Synthesis and Biochemical Characterization of an Analogue of CheY-Phosphate, a Signal Transduction Protein in Bacterial Chemotaxis†

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ABSTRACT: CheY is a signal transduction protein of the bacterial chemotaxis system that acts as a molecular switch to alter the swimming behavior of the bacterium. When CheY becomes phosphorylated at Asp57, CheY-P_i interacts with flagellar motor proteins, including FliM, to increase the likelihood that the flagellar motor will change its sense of rotation, increasing the frequency of tumbling. The structure of CheY in its dephosphorylated (inactive) state has been intensively investigated. The short lifetime (∼20 s) of the aspartyl phosphate has precluded the complete structural determination of CheY-P_i. We have synthesized an analogue of CheY-P_i by alkylating an aspartate-to-cysteine mutant at position 57 of CheY to add a phosphonomethyl group at Cys57. This analogue, phosphono-CheY, is stable for months. Phosphono-CheY binds to two of the targets of CheY-P_i, FliM and CheZ, in a manner similar to that of CheY-P_i and much better than either unphosphorylated CheY or the unmodified form of D57C CheY. Phosphono-CheY also binds Mg(II) with a dissociation constant of ∼6 mM at neutral pH and moderate salt level. These observations indicate that phosphono-CheY is a good biochemical analogue of CheY-P_i.

Two-component regulatory systems are ubiquitous in bacteria and are present in higher organisms (*1*). These systems employ a histidine autokinase to phosphorylate the cognate response regulator protein. Bacterial chemotaxis is one of the most studied of the physiological responses that use two-component systems. During bacterial chemotaxis, the phosphorylated form of the response regulator CheY plays a central role. The phosphorylated form of CheY binds to the flagellar motor to increase its frequency of cw[1] rotation (*2*) which, in turn, allows the bacterium to modulate the length of smooth swimming periods in response to concentration gradients of chemoattractants and -repellents. CheY-P_i interacts with the flagellar proteins FliM and FliG (*3*, *4*). CheY itself catalyzes the hydrolysis of the aspartyl phosphate; in addition, its hydrolysis is accelerated by CheZ (*5*−*8*).

Although its lability makes determining the structure of CheY-P_i extremely difficult, the ^1H and ^{15}N chemical shifts of the amide groups of CheY-P_i have been assigned (*9*). The changes in chemical shift upon phosphorylation suggest that phosphorylation brings about a conformational change involving residues distant from the phosphorylation site.

Here we describe the synthesis and characterization of an analogue of CheY-P_i made with a combination of site-directed mutagenesis and chemical modification leading to

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[1] Abbreviations: cw, clockwise; ccw, counterclockwise; MCPs, methyl-accepting chemotaxis proteins; CheA, product of the chemotaxis A gene; CheY, product of the chemotaxis Y gene; CheY-P_i, phosphorylated form of CheY; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; AMPPCP, 5′-adenylylmethylene diphosphonate; ATP, adenosine 5′-triphosphate; A_{280}, absorbance at 280 nm; UV, ultraviolet; THF, tetrahydrofuran; LB, Luria broth.

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a stable protein with at least some of the biochemical properties of the normally phosphorylated protein. This analogue behaves like CheY-P_i in its binding interactions with CheZ and FliM, but is much more chemically stable. Thus, the analogue, which we call phosphono-CheY, has much of the biochemical activity of CheY-P_i and is much more amenable to long-term studies of its structure. The chemical modification may also prove useful for structural studies of other response regulators of the numerous two-component systems, especially those in which the aspartyl phosphate is considerably more labile than in CheY.

MATERIALS AND METHODS

Materials

AMPSO was from Research Organics, Inc. Di-*tert*-butyl phosphite was from Alfa Aesar or was synthesized from PCl_3 and *tert*-butyl alcohol as previously described (*10*). Triethylamine was stirred in the presence of CaH_2 and distilled. Immobilized monomeric avidin and iodoacetyl-LC-biotin were obtained from Pierce Chemical. All other reagents were reagent-grade and were used as supplied.

