Catalytic Mechanism of Phosphorylation and Dephosphorylation of CheY: Kinetic Characterization of Imidazole Phosphates as Phosphodonors and the Role of Acid Catalysis†

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ABSTRACT: Kinetic and equilibrium measurements of phosphotransfer events involving CheY carried out over a range of pH conditions elucidated several features of the phosphotransfer mechanism. Using tryptophan fluorescence intensity measurements as a monitor of phosphorylation, we showed that phosphorylation using small molecule phosphodonors occurred by fast association of CheY with the phosphodonor, followed by rate-limiting phosphotransfer. Two previously uncharacterized phosphodonors, monophosphoimidazole and diphosphoimdazole, were able to phosphorylate CheY at a concentration about 6-fold lower than that of the previously described phosphodonors acetyl phosphate and phosphoramidate. This was shown to be due to tighter binding of the imidazole phosphates to CheY and implied the presence of binding interactions between CheY and the imidazole group. The ability of CheY to autophosphorylate through the pH range of 5−10 differed for various phosphodonors. Acetyl phosphate and diphosphoimidazole were unaffected by pH over this range, whereas phosphoramidate and monophosphoimidazole showed a steep dependence on pH with a loss of phosphorylation ability at about pH 7.4 (midpoint) for monophosphoimidazole and pH 7.8 (midpoint) for phosphoramidate. This behavior correlated with the loss of the positive charge on the nitrogen atom in the nitrogen−phosphorus bond in both monophosphoimidazole and phosphoramidate and implied that CheY was not capable of donating a proton to the leaving group in phosphotransfer with small molecules. The rate of phosphotransfer from [^{32}P]CheA-phosphate to wild type CheY also decreased markedly (>150 times) between pH 7.5 and 10. Because the mutant CheY proteins K109R and T87A showed the same pH dependence as the wild type, the loss of activity in the alkaline range could not be attributed to deprotonation of either of these active site residues. This observation, combined with the moderate decreases in phosphotransfer rates for these mutants relative to that of wild type CheY, indicated that it is unlikely that either Thr87 or Lys109 plays a direct role in the catalysis of phosphotransfer. Finally, we showed that the rate of autodephosphorylation of CheY was independent of pH over the range of 4.5−11. Together, these studies led to a model with CheY playing a largely entropic role in its own phosphorylation and dephosphorylation.

Signal transduction via two-component regulatory pathways is emerging as an important mechanism of signal transduction in both prokaryotic and eukaryotic systems (reviewed in refs 1 and 2). The chemotaxis pathways of the bacteria *Escherichia coli* and *Salmonella* are arguably the most well-studied of the two-component systems, with every known component purified and a large base of genetic, biochemical, and structural studies. In this system, the sensor kinase, CheA, is present as part of a multimeric complex on the interior of the cell membrane with three other proteins: a membrane-spanning receptor, an auxiliary protein (CheW) which couples the receptor to CheA, and a response regulator protein CheY (*3*). The occupancy of the receptor by the ligand dictates the autokinase activity of CheA (*4*, *5*). CheA autophosphorylates on the $N^{\epsilon 2}$ atom of His48 (*6*−*8*), and the phosphoryl group is subsequently tranferred to the Asp57 side chain of CheY (*9*). CheY-P[1] dissociates from the complex (*3*) and binds to the flagellar switch (*10*) to alter the swimming behavior of the bacterium. CheY catalyzes its own dephosphorylation, and the protein CheZ greatly enhances the rate of dephosphorylation of CheY-P (*11*−*13*).

Central to the phosphorelay pathway in chemotaxis, and by analogy to all two-component systems, is the transfer of the phosphoryl group from the histidyl residue of the sensor kinase, CheA, to the aspartyl residue of the response regulator, CheY. Some fundamental features of the mechanism of the transfer have been deduced from both structural and biochemical studies. Early studies showed that a divalent cation, optimally Mg^{2+}, is essential to the phosphotranfer reaction (*14*), and the ensuing high-resolution crystal structure of CheY chelated with Mg^{2+} showed details of the acid-lined Mg^{2+} binding site on CheY (*15*). This structure laid the groundwork for a proposed phosphotransfer mechanism whereby the oxygen atoms of the phosphoryl group on the phosphodonor directly chelate the Mg^{2+} ion followed by nucleophilic attack by the proximal Asp57 carboxylic acid side chain (*15*). Another significant finding was the observation that the small molecules acetyl phosphate, phosphoramidate, and carbamoyl phosphate react with CheY to phosphorylate specifically at Asp57 (*16*). This suggested that CheY must furnish at least part of the enzymatic machinery in the phosphotransfer process from CheA-P. The

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[1] Abbreviations: CheY-P, CheY-phosphate; CheA-P, CheA-phosphate.

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autocatalytic dephosphorylation of CheY-P shares essential similarities with the phosphotransfer reaction. This reaction is also Mg^{2+}-dependent (*14*), and its chemistry also involves nucleophilic substitution at a phosphoryl group. Therefore, it is likely that the mechanism of autodephosphorylation of CheY-P has features in common with phosphotransfer to CheY.

Despite consensus on the general nature of the Mg^{2+}-centered nucleophilic substitution events in phosphotransfer to and from CheY, there are still questions which remain to be answered with regard to the nature of these phosphotransfer events. Which residues of CheY and/or CheA participate in the catalysis? Are the mechanisms of phosphotransfer by small molecules and CheA-P the same? Why can some small molecules serve as phosphodonors to CheY and not others? What is the role of general acid catalysis in this reaction? With regard to this last question, chemical studies have shown that a positive charge on the nitrogen atom of phosphoramidate compounds such as phosphoramidate (*17*, *18*), phosphoimidazoles (*17*), and creatine phosphate (*19*) is essential for nucleophilic substitution to occur at the phosphorus atom. It follows that protonation of the His48 side chain of CheA-P is a prerequisite for phosphotransfer to CheY. It has been suggested that a residue on CheY, such as conserved active site residue Thr87 or Lys109, may act as a general acid and donate a proton to provide the necessary positive charge on the nitrogen atom of the substrate (*20*).

In this paper, we elucidated several mechanistic features of phosphorylation and dephosphorylation of CheY. Basic kinetic features of the phosphorylation of CheY with small molecules were characterized. This allowed a detailed analysis of the interaction of CheY with the previously uncharacterized phosphodonors monophosphoimidazole and diphosphoimidazole, which are chemically similar to the physiological phosphodonor histidyl phosphate on CheA. Finally, the role of general acid catalysis in phosphotransfer events to CheY by both small molecules and CheA-P as well as in the catalytic autodephosphorylation of CheY-P was assessed.

