Effects of Phosphorylation, Mg^{2+}, and Conformation of the Chemotaxis Protein CheY on Its Binding to the Flagellar Switch Protein FliM†

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ABSTRACT: CheY is the response regulator of bacterial chemotaxis. Previously, we showed that CheY binds to the flagellar switch protein FliM and that this binding is increased upon phosphorylation of CheY [Welch, M., Oosawa, K., Aizawa, S.-I., & Eisenbach, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 8787–8791]. Here, we demonstrate that it is the phosphorylated conformation of CheY, rather than the phosphate group itself, that is recognized and bound by FliM. We found that subsequent to the phosphorylation of CheY, Mg^{2+} was not required for the binding of CheY to FliM. However, phosphorylation of CheY did cause a change in the coordination properties of Mg^{2+} in the acid pocket of the protein. This change in the coordination of Mg^{2+} required the presence of the absolutely conserved residue Lys109. When Lys109 was substituted by arginine, the resulting CheY protein was unable to adopt an active conformation upon phosphorylation, and the protein was not bound by FliM. Surprisingly, the CheY13DK mutant protein, which is active *in vivo* but cannot be phosphorylated *in vitro*, exhibited only a low level of FliM binding activity, suggesting that its ability to cause clockwise rotation in the cell is not due to a constitutively high level of FliM binding. On the basis of these findings, we propose a mechanism for CheY activation by phosphorylation.

The essence of bacterial chemotaxis lies in modulation of the direction of flagellar rotation (Larsen *et al.*, 1974). The CheY protein plays a central role in this process in its capacity as a clockwise (CW)[1] rotation signal (Clegg & Koshland, 1984; Ravid *et al.*, 1986). CheY is a freely diffusible cytoplasmic protein which is phosphorylated by a receptor-coupled kinase (CheA) in response to changes in the ligand occupancy of receptors at the cell surface (Schuster *et al.*, 1993). Attractants suppress CheY phosphorylation while repellents are believed to enhance it (Borkovich *et al.*, 1989). Phosphorylated CheY binds to FliM (Welch *et al.*, 1993), which is one of the proteins that constitute the switch complex of the flagellar motor, and elicits CW rotation (Clegg & Koshland, 1984; Ravid *et al.*, 1986). [The default mode of rotation is counterclockwise (CCW) (Eisenbach & Adler, 1981; Eisenbach *et al.*, 1990; Parkinson & Houts, 1982; Ravid & Eisenbach, 1984; Wolfe *et al.*, 1987).] The mechanism by which CW rotation is generated subsequent to binding is currently not understood.

CheY is a member of a larger family of homologous proteins (Parkinson & Kofoid, 1992; Stock *et al.*, 1985; Volz, 1993), all of which are involved in responding to changes in the extracellular environment. The members of this family are characterized by absolute conservation of three residues, corresponding to residues Asp13, Asp57 (the site of phosphorylation), and Lys109 in CheY (Bourret *et al.*, 1990; Lukat *et al.*, 1991). In the three-dimensional structure of CheY, the acidic residues map into close proximity, creating a constellation known as the acid pocket (which also includes Asp12) (Stock *et al.*, 1989; Volz & Matsumura, 1991). The nearby Lys109 residue is constrained by bonding with the carboxyl group of Asp57. The acid pocket binds a single divalent metal ion (Lukat *et al.*, 1990), probably Mg^{2+} *in vivo* (Needham *et al.*, 1993), which is essential for phosphotransfer from phosphorylated CheA to CheY. Phosphorylation of the Asp57 carboxyl moiety transmits a long range conformational change through the molecule but does not significantly displace the Lys109 side chain (Drake *et al.*, 1993).

In our earlier work, we showed that CheY binds directly to the FliM component of the switch complex [no binding was observed to either of the other two switch proteins, FliG and FliN (Welch *et al.*, 1993)]. This binding was increased under conditions which generated a constant steady-state level of CheY phosphorylation. However, since CheY phosphorylation requires the presence of Mg^{2+}, we could not distinguish whether CheY phosphorylation *per se* was responsible for the increase in FliM binding, or whether CheY phosphorylation *and* Mg^{2+} were required for the binding. The purpose of this study was to investigate the roles of Mg^{2+}, phosphorylation, and the conserved residues of CheY in CheY–FliM binding.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Protein Purification and Radiolabeling. Radiolabeled FliM was prepared from *Escherichia coli* strain BL21(DE3)pLysS (Studier *et al.*, 1990) carrying plasmid pKOT179 (overproducing FliM + FliN) as previously described (Welch *et al.*, 1993), except that in some cases FliM was labeled using L-[4,5-^{3}H]leucine (190 Ci/mmol) instead of L-[U-^{14}C]leucine (300 mCi/mmol). Both radiolabeled products were obtained from Amersham. FliM composed >70% of the total protein in the labeled preparations as estimated by gel scanning densitometry of wet (stained) gels within the linear region of the densitometer response.

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[1] Abbreviations: CCW, counterclockwise; CheY~P, phosphorylated CheY; CW, clockwise.