Methods

Synthesis of Trifluoromethanesulfonyl Hydroxymethylenephosphonic Acid. This compound was synthesized using a modification of a previous synthesis (*11*, *12*). Di-*tert*-butyl phosphite (25 g, 129 mmol) was added to 25 mL of THF and cooled to about −55 °C with a dry ice/2-propanol bath. A 1 M solution of lithium bistrimethylsilylamide (141 mL) was added dropwise while the mixture was being stirred. The mixture was then stirred at 0 °C, and chlorotrimethylsilane (15.4 g, 141 mmol) was added dropwise while the stirring was continued. Dry paraformaldehyde (7.7 g, 260 mmol) was added and the mixture warmed to 45−50 °C and stirred for about 2 h. The mixture was filtered through Celite, and the volatiles were removed with rotary evaporation. A small amount of toluene was added, and the solution was again evaporated. A solution of CsF (19.6 g, 129 mmol) in methanol was added while the stirring was continued and the methanol removed with rotary evaporation. The product was dissolved in 50 mL of $CHCl_3$, and the salts were extracted with 50 mL of $NaHCO_3$. The chloroform layer was dried with $MgSO_4$ and filtered, and the chloroform was removed using rotary evaporation followed by stripping with toluene. Di-*tert*-butyl hydroxymethylenephosphonate was recrystallized from hexanes, and a sample (0.77 g, 3.4 mmol) was dissolved in 5 mL of dry CH_2Cl_2. Triethylamine (0.49 g, 4.8 mmol) was added, and the solution was stirred at −60 °C. Triflic anhydride (1.16 g, 4.12 mmol) in 5 mL of CH_2Cl_2 was added dropwise. Then the mixture was stirred at −30 °C for about 50 min and then cooled to −60 °C. The solution was poured onto 20 mL of cold, saturated $NaHCO_3$, and 10 mL of CH_2Cl_2 was added. The dichloromethane layer was extracted again with bicarbonate and was dried with $MgSO_4$. The mixture was filtered, and the dichloromethane was removed with rotary evaporation. The intermediate was placed on a vacuum pump for several hours to remove the *tert*-butyl groups. Free trifluoromethanesulfonyl hydroxymethylenephosphonic acid hydrolyzes at pH 7 with a half-life of roughly 5 min, as observed by ^{31}P NMR spectroscopy (δ_P = 6.6 ppm). It appears to be stable when stored dry and in the cold.

Protein Purification. All purification steps were performed near 5 °C, and the pH values of buffers were adjusted at room temperature. The *cheY* gene in plasmid pRL22 was subjected to site-directed mutagenesis (*13*) to produce an aspartate-to-cysteine mutation of CheY and confirmed by DNA sequencing. *Escherichia coli* strain 594 was transformed by standard methods. Growth of this transformed strain in minimal medium with glucose and $[^{15}N]NH_4Cl$ was performed essentially as described (*14*). D57C CheY was found primarily in inclusion bodies, as has sometimes been seen with other mutants of CheY (*15*, *16*). The inclusion bodies were washed, and the protein was solubilized and refolded using a modification of a protocol developed for p21 *ras* (*17*). Specifically, cells were suspended in 50 mM Tris (pH 7.5), 1 mM EDTA, and 0.2 mM PMSF and disrupted three times using a French press at ∼1000 psi. The cell debris was spun at 16 000 rpm in a JA-20 rotor for 30 min. The pellet was resuspended in 30% sucrose and 10 mM EDTA (pH 7.5) with a Dounce tissue homogenizer. The mixture was spun at 15 500 rpm for 45 min, and the pellet was homogenized in 1% Triton X-100, 50 mM Tris (pH 7.5), and 5 mM EDTA. The mixture was spun at 16 000 rpm in a JA-20 rotor for 50 min, and the pellet was gently resuspended in deionized (*17*) 6 M urea buffered with 50 mM Tris (pH 7.5), 1 mM EDTA, and 10 mM DTT. After incubation for at least 90 min, the mixture was spun at 17 000 rpm for 50 min. The supernatant was saved, and the pellet was again resuspended with buffered urea and spun again. All of the supernatants were examined using SDS−PAGE. Typically, the first two urea supernatants were combined and diluted with buffered 6 M urea to a final protein concentration of approximately 0.5 mg/mL.

To effect refolding, the protein was dialyzed twice against 20 mM Tris (pH 7.5), 10 mM β-mercaptoethanol, and 0.5 mM EDTA and twice against 5 mM Tris (pH 7.5), 1.4 mM β-mercaptoethanol, and 0.2 mM EDTA. A 2.4 cm × 10.5 cm column of DE-52 was prepared in the chloride form and equilibrated with 5 mM Tris (pH 7.5), 1.4 mM β-mercaptoethanol, and 0.2 mM EDTA. The buffered protein solution was applied, and then the column was rinsed at 1 mL/min with starting buffer until the A_{280} of the effluent was close to that of the starting buffer. Next, a 1 L gradient of 0 to 400 mM NaCl in the same buffer was started, and fractions were collected and pooled on the basis of A_{280} and homogeneity, as assessed by SDS−PAGE.

Soluble D57C CheY was also obtained from cells grown at 30 °C in LB to reduce formation of inclusion bodies and purified as previously described (*18*).

DTNB Assay. Protein thiol content was assayed by reaction with 1 mM DTNB in 100 mM potassium phosphate (pH 7.28) and 1 mM EDTA (*19*). The reaction was monitored by reading the absorbance of the DTNB solution at 410 nm before and after the addition of the protein. The reaction was found to be essentially complete within 15 min. Any thiol which reacted during the mixing time was assumed to result from residual DTT and was ignored.

