylesterase Activity of CheB[†]

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ABSTRACT: Response regulators are activated to elicit a specific cellular response to an extracellular stimulus via phosphotransfer from a cognate sensor histidine kinase to a specific aspartate residue. Phosphorylation at the conserved aspartate residue modulates the activity of the response regulator. Methylesterase CheB is a two-domain response regulator composed of a regulatory domain and an effector domain with enzymatic activity. CheB functions within the bacterial chemotaxis pathway to control the level of chemoreceptor methylation. In its unphosphorylated state, the regulatory domain inhibits methylesterase activity of the effector domain. Phosphorylation of the regulatory domain leads to an enhancement of methylesterase activity through a relief of inhibition and a stimulatory effect on catalysis. CheB is a useful model protein for understanding the effects of phosphorylation of the regulatory domain on interdomain interactions and stimulation of enzymatic activity of the effector domain. Kinetic analyses of CheB activation indicate that the basis for the nearly 100-fold methylesterase activation upon phosphorylation is due to a change in the catalytic rate constant for the methylesterase reaction. It is also shown that the P2 domain of histidine kinase CheA inhibits the methylesterase activity of CheB and that this inhibition is decreased upon phosphorylation of CheB. Finally, studies of methylesterase catalysis by the free catalytic domain in the presence and absence of the regulatory domain have enabled detection of an association between the two domains in the absence of the linker.

A widespread type of signaling in prokaryotes and certain eukaryotes, referred to as "two-component" signaling, involves stimulus−response coupling that is mediated through a histidine protein kinase and a response regulator (*1*, *2*). In archetypal systems, environmental stimuli regulate the activities of the histidine kinase, thereby controlling the level of phosphorylated response regulators that in turn effect the output responses. Bacterial chemotaxis is a well-characterized "two-component" system with a histidine kinase, CheA, and two response regulators, CheB and CheY (*3*, *4*). Chemoreceptors regulate the autokinase activity of CheA via a mechanism that is dependent upon ligand binding and the methylation state of the receptor (*5*). Methylesterase CheB acts together with methyltransferase CheR to control the level of receptor methylation, thereby contributing to adaptation to the levels of chemoeffectors. CheB catalyzes the deamidation of specific glutamine residues (*6*) and the demethylation of methylglutamates introduced by CheR (*7*) in the cytoplasmic region of chemoreceptors.

CheB is a two-domain response regulator with an N-terminal regulatory domain homologous to CheY and a C-terminal effector domain with methylesterase activity (*8*). The regulatory domain exerts an inhibitory effect on the catalytic activity of the effector domain. This has been demonstrated by removal of the N-terminal domain, which results in a 10-fold increase in methylesterase activity (*8*, *9*). Phosphorylation at a single aspartate residue within the regulatory domain results in a stimulation of methylesterase activity (*10*) above that seen for removal of the N-terminal domain alone (*11*). The activation of CheB upon phosphorylation therefore results from both a relief of inhibition and a stimulation of methylesterase catalysis. The structure of methylesterase CheB has provided the structural basis for the inhibitory role of the regulatory domain in the unphosphorylated state, wherein positioning of the regulatory domain partially occludes the methylesterase active site residues in the effector domain, thereby sterically blocking access of substrate to the active site (*12*). However, the absence of a structure of phosphorylated CheB has precluded structural characterization of the phosphorylated state. Although there is evidence for phosphorylation-dependent conformational changes within the regulatory domain (*13*), details of the mechanism for phosphorylation-dependent activation of methylesterase CheB are unclear.

In this study, we have used kinetic analyses of the methylesterase reaction catalyzed by unphosphorylated and phosphorylated CheB to characterize the stimulatory effects of phosphorylation on methylesterase activity. Furthermore, we have used the assay to examine the binding of CheB with

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CheA and probe interdomain interactions between the uncoupled, free regulatory, and effector domains in both unphosphorylated and phosphorylated states. Our results indicate that in addition to stimulating methylesterase catalysis, phosphorylation enhances methylesterase activity through a reduction in binding affinity between CheB and the P2 domain of CheA. We have further probed the interactions between the regulatory and catalytic domains of CheB in the absence of the linker by adding different concentrations of regulatory domain in the unphosphorylated and phosphorylated states. The free regulatory domain mediates only a partial inhibition of the activity of the effector domain relative to the activity of intact, unphosphorylated CheB, suggesting a potential role for the linker in the regulation of enzymatic activity in CheB.

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. *S*-Adenosyl [^{3}H]-L-methionine (10.8 Ci/mmol) was purchased from DuPont−New England Nuclear. 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. All other materials were of reagent grade from standard commercial sources. Liquid scintillation counting was performed with a Beckman scintillation spectrometer (model LS650). Ultraviolet and visible spectroscopy was performed 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)] (*14*) containing plasmid pME98 (obtained from the laboratory of J. Stock, Princeton University, Princeton, NJ), a pUC12 derivative with a cassette containing *Salmonella enterica* serovar Typhimurium *tar*, was used for expression of Salmonella aspartate receptor Tar. *E. coli* HB101 (*15*) was used as a host strain for expression of Salmonella methyltransferase CheR from plasmid pME43 (*16*) and for expression of intact Salmonella CheB from plasmid pME30 (*17*), a derivative of pUC12 with an *Eco*RI−*Hin*dIII cassette containing the *cheB* gene. *E. coli* DH5α (*18*) was used as a host strain for expression of the catalytic domain of Salmonella *cheB* (residues 147−349), CheBc,[1] from plasmid pCP1 (*19*). All plasmid-containing strains except HCB437 were grown at 37 °C in LB medium containing 40 μg/mL ampicillin. For growth of HCB437, 25 μg/mL kanamycin was included in the medium and cells were grown at 30 °C. The P2 domain of Salmonella CheA was isolated from *E. coli* E507 (*20*) cells containing pAS7, a T7 expression vector derived from pJES307 (*21*) containing an insert encoding residues Asp 161−Val 236 of Salmonella CheA with flanking initiator methionine and stop codons.

