Synthesis and Biochemical Characterization of a Phosphorylated Analogue of the Response Regulator CheB†

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ABSTRACT: CheB is a response regulator protein in the bacterial chemotaxis two-component signal transduction pathway. Methylesterase CheB functions together with methyltransferase CheR to modulate the level of glutamate methylation in transmembrane chemoreceptors in response to environmental stimuli. The level of glutamate methylation in turn indirectly controls the direction of flagellar rotation. Like most two-component response regulators, CheB is activated in vivo by phosphorylation of a single aspartate, Asp 56, in its regulatory domain. Extensive biochemical and crystallographic studies have been completed on the inactive, unphosphorylated form of CheB. Because of the inherent lability of aspartyl phosphate bonds and the intrinsic phosphatase activity of CheB, the activated, phosphorylated form of CheB cannot be isolated for further characterization. We present a synthetic scheme to prepare an analogue of phosphorylated CheB using site-specific mutagenesis and chemical modification strategies. Initially, the two native cysteines found in CheB were substituted by serines and a cysteine was substituted for Asp 56 to yield D56C/C207S/C309S CheB. The unique cysteine in the substituted form of CheB was modified by sodium thiophosphate, Na_3SPO_3, using two sequential disulfide bond exchange reactions. The analogue, D56C/C207S/C309S CheB−SPO_3, contained a thiophosphate group covalently bonded to the protein through a disulfide linkage at residue 56. Mass spectrometry showed that the protein was singly modified. Reverse phase chromatography showed that greater than 95% of the protein was modified under optimized conditions and that the analogue had a half-life of 28 days. In in vitro methylesterase assays in the presence of Mg^{2+}, the analogue exhibited activity equivalent to that of fully phosphorylated C207S/C309S CheB. Thus, D56C/C207S/C309S CheB−SPO_3 is a stable analogue that may be useful for characterization of the active form of CheB.

Two-component signal transduction pathways are prevalent in bacteria and also are present in some archaea and eukaryotes (*1*, *2*). These multiprotein systems include at least one histidine kinase and one response regulator. Response regulators comprise a large and functionally diverse family of proteins that are involved in a wide variety of phosphotransfer-dependent signal transduction pathways (*1*). In the two-component system of bacterial chemotaxis, the autophosphorylating histidine kinase CheA is the phosphoryl donor for two response regulator proteins, CheY and CheB (*3*). Methylesterase CheB functions together with methyltransferase CheR to modulate the level of transmembrane chemoreceptor methylation. While CheR introduces methyl groups to specific glutamate residues in the chemoreceptors, CheB catalyzes the demethylation of the same methylated glutamate residues in a phosphorylation-dependent manner (*4*).

CheB is a two-domain protein consisting of an N-terminal regulatory domain juxtaposed to a C-terminal effector domain forming a cleft that contains the methylesterase active site (*5*). The N-terminal regulatory domain of CheB shares structural homology with the family of response regulator proteins. These domains regulate the function of a variety of effector domains in a phosphorylation-dependent manner (*6*). In CheB, the C-terminal effector domain is a methylesterase that belongs to the class of serine hydrolases that contain active site catalytic triads consisting of serine, histidine, and aspartate residues (*7*). The regulatory and effector domains interact with each other to form an

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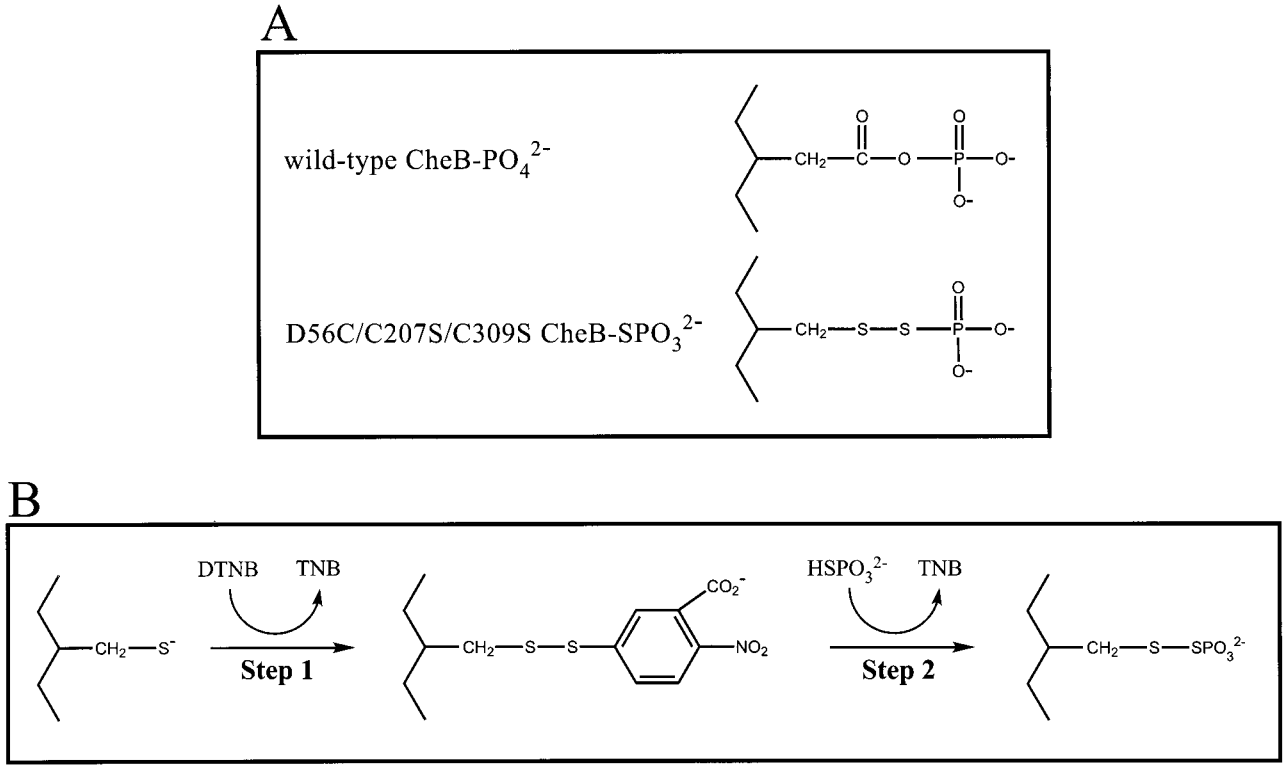