HPLC Analysis. The extent of protein modification was quantitated by reversed-phase HPLC on a 2.1 mm × 250 mm Vydac C-18 column (The Separations Group). Mobile phase A was 0.1% TFA; mobile phase B was 70% aceto-

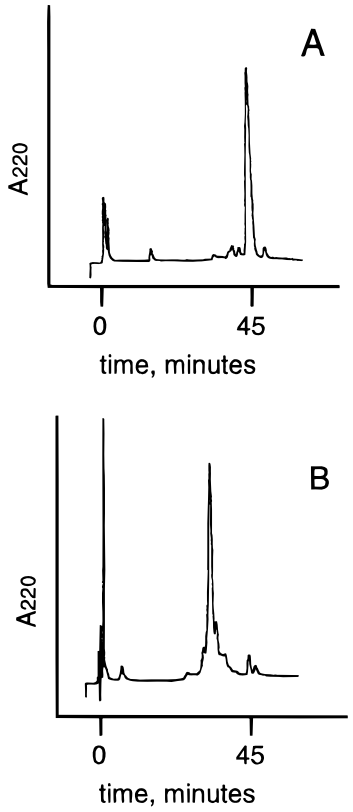


FIGURE 1: HPLC chromatogram of phosphono-CheY after purification. The sample was eluted from a Vydac C-18 column (2.1 mm × 250 mm) at 0.2 mL/min. The gradient was 45.5 to 52.5% acetonitrile in 0.1% TFA over 1 h. Peaks were detected by absorbance at 220 nm: (A) chromatogram for unmodified D57C CheY, with a retention time of 44.4 min; and (B) chromatogram for phosphono-CheY, with a retention time of 34.9 min.

nitrile in ~0.08% TFA, and the effluent was monitored at 220 nm. The gradient was 65 to 75% mobile phase B over 1 h, and the flow rate was 0.2 mL/min. Typically, alkylated D57C CheY eluted ~10 min ahead of the unmodified protein, as shown in Figure 1.

Alkylation. A 500 μL aliquot of D57C CheY (950 μM) was treated with DTT for at least 10 min. It was passed through two Penefsky columns (*20*) which had been equilibrated with 250 mM AMPSO·KOH (pH 9.05) and 1 mM EDTA. The protein concentration was determined by UV spectrophotometry using an ϵ_{280} of 8250 (*14*), and the free thiol content was determined by DTNB assay. $CaCl_2$ (2 M stock) was added to a final concentration of 100 mM. Trifluoromethanesulfonyl hyroxymethylenephosphonic acid (12 mg) was quickly dissolved in 20 μL of triethylamine and 20 μL of absolute ethanol. The solution was immediately added to the protein and the mixture incubated at room temperature for 60−120 min. The precipitate was removed by centrifugation. The supernatant was treated with 10 mM DTT for approximately 15 min, and the protein was passed over a PD-10 column (Pharmacia) or two Penefsky columns which had been equilibrated with 50 mM AMPSO (pH 9.05) and 0.2 mM EDTA. The alkylated protein was assayed for residual free thiol content either before the reduction by DTT or after the reduction and change of buffer. A sample of ~5 μg was taken for HPLC analysis.

To remove residual unalkylated D57C CheY, a fresh solution of 26 mM iodoacetyl-LC-biotin (Pierce Chemical Co.) in dimethyl sulfoxide was prepared with slight warming to effect complete dissolution. This was added to the protein to give a final concentration of iodoacetyl-LC-biotin of 800 μM and the mixture incubated for 3−12 h in the dark. Some reaction mixtures were gently swirled. The protein was passed over a PD-10 column equilibrated with phosphate-buffered saline containing 0.2 mM EDTA and 0.02% sodium azide. The protein was collected and applied to a 5 mL portion of immobilized monomeric avidin which had been prewashed according to the manufacturer's directions. The first ~20 mL of column effluent was collected and concentrated, and a 5 μg sample was taken for HPLC analysis. Typically, the protein was stored in 50 mM MOPS·KOH (pH 6.9), 0.02% sodium azide, 0.1−1 mM EDTA, and 5 mM DTT. The avidin column was washed with the same buffer containing biotin to elute biotinylated protein, washed with 100 mM glycine (pH 2.8), and finally rinsed with phosphate-buffered saline, 0.2 mM EDTA, and 0.02% azide. The purity of the final product was assessed by HPLC. No changes in the chromatogram were noticed when the protein was sampled over the course of several weeks, suggesting that phosphonate modification is not very labile.

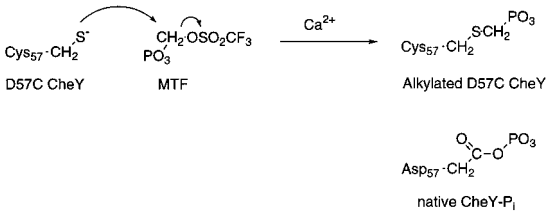
Partial purification could also be achieved by reacting the crude protein mixture with DTNB, and then exchanging the protein into 20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.02% sodium azide (elution buffer). The protein mixture was applied to an extensively washed column of immobilized glutathione (Pharmacia), and the mixture was gently rocked for about 15 h. Alternatively, the column was prepared as the mixed disulfide with 2,2′-dipyridyl disulfide and extensively washed with elution buffer, and the reduced protein in elution buffer was applied to the glutathione column and rocked for about 15 h. In either procedure, the unbound protein was eluted and concentrated.