Methylesterase Assay. *E. coli* membranes containing Salmonella aspartate receptors 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 as described previously (*8*). Methylation of Tar with CheR was carried out for a period of time such that there was no further increase in methylation as determined by incorporation of ^{3}H in Tar. There are two sites of methylation available per Tar. Under our reaction conditions for methylation, >90% of the estimated Tar present in membrane extracts was found to be methylated with two [^{3}H]methyl groups incorporated per Tar molecule. The two preparations of [*methyl*-^{3}H]Tar used for this study had specific activities of 500 and 620 cpm/pmol of [^{3}H]-methyl groups, respectively. Methylesterase activity was assayed by incubating the membrane-bound [*methyl*-^{3}H]Tar with methylesterase CheB (0.2 μM or as indicated) or CheBc (0.2 μM) in 50 mM phosphate buffer, 1 mM EDTA, and 2.8 mM βME, pH 7.0, containing 25 mM $MgCl_2$ or 1 mM EDTA as described previously (*11*). To assay the activity of CheB upon phosphorylation, CheB (0.2 μM) was first preincubated with 50 mM phosphoramidate [synthesized as its ammonium salt according to the method of Sheridan et al. (*22*)] and 25 mM $MgCl_2$ in 50 mM phosphate buffer and 2.8 mM βME, pH 7.0, in the absence of EDTA for 30 s prior to initiation of the reaction. Reactions were initiated by the addition of membrane extracts containing [*methyl*-^{3}H]-Tar containing 25 mM $MgCl_2$ and 50 mM phosphoramidate. At indicated times, 5-μL aliquots of the reaction were removed and quenched by the addition of 30 μL of 5 N acetic acid and applied to filter paper (Whatman), which was then suspended over scintillation fluid, and volatile [^{3}H]methanol was determined using a vapor diffusion assay. The sealed vials were equilibrated for at least 12 h of incubation at 25 °C, and [^{3}H]methanol released in the reaction was then measured using a liquid scintillation spectrometer. The specific activity of the methylated Tar was used to determine esterase activity in millimoles of [^{3}H]methanol per mole of esterase per second. The concentration of [^{3}H]methyl groups in the sample of substrate was determined by hydrolysis of a 5-μL aliquot of [*methyl*-^{3}H]Tar by 0.1 N sodium hydroxide and the subsequent measurement of the volatile counts of [^{3}H]methanol described earlier (*11*).

Saturation experiments with esterase and [*methyl*-^{3}H]-Tar were carried out using increasing concentrations of [*methyl*-^{3}H]Tar to 30 μM. Aliquots of membrane extracts containing [*methyl*-^{3}H]Tar were thawed and equilibrated to 25 °C prior to initiation of the methylesterase reaction. The total volume of the membrane fraction added to the reaction was kept constant under various substrate concentrations by the addition of compensatory membranes prepared from bacterial cells that did not express Tar. The values for the Michaelis constant (K_M) determined from a nonlinear curve fit of a plot of the specific activity versus concentration of [*methyl*-^{3}H]Tar are represented in terms of the concentration of [^{3}H]methyl groups in the membrane fraction containing [*methyl*-^{3}H]Tar.

Protein Expression and Purification. CheBc protein was expressed and purified according to the method of West et

[1] Abbreviations: βME, β-mercaptoethanol; CheBc, C-terminal domain (147−349) of Salmonella CheB; Tm CheBn, N-terminal domain (1−140) of *T. maritima* CheB; Tm CheBc, C-terminal domain (149−344) of *T. maritima* CheB; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; IPTG, isopropyl 1-thio-β-D-galactopyranoside; MeOH, methanol; PA, phosphoramidate; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reversed phase high-performance liquid chromatography; Salmonella, *Salmonella enterica* serovar Typhimurium; SDS, sodium dodecyl sulfate.

al. (*19*). CheB was expressed and purified by following a modification of the procedure of Simms et al. (*8*) as previously described (*11*). Purified CheB and CheBc were precipitated with ammonium sulfate, and the precipitates were aliquoted and stored at 4 °C. CheB, stored in 50% saturated ammonium sulfate, was prepared for analysis by overnight dialysis against 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, and 2.8 mM βME, pH 8.0. Concentrations of purified proteins were estimated by measuring the absorbance at 280 nm for CheB, $\epsilon_{280} = 10900\ M^{-1}\ cm^{-1}$, and CheBc, $\epsilon_{280} = 9600\ M^{-1}\ cm^{-1}$. Extinction coefficients were estimated on the basis of the tryptophan and tyrosine contents of the proteins (*23*).

Purification of CheA P2 Domain. E. coli cells were grown to mid-log phase at 37 °C in L broth containing 50 μg/mL ampicillin. Protein expression was induced by the addition of 0.5 mM IPTG, and incubation was continued for 3 h. Cells (yield ~9 g of wet weight) were resuspended in 0.1 M sodium phosphate, pH 7.0, and disrupted by sonication. The cell lysate was clarified by ultracentrifugation. Ammonium sulfate was added to 40% saturation, and the resulting precipitate was removed by centrifugation. The supernatant was brought to 70% saturation with ammonium sulfate, and the P2 domain was recovered in the precipitate. Following centrifugation, precipitated protein was resuspended in a minimal volume of 25 mM piperazine, pH 6.0, and 50 mM NaCl and dialyzed against the same buffer (2 × 3 L). The dialysate was filtered through a 0.2 μM filter and applied to two tandem HiLoad Q-Sepharose (Amersham Pharmacia Biotech) anion exchange columns (10 mL total bed volume) equilibrated with 25 mM piperazine, pH 6.0, and 50 mM NaCl. The column was washed with 25 mL of buffer, and the protein was eluted in a 100-mL gradient of 50 mM−0.5 M NaCl in 25 mM piperazine, pH 6.0. Fractions were analyzed on 20% SDS−polyacrylamide gels, and those containing P2 were pooled and concentrated with an Amicon Centriprep-3 filtration unit. The concentrated P2-containing pool was applied to a HiLoad Superdex 75 26/60 (Amersham Pharmacia Biotech) gel filtration column equilibrated with 0.1 M sodium phosphate, pH 7.0. Fractions were collected and analyzed by SDS−PAGE. P2-containing fractions were pooled, and the final protein concentration was estimated by Bradford dye-binding assays (Bio-Rad Laboratories).