MATERIALS AND METHODS

Chemicals. Acetyl phosphate (potassium, lithium salt) was purchased from Aldrich. We determined that the purity of this product (*21*) ranges from 70 to 83% (w/w), depending on the length of storage, and all concentration calculations were adjusted to correct for purity. The buffer salts bis-tris propane, MES, CAPS, and HEPES were from Sigma Biochemicals. The potassium salt of phosphoramidate was synthesized using a published method (*22*), and the calcium salts of monophosphoimidazole and diphosphoimidazole were prepared as described (*23*). The identities and degree of purity of the synthesized compounds were confirmed by a combination of ^{1}H and ^{31}P NMR using a Bruker AMX 500 instrument. Phosphoramidate gave a ^{31}P chemical shift of -1.24 ppm relative to P_i at pH 8.0. The analysis showed that the compound was 92−98% pure, with the remaining phosphorus being inorganic phosphate. Monophosphoimidazole gave a characteristic ^{1}H spectrum (chemical shifts of 8.19, 7.28, and 7.17 ppm at 1:1:1) with 5% contaminating imidazole (7.56 and 6.92 ppm at a 2:1 ratio) in the preparation. Diphosphoimidazole had ^{1}H chemical shifts at 8.36 and 7.31 (2:1) ppm, respectively. Monophosphoimidazole and diphosphoimidazole gave ^{31}P chemical shifts of -6.9 and -7.1 ppm, respectively, relative to P_i at pH 8.0. The ^{31}P spectra of both monophosphoimidazole and diphosphoimidazole showed that 85% of the phosphorus was in the desired compound with the rest in the other phosphoimidazole. The formula weights used for the calcium salts of monophosphoimidazole and diphosphoimidazole were 222 and 333 g/mol, respectively (*23*).

Proteins. *E. coli* wild type CheY was purified from overexpressing strain K0641recA/pRBB40 (*24*) as previously described (*25*). CheY mutant proteins K109R (*26*) and T87A (J. L. Appleby and R. B. Bourret, in preparation) were purified in a manner identical to that of the wild type protein. CheA was purified from strain K0685/pDV4 (*27*) according to published methods (*25*). [^{32}P]CheA-P was prepared essentially as described (*25*). High specific activity was achieved by incubation of CheA (17 μM monomer) with [γ-^{32}P]ATP (420 μM, 10.0 Ci/mmol), 35 mM KCl, 3.5 mM $MgCl_2$, and 35 mM Tris (pH 8.0) for 1 h at room temperature. The phosphorylated protein was then precipitated with 45% ammonium sulfate and gel filtered on G-100 Sephadex to remove residual ATP. The purified [^{32}P]CheA-P had a specific activity of 6×10^{15} cpm/mol. The concentrations of CheY and CheA were determined by the Biorad DC Protein Assay (Folin analysis). The exact concentration of [^{32}P]CheA-P was not required for the pseudo-first-order kinetic analysis used here. However, to ensure CheY:CheA-P ratios of at least 5:1, the upper limit of the CheA-P concentration was determined by assuming an 80% recovery of protein in the phosphorylation procedure and assuming that all of the CheA was phosphorylated. Previous estimations of the phosphorylation efficiency for CheA under similar reaction conditions range from 16% (*28*) to 100% (*27*).

Fluorescence Experiments. Fluorescence measurements were made on a Perkin-Elmer LS-50B spectrofluorimeter, and Perkin-Elmer FLDM software was used to operate the instrument and analyze data. All samples were continuously stirred with a built-in magnetic stirring device and kept at a constant temperature with a circulating water bath. The tryptophan fluorescence intensity was measured at an excitation wavelength of 292 nm and an emission wavelength of 346 nm with slit widths of 4 and 7 nm for excitation and emission, respectively.

For kinetic measurements, CheY (1−3 μM) was equilibrated in the appropriate buffer to a constant temperature of 13.0 or 15.0 °C. The fluorescence intensity was continuously monitored, and phosphodonor was injected with a Hamilton syringe through an overhead port directly into the cuvette without opening the chamber. Control experiments where a small fluorophore, *N*-acetyltryptophanamide, was injected into buffer showed complete mixing of the sample within 1−3 s of injection. For equilibrium fluorescence measurements of CheY phosphorylation by various phosphodonors, aliquots of the phosphodonor (from a stock solution prepared in the appropriate buffer or water) were sequentially added to CheY (1.25 μM in the appropriate buffer) at 25 °C. The fluorescence was continuously monitored after each addition, and the next addition was made when the intensity had stabilized. For experiments involving acetyl phosphate and phosphoramidate, the buffers were 200 mM buffer salt and 10 mM $MgCl_2$. For titrations involving mono- and diphos-

phoimidazole, 50 mM $MgCl_2$ was added to prevent the Ca^{2+} present in the phosphodonor preparations from displacing Mg^{2+} on CheY.

Analysis of Fluorescence Intensity Measurements. For analysis of time courses of fluorescence intensity change, the initial velocities were measured by applying a linear least-squares analysis to the first portion of the time course (2−3 s to 10−20%). For further analysis, the entire time courses were analyzed by determining the slope of a plot of the natural logarithm of the intensity versus time. This yielded an apparent rate constant, "k_{app}". Further insight into the meaning of k_{app} is included in the Discussion.

For equilibrium titrations, the progress of the phosphorylation reactions was plotted as the total phosphodonor concentration in the cuvette versus $\Delta I/I_o$, where ΔI is the cumulative change in tryptophan fluorescence intensity as a result of addition of the given concentration of phosphodonor and I_o is the intensity of CheY·Mg^{2+} in the absence of phosphodonor. All intensities were corrected for changes in volume which occurred during the titration. Further analysis of the equilibrium fluorescence titrations was carried out by the method of Lukat et al. (*16*). This analysis was based on the following kinetic model, aspects of which were confirmed directly in this paper:

$$\text{CheY} + \text{X-P} \underset{}{\overset{K_s}{\rightleftharpoons}} \text{CheY·X-P} \xrightarrow[\text{X}]{k_2} \text{CheY-P} \xrightarrow[H_2O \quad P_i]{k_3} \text{CheY} \qquad (1)$$

where X-P is the small molecule phosphodonor, K_s is the equilibrium dissociation constant between the phosphodonor and CheY, and k_2 and k_3 are first-order rate constants. A plot of the concentration of the phosphodonor versus $\Delta I/(I - I_{inf})$, where I_{inf} is the saturating intensity in the titration, gives a straight line at low phosphodonor concentrations. The slope of the line is the reciprocal of K_m, where $K_m = K_s k_3/k_2$ and is also equal to the concentration of phosphodonor required to obtain half of the saturating fluorescence change (*16*).

Phosphorylation of CheY with [^{32}P]Acetyl Phosphate. [^{32}P]Acetyl phosphate was synthesized according to a published method (*21*) and had a specific acitivity of 1.7×10^{13} cpm/mol. The yield of acetyl phosphate was determined by comparison of the synthesized product to commercial acetyl phosphate in its ability to phosphorylate CheY, as determined by fluorescence quenching. CheY (46 μM) was incubated with various concentrations of [^{32}P]acetyl phosphate in 100 mM Tris (pH 7.5) and 10 mM $MgCl_2$ in a total volume of 20 μL at room temperature. After 7 min, the reaction was quenched with 5 μL of 5× SDS electrophoresis sample buffer. The samples were electrophoresed on a 20% polyacrylamide gel, and the gel was dried and the radioactivity associated with CheY quantitated by phosphorimaging.