FIGURE 1: Chemical modification of CheB. (A) Comparison of aspartyl-phosphate and of cysteinyl-thiophosphate. Modification of the cysteine with phosphorthioate results in cysteinyl-thiophosphate. This side chain has slightly longer bond lengths (C−O/O−P, 2.9 Å; S−S/S−P, 4.1 Å), more acute bond angles (C−C(O)−O, 116.5°/C(O)−O−P, 124.6°; C−S−S, 100.6°/S−S−P, 102.0°), and a smaller dihedral angle (C−C(O)−O−P, 171°; C−S−S−P, 105°) than the aspartyl-phosphate side chain. Bond length and bond angle values were obtained from the Cambridge database and dihedral angle values are as published by Silversmith and Bourret (*12*). (B) The two-step chemical modification of D56C/C207S/C309S CheB. In step 1, the cysteine thiol was modified by Ellman's reagent (DTNB). In step 2, the TNB group was released from the protein with the incorporation of a thiophosphate group (SPO_3). Bond lengths and angles are not drawn to scale.

interdomain interface consisting of a small hydrophobic core surrounded and stabilized by hydrogen bond interactions and several salt bridges (*5*).

Removal of the regulatory domain results in a constitutively active methylesterase, CheBc, suggesting that the unphosphorylated regulatory domain inhibits the activity of the C-terminal effector domain (*8*). It has been shown that phosphorylation not only relieves inhibition by the regulatory domain (*9*) but also enhances activity 6−7-fold higher over that for CheBc (*10*).

Structural studies of phosphorylated CheB have not been possible because of its extremely short half-life of approximately 2 s at 25 °C (*11*). The marked instability of the aspartyl phosphate bond is a consequence of the inherent lability of acyl phosphate bonds and the intrinsic phosphatase activity of the protein. Creation of an active state analogue through chemical modification was explored as an approach to isolation of the activated protein. Introduction of a unique, reactive residue, such as a cysteine, at the active site of the response regulator allows the introduction of a nonlabile moiety that may trap the protein in its active conformation. Precedents for this approach exist for other response regulator proteins (*12*, *13*).

The amino acid sequence of CheB contains two cysteines. Substitution of the native cysteines by serines and subsequent substitution of Asp 56 with a cysteine places a unique, reactive residue in the phosphorylation active site. Introduction of a thiophosphate group to the thiol of this cysteine would permit the reversible, covalent introduction of a phosphate group to the phosphorylation active site of CheB. Sodium phosphorthioate (Na_3SPO_3)[1] reacts reversibly with protein disulfide bonds (*14*). Thiophosphate modification of a cysteine thiol mimics the natural aspartyl-phosphate in distance, flexibility, and charge, while covalently modifying the active site by reversibly affixing a phosphate (Figure 1A).

In their effort to isolate a chemical analogue of phosphorylated CheY, Silversmith and Bourret incorporated thiophosphate into the active site. Despite successful modification, no activation of CheY was observed (*12*). Unlike CheY, we found that CheB is activated by modification with thiophosphate. This report describes the synthesis by thiophosphate modification and characterization of a stable analogue of phosphorylated CheB.

[1] Abbreviations: Na_3SPO_3, sodium thiophosphate dodecahydrate; AdoMet-SAM, *S*-adenosyl L-methionine; amu, atomic mass units; *β*-ME, *β*-mercaptoethanol; BeF_3^-, beryllium fluoride; C, cysteine; D, aspartate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast performance liquid chromatography; HPLC, high performance liquid chromatography; IPTG, isopropylthio-*β*-D-galactopyranoside; LC/ESI-MS, liquid chromatography/electrospray ionization-mass spectrometry; MeOH, methanol; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl-fluoride; $-PO_4$, phosphate group; PCR, polymerase chain reaction; S, serine; SDS, sodium dodecyl sulfate; SPO_3, thiophosphate; $-SPO_3$, thiophosphate group; TNB, thio(2-nitrobenzoic acid); −TNB, thio(2-nitrobenzoic acid) group; TFA, trifluoroacetic acid; TfOMP, trifluoromethylsulfonyloxymethylphosphonate; Tris·HCl, tris(hydroxylmethyl)aminomethane hydrochloride; WT, wild-type.