Cloning, Expression, and Purification of Thermotoga maritima Full-Length CheB, N-Terminal Domain, and C-Terminal Domain. The primary sequence of the C-terminal domain of CheB (147−349) from Salmonella was used to search the genome of *T. maritima* (*24*) using the search program BLAST (*25*). One sequence of a protein of comparable size (37,623 Da) was obtained with uniform stretches of homology in primary sequence. This sequence corresponds to ORF TM0408 of the *T. maritima* genome. Oligonucleotide primers (18 bp) complementary to the proximal and distal ends of the putative methylesterase gene were designed, and the gene was amplified by PCR using genomic *T. maritima* DNA as template. The amplified gene was inserted into a pUC12 plasmid and transformed into *E. coli* DH5α cells. The nucleotide sequence of the gene was confirmed by sequencing.

The structure of Salmonella methylesterase CheB (*12*) was used to define domain boundaries for the N- (Tm CheBn) and C-terminal (Tm CheBc) domains of *T. maritima* CheB. The N-terminal domain construct was designed to extend up to residue 138 of Salmonella CheB. This covers a portion of the linker and corresponds to residue 140 of the amino acid sequence of *T. maritima* CheB. The C-terminal domain construct was designed with a methionine at the N terminus to encode the region of the protein from residue 147 to the end of the sequence of Salmonella CheB, corresponding to residues 149−344 of the *T. maritima* CheB gene.

E. coli DH5α cells transformed with a pUC12 plasmid encoding either constitutively expressing *T. maritima* CheB, CheBc, or CheBn were grown for 18−20 h at 37 °C. Following growth, cells were harvested by centrifugation. The cell pellet was resuspended in 50 mM phosphate buffer, pH 7.0, and cells were lysed by sonication. The cell lysate was centrifuged at 120000*g* for 1 h at 4 °C. The soluble portion was maintained at 70 °C for 20 min, and the supernatant was removed after centrifugation at 27000*g*. Such a heat denaturation and precipitation of mesophilic proteins from *E. coli* constituted a significant purification step for the more thermostable cloned *T. maritima* proteins. The supernatant was fractionated via precipitation by the addition of 30 g of ammonium sulfate/100 mL of solution with gentle stirring for 30−60 min. The precipitate was collected by centrifugation at 27000*g*, resuspended in a minimal volume of 50 mM Tris-HCl, pH 8.0, and dialyzed against 2 × 4 L of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 2.8 mM βME overnight. As a final purification step for Tm CheB and Tm CheBc, the dialyzed ammonium sulfate fraction was diluted 2-fold with 1 mM EDTA and 2.8 mM βME and applied to an 18 × 2.5 cm Q-Sepharose Fast Flow (Pharmacia) column equilibrated in Tris buffer. The flow-through was collected and analyzed by SDS−PAGE and was stored as an ammonium sulfate precipitate. CheB, stored in ammonium sulfate, was prepared for analysis by overnight dialysis against 50 mM potassium phosphate and 2.8 mM βME, pH 8.0. Tm CheBn was further purified by gel filtration. The concentrated Tm CheBn-containing pool was applied to a HiLoad Superdex 75 26/60 (Amersham Pharmacia Biotech) gel filtration column equilibrated with 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, and 2.8 mM βME, pH 8.0. Fractions were collected and analyzed by SDS−PAGE. Tm CheBn-containing fractions were pooled, and the final protein concentration was estimated by Bradford dye-binding assays (Bio-Rad Laboratories). Association of Tm CheBn and Tm CheBc was analyzed by gel filtration using an SW 2000 column (7.8 × 30 cm) (TOSOHAAS).

Methylesterase Activity of T. maritima CheB, Phosphorylated CheB, and CheBc. Methylesterase activities of Tm CheBc and CheB were assayed under similar conditions as for Salmonella CheB by incubating the membrane-bound [*methyl*-^{3}H]Salmonella Tar with methylesterase CheB (1.0 μM) or CheBc (1.0 μM) at 25 °C. Assays for methylesterase activity of phosphorylated Tm CheB (1.0 μM) in the presence of 50 mM phosphoramidate and 25 mM $MgCl_2$ were performed as described for Salmonella CheB. The activity of Tm CheBc (1.0 μM) was assayed at 50 mM phosphate buffer, pH 7.0. The ability of Tm CheBn to inhibit Tm CheBc was determined at five concentrations of Tm CheBn (0, 1, 10, 25, and 50 μM). Phosphorylation of Tm CheBn was carried out by incubation with 50 mM phosphoramidate and 25 mM $MgCl_2$ for 30 s at 25 °C prior to initiation of the reaction.

RESULTS

Phosphorylation of CheB Results in a Larger Change in k_{cat} than in K_M for the Methylesterase Reaction. To understand the basis for the stimulatory effect of phosphorylation, we determined the kinetic parameters, K_M and k_{cat}, for the methylesterase reaction catalyzed by CheB and phosphorylated CheB. The methylesterase activity of phosphorylated CheB generated in the presence of phosphoramidate and Mg^{2+} not only is a measure of the activity of phosphorylated CheB but also reflects the independent effects of Mg^{2+} on the methylesterase assay. To delineate the stimulatory effect of phosphorylation on methylesterase activity from the effects of Mg^{2+} in the methylesterase assay, saturation experiments with substrate were carried out for CheB in the presence of 25 mM $MgCl_2$ and phosphorylated CheB in the presence of 25 mM $MgCl_2$ and 50 mM phosphoramidate and compared. The substrate used in our methylesterase assays, [*methyl*-^{3}H]-Tar, was purified as a membrane extract as described under Materials and Methods. To demonstrate saturation of enzyme by substrate and discount nonspecific effects resulting from variable concentrations of membranes in the reaction, it was necessary to carry out saturation experiments with substrate under conditions where the concentration of membrane fraction was maintained at a constant level. This was achieved by using equivalent amounts of membrane fractions from cells not expressing Tar as diluent. Previous studies of the methylesterase activities of CheB using similar substrate and assay conditions showed that CheB could not be saturated with substrate (*9*). Our ability to saturate CheB with substrate Tar is likely due to the use of a different plasmid expression vector (pME98) that yields higher Tar incorporation into membranes.