Kinetics of Phosphotransfer from [^{32}P]CheA-P to CheY. Time courses were monitored by following the loss of ^{32}P from [^{32}P]CheA-P in the presence of excess CheY (wild type, K109R, or T87A) by SDS gel electrophoresis and phosphorimaging analysis. All buffers contained the appropriate buffer salt at 150 mM and 10 mM $MgCl_2$. Reactions were carried out in the cold room (4 °C). For the pH studies, the procedure was basically the same for the three CheY variants, but the exact protocol was adjusted to take into account the difference in phosphotransfer rates for the three proteins. For wild type and CheY K109R, reactions for each time point were run separately to allow a maximal number of early (1−10 s) time points. For each time point, [^{32}P]CheA-P (0.15 pmol for wild type CheY and 0.30 pmol for CheY K109R) was diluted into the appropriate buffer and CheY (5.3 pmol for wild type and 10.6 pmol for CheY K109R) was added in a total volume of 16 μL. The reaction was quenched after the desired time (4 s or longer) with the addition of 4 μL of 5× gel sample buffer. Zero time points were done in duplicate by the substitution of buffer for CheY. A portion (16 μL) of each time point was then electrophoresed; the gels were dried, and the radioactivity associated with the CheA band was quantitated by phosphorimaging analysis. For CheY T87A, [^{32}P]CheA-P (8 pmol) was added to the appropriate buffer to give a final volume of 93 μL. An aliquot (8 μL) was removed and mixed with an equal volume of 2× gel sample buffer for a zero time point. CheY T87A (42 pmol in 8 μL) was added, and aliquots were removed at desired times thereafter, the reactions quenched with an equal volume of 2× loading buffer, and the aliquots analyzed for [^{32}P]CheA-P as described above. The apparent rate constant was derived from computer fitting of the time courses assuming a pseudo-first-order process. For determination of the bimolecular rate constants for wild type CheY, CheY K109R, and CheY T87A, phosphotransfer reactions were carried out in parallel using identical concentrations of [^{32}P]-CheA-P (0.015 μM) and CheY (0.075 μM). The rate constant derived from an exponential analysis of these data was divided by the concentration of CheY to give a bimolecular rate constant.

Kinetics of Autodephosphorylation by CheY-P. To measure the rate of autodephosphorylation of CheY-P, the same basic experiment was used as described above for phosphotransfer kinetics with conditions adjusted so that phosphotransfer from [^{32}P]CheA-P to CheY was completed within the first time point (about 5 s). Under these conditions, the autodephosphorylation of [^{32}P]CheY-P could be monitored without contribution from phosphotransfer. Reactions were carried out at room temperature (measured at 24.2 ± 0.2 °C). CheY (400 pmol) was added to [^{32}P]CheA-P (28 pmol) in a total volume of 90 μL of the appropriate buffer. Aliquots (10 μL) were removed at various times and added to 10 μL of 2× loading buffer to quench the reaction. An aliquot was removed before the addition of CheY for a zero time point. Samples were electrophoresed on 15% polyacrylamide gels; the gels were dried, and the relative concentration of CheY-P was determined by phosphorimaging. First-order rate constants were determined by exponential analysis of the autodephosphorylation time courses.

RESULTS

Determination of the Rate-Limiting Step in Phosphorylation of CheY with Small Molecule Phosphodonors. The kinetic model proposed by Lukat et al. (*16*) suggested a fast association of phosphodonor with CheY followed by rate-limiting phosphorylation. However, this model has not been directly verified, and the possibility that association between CheY and phosphodonor is rate-limiting has not been excluded. One hallmark of the proposed model is that it predicts that the rate of phosphorylation of CheY should be saturatable as a function of phosphodonor concentration. Figure 1A shows that the initial velocity of CheY-P formation

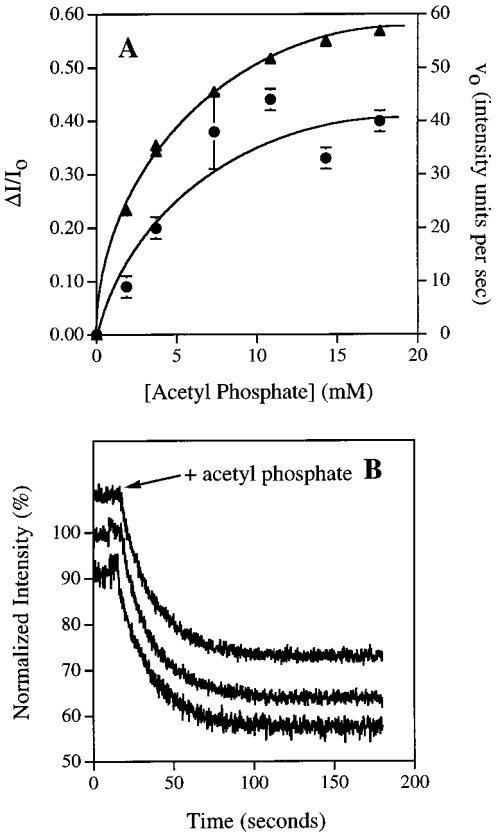


FIGURE 1: Kinetic characterization of CheY phosphorylation with acetyl phosphate. (A) Correlation of the initial velocity of the fluorescence intensity change and the degree of phosphorylation at equilibrium. Aliquots of acetyl phosphate were added to CheY (1.1 μM) at 15 °C. The degree of phosphorylation when equilibrium was reached (▲) is expressed as $\Delta I/I_o$. The saturating value of 0.55 was greater than that observed at 25 °C (Figure 2). The initial velocity of the reaction (●) was measured by applying a linear least-squares analysis to the first portion of the time course. The error bars represent the range of values obtained by choosing slightly different time boundaries to determine v_o. (B) Comparison of the time courses for the approach to steady state phosphorylation at three different CheY concentrations. Shown are time courses for 2.2 μM CheY (top), 1.1 μM CheY (middle), and 0.55 μM CheY (bottom). To facilitate comparison, the time courses were expressed as the percent of the initial fluorescence intensity. The top and bottom time courses were then offset by 10% in either direction so that the full time courses could be visualized. The concentration of acetyl phosphate was 6.3 mM, and the buffer was 100 mM Tris (pH 7.5) and 10 mM $MgCl_2$.

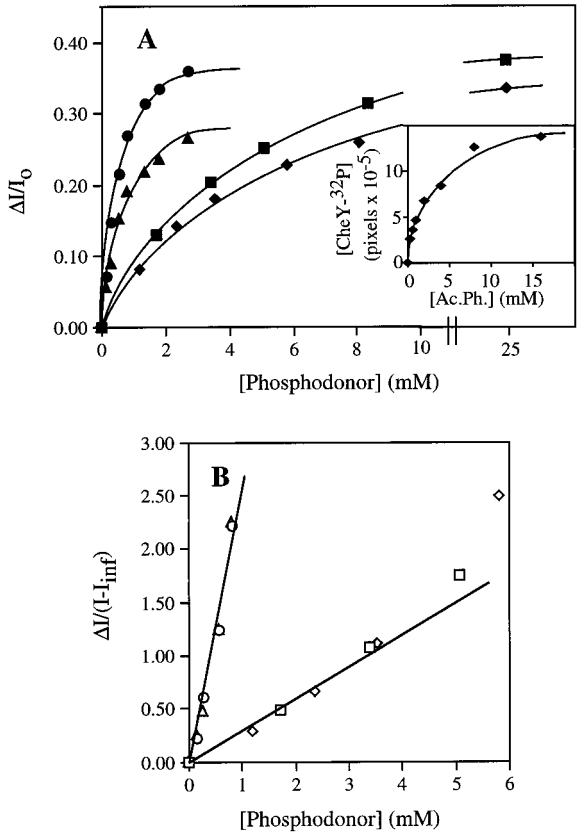


FIGURE 2: Equilibrium titration of CheY with four phosphodonors under optimal pH conditions. The phosphodonors are acetyl phosphate (◆), phosphoramidate (■), monophosphoimidazole (●), and diphosphoimidazole (▲). (A) The change in fluorescence intensity (expressed as $\Delta I/I_o$) upon sequential addition of phosphodonor to a single sample of CheY. The inset shows the phosphorylation of CheY (46 μM) with [^{32}P]acetyl phosphate as a function of acetyl phosphate concentration. The reactions were allowed to come to equilibrium, and the radioactivity in CheY was quantitated by gel electrophoresis and phosphorimaging. (B) Plot of $\Delta I/(I - I_{inf})$ versus phosphodonor concentration obtained from the data in panel A. The slope of this line is equal to the reciprocal of the K_m which is equal to $K_s k_3/k_2$ (eq 1). The buffers were MES at pH 6.6 (phosphoramidate, monophosphoimidazole, and diphosphoimidazole) and Tris at pH 7.5 (acetyl phosphate). All buffers contained 200 mM buffer salt and 10 mM $MgCl_2$.