NMR. ^{31}P NMR spectra were obtained at 121.4 MHz on a Varian INOVA spectrometer. The phosphono-CheY was exchanged into 50 mM MOPS buffer (pH 6.9). The spectral width was 10 000 Hz, the acquisition time 1.6 s, and the relaxation delay 0.1 s. Approximately 18 000 transients were recorded, and protons were decoupled during the acquisition time. Spectra were referenced to 85% H_3PO_4.

Binding of FliM and CheZ. Acetyl [^{32}P]phosphate was synthesized and CheY was phosphorylated, as previously published (*7*, *21*). Identical amounts of wild-type or D57C CheY were mixed with 20 mM acetyl [^{32}P]phosphate in 50 mM Tris·HCl (pH 7.9), 5 mM $MgCl_2$, and 2 mM DTT in a total volume of 20 μL. The reaction mixtures were allowed to stand at room temperature (22 °C) for various times (1−10 min) before the reactions were quenched with an equal volume of 2× SDS−PAGE loading buffer, consisting of 100 mM Tris·HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromophenol, and 20% glycerol. Aliquots of 10 μL each were analyzed by 15% SDS−PAGE and followed by autoradiography. CheY-P_i was stable for approximately 30 min under these conditions. The level of phosphorylation of D57C CheY was less than 5% of that of cellular CheY, in agreement with the lack of a detectable quenching of fluorescence observed with cellular CheY in the presence of phosphoramidate and magnesium.

The assay for binding of CheY to CheZ was carried out as previously described (*22*) with some modifications. CheY beads were suspended to homogeneity in 50 mM Tris·HCl

Scheme 1



(pH 7.9) and dispensed in aliquots of 50 μL in microfuge tubes (about 3 nmol of CheY on the beads). One hundred microliters of the reaction buffer (50 mM Tris·HCl and 5 mM $MgCl_2$) and 35 μL of the stabilizer solution (3.4 M glycerol and 12.5 mM $MgCl_2$) were added. Nonradioactive acetyl phosphate was added from a 1 M stock to a final concentration of 20 mM when needed, and the phosphorylation reactions were allowed to proceed for 2 min at room temperature. Then 3 nmol of purified CheZ was added to each tube as indicated. The final volume of each binding reaction mixture was adjusted to 200 μL with 50 mM Tris·HCl. The reaction mixtures were incubated at room temperature for 10 min. The beads were washed twice with 1 mL aliquots of 50 mM cold Tris·HCl with 5 mM $MgCl_2$ (acetyl phosphate was added to the appropriate wash buffer). A 60 μL aliquot of 2× SDS−PAGE loading buffer was added and the solution mixed at room temperature for 5 min. The beads were boiled for 2 min to remove the bound CheZ from CheY beads. Aliquots of 10 μL each were loaded onto a 15% polyacrylamide gel and subjected to SDS−PAGE. Gels were stained with Coomassie brilliant blue. CheZ bands were scanned by SigmaGel Gel Analysis Software. BSA beads served as controls. The assay for binding of CheY to FliM was the same as that described above, except that FliM was substituted for CheZ.

Fluorescence. Spectra were acquired on a Hitachi F-4500 fluorescence spectrophotometer. The change in fluorescence emission from Trp58 (*23*) was used to monitor the binding of Mg^{2+} to phosphono-CheY. The excitation wavelength was 295 nm with a slit width of 2.5 nm. The emission wavelength was 340 nm with a slit width of 10 nm. $MgCl_2$ (0−30 mM) was added to phosphono-CheY (∼5 μM) in 50 mM MOPS (pH 7.0) and 0.02% sodium azide. To obtain the equilibrium constant, the intensities at various concentrations of divalent metal were fitted to

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K[\mathrm{Mg}]} + \frac{1}{f_a} \qquad (1)$$

where f_a represents the fractional decrease in fluorescence at saturating magnesium concentrations (*24*) and K is the association constant for magnesium ion binding. Binding of Mg^{2+} to unalkylated D57C CheY could not be detected.

RESULTS

Our search for a stable analogue of CheY phosphorylated at D57 led us to attempt to phosphonomethylate Cys57 of D57C CheY (Scheme 1). The alkylation of Cys57 did not proceed with iodomethylenephosphonic acid (*25*), probably owing to the lack of reactivity of the iodide (C. Halkides, unpublished experiments). However, we noted that the diethyl ester of hydroxymethylenephosphonic acid bearing an α-trifluoromethanesulfonyl group is far more reactive toward nucleophilic substitution than the corresponding iodomethylphosphonate diester (*11*). Therefore, we synthesized trifluoromethanesulfonyl hydroxymethylenephosphonic acid (*12*). Trifluoromethanesulfonyl hydroxymethylenephosphonic acid reacts with β-mercaptoethanol incompletely (in competition with hydrolysis), to give the phosphonomethyl derivative of β-mercaptoethanol, as shown by DTNB assay and ^{1}H, ^{13}C, and ^{31}P NMR (data not shown).