A nonlinear curve-fitting program was used to determine the values for the Michaelis constant (K_M) and catalytic rate constant (k_{cat}) for each of the methylesterase reactions catalyzed by unphosphorylated CheB and phosphorylated CheB (Figure 1). The use of partially purified membrane extracts of *E. coli* expressing various levels of substrate Tar in different preparations in our methylesterase assay introduced some variability in the absolute values for the kinetic parameters for the methylesterase reaction catalyzed by CheB. However, the ratios in values for the activity of CheB (+ Mg^{2+}) in the presence and absence of phosphoramidate were constant, allowing comparative analyses of methylesterase catalysis mediated by different states of CheB. The values for the Michaelis constant (K_M) for unphosphorylated and phosphorylated CheB were similar, whereas the catalytic rate constant (k_{cat}) for phosphorylated CheB was significantly greater for phosphorylated CheB than unphosphorylated CheB (+Mg^{2+}).

To delineate the independent effects of Mg^{2+} on methylesterase activity, we performed saturation experiments with CheBc in the absence and presence of Mg^{2+}(Figure 2). Greater concentrations of Tar were required to saturate CheBc in the presence of Mg^{2+}. This is reflected in the increase in K_M for the reaction for CheBc with Mg^{2+}. There was no significant difference in values for k_{cat} for reactions in the presence and absence of Mg^{2+}. Table 1 summarizes the kinetic parameters for CheB and CheBc in the presence and absence of Mg^{2+} and phosphorylated CheB. Mg^{2+} increases both k_{cat} and K_M for CheB, whereas Mg^{2+} increases K_M alone for CheBc.

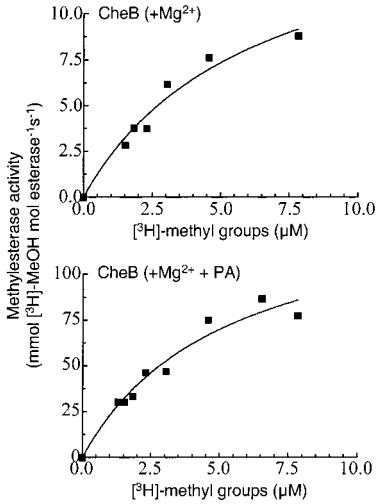

FIGURE 1: Saturation of CheB and phosphorylated CheB with [*methyl*-^{3}H]Tar. Methylesterase activity was determined as described under Materials and Methods by incubating various amounts of membranes containing [*methyl*-^{3}H]Tar with methylesterase in a reaction buffer of 50 mM potassium phosphate, 25 mM $MgCl_2$, 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 0.2 μM methylesterase. Curves were generated using a nonlinear curve fitting program to fit the Michaelis−Menten equation, and values for kinetic parameters with standard errors were calculated for CheB ($K_M = 6.0 \pm 2.0$, $k_{cat} = 16.2 \pm 3.1$) and phosphorylated CheB ($K_M = 5.2 \pm 1.5$, $k_{cat} = 143.6 \pm 22.6$). The standard errors represent the closeness of fit of the data to the Michaelis−Menten equation.

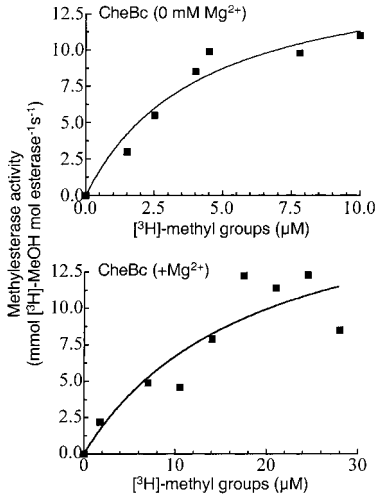

FIGURE 2: Effect of Mg^{2+} on saturation of CheBc with [*methyl*-^{3}H]-Tar. Saturation of CheBc with [*methyl*-^{3}H]Tar was carried out as described in Figure 1. Assays were performed using 0.2 μM CheBc in phosphate buffer, 1 mM EDTA, or 25 mM $MgCl_2$ as indicated. The values for kinetic parameters with standard errors were calculated for CheBc (− Mg^{2+}) ($K_M = 4.2 \pm 1.7$, $k_{cat} = 16.1 \pm 2.8$) and CheBc (+ Mg^{2+}) ($K_M = 18.4 \pm 16$, $k_{cat} = 19.1 \pm 8.3$). The standard errors represent the closeness of fit of the data to the Michaelis−Menten equation.