MATERIALS AND METHODS

Reagents and Apparatus. Ammonium sulfate from ICN and Tris·HCl from United States Biochemical were ultrapure grade. Na_3SPO_3 was purchased from Aldrich. *S*-Adenosyl [^{3}H] L-methionine (10.8 Ci/mmol) was purchased from DuPont-New England Nuclear. Ammonium phosphoramidate was synthesized by the method of Sheridan (*15*). Ecoscint A scintillation fluid was obtained from National Diagnostics and poly(ethylene terephthalate) scintillation vials with screw caps were purchased from Wheaton. Oligonucleotides were synthesized by the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School Biochemistry Department Oligonucleotide Synthesis Facility. Restriction, ligation, and other DNA modification enzymes were purchased from New England Biolabs and Boehringer Mannheim. Native Pfu polymerase was obtained from Stratagene. Sequenase 2.0 DNA polymerase and Sequenase 2.0 GTP sequencing kit were purchased from United States Biochemical. Electrophoresis reagents, Bradford protein assay solution, Bio-Gel P-60 gel filtration matrix, and Bio-Gel P-6 spin columns were obtained from BioRad. HiTrap-SP cation (5 mL) exchange columns and Q Sepharose Fast Flow column matrix were purchased from Pharmacia LKB. All other materials were reagent grade from standard commercial sources.

Mutagenesis of CheB. Overlap-extension PCR site-directed mutagenesis was used to generate all CheB mutants (*16*). The C207S, C309S, and D56C mutations were introduced sequentially using a plasmid that encodes WT *Salmonella enterica* serovar Typhimurium CheB, pME30, as the initial template and native Pfu polymerase (*17*). Each mutation was confirmed by sequencing. All strains and plasmids used have been described previously (*10*).

Protein Purification. WT CheB, mutant CheB, and CheBc proteins were expressed and purified as previously described (*8*, *10*, *18*) with the modification that the Q Sepharose Fast Flow column was 5.5 × 28 cm. Reducing agents were included in all buffers throughout purification and storage. Purity was assessed by SDS−polyacrylamide gels (15%) run on a Mini-PROTEAN II gel electrophoresis system (BioRad) and stained with Coomasie Blue. CheB proteins were precipitated with ammonium sulfate (50% (w/v), and the precipitates were aliquoted and stored at 4 °C with the exception of D56C/C207S/C309S CheB, which was used as purified. CheB stored in ammonium sulfate was resolubilized by overnight dialysis against 50 mM Tris·HCl, 50 mM NaCl, pH 8.0. Concentrations of purified protein were determined by measuring the absorbance at 280 nm [WT CheB and mutants, $\epsilon_{280} = 10\,900\ M^{-1}\ cm^{-1}$, CheBc, $\epsilon_{280} = 9600\ M^{-1}\ cm^{-1}$]. Extinction coefficients were estimated based on the tryptophan and tyrosine contents of the protein (*19*). Coomasie Blue staining of the purified proteins analyzed by SDS−PAGE correlated directly with the UV absorbance quantitation.

Protein Modification. D56C/C207S/C309S CheB in 80 mM potassium phosphate, 1 mM EDTA, 0.5 mM PMSF, 100 mM NaCl, 1 mM DTT, pH 6.0, was incubated in the presence of additional 1 mM DTT at 4 °C for a minimum of 15 min. As CheB was always stored in the presence of reducing agents, this step was included only as a precaution to ensure complete reduction of the cysteine thiol. DTT and NaCl were then removed from the protein using Bio-Gel P6 Spin columns equilibrated in 100 mM Tris·HCl, 1 mM EDTA, pH 8.2. A 90-fold molar excess of 100 mM DTNB prepared immediately prior to use in 100 mM Tris·HCl, 1 mM EDTA, pH 8.2, was added to the pooled protein (200 μM). The reaction was incubated at 0 °C for 30 min. After completion of the reaction, the unreacted DTNB was removed using Bio-Gel P6 spin columns equilibrated with 100 mM Tris·HCl, 1 mM EDTA, pH 8.2. The modified protein, D56C/C207S/C309S CheB−TNB, (120 μM) was pooled. 500 mM Na_3SPO_3 prepared immediately prior to use in 100 mM Tris·HCl, 1 mM EDTA, pH 8.2, was added in a 1000-fold molar excess. The reaction was incubated at 0−4 °C for a total of 13 h. The progress of the reaction was followed by C-8 reverse phase HPLC. Each hour, 50-μL aliquots were removed, and the protein was isolated using Bio-Gel P6 spin columns equilibrated in 100 mM potassium phosphate, 1 mM EDTA, pH 7.0. The protein was used for methylesterase assays, HPLC, and mass spectrometry analyses. Protein concentration was determined by Bradford protein assay.

WT CheB and other mutants of CheB show visible precipitation after two weeks of storage at 4 °C in solution. D56C/C207S/C309S CheB has an accelerated rate of precipitation in comparison to WT CheB and is used directly after purification for that reason. To demonstrate that the activity of the protein observed was attributable to the modification and not to hydrolysis products, analysis of the protein for CheBc content was performed. No significant amount of CheBc was discernible in samples analyzed by HPLC and by Western blotting using anti-CheBc antibodies (data not shown).