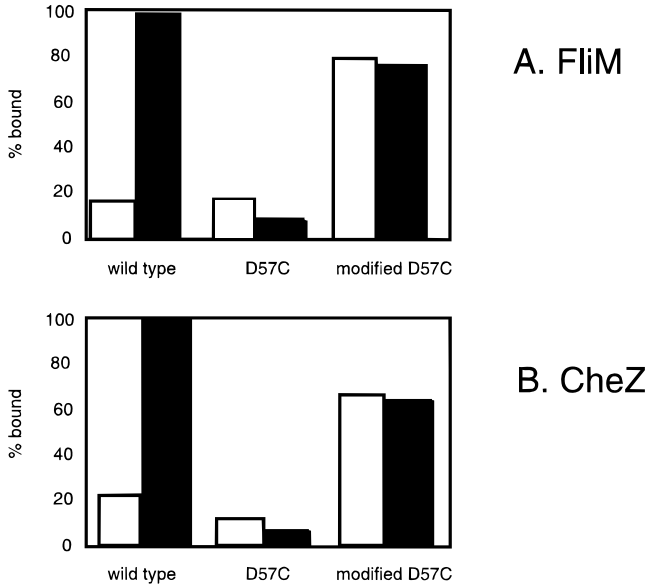


FIGURE 2: Binding of phosphono-CheY to CheZ and to FliM in the presence (filled bars) and absence of acetyl phosphate (open bars). (A) Binding of D57C CheY, phosphono-CheY, and CheY to FliM. The amount of FliM that bound to immobilized CheY was quantitated as described in Materials and Methods. (B) Binding of D57C CheY, phosphono-CheY, and CheY to CheZ. The amount of CheZ that bound to immobilized CheY was quantitated as described in Materials and Methods.

Both the decrease in residual thiol content and the relative areas in HPLC chromatographic peaks were used to characterize the extent of modification at Cys57 by the reagent, and the two techniques gave satisfactory agreement. The presence of the phosphonate group was confirmed using ^{31}P NMR, which showed a broad signal at 17.2 ppm. Alkylation proceeded in good yield only in the presence of divalent metal ions. The yield was typically 75−80% in the presence of calcium ions, and yields decreased in the following order: $Ca^{II} > Ba^{II} > Sr^{II} \sim Zn^{II} \sim La^{III} > Lu^{III} \sim Mg^{II} \sim$ no metal. The ability of various cations to promote alkylation does not seem to correlate with the affinity of binding to D57C CheY. These results suggest that the affinity of the alkylating agent for the active site is increased by metal ions, and that alkylation is facilitated by metals with large sizes and/or large coordination numbers. Attempts to alkylate Cys57 CheY in the presence of urea or guanidinium chloride and to refold the protein were unsuccessful.

Both phosphono-CheY and (unalkylated) D57C CheY were compared with wild-type CheY for their abilities to bind to FliM and CheZ (Figure 2). FliM is one of the proteins of the flagellar motor to which CheY-P_i binds (*3*, *4*, *21*). Figure 2A shows that the phosphorylated form of wild-type CheY binds to FliM much more effectively than wild-type CheY in its unphosphorylated form. This result is in accord with previous studies on FliM, which showed

that Asp57 of CheY is phosphorylated in the presence of acetyl phosphate (*26*) and binding to FliM is thereby enhanced (*2*, *21*). The mutant D57C does not bind strongly to FliM, and acetyl phosphate does not increase its affinity. Acetyl phosphate does not phosphorylate D57C CheY (data not shown). However, phosphono-CheY does bind FliM. Therefore, some or all of the conformational changes which confer an increased affinity for FliM on CheY-P_i are likely to be present in the analogue. Acetyl phosphate has no effect on the binding of phosphono-CheY to FliM, as expected.

Figure 2B shows that phosphorylation of wild-type CheY likewise enhances its interaction with CheZ, which stimulates the phosphatase activity of CheY. Fluorescence depolarization measurements have shown that CheY-P_i binds to CheZ with a dissociation constant of less than 4 μM, but CheY itself binds roughly 2 orders of magnitude more weakly (*27*). The data in Figure 2B on CheY and CheY-P_i are consistent with these results. The mutant D57C CheY does not bind to CheZ in either the absence or presence of acetyl phosphate. On the other hand, phosphono-CheY does bind CheZ, and acetyl phosphate does not increase the amount of CheZ which is bound. These results are entirely parallel to the binding data on FliM (vide supra). A quantitative comparison of the binding affinities of CheY-P_i and phosphono-CheY is difficult with this qualitative assay. Preliminary results using the fluorescence polarization assay of Blat and Eisenbach (*27*) suggest that phosphono-CheY binds to CheZ about 10 times less well than CheY-P_i.