Inhibition of Methylesterase Activity of CheB by P2 Domain Is Decreased upon Phosphorylation. An additional role of the regulatory domain is its interaction with the P2 domain of CheA. To determine the effect of the P2 domain on methylesterase activity, various concentrations of P2 were added to CheB and CheBc and the methylesterase activities

Table 1: Kinetic Parameters for the Methylesterase Reactions[a] Catalyzed by CheB, CheBc, and Phosphorylated CheB[b]

methylesterase	K_M[c]	k_{cat}[d]
CheB ($-$ Mg^{2+})[e]	$<1.8 \pm 0.07$	0.8 ± 0.03
CheB ($+$ Mg^{2+})	4.5 ± 1.5	25.5 ± 9.7
CheBc ($-$ Mg^{2+})	5.6 ± 1.4	19.5 ± 3.4
CheBc ($+$ Mg^{2+})	16.1 ± 2.3	17.6 ± 1.4
(CheB + PA + Mg^{2+})	7.0 ± 0.9	148.0 ± 3.9

[a] Methylesterase activity of CheB and CheBc 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. Effect of Mg^{2+} was carried out with reaction buffer minus EDTA and with 25 mM $MgCl_2$. [b] Phosphorylation of CheB was carried out by preincubation of CheB with 25 mM $MgCl_2$ and 50 mM phosphoramidate (PA) for 30 s in phosphate reaction buffer. [c] Values are expressed as μM of [^{3}H]methyl groups in the [*methyl*-^{3}H]-Tar as described under Materials and Methods. [d] Values are expressed as mmol of [^{3}H]methanol (mol of esterase)$^{-1}$ s^{-1}. Values for K_M and k_{cat} are expressed as mean $\pm$ SD of at least two independent experiments (one each of which is shown in Figures 1 and 2). [e] The low methylesterase activity of CheB ($-$ Mg^{2+}) necessitated use of higher concentrations of CheB (5 μM) to detect methylesterase activity under our assay conditions. Although saturation of CheB with substrate was achieved, the value of K_M determined represents an approximate value and can be expected to be greater than the true value for K_M.

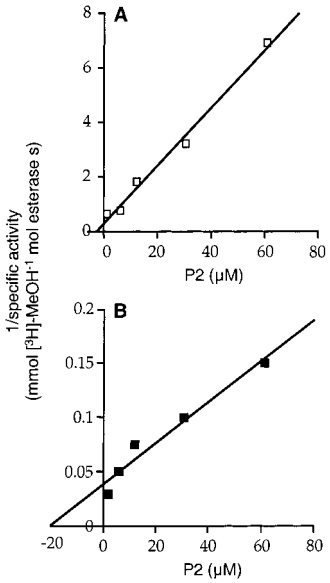

FIGURE 3: Dixon plots [(1/specific activity) vs concentration of P2 domain] for the inhibition by the P2 domain of CheA of the methylesterase activity of (A) CheB (□) and (B) phosphorylated CheB (■). Various concentrations of P2 domain from 1 to 60 μM were used to inhibit CheB (5 μM) and phosphorylated CheB (0.5 μM) in the methylesterase assay as described under Materials and Methods.

were quantitated in the assay. CheB, but not CheBc, was inhibited by P2 (data not shown), providing direct evidence for the previously postulated interaction between P2 and the N-terminal domain (*26*). A reciprocal plot of specific activity of CheB versus concentration of P2 is shown in Figure 3A. The apparent inhibitory constant (K_I) determined from this plot is 2.5 μM. Similar experiments were performed in the presence of 50 mM potassium phosphoramidate and 25 mM Mg^{2+} (Figure 3B). Due to the much lower specific methylesterase activity of CheB relative to that of phosphorylated CheB, concentrations of unphosphorylated CheB and CheB in the presence of phosphoramidate were adjusted so that in each reaction the absolute quantity of MeOH released fell within a range of 200−2000 counts/min. The apparent inhibitory constant (K_I) determined for CheB in the presence of phosphoramidate and Mg^{2+} is 21.3 μM.

Unphosphorylated Regulatory Domain Partially Inhibits Methylesterase Activity of Effector Domain in the Absence of the Linker. To examine interdomain interactions in CheB, we set out to explore the ability of the free regulatory domain to inhibit the methylesterase activity in the absence of the interdomain linker. In contrast to the catalytic domain, which can be stably expressed on its own, many different constructs of the Salmonella CheB regulatory domain that included various lengths of the linker all failed to be stably expressed (A. West and A. Stock, unpublished data). An alternative was to explore the possibility of stably expressing the free effector and regulatory domains from a thermophilic homologue of CheB.

A BLAST search (*25*) of the complete sequenced genome of *T. maritima* (*24*) using the primary sequence of the C-terminal domain of CheB (147−349) from Salmonella revealed the existence of a gene homologous to *cheB* in a locus on the genome separate from the cluster of chemotaxis genes *cheA*, *cheW*, and *cheY* that were previously isolated and characterized (*27*). The gene encodes a protein of comparable size (37,623 Da) and contains conserved residues corresponding to the site of phosphorylation and methylesterase catalytic triad. The sequence shows an overall 37% amino acid identity with Salmonella CheB, with most of the sequence variability occurring within the interdomain linker region. This gene was cloned, sequenced, and expressed in *E. coli* vectors, and the protein was purified as described under Materials and Methods.

The ability of purified *T. maritima* CheB to be phosphorylated in the presence of phosphoramidate and $MgCl_2$ was shown using γ-^{32}P-labeled phosphoramidate (*28*) (data not shown) and by RP-HPLC analyses of samples of phosphorylated and unphosphorylated protein. The homologue was also assayed for methylesterase activity in the unphosphorylated and phosphorylated states by incubation with Salmonella [*methyl*-^{3}H]Tar at 25 °C under conditions identical to those for Salmonella CheB. Phosphorylation of *T. maritima* CheB was carried out in the presence of phosphoramidate and $MgCl_2$ as for Salmonella CheB. Unphosphorylated *T. maritima* CheB was observed to catalyze methyl ester hydrolysis of Salmonella [*methyl*-^{3}H]Tar at 25 °C [specific activity of 0.71 ± 0.02 mmol of [^{3}H]methanol (mol of esterase)$^{-1}$ s^{-1} in phosphate buffer, pH 7.0], and phosphorylation of *T. maritima* CheB in the presence of phosphoramidate and $MgCl_2$ resulted in an increase in methylesterase activity for the phosphorylated state of the enzyme [specific activity of 90 ± 10 mmol of [^{3}H]methanol (mol of esterase)$^{-1}$ s^{-1} in phosphate buffer, pH 7.0]. The *T. maritima* homologue of CheB shows enzymatic properties and activation upon phosphorylation similar to those of Salmonella CheB.