Methylesterase Assay. *Escherichia coli* membranes containing overexpressed *S. enterica* 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 (*20*). Esterase activity was assayed by incubating the membrane-bound [methyl-^{3}H] Tar (620 cpm/pmol methyl group) at 30 °C with methylesterase WT CheB (5 μM), WT CheB−PO_4 (0.5 μM), D56C/C207S/C309S CheB (1 μM), D56C/C207S/C309S CheB−TNB (0.1 μM), D56C/C207S/C309S CheB−SPO_3 (0.1 μM), C207S/C309S CheB (5 μM), C207S/C309S CheB−PO_4 (0.5 μM), or CheBc (0.1 μM) in 50 mM potassium phosphate, 25 mM $MgCl_2$, pH 7.0. To assay the activity of CheB upon phosphorylation, WT CheB (0.5 μM) or C207S/C309S CheB (0.5 μM) was preincubated in the presence of 50 mM phosphoramidate and 25 mM $MgCl_2$, as described previously (*10*) unless otherwise specified, in 50 mM potassium phosphate, pH 7.0, for 30 s prior to initiation of the reaction by the addition of [methyl-^{3}H] Tar. At 45-s intervals over a course of 5.5 min, 5-μL aliquots of the reaction were removed and quenched by addition to 30 μL of 5 N acetic acid, applied to Whatman filter paper attached to the lid of a scintillation vial, and sealed suspended over 2.5 mL of scintillation fluid. Care was taken to ensure that the reaction mixture was an even suspension of membranes before each time point was taken. Volatile counts of [^{3}H] methanol released in the reaction were then measured with a Beckman liquid scintillation spectrometer model LS650 after at least a 12-h incubation at 25 °C. Rates were determined from linear regression fits of the data. The

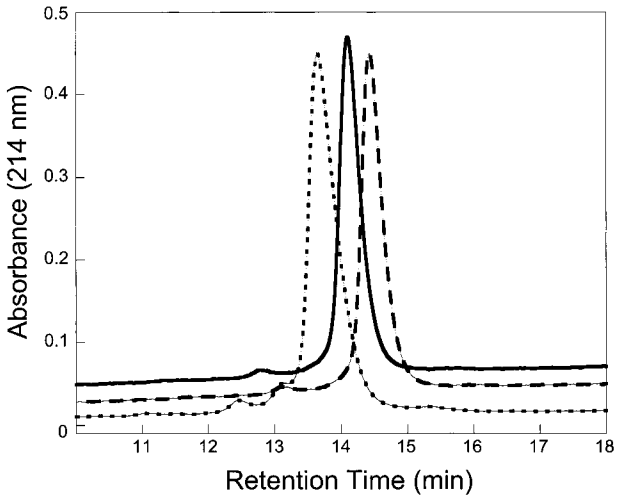

FIGURE 2: HPLC chromatograms of D56C/C207S/C309S CheB (−), D56C/C207S/C309S CheB−TNB (- - -), and D56C/C207S/C309S CheB−SPO_3 (•••). D56C/C207S/C309S CheB−SPO_3 eluted at 13.7 min, D56C/C207S/C309S CheB eluted at 14.2 min, and D56C/C207S/C309S CheB−TNB eluted at 14.6 min. The retention times presented are an average of a minimum of three independent measurements. The overlaid spectra are separated by 0.03 absorbance units for clarity. Approximately 100 μg (2.7 nmol) of protein were analyzed in each run.

specific activity of the methylated Tar was used to determine esterase activity in mmol of [^{3}H] methanol released (mol of methylesterase)$^{-1}$ s^{-1}. The specific activities measured were normalized to the activity of CheBc, which was run as an internal control in all sets of methylesterase assays, to minimize difficulties associated with assaying the range of activities of different forms of CheB and to be able to compare activities obtained with different receptor/membrane preparations. The 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)$^{-1}$ s^{-1}. At all concentrations of CheB used, the initial rates of [^{3}H] methanol released were linear with respect to the concentration of CheB.

HPLC Analysis. The extent of protein modification was quantitated by reverse phase HPLC. The HPLC was performed on a 6000 series system from Hitachi (L6200A Intelligent Pump, L3000 Photodiode Array detector, D6000 Interface, and D7000 HPLC system manager). A Varian C-8 column (4.6 mm × 10 cm) was run with a 0.1% TFA and acetonitrile solvent system (mobile phase A, 0.1% TFA/H_2O; mobile phase B, 0.1% TFA/90% acetonitrile). A gradient of 40−60% mobile phase B in 20 min at a flow rate of 1 mL/min was used for analysis of all samples and ultraviolet detection was performed at both 214 and 280 nm. Typically, D56C/C207S/C309S CheB−SPO_3 eluted at 13.7 min, D56C/C207S/C309S CheB eluted at 14.2 min, and D56C/C207S/C309S CheB−TNB eluted at 14.6 min (Figure 2). The percentage of each component was calculated by comparing the area under the peak of interest to the total area under all peaks.

For the analysis of the stability of D56C/C207S/C309S CheB−SPO_3, the elution patterns of the analogue were collected over 21 days. After removal of any precipitation by centrifugation, 20-μL aliquots (initial concentration 3.2 mg/mL) of the soluble portion of the analogue were injected. Precipitation was observed at 8 days, and approximately 80% of the initial D56C/C207S/C309S CheB−SPO_3 was lost due to precipitation by 21 days.