Magnesium ion is necessary for the phosphorylation of Asp57, as well as its efficient dephosphorylation (*7*, *26*, *28*). Magnesium ion binds unphosphorylated CheY from *Salmonella typhimurium* in a pocket formed by Asp12, Asp13, Asp57, and Asn59. More specifically, Asp13 and Asp57 coordinate via their carboxylate groups, Asn59 coordinates via its backbone carbonyl oxygen, and the carboxylate of Asp12 forms a hydrogen bond to one of the three waters of coordination (*29*). Mg^{2+} binds to CheY with a dissociation constant of 1 mM; it binds to CheY-P_i with a dissociation constant that is estimated to be 0.2 mM (*7*).

The binding of metals changes the fluorescence intensity of Trp58 which lies close to the active site (*23*). The level of binding of Mg^{2+} to phosphono-CheY at pH 7.0 is shown in Figure 3, which is an inverse plot of fluorescence intensity versus [Mg^{2+}]. A binding constant of 6 mM was determined from these data. This value is somewhat lower than that estimated for CheY-P_i, determined at pH 7.9 in 50 mM Tris. The difference in affinity may reflect a change in the protonation state of the phosphonate moiety relative to the natural aspartyl phosphate, due to either the difference in pH values of the titrations or the pK_a values of the phosphoryl oxygen atoms (vide infra).

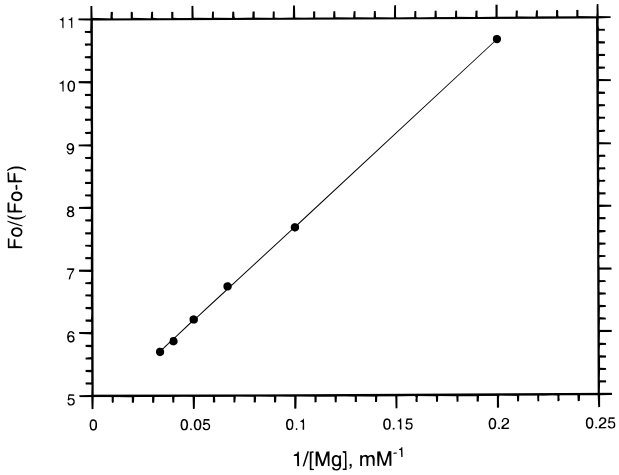


FIGURE 3: Binding of Mg^{2+} to phosphono-CheY, as monitored by the change in fluorescence emission from Trp58 (*23*). The graph shows the ratio of fluorescence intensity in the absense of Mg^{2+} to the change in intensity vs the reciprocal of [Mg^{2+}]. The excitation wavelength was 295 nm with a slit width of 2.5 nm. Data were fitted to eq 1 in the text.

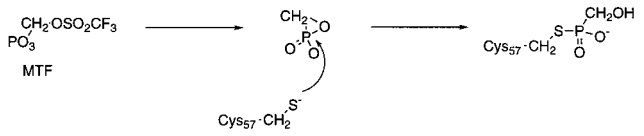
DISCUSSION

The signal transduction protein CheY exists in two signaling states: the inactive (off) state in which Asp57 is a free carboxyl group and the active (on) state in which Asp57 is phosphorylated to become an aspartyl phosphate, a mixed anhydride. Phosphorylation brings about extensive conformational changes in CheY (*9*) and increased affinity for two protein targets of the active form of CheY: FliM and CheZ. Even in the absence of CheZ, the aspartyl phosphate is short-lived, having a half-life of about 20 s (*28*). This lability makes conventional methods of structure determination extremely difficult. Indeed, despite the characterization of more than 100 response regulators, at present, no structure of a response regulator in its active phosphorylated form has been solved.

The lability of the aspartyl phosphate in CheY-P_i led us to consider making a nonhydrolyzable analogue of the labile mixed anhydride by means of site-directed mutagenesis and chemical modification. Phosphonate esters and diphosphonates are nonhydrolyzable inhibitors of many phosphate esters and anhydrides that are enzymatic substrates; for example, AMPPCP is a commonly used analogue of ATP.

We chose to alkylate a cysteine introduced at position 57 (the site of phosphorylation) to produce a novel species, a protein phosphonate. While chemical modification of a cysteine is often used to add fluorescent labels or occasionally to add an aminoethyl extension onto the side chain, we sought to induce a structural change of the mutant D57C CheY that would constitute a gain-of-function transition, a new variation on the theme of chemical modification of proteins. The requirement for particular divalent metals in the alkylation reaction suggests that trifluoromethanesulfonyl hydroxymethylenephosphonic acid (TMSP) is an affinity reagent for D57C CheY. To our knowledge, metal-directed affinity labeling has been demonstrated for only one other enzyme, alcohol dehydrogenase (*30*). The reaction shown in Scheme 1 is similar to the FosA-catalyzed deactivation of the antibiotic phosphonomycin by glutathione, which also requires a divalent metal (*31*). The result of alkylation of D57C CheY with the TMSP is most likely the phosphonate structure shown in Scheme 1. It is possible that TMSP had undergone a Favorskii rearrangement (*39*) to form the isomeric structure shown in Scheme 2. This alternative is difficult to rule out from first principles. However, the 1.9 Å resolution crystal structure of phosphono-CheY (C. Halkides, P. Matsumura, K. Volz, and F. Dahlquist, unpublished experiments) supports the phosphonate structure of Scheme 1, and rules out the adduct structure in Scheme 2. If others use this approach to generate similar phosphonates

Scheme 2



of cysteine residues in other proteins, the Favorskii product should be considered.