Expression constructs for the N- and C-terminal domains of the *T. maritima* homologue were designed using boundaries observed in the structure of Salmonella CheB (Figure 4). Both domains were stably expressed and purified as described under Materials and Methods. The *T. maritima* CheB C-terminal domain (Tm CheBc) exhibited methylesterase activity with a specific activity of 18.6 ± 1.2 mmol of [^{3}H]methanol (mol of esterase)$^{-1}$ s^{-1} in phosphate buffer,

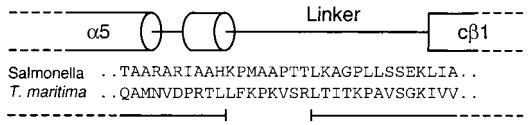

FIGURE 4: Boundaries for regulatory and effector domain constructs of *T. maritima* CheB. Sequences encompassing the linker regions of Salmonella (residues 129−160) and *T. maritima* (residues 131−162) CheB aligned by BLAST (*25*) are shown along with the secondary structure elements that they span based on the crystal structure of Salmonella CheB (*12*). The *T. maritima* N-terminal regulatory domain construct extends from the N terminus to residue 140 of *T. maritima* CheB (residue 138 of Salmonella CheB). The *T. maritima* C-terminal effector domain construct extends from residue 149 (residue 147 of Salmonella CheB) to the C terminus.

pH 7.0 at 25 °C. The *T. maritima* CheB N-terminal domain (Tm CheBn) was active as inferred from its ability to transfer phosphoryl groups from phosphoramidate in the presence of Mg^{2+} (data not shown). RP-HPLC analysis of samples of phosphorylated and unphosphorylated Tm CheBn indicated a shorter retention time for phosphorylated Tm CheBn than for unphosphorylated Tm CheBn, and this assay was used to show that in the presence of phosphoramidate and Mg^{2+}, CheBn could be completely converted to the phosphorylated state (data not shown). Our constructs of the N- and C-terminal domains of *T. maritima* CheB were stably expressed and retained their functional properties. These constructs could therefore be used in experiments probing interactions between the isolated regulatory and effector domains of CheB.

Although the association of Tm CheBn and Tm CheBc was not detectable by gel filtration chromatography (data not shown), we set out to determine if the free N-terminal domain inhibited the methylesterase activity of the free catalytic domain under our conditions for the methylesterase assay at pH 7.0 (Figure 5). The activity of Tm CheBc was measured in the presence of variable concentrations of unphosphorylated and phosphorylated Tm CheBn. There was a 2-fold inhibition of methylesterase activity in the presence of 50 μM unphosphorylated Tm CheBn. The relationship between increased concentrations of unphosphorylated Tm CheBn and activity was not linear, with only a marginal effect on methylesterase activity between the reactions containing 25 and 50 μM Tm CheBn. There was little change in relative methylesterase activity upon addition of phosphorylated Tm CheBn generated in the presence of 50 mM phosphoramidate and 25 mM $MgCl_2$.

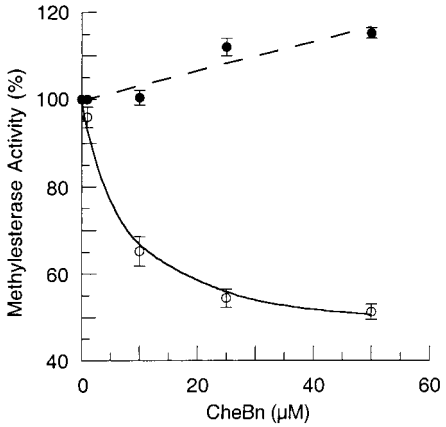

FIGURE 5: Methylesterase activity of Tm CheBc (effector domain) in the presence of Tm CheBn (regulatory domain) in the unphosphorylated (○) and phosphorylated states (●). Methylesterase activity at each concentration of Tm CheBn is shown as a percentage of the activity of Tm CheBc (Tm CheBn = 0). One hundred percent methylesterase activity corresponded to methylesterase activities of 18.6 ± 1.2 and 8.6 mmol of [^{3}H]methanol (mol of esterase)$^{-1}$ s^{-1} for Tm CheBc in the absence and presence of 50 mM phosphoramidate and 25 mM $MgCl_2$, respectively. The lower activity of the latter reflects the ∼2-fold inhibitory effect of 25 mM $MgCl_2$ reported previously (*11*). Methylesterase activity was determined as described under Materials and Methods in a reaction buffer of 50 mM potassium phosphate, 25 mM $MgCl_2$, 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 1.0 μM Tm CheBc. The solid line represents a theoretical curve calculated by assuming a dissociation constant of 6.5 μM for the CheBn−CheBc complex and an activity of the CheBn−CheBc complex equal to 44% of the activity of CheBc. The small effect of addition of CheBn in the presence of phosphoramidate and $MgCl_2$ precluded modeling. The dashed line represents a least-squares fit of the experimental data.

DISCUSSION

The Basis for Increased Methylesterase Activity of CheB upon Phosphorylation Is a Large Change in k_{cat} for the Methylesterase Reaction. Structural studies of the phosphorylated states of the regulatory domains of several response regulators have advanced the understanding of the phosphorylation-induced conformational changes within the regulatory domain (*29*−*32*). There is, however, less understanding of the activation of associated effector domains by their phosphorylated regulatory domains. It is generally assumed that phosphorylation-mediated regulation by these domains is achieved through specific communication between regulatory and effector domains that are modulated by the different conformational states influenced by phosphorylation. Among the family of response regulator proteins, several different types of regulatory mechanisms have been observed. These include relief of inhibition as in NarL (*33*) and CheB (*12*), where the unphosphorylated N-terminal domains sterically inhibit effector domain function, facilitation of dimerization upon phosphorylation of the regulatory domain as in FixJ, PhoB, and NtrC (*34*) and facilitation of heteromeric interactions as in CheY, where phosphorylation promotes interaction with the flagellar motor (*35*).