was saturatable as a function of acetyl phosphate concentration and that the rate changed in a manner proportional to the final degree of phosphorylation (Figure 1A). This behavior supported the proposed model and also eliminated the alternative possibility that association of CheY with phosphodonor was rate-limiting as, in this case, the rate of phosphorylation would continue to increase with increasing phosphodonor concentration. Furthermore, the time courses of phosphorylation, normalized to the concentration of CheY, present were nearly superimposable over a 4-fold concentration range of CheY (Figure 1B). This behavior was also consistent with a system whose rate is dependent on the concentration of an associated complex and subsequent first-order rate constants.

Quantitative Comparison of Various Phosphodonors as Substrates for CheY Phosphorylation. The imidazole derivatives, monophosphoimidazole and diphosphoimidazole, were compared to previously identified phosphodonors acetyl phosphate and phosphoramidate in their abilities to serve as substrates for CheY phosphorylation, using the degree of fluorescence quenching as a monitor of phosphorylation. All four phosphodonors gave similar degrees of quenching at saturation, quenching to 60−75% of their original intensity at 25 °C (Figure 2A). However, the phosphodonors worked under different concentration ranges, with the two imidazole derivatives shifted to concentrations about 6-fold lower relative to those of acetyl phosphate and phosphoramidate. The K_m values for the phosphodonors under the buffer conditions shown in Figure 2 were 3.2 ± 0.4 mM (acetyl phosphate and phosphoramidate) and 0.6 ± 0.1 mM (monophosphoimidazole and diphosphoimidazole) (Figure 2B). The error reflects variation between titrations with a given phosphodonor. Parallel equilibrium titration of CheY with [^{32}P]acetyl phosphate gave a titration curve (Figure 2A, inset) and deduced K_m (2.7 ± 0.5 mM) similar to those given by the fluorescence titration with acetyl phosphate (Figure 2A), thus confirming that the fluorescence change is a valid indicator of the degree of CheY phosphorylation.

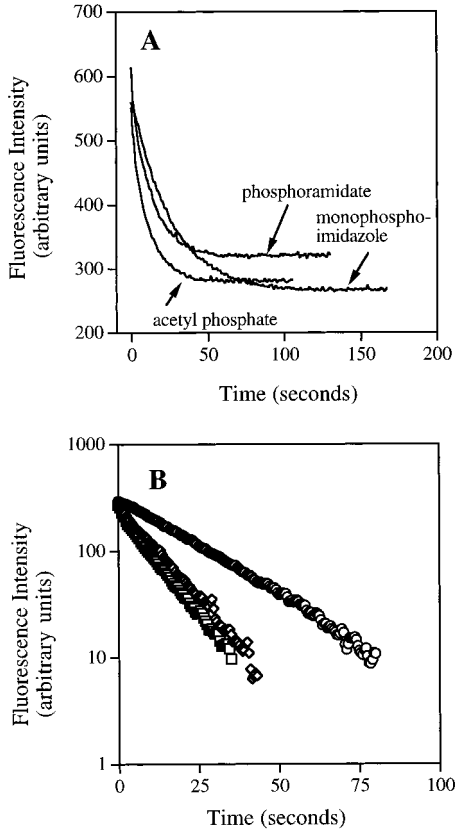


FIGURE 3: Time courses for the fluorescence quench upon reaction of CheY with saturating concentrations of acetyl phosphate, phosphoramidate, and monophosphoimidazole. The concentration of CheY was 1.25 μM, and the temperature was 13.0 °C. The concentrations of phosphodonor added at time zero were 30 mM acetyl phosphate, 30 mM phosphoramidate, and 5 mM monophosphoimidazole. (A) Raw data and (B) exponential analysis of the raw data for phosphorylation time courses shown in panel A with monophosphoimidazole (○), acetyl phosphate (◇), and phosphoramidate (□). Diphosphoimidazole could not be included in this experiment due to solubilty limitations at the concentrations required.

Because the K_m is actually a combination of rate and binding constants ($K_m = K_s k_3/k_2$), a shift in the phosphorylation titration curve to lower concentrations of phosphodonor as seen for the imidazole derivates relative to the other phosphodonors could be due to either enhanced binding of the phosphodonor to CheY (manifested in a lower K_s), faster reaction of the bound phosphodonor with CheY (manifested in a higher k_2), or a combination of these two factors. The rate of autodephosphorylation, k_3, is assumed to be independent of the identity of the phosphodonor and thus could not contribute to the differences. To determine the basis of the shifts observed here for the imidazole phosphates, the k_{app} values for the phosphodonors were measured. Knowledge of k_{app} gives information about k_2 as k_{app} is based solely on k_2 and k_3 (Figure 1 and Discussion). k_{app} was determined by measuring the rate of acquisition of steady state phosphorylation under saturating phosphodonor conditions (10 times the K_m for each phosphodonor). Under saturating conditions, the concentration of associated complex (CheY·X-P) is equal to the initial CheY concentration and is constant for all of the phosphodonors, so any differences in rate should be due to inherent differences in k_2. Figure 3 shows that the two phosphodonors with the higher K_m values, acetyl phosphate and phosphoramidate, actually had faster phosphorylation rates than monophosphoimidazole. This

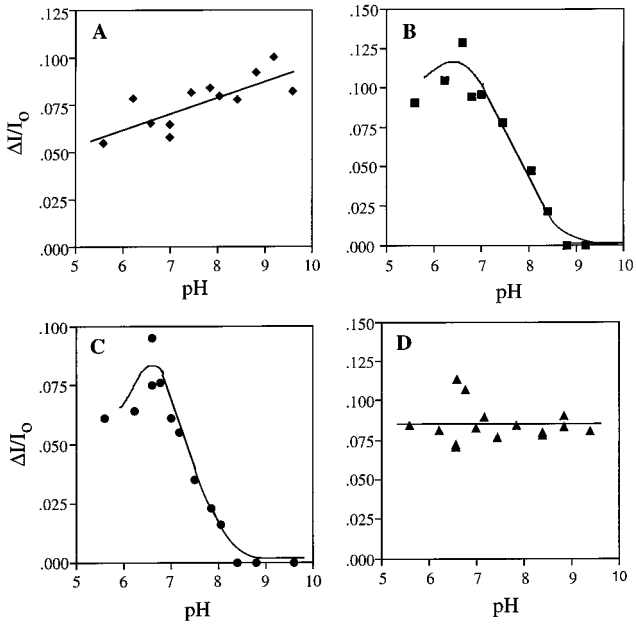
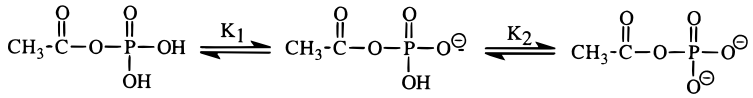
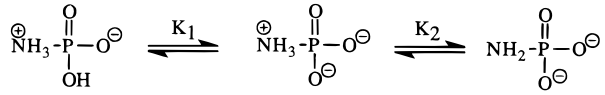
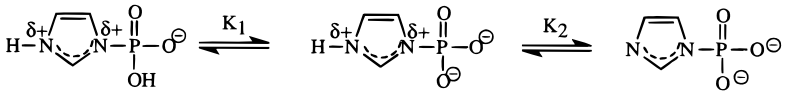
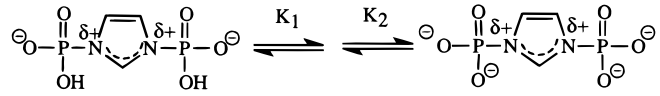