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Wild-type CheY was purified from RP1616 (a Δ*cheZ* mutant received from J. S. Parkinson) carrying plasmid pRL22-(Δ*PvuII*) as previously described (Barak *et al.*, 1992) except that EDTA was omitted from the protocol. Mutant forms of CheY (CheY13DK, CheY57DE, or CheY109KR) were expressed from the appropriate pRBB40 derivative (received from R. B. Bourret and M. I. Simon) in strain K0641*recA* (Bourret *et al.*, 1990) (a Δ*cheY* mutant) and purified in the same way as wild-type CheY. The pure CheY was estimated from its absorbance at 280 nm in 6 M urea using ϵ_{280} = 6970 M^{-1} cm^{-1}. Both wild-type and mutant forms of CheY were coupled to CNBr-activated Sepharose 4B beads (Pharmacia) as previously described (Welch *et al.*, 1993) and stored at 4 °C. The beads were used within 3 weeks of coupling. The extent of immobilization was >98% of the added CheY, as determined by the Bradford technique (Bradford, 1976). Control beads carried BSA instead of CheY (Welch *et al.*, 1993).

Determination of Switch Protein Binding to CheY. Binding assays were carried out as previously described (Welch *et al.*, 1993) with the following modifications. Beads carrying immobilized CheY were suspended to homogeneity in 50 mM Tris-HCl, pH 7.9 (hereafter, Tris buffer), and dispensed in aliquots of 75–100 μL (as indicated) in Eppendorf tubes. Stabilizer mix (40 μL, consisting of 10 mg/mL BSA, 3.4 M glycerol, and 12.5 mM $MgCl_2$ in Tris buffer) was added, and the contents were thoroughly but gently mixed. [^{14}C]FliM (41.5 μM, ~280 dpm/μL) or [^{3}H]FliM (14.3 μM, ~1700 dpm/μL) was added to each tube in the amounts indicated. When necessary, acetyl phosphate (Sigma), or phosphoramidate [prepared according to Sheridan *et al.* (1971)], was added from freshly prepared 0.5 or 1 M stock solutions to the concentrations indicated. The final volume of each reaction mixture was adjusted to 250 μL with Tris buffer. The mixtures were incubated at room temperature (24 °C) for 15 min. The beads were washed once in 1 mL of ice-cold Tris buffer containing 2 mM $MgCl_2$ (±phospho-donor as appropriate).

Determination of the Mg^{2+} Dependence of CheY Binding to FliM. CheY and BSA beads were prewashed in Tris buffer containing 100 mM EDTA followed by washing and equilibration in Tris buffer only. As a control, another batch of CheY (and BSA) beads was treated similarly except that no EDTA was used. Aliquots (100 μL containing 7 μg of CheY/mg dry weight of beads, 5 nmol of immobilized CheY in total) of bead suspension were mixed with 40 μL of stabilizer mix identical to that described above except without Mg^{2+}. $MgCl_2$ was added back to the reaction mixture from concentrated stock solutions to the indicated final concentrations. [^{3}H]-FliM (30 μL, containing 0.43 nmol of FliM, 52 000 dpm) was added and the volume adjusted to 250 μL with Tris buffer. When necessary, phosphorylating conditions were achieved by addition of phosphoramidate to a final concentration of 20 mM. The mixture was incubated at 24 °C for 15 min before pelleting the beads and washing as described above. The wash solutions contained the appropriate concentration of $MgCl_2$ (±20 mM phosphoramidate as appropriate).

Preparation of Phosphorylated CheY Beads Depleted of Mg^{2+}. CheY beads (500 μL, containing 40 nmol of CheY; 8 μg of CheY/mg dry weight of beads) or BSA beads were mixed with 500 μL of Tris buffer containing acetyl phosphate (36 mM) and $MgCl_2$ (4 mM). After 2 min at room temperature, 500 μL of ice-cold 300 mM EDTA was added, and the beads were pelleted by centrifugation. The bead pellet was washed once in ice-cold 100 mM EDTA (in Tris buffer) and then 3 times in ice-cold 75 mM EDTA. Finally, the beads were washed 5 times in ice-cold 2 mM EDTA. The beads were pelleted by centrifugation, and the supernatant was removed until the meniscus just reached the top of the bead pellet (which occupied a packed volume of 300 μL). The bead pellets were then resuspended in 200 μL of ice-cold Tris buffer containing 2 mM EDTA and dispensed into separate aliquots (of 100 μL volume) to assay FliM binding. Subsequent to this step, all operations were timed and completed within 20 min. As controls, CheY and BSA beads not pretreated with acetyl phosphate were washed as above in either EDTA or Tris buffer (without EDTA) before resuspending in 2 mM EDTA. Binding assays consisted of CheY beads (containing 8 nmol of immobilized CheY), stabilizer mix (40 μL, identical to that described under Experimental Procedures except without $MgCl_2$) and [^{3}H]FliM (30 μL, 0.43 nmol of FliM) in a final volume of 250 μL. When phosphorylating conditions were required, $MgCl_2$ (5 mM) and acetyl phosphate (18 mM) were added. The binding experiments were carried out at room temperature (21 °C).

CheY Phosphorylation. Acetyl [^{32}P]phosphate was synthesized according to Stadtman (1957). Aliquots of acetyl [^{32}P]phosphate were mixed with 5 nmol of CheY (wild type or mutant) in the presence of 2 mM $MgCl_2$ in a final volume of 20 μL. The reaction mixture was allowed to stand at room temperature (21 °C) for 12 min before being terminated with 5 μL of 5× concentrated electrophoresis sample buffer. Samples of 10 μL were immediately run on 15% SDS–polyacrylamide gels. The CheY band was visualized by brief staining in Coomassie Blue G-250 at 0 °C followed by destaining at the same temperature. The bands were cut out and dissolved overnight at 80 °C in 0.8 mL of 30% hydrogen peroxide, and the radioactivity was measured by scintillation counting. Standards of acetyl [^{32}P]phosphate, appropriately diluted, were counted with the (dissolved) background gel slices. To determine the Mg^{2+} dependence of CheY phosphorylation, the same method was used except that the reaction mixture consisted of CheY (5 nmol), 20 mM acetyl [^{32}P]-phosphate (2×10^{14} cpm/mol), and $MgCl_2$ (at the appropriate final concentration) in a reaction volume of 20 μL.