Phosphono-CheY is expected to have some steric and electronic differences when compared with CheY-P_i. The substitution of sulfur for the carbonyl carbon atom will result in longer bond lengths in the analogue. The S−CH−P bond angle in alkylated D57C is almost certainly more acute than the C(O)−O−P bond angle in CheY-P_i, and this is expected to compensate partially for the longer bond lengths in the analogue (*32*). Phosphonate esters and diphosphonates are generally weaker acids than the corresponding phosphate esters and anhydrides; for example, AMPPCP is a weaker acid than ATP by 1.5 pH units (*32*). The second dissociation constant of acetyl phosphate appears to be 4.0−4.5 on the basis of its rate of hydrolysis (*33*), which may be taken as a rough estimate of the pK_a of the aspartyl phosphate in CheY-P_i. The pK_a values of phosphonate esters with a slightly electron-withdrawing group fall in the range of 6−7 (*34*). However, the pK_a values of the phosphoryl group of CheY-P_i and phosphono-CheY are not known, and both are likely to be perturbed from model compounds by the presence of Asp12, Asp13, Lys109, and Mg^{2+}. Therefore, the properties of the analogue may not mimic those of CheY-P_i at low pH as well as they do at high pH. The apparent affinity of phosphono-CheY for Mg^{2+} is likely to be lower at low pH as well. In addition, the strengths of hydrogen bonds to the phosphoryl group of phosphono-CheY may be altered relative to those of CheY-P_i by the difference in their pK_a values.

CheY-P_i interacts at least with FliM and possibly other components of the flagellar motor to increase the likelihood that the motor will rotate cw. Therefore, an important test for an analogue of CheY-P_i is how well it binds FliM. Figure 2A indicates that phosphono-CheY is similar to CheY-P_i in the amount of FliM it binds. In contrast, the only known activating mutant of CheY, D13K CheY, binds FliM only marginally better than CheY (*7*). The mutant D13K CheY is very poorly phosphorylatable, yet this mutant displays a tumbly phenotype that is more pronounced when the gene is present in multiple copies than when it is present in a single copy (*35*).

CheY-P_i also binds to CheZ more strongly than CheY does. CheZ accelerates the hydrolysis of CheY-P_i but not that of the complex between CheY-P_i and FliM (*8*). This observation is consistent with the fact that the binding surfaces on CheY for these two proteins overlap (*36*). It seems reasonable that binding of CheY-P_i to FliM precludes (is competitive with) binding to CheZ. Phosphono-CheY binds to CheZ more readily than wild-type CheY, D13K CheY (*37*), or D57C CheY, and only slightly less well than CheY-P_i (Figure 2B). This result implies that the conformation adopted by phosphono-CheY resembles the conformation adopted by CheY-P_i.

Magnesium ion is required as a cofactor for phosphorylation of CheY (*26*), and Mg^{2+} binds to CheY-P_i with a binding constant that is estimated to be 0.2 mM (*7*). Although magnesium ion does not have a key role in the binding of CheY-P_i to FliM, magnesium ion increases the affinity of CheY-P_i for CheZ (*37*). Mg^{2+} binds to phosphono-CheY at pH 7.0 roughly 1 order of magnitude more weakly than it binds to CheY-P_i at pH 7.9. The coordination schemes of the divalent metal ions in either protein are not yet known, so the basis for the modest decrease in affinity evidenced by phosphono-CheY is not known. However, it may be related to the putative increase in the pK_a of the phosphonate relative to that of the aspartyl phosphate (vide supra).

While these three binding assays do not encompass all aspects of the biological functions of CheY-P_i, they imply that phosphono-CheY is a good analogue of CheY-P_i. Indeed, the comparison of our results to those for D13K CheY (*35*) suggests that phosphono-CheY more closely matches CheY-P_i in conformation than D13K CheY. The latter generates a tumbly phenotype but cannot be phosphorylated and does not bind FliM (*7*) or CheZ well (*37*); therefore, it is not a close analogue of CheY-P_i.

It is possible that phosphono-CheY undergoes only some of the conformational changes that CheY does when the latter becomes phosphorylated. D57C CheY may not undergo the same conformational change upon alkylation as native CheY does upon phosphorylation, yet the binding of phosphono-CheY to FliM and CheZ implies that phosphono-CheY undergoes the most biologically important conformational changes.

The alkylation of a cysteine at the site of phosphorylation is potentially a general strategy for studying the active form of response regulators. It may be particularly valuable for those response regulators which have rapid dephosphorylation rates, such as CheB (*38*). We hope that this methodology will be actively pursued with other response regulators.