FIGURE 4: Effect of pH on the ability of CheY to use various phosphodonors. Equilibrium titrations were carried out at 25 °C for each phosphodonor under various pH conditions. The phosphorylation ability was expressed as the $\Delta I/I_o$ upon addition of a low concentration of phosphodonor (1.7 mM for acetyl phosphate and phosphoramidate, 0.14 mM for monophosphoimidazole, and 0.28 mM for diphosphoimidazole): (A) acetyl phosphate, (B) phosphoramidate, (C) monophosphoimidazole, and (D) diphosphoimidazole. The buffers were bis-tris propane (pH 8.0−9.7), Tris (pH 7.4−7.8), HEPES (pH 6.6−7.2), and MES (pH 5.6−6.6). The buffers were 200 mM buffer salt and 10 mM $MgCl_2$.

lack of correlation between k_{app} and K_m values for the phosphodonors (cf. Figures 2 and 3) indicated that the difference in K_m values mainly reflects differences in K_s values for the phosphodonors. This implies that the active site of CheY contains determinants which are involved in binding interactions with the imidazole group of the phosphodonor. It follows that these interactions also occur with the physiologic substrate CheA His48-P. That the logarithmic analysis of the time courses showed excellent agreement with linearity (Figure 3B) was an indication of a first-order rate-limiting step which was further evidence in support of the kinetic model described above. The k_{app} values at 13 °C derived from this analysis (Figure 3B) were 0.081 s^{-1} for acetyl phosphate, 0.091 s^{-1} for phosphoramidate, and 0.040 s^{-1} for monophosphoimidazole.

Effect of pH on Phosphorylation of CheY by Various Phosphodonors. Equilibrium fluorescence titration of CheY with the four phosphodonors acetyl phosphate, phosphoramidate, monophosphoimidazole, and diphosphoimidazole was carried out over a range of pH conditions. The phosphorylating ability at a given pH was assessed by calculating the degree of phosphorylation ($\Delta I/I_o$) as a result of adding a low concentration of phosphodonor (less than $^1/_2$ the K_m for each phosphodonor). At low phosphodonor concentrations, the change in fluoresence intensity was approximately proportional to the amount of phosphodonor added and so was a sensitive measure of phosphorylation ability. The results showed fundamental differences between the phosphodonors (Figure 4). Two of the phosphodonors, acetyl phosphate and diphosphoimidazole, were nearly unaffected by pH over the range of 5−10 (Figure 4A,D). In

Table 1: Relevant Protonation States of the Phosphodonors Used in These Studies[a]

Phosphodonor	Structures	pK1	pK2	Ref.
acetyl phosphate		1.2	4.9	29
phosphoramidate		2.7	7.9-8.2[b]	30
mono-phosphoimidazole		N/A	7.0	17
diphosphoimidazole		N/A	N/A	

[a] Each species is represented by the dominant resonance form. [b] Variability represents different published values at ambient temperature.

contrast, phosphoramidate and monophosphoimidazole showed a steep, sigmoidal pH dependence on phosphorylation ability over a narrow pH range (Figure 4B,C). For both of these compounds, phosphorylation occurred at acidic pH and was abolished as the pH increased, indicating that protonation of a functional group was necessary for efficient phosphorylation of CheY. The midpoint pH's for the transitions were about 7.4 for monophosphoimidazole and 7.8 for phosphoramidate.

The fact that two of the donors (diphosphoimidazole and acetyl phosphate) phosphorylated CheY in a pH-independent fashion implied that the steep dependence observed with the other two donors is due to protonation events on the donors themselves as opposed to protonation events on CheY. Insight into the molecular basis for the varying behavior between the phosphodonors can be achieved by consideration of the different protonation events which would occur through the pH range studied (Table 1). Acetyl phosphate loses its two phosphoryl hydrogens with pK_a values of 1.2 and 4.9 (*29*), so therefore should undergo no change in protonation state between pH 5.5 and 10. Each of these deprotonation events results in an increase in negative charge on the phosphoryl group but does not affect the rest of the molecule. This behavior contrasts with that of the phosphoramidate compounds which possess novel zwitterionic structures at intermediate pH's. The monoanionic structures of phosphoramidate and monophosphoimidazole are zwitterions with a positive charge on the nitrogen atom and a dinegative phosphoryl group. Loss of this proton with a pK_a of 7.9−8.2 for phosphoramidate (*30*) and 7.0 for monophosphoimidazole (*17*) results in the dianionic species which has a neutral nitrogen atom. In contrast, diphosphoimidazole, because of the extra bond on the other imidazole nitrogen, maintains the positive charge on the nitrogen atom even after removal of all of the phosphoryl hydrogens.

Taking the known protonation states of the phosphodonors into account (Table 1), as well as the pH dependence of CheY phosphorylation exhibited by the phosphodonors (Figure 4), it is clear that phosphoramidate and monophosphoimidazole lose their positive charge on the nitrogen over the same approximate pH range as they lose their ability to phosphorylate CheY. Diphosphoimidazole, which possesses a positive charge on the nitrogen atoms over the entire pH range, can phosphorylate CheY equally well over the entire pH range. Acetyl phosphate, which represents a different class of phosphodonors as it lacks the nitrogen−phosphorus linkage, has no protonation requirements in the range tested. Therefore, there is a direct correlation of the ability of CheY to phosphorylate with a phosphoramidate phosphodonor and the presence of a positive charge on the nitrogen atom in the nitrogen−phosphorus bond. Chemical studies have shown that protonation of the nitrogen atom involved in a nitrogen−phosphorus bond is essential for nucleophilic substitution reactions at the phosphorus (*17*, *18*). Therefore, CheY is incapable of supplying a proton in its reaction with small molecule phosphodonors and consequently does not contain a residue which acts as a general acid catalyst during this process.