The extent of WT CheB and C207S/C309S CheB phosphorylation under methylesterase assay conditions was measured by reverse phase HPLC. This HPLC was performed on a 7000 series system from Hitachi (L7100 Intelligent Pump, L7455 Photodiode Array detector, D7000 Interface, and D7250-P autosampler). A Rainin Dynamax C-18 column (4.6 mm × 10 cm) was run with a 0.1% TFA and acetonitrile solvent system (mobile phase A, 0.1% TFA/H_2O; mobile phase B, 0.1% TFA/100% acetonitrile). A gradient of 40−60% mobile phase B in 20 min at a flow rate of 1.0 mL/min was used for analysis of all samples, and ultraviolet detection was performed at 220 nm. To analyze unphosphorylated WT and C207S/C309S CheB, a 100-μL sample containing 15 μg of CheB in 50 mM potassium phosphate, 25 mM $MgCl_2$, pH 7.0, was injected onto the column. To analyze phosphorylated WT and C207S/C309S CheB, an identical sample was incubated in the presence of 50 mM phosphoramidate for 100−114 s prior to injection onto the column. Typically, WT CheB eluted at 12.4 min, WT CheB−PO_4 eluted at 12.1 min, C207S/C309S CheB eluted at 12.2 min, and C207S/C309S CheB−PO_4 eluted at 11.8 min. The percentage of each component was calculated by comparing the area under the peak of interest to the total area under all peaks.

Mass Spectrometry. LC/ESI-MS spectra were obtained on a MAT 90 double focusing mass spectrometer. Mass spectrometric analysis was performed 24−72 h after completion of the modification reaction. Protein in 100 mM potassium phosphate, 1 mM EDTA, pH 7.0, was injected onto the reverse phase column component of the LC/ESI-MS and subsequently eluted with acetonitrile into the mass spectrometer's ionization chamber.

RESULTS

Chemical Modification of CheB. D56C/C207S/C309S CheB was modified by Na_3SPO_3 in a two-step reaction (Figure 1B). In step 1, DTT-treated D56C/C207S/C309S CheB was modified by Ellman's reagent, DTNB. Greater than 90% of D56C/C207S/C309S CheB incorporated TNB as assessed by reverse phase HPLC (Figure 2). Excess DTNB and unincorporated TNB were then removed using a BioRad Bio-gel P-6 spin column. In step 2, sodium thiophosphate, Na_3SPO_3, was introduced to D56C/C207S/C309S CheB−TNB (Figure 1B). D56C/C207S/C309S CheB−TNB modification by thiophosphate was greater than 95% complete after 4 h as measured by reverse phase chromatography. The loss of D56C/C207S/C309S CheB−TNB was inversely proportional to the formation of the D56C/C207S/C309S CheB−SPO_3 (Figure 3).

Single Thiophosphate Incorporation. LC/ESI-MS was used to identify the increase in mass of D56C/C207S/C309S CheB upon chemical modification. Mass spectrometric analysis of the reaction mixture identified masses of D56C/C207S/C309S CheB, D56C/C207S/C309S CheB−TNB, and D56C/C207S/C309S CheB−SPO_3 (Table 1). There were no higher molecular masses observed in the mixture correlating to multiple modifications. The mass of the D56C/C207S/C309S CheB mutant was 40 amu less than the mass of the WT CheB. This reflects the net loss of a sulfhydryl group

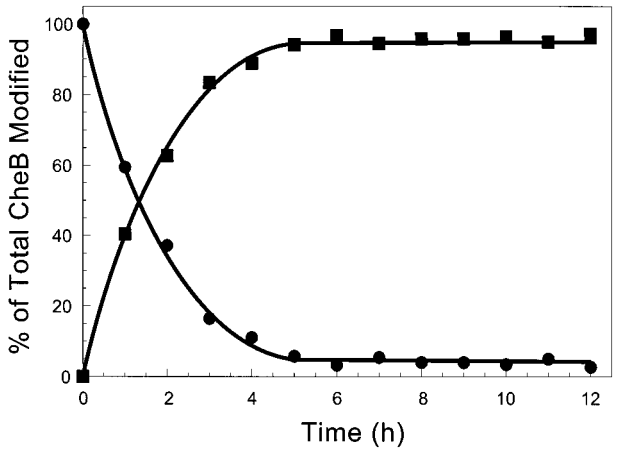

FIGURE 3: Time course of D56C/C207S/C309S CheB−SPO_3 synthesis. The formation of D56C/C207S/C309S CheB−SPO_3 (■) and the consequential loss of D56C/C207S/C309S CheB−TNB (●) was monitored by reverse phase HPLC ($\lambda = 214$ nm). The percent of CheB modified was determined as described in Materials and Methods.