The N-terminal domain of CheB plays dual regulatory roles, functioning to inhibit methylesterase activity in the unphosphorylated state while having a stimulatory effect upon phosphorylation. We previously reported that the methylesterase activities of CheBc and CheB in the presence of phosphoramidate and Mg^{2+} were 10- and 70-fold higher than that of the unphosphorylated CheB (*11*). Accounting for the observation that ∼65% of CheB exists in the phosphorylated state under our conditions of phosphorylation in the presence of phosphoramidate and Mg^{2+}(*13*), phosphorylation results in an ∼100-fold increase in methylesterase activity relative to unphosphorylated CheB (*13*). Assuming that the isolated catalytic domain represents the activity of the uninhibited state, the 100-fold increase results from relief of inhibition, yielding a 10-fold increase in activity along with an additional 10-fold increase in activity coming through a stimulatory effect.

The structure of intact unphosphorylated methylesterase CheB suggested a mechanism for steric inhibition of methylesterase activity of CheB by the unphosphorylated N-terminal domain (*12*). It was initially postulated that phosphorylation of the N-terminal domain might result in a significant repositioning of the two domains relative to each other, allowing access of the methylglutamates of the chemoreceptors to the active site of the enzyme. However, it has been shown that the linker and regulatory domain remain associated with the catalytic domain upon phosphorylation with only subtle changes at the interdomain interface (*36*).

The basis for the stimulatory effect on methylesterase activity generated by phosphorylation of the regulatory domain is not readily apparent. A possible role for the phosphorylated regulatory domain in mediating binding interactions with the receptor can be envisioned on the basis of similarities in the structures of the two chemoreceptor modification enzymes, methyltransferase CheR and CheB. CheR, like CheB, is a two-domain protein with the methyltransferase active site located at the interface between the two domains and with an N-terminal regulatory domain that plays a role in substrate recognition (*12*). However, our kinetic analysis indicates that the basis for the stimulatory effect of phosphorylation on methylesterase activity results from changes in k_{cat} alone. The absence of any significant difference in K_M for both CheB and phosphorylated CheB suggests that phosphorylation does not result in an increased affinity for binding the receptor substrate. This does not necessarily exclude a role for the N-terminal domain in interaction with the receptor substrate. For instance, an increase in k_{cat} upon phosphorylation might reflect the ability of the phosphorylated N-terminal domain to properly orient the receptor substrate, thereby improving catalysis. It is also possible that enhanced binding of phosphorylated CheB to the receptor methylation region does occur, but that this is reflected in an altered k_{cat}, whereas the K_M reflects the previously documented tethering of CheB to a pentapeptide at the C terminus of the receptor (*37*), an interaction that may not be affected by phosphorylation. Alternatively, enhancement of methylesterase catalysis upon phosphorylation may result from allosteric effects on the active site as a consequence of propagated conformational changes from the site of phosphorylation. These effects and others, separately or in combination, could explain the stimulatory effect of phosphorylation on the methylesterase activity of CheB.

Our results also enable delineation of the independent effects of Mg^{2+} on the methylesterase activity in CheB. Mg^{2+} has previously been shown to have an inhibitory effect on CheBc, presumably affecting methylesterase catalysis (*11*). The basis for this inhibition of CheBc by Mg^{2+} is an increase in value for K_M for the reaction. In contrast, Mg^{2+} binding at the site of phosphorylation in intact CheB enhances methylesterase catalysis, presumably as a result of conformational changes that have been observed upon Mg^{2+} binding (*13*). The value of K_M for the reaction catalyzed by CheB in the presence of Mg^{2+} is larger than that for CheB in the absence of Mg^{2+}, suggesting that the stimulatory effect of Mg^{2+} binding at the site of phosphorylation offsets the nonspecific inhibitory effect of Mg^{2+}. Optimum phosphorylation of CheB requires high concentrations of Mg^{2+}. Activation of methyl ester hydrolysis observed in the presence of phosphoramidate and Mg^{2+} binding to CheB as measured by our assay therefore reflects both the stimulatory effect of phosphorylation and the inhibitory effect of Mg^{2+} on methylesterase catalysis. This is reflected in a slight increase in values of K_M and a greater increase in values of k_{cat} for phosphorylated CheB generated in the presence of phosphoramidate and $MgCl_2$, relative to unphosphorylated CheB.

Role of the Regulatory Domain in Mediating Interactions with the P2 Domain of CheA. In addition to regulating the methylesterase activity of the catalytic domain, the N-terminal domain of CheB is also involved in mediating interactions with CheA. Intact CheA has been shown to inhibit CheB, but not CheBc, suggesting that this inhibition cannot be localized to an interaction involving the C-terminal domain of CheB alone (*26*). CheB and CheY have been shown to exhibit competitive binding to CheA, suggesting that the binding surfaces for CheB and CheY on CheA overlap, and the binding of CheB and CheY has been localized to a fragment of CheA that contains the phosphotransfer P1 and P2 domains (*38*). Biochemical studies have shown that the P2 domain is the primary determinant for binding CheY, and P2 has been postulated to bind CheB as well (*39*, *40*). Our analysis of the effect of P2 on methylesterase activity of CheB confirms that P2 inhibits the methylesterase activity of intact CheB but not of CheBc, presumably by binding to its N-terminal domain. This binding might inhibit methylesterase activity by sterically interfering with the ability of the methylesterase to bind its receptor substrate or through allosteric effects on the active site as a consequence of conformational changes induced by binding of P2 to CheB. In this regard, alterations in the structure of CheY have been observed in the crystal structures of the CheY−P2 complex (*41*, *42*).