Fluorescence Quenching. Mg^{2+} binding to CheY was determined according to Lukat *et al.* (1990) using a Perkin Elmer LS50B luminescence spectrometer with excitation and emission wavelengths set at 295 and 346 nm, respectively. CheY (8–20 μmol, depending on the experiment) was diluted into 600 μL of Tris buffer containing the appropriate concentration of $MgCl_2$, and the fluorescence was recorded. Subsequently, phosphoramidate was added from a freshly prepared 1 M stock solution to a final concentration of 20–33 mM (depending on the experiment) and the fluorescence was again recorded. The level of CheY phosphorylation was considered to be proportional to the enhancement of fluorescence quenching in the presence of the phospho-donor [Lukat *et al.* (1992); M. Welch and M. Eisenbach, unpublished observations]. At high concentrations of Mg^{2+} (>50 mM $MgCl_2$), the fluorescence signal, which until this point was tending toward a minimum, began to increase again. Control experiments using L-tryptophan (1.7 μM) instead of CheY showed that this was a nonspecific effect, and the data were corrected accordingly. It should be noted that the added phospho-donors remained soluble in high Mg^{2+} concentrations (no change in light scattering at 590 nm was observed upon the addition of the donors to increasing Mg^{2+} concentrations). Experiments were carried out at room temperature (21 °C).

Preparation of Mg^{2+}-Depleted CheY~P for Fluorescence Studies. CheY (149 nmol in 70 μL) was mixed with an equal

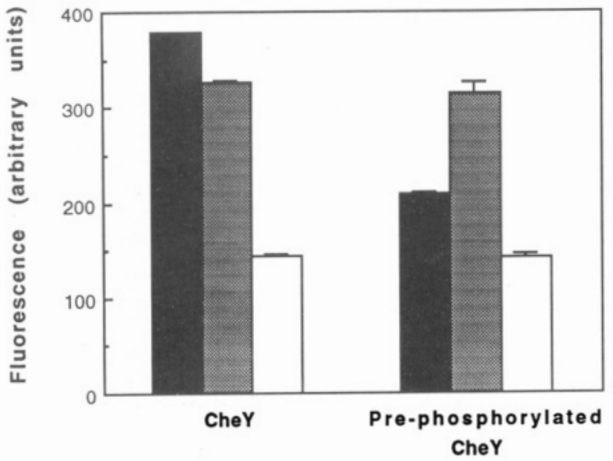

FIGURE 1: Depletion of Mg^{2+} from phosphorylated CheY traps the protein in its active conformation. Mg^{2+}-depleted CheY and Mg^{2+}-depleted CheY~P (denoted in the figure as CheY and prephosphorylated CheY, respectively) were prepared as described under Experimental Procedures. The cuvettes contained 100 mM EDTA (black columns), 2 mM $MgCl_2$ (gray columns), or 2 mM $MgCl_2$ plus 18 mM acetyl phosphate (white columns). Addition of acetyl phosphate to the cuvettes containing EDTA resulted in no change in fluorescence. The data are the means (±SD) of two experiments carried out with the same batch of purified CheY. The results with another batch of CheY were comparable.

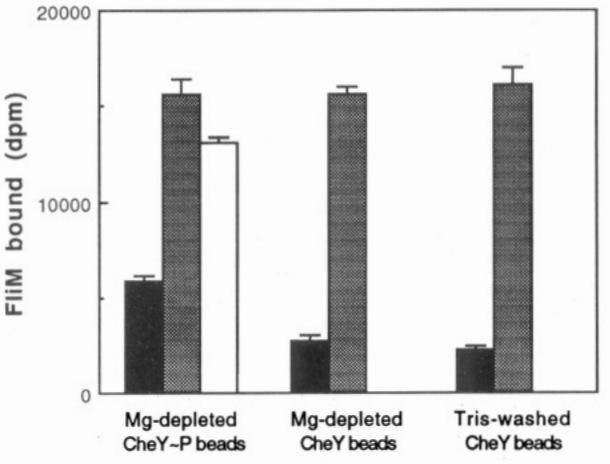

FIGURE 2: Mg^{2+}-depleted CheY~P retains its ability to bind to FliM. Mg^{2+}-depleted CheY~P beads, Mg^{2+}-depleted nonphosphorylated CheY beads, and control (Tris-washed) CheY beads were prepared and assayed for binding as described under Experimental Procedures. The results are the average of three independent experiments (±SD) and show the *net* amount of FliM bound to each of the CheY bead samples (i.e., after subtracting the amount of FliM bound to the appropriately treated BSA beads). Black columns, FliM bound in the absence of Mg^{2+}; gray columns, FliM bound under phosphorylating conditions; white column, FliM bound to Mg^{2+}-depleted CheY~P corrected for dephosphorylation as described under Experimental Procedures.