Effect of pH on the Phosphotransfer from CheA-phosphate to CheY. Although general acid catalysis by a CheY residue does not appear to play a role in phosphorylation with small molecules, the possibility remains that the interaction of CheY with CheA causes conformational shifts on CheY which put an acidic residue in a position so that proton donation can occur in the protein−protein phosphotransfer. To investigate the role of general acid catalysis by a CheY residue or other protonation events in the phosphotransfer from CheA-P to CheY, the pH dependence of the kinetics of phosphotransfer was studied. The rate of phosphotransfer from [^{32}P]CheA-P to CheY decreased a minimum of 150 times between pH 7.5 and 10 (Figure 5A), indicating that protonation of at least one residue with a pK_a within this range on either CheY or CheA-P is required for optimal phosphotransfer kinetics. Rate constants at pH values of less

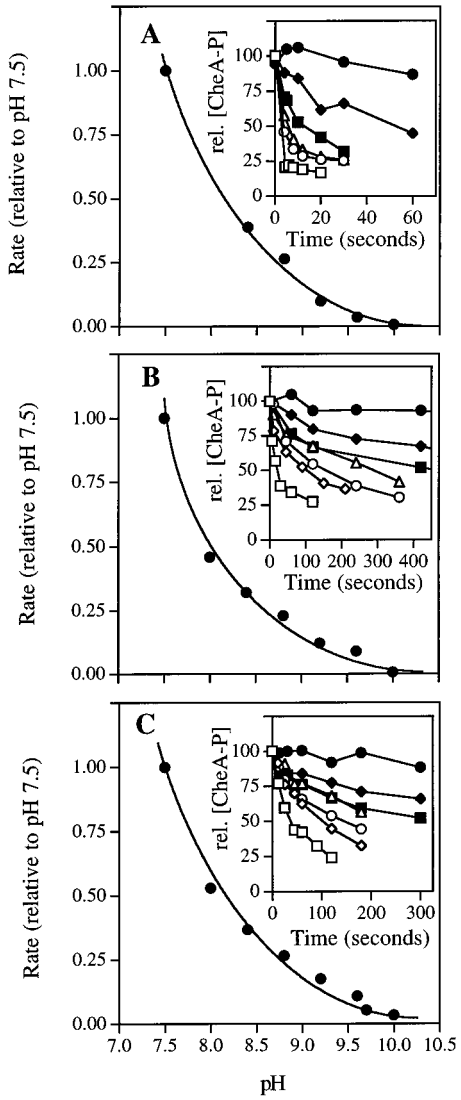


FIGURE 5: Effect of pH on the kinetics of phosphotransfer from [^{32}P]CheA-P to wild type and mutant CheY. The phosphotransfer time courses were analyzed on the basis of pseudo-first-order kinetics. The rate constants, expressed relative to the rate constant at pH 7.5 for each mutant, were plotted as a function of pH. The time courses are shown in the insets with pH 7.5 (□), 8.0 (◇), 8.4 (○), 8.8 (△), 9.2 (■), 9.6 (◆), and 10.0 (●). (A) Wild type CheY where [CheY] is 0.27 μM and [CheA-P] is 0.01 μM. (B) CheY K109R where [CheY K109R] is 0.54 μM and [CheA-P] is 0.02 μM. (C) CheY T87A where [CheY T87A] is 0.40 μM and [CheA-P] is 0.09 μM. Note the different time scales on the time courses. The buffers were CAPS (pH 9.7−10.1) and bis-tris propane (pH 7.5−9.6) and contained 150 mM buffer salt and 10 mM $MgCl_2$. The reaction temperature was 4 °C.

than 8.0 could not be accurately assessed as the reactions were nearly complete by the first time point. To evaluate the possibility that this trend was due to protonation events on CheY, the phosphotransfer properties of two mutants of CheY, CheY K109R, and CheY T87A were characterized. Thr87 and Lys109 are the residues on CheY which are the most likely candidates to play a direct role in catalysis (*20*). Both of these residues are conserved in the response regulator family, and are located in the active site (*31*). Furthermore, both possess functional groups which are capable of proton donation with pK_a values which could potentially be in the alkaline range considered here. Mutant proteins CheY K109R and T87A had decreased phosphorylation rates relative to that of wild type CheY. CheY K109R showed

Table 2: Phosphotransfer Rates from CheA-P to Wild Type and Mutant CheY at 4 °C[a]

CheY	k (M^{-1} s^{-1})
wild type	2.4×10^6
K109R	5.8×10^5
T87A	1.6×10^5 [b]

[a] Rate constants were derived from time courses for reaction of 0.015 μM CheA-P and 0.075 μM CheY. [b] Phosphotransfer from CheA-P to CheY T87A appeared to come to equilibrium with about half of the phosphate remaining on CheA-P.

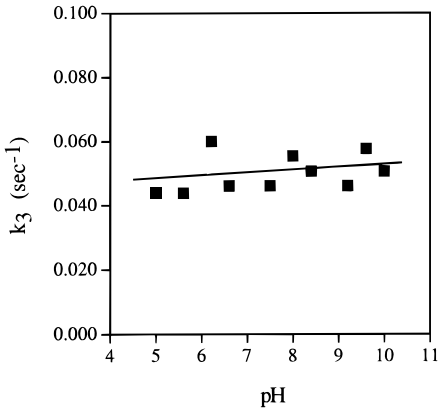


FIGURE 6: Effect of pH on the rate constant for autodephosphorylation of CheY-P. The temperature was 24.0 ± 0.2 °C.

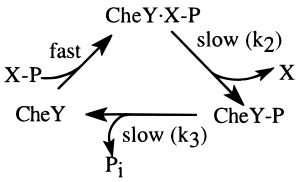
an approximately 4-fold slower rate than wild type CheY (Table 2). This agrees well with the approximately 7-fold difference between CheY 109KR and wild type CheY previously reported (*32*). CheY 87TA gave a rate constant about 15-fold slower than that of the wild type protein (Table 2). Despite the moderately decreased rate constants, the rates of phosphotransfer to both CheY T87A and CheY K109R showed a dependence on pH which was indistinguishable from that of wild type (Figure 5). Therefore, it is unlikely that the decrease in phosphotransfer rates between pH 7.5 and 10.0 seen in wild type CheY can be attributed to deprotonation of Thr87 or Lys109. The loss of activity throughout this range for phosphotransfer to wild type CheY is thus probably due to a deprotonation event on CheA-P.

pH Dependence of CheY-P Autodephosphorylation. Because of the probable overlap of mechanistic features between phosphorylation and autodephosphorylation of CheY, investigating the effect of pH on the rate of autodephosphorylation of CheY-P was of interest. The first-order rate constant of CheY-P autodephosphorylation (k_3) showed little or no dependence on pH between the range of pH 4.5−10.5 (Figure 6). Furthermore, the kinetics gave excellent correlation with first-order decomposition ($r = 0.99-1.00$), and k_3 values agreed with published autodephosphorylation rates (*13*, *32*). Therefore, there are no residues on CheY with pK_a values in the range of 4.5−10.5 whose protonation state plays a role in the autodephosphorylation mechanism of CheY.