Table 1: Liquid Chromatography/Electrospray Ionization-Mass Spectrometry (LC/ESI-MS) of WT CheB, D56C/C207S/C309S CheB, D56C/C207S/C309S CheB−TNB, and D56C/C207S/C309S CheB−SPO_3

protein	mass[a] (amu)	Δ[b] (amu)	mass fragment assignment
wild-type CheB	37421	40	$M^+{}_{WT}$[c]
D56C/C207S/C309S CheB	37381		M^+
D56C/C207S/C309S CheB + TNB	37381		M^+
	37581	200	$M^+ + TNB$
D56C/C207S/C309S CheB−TNB + SPO_3	37382	1	$M^+ + 1H$
	37580	198	$M^+ + TNB - 2H$
	37493	112	$M^+ + SPO_3 + 1H$

[a] The determined masses are in atomic mass units (amu). [b] Fragment assignments were made based on the mass differences (Δ) between the parent protein, D56C/C207S/C309S CheB, (M^+) and the observed masses of the other protein species. The theoretical mass of a TNB group is 200 amu and the theoretical mass of an SPO_3 group is 111 amu. [c] Mass fragment assignment for $M^+{}_{WT}$ indicates the mass of D56C/C207S/C309S CheB plus the mass of a cysteine side chain, plus the mass of an aspartate side chain, minus the mass of two serine side chains, and minus the mass of four protons.

(cysteine) and of a carboxyl group (aspartate) and the gain of two hydroxyl groups (serine). 200 amu is the calculated mass of a TNB group. 111 amu is the calculated mass of an SPO_3 group.

Stability. Hydrolysis of D56C/C207S/C309S CheB−SPO_3 stored at 4 °C was monitored by reverse phase HPLC over a period of 21 days. From these data, a $t_{1/2}$ of 28 days was calculated for soluble-modified protein (Figure 4). However, it should be noted that precipitation of the protein was visible after 8 days, and by 21 days only 20% of the initial protein remained in solution. Some precipitation of WT CheB was also seen over this time. No attempts have yet been made to determine whether solution conditions can be found that improve the solubility and/or stability of the modified CheB protein.

Biochemical Properties of D56C/C207S/C309S CheB−SPO_3. Reverse phase HPLC and methylesterase assays were used to follow the reaction of D56C/C207S/C309S CheB-TNB with Na_3SPO_3 over time. The reaction was terminated

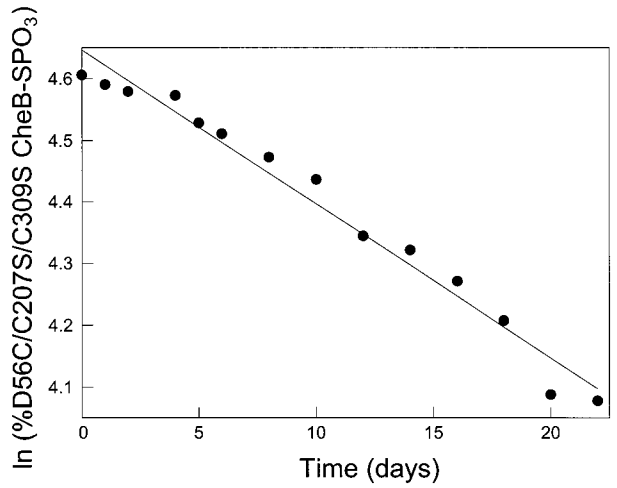

FIGURE 4: Half-life of the D56C/C207S/C309S CheB−SPO_3. The stability of the analogue was determined by measuring the percent D56C/C207S/C309S CheB−SPO_3 by reverse phase HPLC ($\lambda = 214$ nm). The amount of D56C/C207S/C309S CheB−SPO_3 (%) was determined by measuring the area under the peak of interest with respect to the total area under all peaks. The half-life of D56C/C207S/C309S CheB−SPO_3 was 28 days. Significant loss of the initial D56C/C207S/C309S CheB−SPO_3 to precipitation was noted.

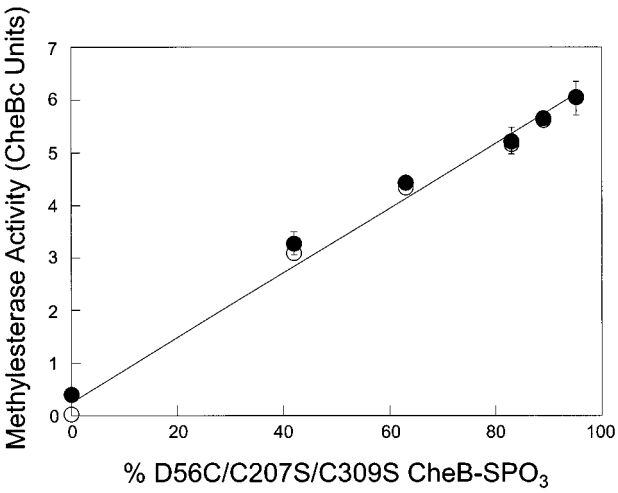

FIGURE 5: Methylesterase activity of D56C/C207S/C309S CheB−SPO_3 monitored as a function of protein modification. Total methylesterase activity (●) was determined and methylesterase activity of D56C/C207S/C309S CheB−SPO_3 (○) was calculated by subtracting the contribution of activity of D56C/C207S/C309S CheB−TNB from the total activity. Activities are plotted vs the extent of thiophosphate modification of D56C/C207S/C309S CheB. Methylesterase activity and percent modification were determined as described in Materials and Methods.

at hourly intervals by removing excess reagents with Biogel P-6 (BioRad) spin columns. The extent of modification was quantitated by HPLC. An in vitro methylesterase assay was used to measure the activity (*8*). A direct correlation was observed between methylesterase activity and the extent of thiophosphate modification (Figure 5). Addition of Na_3SPO_3 to D56C/C207S/C309S CheB directly did not result in incorporation of thiophosphate, and no nonspecific effects on the methylesterase reaction were observed. No significant amount of CheBc was discernible in samples upon analysis by Western blot using antibodies to CheBc and by HPLC. Therefore, increase in methylesterase activity can be attributed to thiophosphate modification.