volume of Tris buffer containing 4 mM Mg^{2+} plus 36 mM acetyl phosphate, or, as a control, Tris buffer only without additions. Aliquots (5 μL, containing 5.3 nmol of CheY) of these mixtures were placed in Eppendorf tubes and diluted by injecting 700 μL of 100 mM EDTA (in Tris buffer) or 2 mM $MgCl_2$ (in Tris buffer). The resulting solutions were then transferred to quartz cuvettes (1 mL volume), and the fluorescence was measured. When steady-state phosphorylating conditions were required, acetyl phosphate was added to the cuvettes (18 mM final concentration). To measure the decay rate of Mg^{2+}-depleted CheY~P, the same procedure was followed except that 700 μL of 2 mM EDTA were used to dilute the 5 μL sample of phosphorylated CheY [considering a K_d of 2×10^{-9} M for $Mg{\cdot}EDTA^{2-}$ (Welcher, 1958), this concentration of EDTA should effectively chelate all the Mg^{2+} present, both free and CheY-bound]. The sample was then transferred into a cuvette and the fluorescence recorded at intervals of 2 min.

RESULTS

To examine the roles of phosphorylation and Mg^{2+} on CheY–FliM binding, we studied whether phosphorylated CheY (CheY~P), which had been depleted of Mg^{2+}, retained its active conformation and, as a consequence of this, its ability to bind FliM. To do this, we employed two complementary approaches: monitoring the Trp58 fluorescence of CheY [which is sensitive to the conformation of the protein and has been shown to be quenched when the protein is activated by phosphorylation (Lukat *et al.*, 1992)] and measuring directly the binding of CheY to FliM.

Effects of Mg^{2+} and Phosphorylation on Trp58 Fluorescence. Samples of CheY were incubated with either buffer alone or buffer containing Mg^{2+} and acetyl phosphate (as a phospho-donor). Subsequently Mg^{2+} was removed by addition of excess EDTA, yielding Mg^{2+}-depleted CheY and Mg^{2+}-depleted CheY~P, respectively, and the fluorescence of each was measured. As shown in Figure 1 (black columns), the fluorescence of Mg^{2+}-depleted CheY~P was substantially quenched relative to that of Mg^{2+}-depleted CheY. Such a difference was not observed when EDTA was replaced with Mg^{2+} (dephosphorylating conditions, gray columns) or with Mg^{2+} plus acetyl phosphate (phosphorylating conditions, white columns). The quenching upon the addition of Mg^{2+} and the further quenching upon phosphorylation (Figure 1, left group of columns) were reported earlier (Lukat *et al.*, 1990, 1992; Needham *et al.*, 1993).

Effects of Mg^{2+} and Phosphorylation on CheY Binding to FliM. In the second approach to determine whether Mg^{2+}-depleted CheY~P retains it activity, we compared its ability to bind FliM with that of active CheY (i.e., in the presence of Mg^{2+} and under conditions of steady-state phosphorylation). Bead-bound CheY~P (prepared by incubating CheY beads with Mg^{2+} plus acetyl phosphate as described under Experimental Procedures) was depleted of Mg^{2+} by washing in excess EDTA (100 mM). As a control, nonphosphorylated CheY beads were put through the same EDTA wash procedure. Another control (for the possibility that the high concentration of EDTA might affect the integrity of the CheY beads) was CheY beads washed in Tris buffer in the absence of EDTA. All three sets of beads were then suspended in 2 mM EDTA (to keep the ionic strength low), and the binding of FliM to each set of beads was assayed under two conditions: (*i*) in the absence of Mg^{2+} (i.e., in the presence of 2 mM EDTA) and (*ii*) under steady-state phosphorylating conditions (i.e., in the presence of Mg^{2+} and acetyl phosphate). In the absence of Mg^{2+}, the amount of FliM bound to Mg^{2+}-depleted CheY~P beads was higher than the amount bound to the two sets of control beads (Figure 2, black columns). Under steady-state phosphorylating conditions, the binding was comparable in all three sets of beads (gray columns). In line with the observation of Sanders *et al.* (1989), we found in a separate experiment that CheY~P is spontaneously dephosphorylated even in the presence of 2 mM EDTA. This was done by measuring the change in fluorescence quenching of Mg^{2+}-depleted CheY~P as a function of time. From this we calculated that the half-lifetime of CheY~P under these conditions was 12 min. When we took this dephosphorylation into consideration and calculated the relative level of phos-

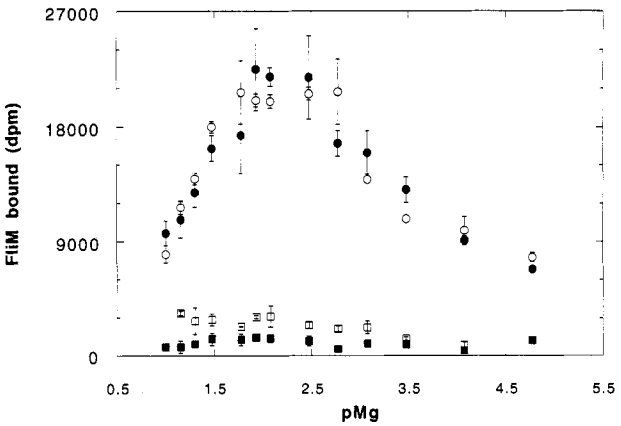

FIGURE 3: Mg^{2+} dependence of FliM binding to CheY. $MgCl_2$ was titrated against a fixed amount of FliM and a fixed amount of CheY beads, and the amount of FliM bound was determined as described under Experimental Procedures. Squares, FliM bound in the absence of phosphorylation; circles, FliM bound under phosphorylating conditions. Black symbols relate to CheY beads prewashed in Tris buffer; white symbols relate to CheY beads prewashed in EDTA (i.e, depleted of Mg^{2+}).