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REFERENCES

1. Swanson, R. V., and Simon, M. I. (1994) *Curr. Biol. 4*, 234−237.
2. Welch, M., Oosawa, K., Aizawa, S.-I., and Eisenbach, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8787−8791.
3. Roman, S. J., Meyers, M., Volz, K., and Matsumura, P. (1992) *J. Bacteriol. 174*, 6247−6255.
4. Sockett, H., Yamaguchi, S., Kihara, M., Irikura, V. M., and Macnab, R. M. (1992) *J. Bacteriol. 174*, 793−806.
5. Hess, J. F., Oosawa, K., Kaplan, N., and Simon, M. I. (1988) *Cell 53*, 79−87.
6. McNally, D. F., and Matsumura, P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6269−6273.
7. Welch, M., Oosawa, K., Aizawa, S.-I., and Eisenbach, M. (1994) *Biochemistry 33*, 10470−10476.
8. Bren, A., Welch, M., Blat, Y., and Eisenbach, M. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 10090−10093.
9. Lowry, D. F., Roth, A. F., Rupert, P. B., Dahlquist, F. W., Moy, F. J., Domaille, P. J., and Matsumura, P. (1994) *J. Biol. Chem. 269*, 26358−26362.
10. Goldwhite, H., and Saunders, B. C. (1957) *J. Chem. Soc.*, 2409−2412.
11. Phillion, D. P., and Andrew, S. S. (1986) *Tetrahedron Lett. 27*, 1477−1480.

12. Phillion, D. P. (1988) in *(Phosphonomethyl)trifluoromethyl sulfonates*, Monsanto Co.
13. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
14. Moy, F. J., Lowry, D. F., Matsumura, P., Dahlquist, F. W., Krywko, J. E., and Domaille, P. J. (1994) *Biochemistry 33*, 10731−10742.
15. Filimonov, V., Prieto, J., Martinez, J. C., Bruix, M., Mateo, P., and Serrano, L. (1993) *Biochemistry 32*, 12906−12920.
16. Stock, A. M., Mottonen, J. M., Stock, J. B., and Schutt, C. E. (1989) *Nature 337*, 745−749.
17. Halkides, C., Farrar, C. T., Larsen, R. G., Redfield, A. G., and Singel, D. J. (1994) *Biochemistry 33*, 4019−4035.
18. Matsumura, P., Rydel, J. J., Linzmeier, R., and Vacante, D. (1984) *J. Bacteriol. 160*, 36−41.
19. Riddles, P. W., Blakeley, R. L., and Zerner, B. (1983) *Methods Enzymol. 91*, 49−60.
20. Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527−530.
21. Zhu, X., Amsler, C. D., Volz, K., and Matsumura, P. (1996) *J. Bacteriol. 178*, 4208−4215.
22. Blat, Y., and Eisenbach, M. (1996) *J. Biol. Chem. 271*, 1232−1236.
23. Needham, J. V., Chen, T. Y., and Falke, J. J. (1993) *Biochemistry 32*, 3363−3367.
24. Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.
25. Pitre, D., and Grabitz, E. B. (1966) *J. Prakt. Chem. 32*, 317−319.
26. Lukat, G., McCleary, W. R., Stock, A. M., and Stock, J. B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 718−722.
27. Blat, Y., and Eisenbach, M. (1994) *Biochemistry 33*, 902−906.
28. Lukat, G. S., Stock, A. M., and Stock, J. B. (1990) *Biochemistry 29*, 5436−5442.
29. Stock, A. M., Martinez-Hackert, E., Rasmussen, B. F., West, A. M., Stock, J. B., Ringe, D., and Petsko, G. A. (1993) *Biochemistry 32*, 13375−13380.
30. Dahl, K. H., McKinley-McKee, J. S., Beyerman, H. C., and Noordam, A. (1979) *FEBS Lett. 99*, 308−312.
31. Bernat, B. A., Laughlin, L. T., and Armstrong, R. N. (1997) *Biochemistry 36*, 3050−3055.
32. Yount, R. (1975) *Adv. Enzymol. 43*, 1−56.
33. Koshland, D. E., Jr. (1952) *J. Am. Chem. Soc. 74*, 2286.
34. Martell, A. E., and Smith, R. M. (1982) *Critical Stability Constants*, Plenum Press, New York.
35. Bourret, R. B., Drake, S. K., Chervitz, S. A., Simon, M. I., and Falke, J. J. (1993) *J. Biol. Chem. 268*, 13089−13096.
36. Zhu, X., Volz, K., and Matsumura, P. (1997) *J. Biol. Chem. 272*, 23758−23764.
37. Blat, Y., and Eisenbach, M. (1994) *Biochemistry 33*, 902−906.
38. Stewart, R. C. (1993) *J. Biol. Chem. 268*, 1921−1930.
39. March, J. (1985) *Advanced Organic Chemistry*, John Wiley & Sons, New York.

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