DISCUSSION

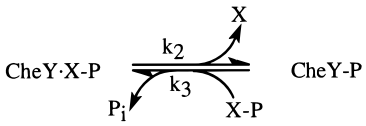
Implications of the Kinetic and Equilibrium Properties of CheY Phosphorylation by Small Molecules. The kinetic data presented here for the reaction of CheY with small molecule phosphodonors are in agreement with a model which includes fast association of CheY with the phosphodonor in forming

an associated complex, followed by rate-limiting phosphorylation and subsequent dephosphorylation. With the confirmation of the rate-limiting steps in this model, the scheme (eq 1) can be rewritten as



(2)

Under saturating phosphodonor concentrations, this scheme predicts that the concentration of CheY is negligible and the scheme can be further simplified to the following equilibrium



(3)

This simplification is analogous to that made in the kinetic analysis of chymotrypsin (*33*). This scheme (eq 3) has several interesting implications. In such a system, the first-order rate constant for the rate of acquisition of equilibrium (k) starting from nonequilbrium conditions is (*33*)

$$k = k_2 + k_3 \tag{4}$$

Therefore the k_{app} values measured here, the rate constants for the fluorescence intensity change upon phosphorylation of CheY by small molecule phosphodonors, should be equal to the sum of k_2, the rate constant for the phosphorylation step by that donor, and k_3, the rate constant for autodephosphorylation. Because the value of k_3 is known (0.048 s^{-1} at 25 °C; Figure 6), k_2 values can be calculated. For example, at saturating acetyl phosphate concentrations, the k_{app} value is 0.081 s^{-1} at 13 °C (Figure 1A) which corresponds to 0.22 s^{-1} at 25 °C (E_a for k_{app} is 13.9 kcal/mol; R. E. Silversmith, unpublished results). Solving eq 4 for k_2 gives a value of 0.17 s^{-1} for acetyl phosphate. Another implication of eq 4 is that the equilibrium constant would be [CheY-P]/[CheY·X-P] = k_2/k_3. In the case of acetyl phosphate (k_2 = 0.17 s^{-1} and k_3 = 0.048 s^{-1}), the value of this equilibrium constant is 3.5. Therefore, at saturating acetyl phosphate concentrations, this model predicts that about three-fourths of the CheY molecules would be phosphorylated. The rest would be in a noncovalent complex with phosphodonor. Finally, the K_s for acetyl phosphate would be 11 mM ($K_m = K_s k_3/k_2$). Similar calculations can be made for the other phosphodonors.

The K_m value for acetyl phosphate determined here (3.2 mM) was significantly greater than the published value for *Salmonella* CheY [0.7 mM (*16*)] which was determined by the identical method of fluorescence titration. This was in contrast to two other phosphodonors which gave much closer values in the two studies. The K_m values for carbamyl phosphate were 1.2 mM (*16*) versus 1.9 mM (R. E. Silversmith, unpublished results) and for phosphoramidate were 1.7 mM (*16*) versus 3.2 mM (this work). The possibility that this disparity was due to an inherent difference between the CheY proteins from *E. coli* and *Salmonella*, which differ by only three out of 128 amino acids (*34*, *35*), was eliminated as *Salmonella* CheY gave a K_m value similar to that of the *E. coli* protein in this lab (data not shown). Furthermore, the purity of the commercial acetyl phosphate was directly assayed to exclude the possibility that the higher K_m was due to an impure source of phosphodonor. Although the source of this disparity in K_m values is unclear at this time, uncertainty in this value may have implications for the importance of phosphorylation of CheY by acetyl phosphate *in vivo*. *In vivo* concentrations of acetyl phosphate range from 0.04 to 1.3 mM depending on the carbon source and growth phase of the cells (*36*).

That the imidazole phosphates served as phosphodonors at molar concentrations much lower than those of the other organophosphates was shown to be due to enhanced binding of the phosphodonors to the CheY active site. This result broadens our perception of the active site of CheY. It indicates that, in addition to interactions between the active site Mg^{2+} ion and the phosphoryl oxygens on the phosphodonor, there are also attractive interactions between residues on CheY and the imidazole functional group of phosphoimidazoles. Insight into which residues on CheY are involved in these interactions could be achieved by assessment of the abilities of active site mutants of CheY to use imidazole phosphates as phosphodonors.

Effective versus Ineffective Phosphodonors for CheY. What makes one phosphocompound capable of serving as a phosphodonor for CheY and not another? While there is a subset of molecules which can phosphorylate CheY (phosphoanhydrides such as carbamoyl phosphate and acetyl phosphate and phosphoramidates such as phosphoramidate, monophosphoimidazole, and diphosphoimidazole), many organophosphates are incapable of acting as phosphodonors. To date, inactive phosphocompounds appear to fall into three categories: (1) phosphomonoesters such as serine phosphate, phosphoethanolamine, and phosphoenolpyruvate (R. E. Silversmith, unpublished data), (2) derivatives of pyrophosphate such as ATP and ADP (*16*), and (3) guanidinium phosphates such as creatine phosphate (*16*) and arginyl phosphate (R. E. Silversmith, unpublished). Here, we have shown that CheY is not able to provide the necessary proton for phosphotransfer from phosphoramidate and imidazole phosphate. That is, CheY is only able to use phosphoramidates as phosphodonors under conditions where they themselves are capable of undergoing nucleophilic substitution. Upon inspection of the other molecules that can and cannot serve as phosphodonors, it appears that this correlation can be extended. It appears that CheY is able to autophosphorylate using phosphodonors which are themselves susceptible to nucleophilic attack (such as hydrolysis) at a reasonable rate. Phosphomonoesters are kinetically stable to nucleophilic attack, indicated by their stability to hydrolysis at neutral pH (*37*). However, phosphoanhydrides such as acetyl phosphate and carbamoyl phosphate have moderate rates of hydrolysis at neutral pH (*38*). It is probable that ATP, ADP, and PP_i, although kinetically capable of hydrolysis at neutral pH, cannot effectively bind CheY due to electrostatic repulsion from their high negative charge density. These molecules also bind Mg^{2+} with high affinity so may compete with CheY for Mg^{2+}, thereby preventing phosphotransfer. It is also clear why creatine phosphate and arginyl phosphate, although phosphoramidates, cannot serve as phosphodonors for CheY. Both of these compounds possess a positively charged nitrogen atom only at acidic pH [the pK_a of creatine phosphate is 4.5 (*19*)] so phosphorylation could not occur

at the pH's used in these studies. Conversely, we predict that pyridine phosphate and picoline phosphate, phosphoramidate compounds that have been shown to act as phosphodonors for the related response regulator Spo0F (*39*), would act as phosphodonors for CheY in a pH-independent fashion because the nitrogen atoms in these compounds are positively charged without binding a proton (as in the case with diphosphoimidazole). Finally, it should also be recognized that only phosphodonors which have adequately high k_2 values would result in accumulation of CheY-P. There may be some phosphomolecules that are capable of binding and undergoing slow phosphotransfer, but are unable to accumulate CheY-P because the phosphorylation rate is too slow relative to the dephosphorylation rate.

Catalytic Role of CheY in Phosphotransfer from CheA-P. The observation that mutation of Lys109 or Thr87, the two conserved active site residues in CheY which are prime candidates for a direct role in catalysis, resulted in relatively modest decreases in what is an extremely fast reaction for the wild type CheY agrees with studies which have suggested no direct role in phosphotransfer for Lys109 (*32*) and Thr87 (*40*). Because neither of these residues is essential for catalysis, along with the prior conclusions that CheY is capable of phosphorylation only with labile phosphates and does not serve as an acid catalyst in phosphotransfer, it is likely that a major component of the role that CheY plays in catalysis is to serve as a template to bring the Asp57 carboxyl group and the phosphodonor close together and in an optimal orientation for reaction. Mg^{2+} has very specific stereochemical requirements for its ligands (*41*) and has been shown to be used to achieve optimal orientation of reacting molecules in other enzymes (*41*). In addition to this entropic role for CheY, direct chelation of the phosphoryl group of the phosphodonor to Mg^{2+} would polarize the P−O(N) bond, making it more susceptible to nucleophilic attack. Because of their modest but finite effect on rate, it is probable that the active site residues Lys109 and Thr87 contribute to catalysis indirectly. Possible roles for these residues could be stabilization of the transition state for the phosphorylation reaction, as has been suggested previously for Lys109 (*32*), or participation in binding interactions with the phosphodonor molecule.