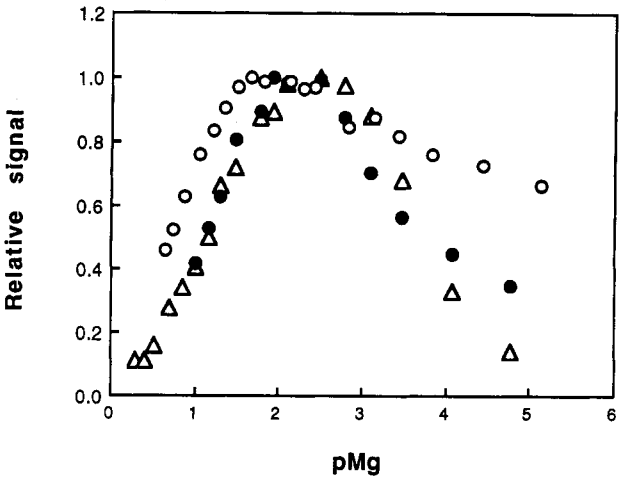

FIGURE 4: Mg^{2+} dependence of CheY phosphorylation and of FliM binding to CheY under phosphorylating conditions is similar. The Mg^{2+} dependence of FliM binding to the Tris- and EDTA-washed CheY beads (under phosphorylating conditions) in Figure 3 was averaged (black circles) and normalized relative to the maximal amount of FliM bound. The Mg^{2+} dependence of CheY phosphorylation, measured either by direct labeling with acetyl [^{32}P]phosphate (white circles) or by fluorescence quenching (triangles), was similarly normalized. In the case of acetyl [^{32}P]phosphate, when the relative phosphorylation level was 1, we calculated that 36% of the CheY molecules were phosphorylated. Error bars are omitted for clarity but did not exceed ±5% in the experiments where CheY phosphorylation was measured (by either method).

phorylation at the initiation of the binding assay, the value of binding was close to that under steady-state phosphorylating conditions (Figure 2, white column). (All other columns were under steady-state conditions, and therefore the phosphorylation level did not change with time.)

The results obtained in the above two approaches indicate that, even in the absence of Mg^{2+}, phosphorylated CheY retains its FliM binding activity (i.e., its active conformation).

Dependence of CheY Binding to FliM on the Concentration of Mg^{2+}. Now that we found that Mg^{2+} is not obligatory for maintaining the active conformation of CheY~P, we investigated whether it can modulate the extent of CheY–FliM binding. For this purpose, we measured FliM binding to CheY beads at various Mg^{2+} concentrations. Since CheY purified under our conditions (i.e., in the absence of EDTA) has been reported to contain endogenous Mg^{2+} (Kar *et al.*, 1992), we used two sets of CheY beads: beads which either had or had not been depleted of Mg^{2+} (by prewashing in EDTA). Under nonphosphorylating conditions, the FliM binding to both sets of beads was low, as expected (Figure 3, squares). The binding of FliM to the Mg^{2+}-depleted CheY beads (white squares), but not to the nondepleted beads (Tris-washed beads; black squares), showed a weak dependence on Mg^{2+} concentration. The different behavior of the two sets of CheY beads is consistent with the observations of Kar *et al.* (1992) showing that the conformation of EDTA-treated CheY is subtly different than that of CheY pretreated with Tris only. In contrast, under steady-state phosphorylating conditions, the binding of FliM to both the Mg^{2+}-depleted and nondepleted CheY beads (Figure 3, white and black circles, respectively) showed a strong and similar dependence on the Mg^{2+} concentration. The strong dependence of the binding under phosphorylating conditions on the Mg^{2+} concentration may reflect the dependence of CheY phosphorylation and/or the binding itself on the Mg^{2+} concentration. To distinguish between these possibilities, we examined the dependence of CheY phosphorylation on the Mg^{2+} concentration.

Dependence of CheY Phosphorylation on the Mg^{2+} Concentration. The relative level of CheY phosphorylation at different concentrations of Mg^{2+} was measured by both direct labeling and fluorescence quenching. Since in the above experiments we used acetyl phosphate and phosphoramidate as phospho-donors, we measured the phosphorylation with each of them. With acetyl [^{32}P]phosphate as a phospho-donor, there was a moderate increase in the phosphorylation level of CheY with increasing Mg^{2+} concentrations (i.e., decreasing pMg), reaching a maximum at 20–30 mM (Figure 4, white circles). At higher Mg^{2+} concentrations (low pMg), there was a rather sharp decrease in the phosphorylation level. Qualitatively similar results were obtained with phosphoramidate as a phospho-donor (Figure 4, white triangles). In this case, we could not measure phosphorylation by direct labeling because of the unavailability of radioactive precursors for the synthesis of phosphoramidate. Instead we used fluorescence quenching. The Mg^{2+} dependence of the phosphorylation-related fluorescence quenching was very similar to that of FliM binding to CheY in the presence of phosphoramidate (Figure 4, black circles). This suggests that the Mg^{2+} dependence of FliM binding to CheY under phosphorylating conditions actually reflects the Mg^{2+} dependence of CheY phosphorylation.

Binding of Selected CheY Mutants to FliM. The results above show that CheY phosphorylation alone is sufficient to facilitate its binding to FliM. They also show that upon phosphorylation, the acid pocket undergoes a significant conformational change which does not require Mg^{2+} for its maintenance. In order to determine whether FliM recognizes the *conformation* of phosphorylated CheY or the phosphoaspartate moiety itself, and in order to examine the role of the highly conserved acid pocket residues in the mechanism of CheY activation, we studied the binding of selected CheY mutants to FliM.