Table 2: Methylesterase Activity of Wild-Type, Mutant, and Modified CheB Proteins in the Presence and Absence of Mg^{2+}

protein	methylesterase activity[a] $-Mg^{2+}$	$+Mg^{2+}$
wild-type CheB	0.2 ± 0.1	0.4 ± 0.1
wild-type CheB + phosphoramidate/Mg^{2+} [b]		7.0 ± 0.6
wild-type CheBc	1.0	0.8
D56C/C207S/C309S CheB	0.2 ± 0.0	0.4 ± 0.1
D56C/C207S/C309S CheB−TNB	1.1 ± 0.4	0.4 ± 0.0
D56C/C207S/C309S CheB−SPO_3	4.0 ± 0.3	6.0 ± 0.1
C207S/C309S CheB + phosphoramidate/Mg^{2+} [b]		4.3 ± 0.9

[a] Methylesterase activity of wild-type and mutant CheB proteins were determined by incubating membranes containing [methyl-^{3}H]Tar with methylesterase in a reaction buffer of 50 mM potassium phosphate, pH 7.0, with or without 25 mM Mg^{2+} as described in Materials and Methods. Methylesterase activities are expressed relative to that of CheBc. The 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)$^{-1}$ s^{-1}. Values are represented as the mean ± SD of at least two independent experiments. [b] 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 before the addition of substrate. Under these steady-state conditions, approximately 70% of CheB is phosphorylated (see Results).

In the unphosphorylated state, WT CheB (0.4 CheBc units) and D56C/C207S/C309S CheB (0.4 CheBc units) show the same methylesterase activities (Table 2). Under conditions for phosphorylation, both WT CheB (7 CheBc units) and C207S/C309S CheB (4.3 CheBc units) showed significantly greater activity than the unphosphorylated proteins. The activity for the D56C/C207S/C309S CheB−SPO_3 with Mg^{2+} (6.0 CheBc units) was found to be 1.4-fold greater than that for C207S/C309S CheB under phosphorylating conditions. The levels of phosphorylation of CheB and C207S/C309S CheB under similar conditions were determined to be 70.7 ± 1.7% and 73.8 ± 1.5%, respectively (data not shown). TNB modification of D56C/C207S/C309S CheB (1.1 CheBc units) resulted in partial activation, and addition of Mg^{2+} decreased the activity of D56C/C207S/C309S CheB−TNB (0.4 CheBc units).

DISCUSSION

D56C/C207S/C309S CheB−SPO_3 is a Good Biochemical Analogue of Phosphorylated CheB. Comparison of the activities of different mutant and activated forms of CheB requires several considerations. Because of the short lifetime of phosphorylated CheB, it is not possible to achieve a steady-state level of 100% phosphorylation under conditions of the methylesterase assay. Previous analyses have indicated a level of approximately 65% phosphorylation under such conditions (*21*). Further complexity can potentially arise in considering CheB mutant proteins, since it has been shown for some response regulators that mutations in the effector domain can affect the level of phosphorylation of the regulatory domain by perturbing either phosphotransfer or dephosphorylation (*22*). However, this is not the case for the cysteine-substituted CheB proteins used in this study. Under steady-state conditions of the methylesterase assay, both WT CheB and C207S/C309S CheB were shown by HPLC analyses to have similar levels of phosphorylation. Thus, with methylesterase activity 1.4-fold greater for the analogue (D56C/C207S/C309S CheB−SPO_3) than for the cysteine-substituted CheB in the presence of phosphoramidate and Mg^{2+}, the analogue has almost exactly the methylesterase activity expected for 100% phosphorylated C207S/C309S CheB.

The activity of thiophosphorylated CheB (D56C/C207S/C309S CheB−SPO_3) is slightly less than that expected for fully phosphorylated WT CheB. This appears to be attributable to the cysteine substitutions in the catalytic domain. Substitution of these cysteine residues with alanine has previously been shown to decrease methylesterase activity of CheB (*7*). In this study, the activity of C207S/C309S CheB in the presence of phosphoramidate and Mg^{2+} was 1.6-fold less than that of WT CheB assayed under the same conditions, although both proteins have been shown to be phosphorylated to the same extent. Thus, it appears that the cysteine substitutions in the C-terminal domain of CheB mildly affect the activation of methylesterase activity by the phosphorylated regulatory domain. Notably, the 1.6:1 ratio of activities of phosphorylated WT CheB:phosphorylated C207S/C309S CheB are in close agreement with the 1.6:1 ratio of activities of 100% phosphorylated WT CheB: thiophosphorylated CheB (D56C/C207S/C309S CheB−SPO_3). This suggests that the minor differences in the activities of the phosphorylated and thiophosphorylated proteins result primarily from the cysteine substitutions within the catalytic domain of the latter. Thus, D56C/C207S/C309S CheB−SPO_3 appears to be a good mimic of phosphorylated CheB. Furthermore, the 28-day half-life of the analogue represents a significant stabilization of the activated CheB relative to the 2-s half-life of the phosphoaspartate-containing CheB protein.