The apparent inhibitory constant of P2 with respect to methylesterase activity (K_I = 2.5 μM) is similar to the binding constant determined for CheB and the fragment of CheA containing the P1 and P2 domains (3.2 μM) (*38*). We have observed an ~8-fold increase in the inhibitory constant upon phosphorylation of CheB, presumably reflecting a decrease in binding of P2 to phosphorylated CheB. Analogously, phosphorylation of CheY has been shown to result in a 3-fold reduction in binding to the P2 domain of CheA (*38*).

Structures of the complex between CheY and P2 (*41*, *42*) show an extensive intermolecular interface involving the α4-β5-α5 region of CheY. Despite the competitive binding of CheB and CheY to CheA, it appears that binding occurs through different surfaces of the conserved regulatory domains. There is very little conservation between the primary sequences of CheB and CheY for the residues identified as being involved in interaction with P2 in CheY. Furthermore, the corresponding α4-β5-α5 surface in CheB is inaccessible, being a part of the interdomain interface in CheB (*12*).

Association of Regulatory and Effector Domains in the Absence of the Linker Results in Partial Inhibition of Methylesterase Activity. Response regulators are highly modular proteins with conserved regulatory domains coupled to diverse effector domains, the activities of which are modulated in a phosphorylation-dependent manner. Structural analyses of the unphosphorylated states of two intact response

regulators, NarL (*33*) and CheB (*12*), reveal highly specific inhibitory interactions between the regulatory and effector domains that are presumably altered by phosphorylation of the regulatory domain. Thus, at least for some response regulators, specific interdomain contacts appear to be essential in defining the regulation of effector domain activity by the regulatory domain.

The structure of CheB shows a tightly packed domain interface involving residues from the N- and C-terminal domains as well as residues from the N-terminal region of the linker, containing a short helical segment (*12*). This extensive interface suggests that the free regulatory and effector domains might associate in solution. Although no complex is detected by size exclusion chromatography, methylesterase activity assays provide evidence of domain interactions. Increasing concentrations of N-terminal domain cause increased inhibition, but the relationship is nonlinear. This suggests saturation in binding of the free effector domain and is consistent with a dissociation constant of 5−10 μM. However, even at saturated binding, the extent of inhibition is only ∼2-fold, substantially less than the ∼20-fold inhibition observed in intact CheB. Phosphorylated CheBn does not inhibit CheBc but has little, if any, stimulatory effect. Thus, the association of the free regulatory and effector domains is insufficient to recapitulate the enzymatic regulation that occurs in the full-length protein.

Although our regulatory domain construct of *T. maritima* CheB included portions of the linker region that form a helical turn in the structure of Salmonella CheB, it is possible that in our truncated constructs, these segments do not adopt the unique conformation seen in the intact protein. Thus, the domain interface formed by the intermolecular interaction of the isolated N- and C-terminal domains may differ from that of the intact protein. A specific role for the linker notwithstanding, covalent coupling of the regulatory and catalytic domains would enhance interdomain association. Our data demonstrate the importance of the linker region, but whether the linker serves merely as a tether or whether it plays a more specific role in intramolecular signaling remains an open question.

Docking of CheB to the Receptor−CheW−CheA Complex. An additional aspect of the regulation of the methylation enzymes CheB and CheR is the clustering of the receptors with histidine kinase CheA and the adaptor protein CheW at the poles of the cells (*43*). The docking of CheB to the receptor−CheW−CheA complex through the P2 domain and tethering of CheR to the receptor results in increased local concentrations of substrate for both methylation enzymes. While CheR remains tethered to the receptor, the binding of CheB to CheA is modulated by the phosphorylation state of CheB. An additional level of complexity in the regulation of CheB is introduced through the ability of CheB to be attached to the pentapeptide tail of the receptor, which serves as the docking site for CheR (*37*). Phosphorylation of CheB reduces the affinity of CheB for CheA. This presumably allows active phosphorylated CheB to diffuse to the methylation region of the chemoreceptor and subsequently to catalyze the demethylation of methylglutamates of chemoreceptors.

Modeling studies of the chemotactic signaling pathway have assumed that the receptor−CheW−CheA complexes exist in a dynamic equilibrium between two conformational states that are regulated both by ligand occupancy and by the methylation state of the receptor (*44*, *45*). The methylation enzymes have further been postulated to catalyze enzymatic reactions exclusively on one or another conformation of the receptor−CheW−CheA complex. Our data support the hypothesis that CheB is a highly regulated enzyme with phosphorylation altering an equilibrium between CheA-bound CheB with lower methylesterase activity and free phosphorylated CheB with high methylesterase activity. An additional level of regulation of methylesterase activity may be contributed by the receptor substrate itself.

Phosphorylation-Dependent Regulation of Methylesterase CheB. Structural and biochemical studies of diverse enzymes in the unphosphorylated and phosphorylated states have enabled detailed understanding of the molecular basis for control of protein function by phosphorylation. Some of the mechanisms for describing the effect of phosphorylation on enzyme function include electrostatic effects on the protein resulting from introduction of a phosphate moiety as in isocitrate dehydrogenase (*46*) and allosteric activation of the enzyme upon phosphorylation as in glycogen phosphorylase (*47*). Other mechanisms involve regulation of enzyme activity through disruption of inhibitory interactions as seen in the Src family of kinases (*48*) and the SH2-regulated tyrosine phosphatase SHP-2 (*49*). Phosphorylation in CheB mediates the ∼3 orders of magnitude increase in methylesterase activity from the inactive CheA-bound state to the free activated state of the enzyme. Our studies suggest that phosphorylation mediates sophisticated control of CheB activity that presumably involves a combination of effects described in other enzymes. These include a decrease in binding interactions between the phosphorylated regulatory domain and the P2 domain of CheA. The phosphorylation-dependent activation of CheB also results from a relief of inhibition as a consequence of alterations in interdomain contacts between the regulatory and effector domains. Further stimulation of methylesterase activity upon phosphorylation is achieved through enhanced methylesterase catalysis.

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