The mutant forms of CheY we examined were CheY13DK which is CW-biased *in vivo* but is only slightly phosphorylated *in vitro*, CheY109KR which is exclusively CCW *in vivo* in spite of being highly phosphorylated *in vitro*, and CheY57DE which is also exclusively CCW *in vivo* but is nonphosphorylatable *in vitro* (Bourret *et al.*, 1990; Lukat *et al.*, 1991). Identical amounts of wild-type and mutant CheY proteins were separately immobilized onto CNBr-activated Sepharose

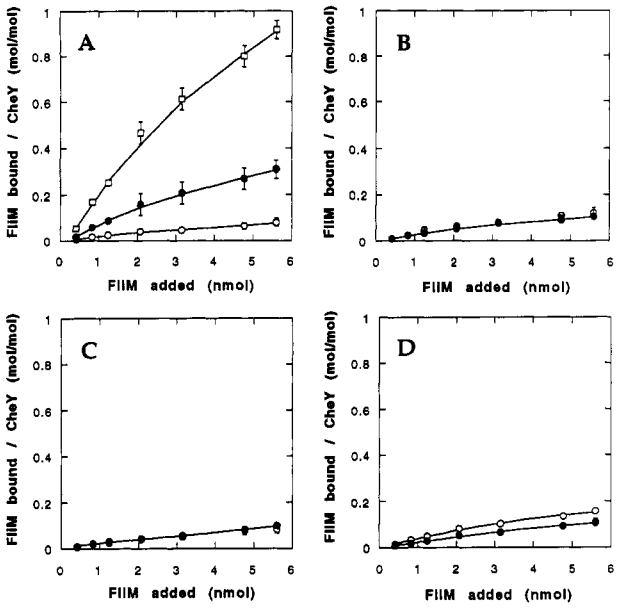

FIGURE 5: Binding of FliM to CheY mutant proteins. Identical amounts of CheY (mutant and wild type) were immobilized onto CNBr-activated Sepharose CL-6B beads (8.7 μg of CheY/mg dry weight of beads). Aliquots of these beads (75 μL volume, containing 3.5 nmol of CheY) were mixed with 40 μL of stabilizer mix and 5–100 μL of [^{14}C]FliM (41.5 μM, 280 dpm/μL). Acetyl phosphate was added to a concentration of 18 mM when necessary. The final volume of the reaction mix was adjusted to 250 μL with Tris buffer. The amount of FliM bound to the beads was determined as described under Experimental Procedures. The level of CheY phosphorylation under these conditions was determined separately using acetyl [^{32}P]-phosphate (see Experimental Procedures). The data show the net amount of FliM bound to the CheY beads (i.e., after subtracting the FliM bound to the BSA control beads). White circles, FliM binding under nonphosphorylating conditions; black circles, FliM binding in the presence of acetyl phosphate. (A) Wild-type CheY beads (white squares indicate data corrected for FliM bound per phosphorylated CheY); (B) CheY57DE beads; (C) CheY109KR beads (since phosphorylating conditions did not change the amount of FliM bound to these beads, we did not correct the data for the amount of FliM bound per phosphorylated CheY109KR); (D) CheY13DK beads.

beads. The ability of each species of CheY to bind FliM was then measured under both nonphosphorylating and phosphorylating conditions (Figure 5A–D, white and black circles, respectively). Under nonphosphorylating conditions, none of the mutants were able to bind FliM to a degree much greater than that of the nonphosphorylated wild-type protein. One particularly surprising result was that the CheY13DK mutant protein bound FliM to a level only marginally greater than that of the nonphosphorylated wild-type CheY (under phosphorylating conditions, the binding of FliM to CheY13DK even decreased somewhat). Under phosphorylating conditions, only wild-type CheY exhibited an increase in FliM binding. In this regard, it is interesting to note that the CheY109KR mutant protein, which was stably phosphorylated (Table 1), did not increase its binding to FliM in the presence of acetyl phosphate. This suggests that FliM does not recognize the phosphate group on CheY, but rather a conformation of CheY which is induced upon phosphorylation.

To assess the validity of our earlier conclusions, we measured (using fluorescence) the ability of the mutant CheY proteins to bind Mg^{2+}. Neither CheY13DK nor CheY57DE bound Mg^{2+} (Table 1). Since Mg^{2+} is necessary for phosphorylation, this finding may explain why these proteins are not phosphorylated. In line with this possibility, the CheY109KR mutant protein (which is phosphorylated) did bind Mg^{2+}. However, unlike the wild-type protein, the Mg^{2+}-dependent

Table 1: Biochemical Properties of CheY Mutant Proteins

	wild type	CheY13DK	CheY57DE	CheY109KR
phenotype[a]	CW	CW	CCW	CCW
phosphorylation[b]	+	–	–	+
fluorescence quenching by Mg^{2+}	+	–	–	+
fluorescence quenching by AcP[c]	+	–	–	–
FliM binding	+	–	–	–

[a] The phenotype of each strain refers to the direction of flagellar rotation in gutted cells [strain RP1091, Δ(*cheA-cheZ*] containing plasmids encoding the appropriate CheY derivative. [b] Phosphorylation was measured by direct labeling using acetyl [^{32}P]phosphate. [c] Fluorescence quenching by acetyl phosphate in the presence of Mg^{2+}.

fluorescence quenching of CheY109KR was not enhanced under phosphorylating conditions (Table 1). This result appears to indicate that (*i*) the physical presence of a phosphate group in the acid pocket *per se* does not cause fluorescence quenching and (*ii*) in CheY109KR phosphorylation is uncoupled from a change in the environment of the acid pocket (see Discussion).