An unexpected observation was the partial activation upon TNB modification of D56C/C207S/C309S CheB to a level seen in CheBc. The large aromatic moiety presumably nonspecifically disrupts the structure of the N-terminal domain enough to result in similar activity to that observed for the isolated C-terminal methylesterase domain.

The effect of Mg^{2+}, which is required for the phosphorylation of WT CheB (*10*, *23*), on the methylesterase activity of D56C/C207S/C309S CheB−SPO_3 was investigated (Table 2). The presence of Mg^{2+} increased the activity of the thiophosphate analogue. However, the same concentration of Mg^{2+} decreased the activity of D56C/C207S/C309S CheB−TNB. This difference in the effect of Mg^{2+} on the activity of D56C/C207S/C309S CheB−SPO_3 and D56C/C207S/C309S CheB−TNB suggests a different mode of activation and is consistent with the assumption of specific versus nonspecific activation for the SPO_3 and TNB moieties, respectively.

Comparison to Other Chemical Analogues of Phosphorylated Response Regulator Proteins. Three chemical analogues of phosphorylated CheY have been reported. The first analogue was synthesized by modification of D57C CheY with phosphorthioate (*12*) analogous to the modification of CheB in this study. No enhanced activity was demonstrated for thiophosphorylated CheY upon biochemical characterization. The second phosphorylated CheY analogue was synthesized by modification of D57C CheY with trifluoromethylsulfonyloxymethylphosphonate (TfOMP). In contrast to the modification of D57C CheY with phosphorthioate, methylphosphonate-CheY was determined to be activated

(*13*). However, TfOMP modification of CheB under the same conditions used for CheY was not successful. TfOMP with or without Ca^{2+}, Ba^{2+}, Mg^{2+}, and Mn^{2+} resulted in the complete precipitation of D56C/C207S/C309S CheB as determined by the loss of all methylesterase activity from the soluble portion of the reaction (R. Saxl, unpublished results). Third, beryllium fluoride (BeF_3^-) has been shown to mimic the phosphorylated state of several response regulator proteins including NtrC, CheY, OmpR, and Spo0F (*24*). However, under a range of similar modification conditions, stimulation of CheB methylesterase activity was not observed (G. Anand, unpublished results).

Response regulator proteins have been postulated to exist in a dynamic equilibrium between their active and inactive conformations with phosphorylation shifting the equilibrium toward the active conformation (*25*, *26*). Mutations in the unphosphorylated regulatory domain that activate the response regulator by presumably shifting the dynamic equilibrium to favor the phosphorylated state have been identified for most response regulators. It has been observed that an activating mutation in one response regulator does not necessarily result in activation for an analogous mutation for another response regulator. For instance, mutation of the site of phosphorylation in NtrC (D54E) is found to be partially active (*27*), but the analogous mutation (D56E) does not activate CheB (R. Saxl, unpublished results). Together, the chemical activation and mutagenesis data suggest that different response regulator proteins have different sensitivities to "activating" moieties; that the ability of these extrinsic factors to shift the equilibrium toward the active conformation is specific to the protein.

Suitability for Structural Studies. Structures of regulatory domains of activated response regulators CheY (*28*, *29*), NtrC (*30*), Spo0A (*31*), and FixJ (*32*) have recently been determined. A clear picture of the conformational changes that occur upon phosphorylation at the active site aspartate residue has emerged from these studies. However, since the majority of response regulator proteins have two or more domains (*6*), a significant question regarding the phosphorylation-dependent activation of response regulators remains. A complete understanding requires a description of how conformational changes within the regulatory domain are communicated to the effector domain, resulting in its activation. While the mechanism of interdomain regulation in response regulators is likely to vary in different proteins, the thiophosphorylated CheB analogue of phosphorylated CheB provides potential for structural characterization of one example of an activated multidomain response regulator protein.

CONCLUSIONS

Two aspects of phosphorylation-dependent activation of CheB are relief of steric inhibition mediated by the unphosphorylated regulatory domain and stimulation of methylesterase catalysis by the phosphorylated regulatory domain. Relief of inhibition can be achieved by removal of the regulatory domain of CheB (CheBc) which results in ~10-fold activation of methylesterase activity (*8*). However, an additional ~10-fold activation occurs in phosphorylated intact CheB, presumably resulting from specific interactions between the phosphorylated regulatory domain and the catalytic domain (*10*, *21*).

We have observed both nonspecific and specific activation with our chemical modification of CheB. With an activity similar to that for CheBc, D56C/C207S/C309S CheB−TNB represents a partially active form of CheB that presumably reflects the nonspecific relief of inhibition alone. D56C/C207S/C309S CheB−SPO_3, with an activity analogous to that of phosphorylated C207S/C309S CheB, has an activity that represents an enhancement of methylesterase activity over that seen for CheBc. Additionally, Mg^{2+} was found to reduce the level of methylesterase activity observed for D56C/C207S/C309S CheB-TNB as seen for CheBc, while enhancing that observed for D56C/C207S/C309S CheB−SPO_3, further contrasting the nonspecific and specific activation observed with the two chemical modifications of CheB. Thus, thiophosphate modification of CheB appears to recapitulate the activation associated with phosphorylation of CheB.

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