The fact that the rate of autodephosphorylation of CheY-P was independent of pH is consistent with the notion that its mechanism is similar to the phosphotransfer mechanism, where all pH effects could be attributed to the phosphodonor. An analogous entropic role for Mg^{2+} in autodephosphorylation could be direct chelation of the phosphoryl group and of a water molecule. This would orient the reacting groups for nucleophilic attack and also polarize the water molecule, making it a better nucleophile.

pK_a of His48-P. With the probable exclusion of a residue on CheY as a general acid, the question arises as to the source of the necessary proton in the phosphotransfer process from CheA-P. There are two remaining possibilities. (1) His48-P is protonated to a significant extent at physiologic pH and so does not require a proton donor in phosphotransfer, or (2) a residue on CheA acts as a proton donor. Distinguishing between these possibilities requires knowledge of the pK_a of His48-P. Preliminary NMR studies have suggested that the pK_a of CheA His48-P is 8.0 (*8*). Although this value was determined indirectly and is not conclusive, a pK_a of 8.0 would imply that more than half of the His48 groups in CheA-P would be protonated at physiologic pH and may contraindicate the need for general acid catalysis in the phosphotransfer. Further direct determination of the pK_a of this residue will establish whether the proton required for phosphotransfer is present due to an adequately high pK_a value for His48-P or whether it is supplied by a general acid on CheA.

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REFERENCES

1. Appleby, J. L., Parkinson, J. S., and Bourret, R. B. (1996) *Cell 86*, 845−848.
2. Hoch, J. A., and Silhavy, T. J., Eds. (1995) *Two-Component Signal Transduction*, ASM Press, Washington, DC.
3. Schuster, S. C., Swanson, R. V., Alex, L. A., Bourret, R. B., and Simon, M. I. (1993) *Nature 365*, 343−347.
4. Borkovich, K. A., and Simon, M. I. (1990) *Cell 63*, 1339−1348.
5. Ninfa, E. G., Stock, A., Mowbray, S., and Stock, J. (1991) *J. Biol. Chem. 266*, 9764−9770.
6. Hess, J. F., Bourret, R. B., and Simon, M. I. (1988) *Nature 336*, 139−143.
7. Surette, M. G., Levit, M., Liu, Y., Lukat, G., Ninfa, E. G., Ninfa, A., and Stock, J. B. (1996) *J. Biol. Chem. 271*, 939−945.
8. Zhou, H., and Dahlquist, F. W. (1997) *Biochemistry 36*, 699−710.
9. Sanders, D. A., Gillece-Castro, B. L., Stock, A. M., Burlingame, A. L., and Koshland, D. E., Jr. (1989) *J. Biol. Chem. 264*, 21770−21778.
10. Welch, M., Osawa, K., Aizawa, S.-I., and Eisenbach, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8787−8791.
11. Hess, J. F., Oowasa, K., Kaplan, N., and Simon, M. I. (1988) *Cell 53*, 79−87.
12. Stock, A. M., Wylie, D. C., Mottonen, J. M., Lupas, A. N., Ninfa, E. G., Ninfa, A. J., Schutt, C. E., and Stock, J. B. (1988) *Cold Spring Harbor Symp. Quantum Biol. 53*, 49−57.
13. Hess, J. F., Bourret, R. B., Oosawa, K., Matsumura, P., and Simon, M. I. (1988) *Cold Spring Harbor Symp. Quantum Biol. 53*, 41−48.
14. Lukat, G. S., Stock, A. M., and Stock, J. B. (1990) *Biochemistry 29*, 5436−5442.
15. Stock, A. M., Martinez-Hackert, E., Rasmussen, B. F., West, A. H., Stock, J. B., Ringe, D., and Petsko, G. A. (1993) *Biochemistry 32*, 13375−13380.
16. Lukat, G., McCleary, W. R., Stock, A. M., and Stock, J. B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 718−722.
17. Jencks, W. P., and Gilchrist, M. (1965) *J. Am. Chem. Soc. 87*, 3199−3209.
18. Benkovic, S. J., and Sampson, E. J. (1971) *J. Am. Chem. Soc. 93*, 4009−4016.
19. Allen, G. W., and Haake, P. (1976) *J. Am. Chem. Soc. 98*, 4990−4996.
20. Stock, J. B., Surette, M. G., Levit, M., and Park, P. (1995) in *Two-Component Signal Transduction* (Hoch, J. A., and Silhavy, T. J., Eds.) pp 25−51, American Society for Microbiology, Washington, DC.
21. Stadtman, E. R. (1957) *Methods Enzymol. 3*, 228−231.
22. Sheridan, R. C., McCullough, J. F., and Wakefield, Z. T. (1972) *Inorg. Synth. 13*, 23−26.
23. Rathlev, T., and Rosenberg, T. (1956) *Arch. Biochem. Biophys. 65*, 319−339.
24. Bourret, R. B., Hess, J. F., and Simon, M. I. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 41−45.
25. Hess, J. F., Bourret, R. B., and Simon, M. I. (1991) *Methods Enzymol. 200*, 188−204.

26. Bourret, R. B., Drake, S. K., Chervitz, S. A., Simon, M. I., and Falke, J. J. (1993) *J. Biol. Chem. 268*, 13089−13096.
27. Hess, J. F., Oosawa, K., Matsumura, P., and Simon, M. I. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7609−7613.
28. Stewart, R. C. (1997) *Biochemistry 36*, 2030−2040.
29. Lipmann, F., and Tuttle, L. C. (1947) *Arch. Biochem. 13*, 373−377.
30. Perrin, D. D., Ed. (1982) *Ionization Constants of Inorganic Acids and Bases in Aqueous Solution*, 2nd ed., Pergamon Press, Oxford.
31. Volz, K. (1993) *Biochemistry 32*, 11741−11753.
32. Lukat, G. S., Lee, B. H., Mottonen, J. M., Stock, A. M., and Stock, J. B. (1991) *J. Biol. Chem. 266*, 8348−8354.
33. Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman and Co., New York.
34. Stock, A. M., Koshland, D. E., Jr., and Stock, J. B. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7989−7993.
35. Matsumura, P., Rydel, J. J., Linzmeier, R., and Vacante, D. (1984) *J. Bacteriol. 160*, 36−41.
36. McCleary, W. R., and Stock, J. B. (1994) *J. Biol. Chem. 269*, 31567−31572.
37. Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, McGraw-Hill, New York.
38. Koshland, D. E., Jr. (1952) *J. Am. Chem. Soc. 74*, 2286−2292.
39. Zapf, J. W., Hoch, J. A., and Whitely, J. M. (1996) *Biochemistry 35*, 2926−2933.
40. Ganguli, S., Wang, H., Matsumura, P., and Volz, K. (1995) *J. Biol. Chem. 270*, 17386−17393.
41. Williams, R. J. P. (1993) in *Magnesium and the Cell* (Birch, N. J., Ed.) pp 15−30, Academic Press, London.

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