DISCUSSION

The main observations made is this study are as follows: (*i*) In wild-type CheY, the phosphorylated protein retains its ability to bind to FliM even after removal of Mg^{2+} by EDTA treatment. (*ii*) CheY phosphorylation causes a change in the conformation of the acid pocket; this conformation does not require Mg^{2+} for its preservation. (*iii*) Mutation of the acid pocket region residues in CheY greatly diminishes the ability of these mutant proteins to bind to FliM. Below we discuss each of these observations in depth.

Mg^{2+} Is Not Required for the Binding of CheY~P to FliM. The observation that CheY~P retains its ability to bind to FliM even after removal of Mg^{2+} by EDTA treatment (Figure 2) indicates that Mg^{2+} is not required for the binding of CheY~P to FliM. We did find that FliM binding to CheY exhibited a marked dependence on Mg^{2+} concentration (Figure 3) but this was found to correlate with the Mg^{2+} dependence of CheY phosphorylation, measured by two independent assays (Figure 4). Collectively, these results suggest that once phosphorylated, the Mg^{2+} bound in the acid pocket of CheY becomes dispensable for the binding of CheY to FliM. We conclude that phosphorylation alone is both necessary and sufficient for promoting the binding of wild-type CheY to FliM [but it is apparently not sufficient for facilitating CW rotation (Barak & Eisenbach, 1992)].

Trp58 Fluorescence Reflects the Conformation of CheY. The fluorescence quenching of Trp58 was used in the past for monitoring metal ion binding to CheY (Lukat *et al.*, 1990) and phosphorylation of CheY by low-molecular-weight phospho-donors (Lukat *et al.*, 1992). In our experiments, the fluorescence quenching was largely maintained even after removal of Mg^{2+} from CheY~P by EDTA treatment (Figure 1). This suggests that the phosphorylation-related fluorescence quenching reflects either the presence of the phosphate group on CheY or a change in the local conformation of CheY in the vicinity of Trp58 (i.e., in the acid pocket). The finding that the fluorescence of CheY109KR was not quenched upon phosphorylation (Table 1) favors the latter possibility. Since, unlike wild-type CheY, the CheY109KR mutant protein did not bind to FliM even when phosphorylated (Figure 5A *vs* 5C), we conclude that the quenching of Trp58 fluorescence reflects a conformational change that is a necessary prelude to CheY activation.

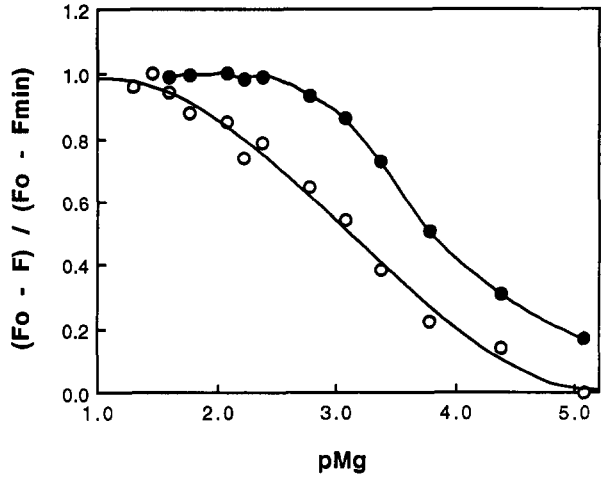

FIGURE 6: Phosphorylation of CheY increases its affinity for Mg^{2+}. The Mg^{2+} dependence of CheY fluorescence was measured as described under Experimental Procedures. The fluorescence quenching due to Mg^{2+} was plotted as the fraction of the maximal quenching observed, i.e., $(F_o-F)/(F_o-F_{min})$, where F_o is the initial fluorescence in the absence of Mg^{2+}, F is the fluorescence at any given concentration of Mg^{2+}, and F_{min} is the fluorescence at saturating concentrations of Mg^{2+}. F_{min} was determined by extrapolation to infinite Mg^{2+} concentration in plots of $1/(F_o - F)$ vs $1/[Mg^{2+}]$. White circles, CheY only; black circles, CheY plus phosphoramidate. The results are the mean values from three independent experiments. The standard deviations did not exceed ±2% of the value of the mean.

Phosphorylation of CheY Changes Its Mg^{2+} Binding Properties. The acid pocket of CheY is known to coordinate the Mg^{2+} in a rigid octahedral geometry (Stock *et al.*, 1993). The introduction of a relatively bulky and highly charged phosphate moiety into the acid pocket would be expected to cause some restructuring of the acid pocket and, in particular, of the ligands coordinating the Mg^{2+} (Stock *et al.*, 1992). If this is the case, we might expect that the binding of Mg^{2+} to phosphorylated CheY might change under phosphorylating conditions. To test this prediction, we transformed the data of the Mg^{2+}-dependent fluorescence quenching reported above so that the apparent binding constants can be calculated (Figure 6). Under nonphosphorylating conditions (white circles), the half-maximal quenching occurred at a Mg^{2+} concentration of 1 mM, which is consistent with earlier estimates of the K_d for the CheY·Mg^{2+} complex (Lukat *et al.*, 1990). Under phosphorylating conditions, generated by the addition of phosphoramidate (black circles), the half-maximal quenching was shifted to a lower Mg^{2+} concentration (~0.2 mM), in line with the prediction. Since the CheY109KR mutant protein does not exhibit phosphorylation-dependent fluorescence quenching (Table 1), we further suggest that the Lys-109 residue plays some role in this structural rearrangement.

Mutation of the Conserved Acid Pocket Residues Severely Reduces the Ability of CheY To Bind to FliM. Our data are in line with the notion that the conserved residues in the acid pocket are crucial for the mechanism of activation of CheY. Thus, the lack of FliM binding capability in CheY57DE and CheY109KR correlates with their inability to elicit CW rotation when expressed *in vivo*, and reinforces our earlier conclusions that both phosphorylation and the Lys109 residue are required if the protein is to adopt the active conformation. Furthermore, the observation that CheY109KR also did not bind well to FliM, in spite of being phosphorylated, indicates that the physical presence of a phosphate group in the acid pocket is not sufficient to promote switch binding. Our data do not exclude the previously suggested possibility (Bourret *et al.*, 1993) that Lys109 plays a direct role in the interaction between CheY and FliM. However, in view of the fact that this residue is required for the phosphorylation-dependent active conformation of CheY (Table 1 and text above), we feel that this possibility is less likely.

CheY13DK was able to bind FliM only marginally better than nonphosphorylated wild-type CheY, and certainly to a much lower level than phosphorylated wild-type CheY. Our data thus indicate that the CW-causing activity of CheY13DK *in vivo* cannot be attributed to the affinity of this protein for FliM. Two potential reasons may account for the CW-causing activity of CheY13DK *in vivo*. (*a*) An intracellular level of CheY13DK higher than that of wild-type CheY~P. This potential reason is consistent with the observations of Bourret *et al.* (1993), who noted that CheY13DK, when expressed at physiological levels (i.e., from the chromosome under the control of the wild-type promoter), was significantly less proficient at generating CW rotation than wild-type CheY~P (expressed in a Δ*cheZ* host). (*b*) The low binding of CheY13DK to FliM may be compensated by a high intrinsic CW-causing activity in those few molecules which do happen to bind to the switch (see below). This supports the contention that CheY binding to the switch and the subsequent generation of CW rotation may be separable processes, and is in line with previous observations of Kuo and Koshland (1989) indicating that the CCW to CW switching involves two or more kinetically-resolvable steps.

Implications for the Mechanism of CW Generation. The results obtained in this study indicate that the processes of CheY activation and CW generation by the switch complex may be broken down into three basic steps.

In the first step, CheY (with bound Mg^{2+}) is phosphorylated on Asp57. The introduction of the phosphate moiety into the confines of the acid pocket results in a structural reorganization of the ligands coordinating the Mg^{2+} or phosphate group. Our data suggest that this restructuring requires the presence or participation of the Lys109 residue. Such a restructuring is consistent with the result of Drake *et al.* (1993), who showed, using ^{19}F NMR studies on 4F-Phe-labeled CheY, that the 4F-Phe14 resonance (which is sensitive to the acid pocket environment) is significantly perturbed when the protein is phosphorylated.

In the second step, we suggest that the phosphorylated CheY acquires a new conformation. This step occurs only after the localized restructuring in the acid pocket, and results in the exposure of the FliM binding surface of CheY. [The existence of a FliM binding surface has been inferred from genetic analyses (Roman *et al.*, 1992; Sockett *et al.*, 1992).] This suggestion is supported by the long-range conformational change known to occur in CheY subsequent to phosphorylation (Drake *et al.*, 1993), and by genetic analyses which indicated that the *cheY* suppressors defining the FliM binding surface of CheY are specifically altered in their ability to transduce signals to the switch at some point subsequent to phosphorylation (Roman *et al.*, 1992). Our data indicate that once this activated conformation has been achieved, Mg^{2+} plays no further role in its maintenance.

In the third and final step, the activated CheY is bound, *via* its signaling surface, by the FliM component of the switch complex. Once bound to FliM, we suggest that the restructured acid pocket region in CheY is involved in the generation of CW rotation by the switch. The latter proposal comes from two observations. First, CheY13DK is active in causing CW rotation, but does not bind well to FliM. Second, experiments using ^{19}F NMR have shown that wild-type

CheY~P and CheY13DK may share a common structural feature; the acid pocket sensitive resonance (4F-Phe14) in both proteins is displaced (relative to non-phosphorylated CheY) to a similar extent (Bourret *et al.*, 1993; Drake *et al.*, 1993), indicating that these proteins may adopt similar conformations in their respective acid pockets. This common feature of both CheY13DK and wild-type CheY~P presumably accounts for their CW-causing activity.

A Common Activation Mechanism in CheY and Ras? The mechanistic implications of this model may extend to other signal-transducing systems. CheY is structurally homologous to Ras p21 (Artymiuk *et al.*, 1990; Chen *et al.*, 1990). This has led to speculation that the two proteins might share a common mechanism. In the case of Ras p21, high-resolution structures are available for both the active and inactive forms of the protein (Milburn *et al.*, 1990). A comparison between these structures shows that during activation, a conformational change occurs, exposing a signaling surface. The trigger for this conformational change is an alteration in the coordination of Mg^{2+} in the active site (Schlichting *et al.*, 1990). This mechanism of activation is very similar to the mechanism proposed by us for CheY.

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