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Phosphorylation-dependent binding of a signal molecule to the flagellar switch of bacteria

(chemotaxis/signal transduction/bacterial flagella/CheY/switch proteins)

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ABSTRACT **Regulation of the direction of flagellar rotation is central to the mechanism of bacterial chemotaxis. The transitions between counterclockwise and clockwise rotation are controlled by a "switch complex" composed of three proteins (FliG, FliM, and FliN) and located at the base of the flagellar motor. The mechanism of function of the switch is unknown. Here we demonstrate that the diffusible clockwise-signal molecule, the CheY protein, binds to the switch, that the primary docking site is FliM, that the extent of CheY binding to FliM is dependent upon the phosphorylation level of CheY, and that it is unaffected by the other two switch proteins. This study provides a biochemical demonstration of binding of a signal molecule to the bacterial switch and demonstrates directly that phosphorylation regulates the activity of this molecule.**

Bacteria respond to changes in the composition of their environment by modulating the direction of flagellar rotation, enabling them to swim toward attractants or away from repellents (1–3). The events leading to this process involve a signal transduction pathway composed of membrane-bound receptors and cytoplasmic proteins (for reviews, see refs. 4–7). Repellents are thought to activate CheA, a receptor-coupled kinase which phosphorylates CheY. Conversely, attractants deactivate CheA and thereby reduce the phosphorylation level of CheY (8, 9). In this way, the flux of information from the receptors is integrated into a common form: phosphorylated CheY. Changes in the phosphorylation level of CheY are believed to be sensed by a group of proteins located on the cytoplasmic face of the flagellar motor known as the "switch complex" (10), which causes the motor to adopt either a clockwise bias (if levels of phosphorylated CheY are high) or a counterclockwise bias (when phosphorylated CheY is low) (11). The mechanism of switch function is not known. Overproduction of CheY in a strain lacking all other chemotaxis proteins (12–14) or insertion of purified CheY into cell envelopes devoid of cytoplasm (15) generated clockwise rotation, indicating a direct interaction between CheY and the switch complex. A similar conclusion was obtained from an elegant genetic approach designed to detect interactions between chemotaxis proteins (10, 16–18). The recent cloning and expression to high levels of the proteins constituting the *Salmonella typhimurium* switch complex (FliG, FliM, and FliN) (K.O., T. Ueno, and S.-I.A., unpublished work) opened up the possibility to examine the CheY–switch interaction biochemically. In this study we took advantage of this development to identify directly the docking site of CheY on the switch and to establish the role of CheY phosphorylation in signal transduction.

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MATERIALS AND METHODS

Preparation of CheY Beads. CheY beads and control beads were prepared in parallel under identical conditions. Two samples (1-g dry weight each) of CNBr-activated Sepharose 4B beads (Pharmacia) were washed five times in 14 ml of 1 mM HCl and then three times in coupling buffer (0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3). CheY [5–45 mg, determined from absorbance at 280 nm by using a molar extinction coefficient of 6970 $M^{-1}\cdot cm^{-1}$ (19)] purified from *Escherichia coli* (11) was dialyzed twice against 2 liters of coupling buffer and added to one tube. An equal volume of coupling buffer was added to the other tube (containing control beads). Both tubes were mixed end-over-end overnight at 4°C. In this way 95–99% of the CheY became covalently immobilized. Bovine serum albumin (BSA, 20 mg), previously dialyzed against coupling buffer, was then added to both tubes in order to block unreacted cyano groups, and the incubation with mixing continued for 5–10 hr at 4°C. To complete the coupling, additional BSA (20 mg), this time in 0.1 M Tris·HCl (pH 7.9), was added to both tubes and incubated further for 5–10 hr. Finally the beads were washed in 50 mM Tris·HCl (pH 7.9) and stored at 4°C. Prior to use in the binding assay, the beads were washed and resuspended in 8–12 ml of Tris buffer. CheY57DE was produced and isolated as described earlier (20) from plasmid pRBB40.57DE (21), which was received from R. Bourret and M. Simon, and then immobilized as wild-type CheY.

Preparation of Specifically Radiolabeled Switch Proteins. *E. coli* strain BL21(DE3)/pLysS (22), carrying one of the plasmids pKOT113 (for overproducing FliG), pKOT177 (FliN), or pKOT179 (FliM plus FliN), was grown to OD_{590} = 0.5 in Luria broth and induced with isopropyl β-D-thiogalactopyranoside (IPTG, 1 mM) for 30 min. Specific radiolabeling of the cells was performed essentially as described (23). The cells (100 ml) were harvested, washed three times in T7 expression medium [H-1 minimal medium (24) supplemented with 1% (vol/vol) glycerol, 1 mM IPTG, and 0.01% (wt/wt) each amino acid except leucine], and resuspended to a final volume of 10 ml. Rifampicin was added (200 μg/ml) to 2-ml aliquots of this suspension and incubated for 20 min with good aeration by rapid agitation. Next, L-[^{14}C]leucine (10 μCi; 1 μCi = 37 kBq) was added to each sample and incubation continued for 1 hr. The cells were then harvested and washed once in T7 expression medium. In parallel, nonlabeled cells were prepared by inducing the same strains with IPTG in Luria broth for 2 hr. The radiolabeled cells were mixed with a large (>10-fold) excess of nonlabeled cells, the latter serving as a source of carrier protein in order to reduce losses of radiolabeled protein during extraction. This mixture of cells was resuspended in 50 ml of 50 mM Tris·HCl (pH 7.9) and sonicated on ice to completion. The particulate fraction

Abbreviation: BSA, bovine serum albumin.

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was separated from the soluble fraction by centrifugation (100,000 × *g* at 4°C for 90 min). FliN was obtained exclusively from the soluble fraction of cells containing pKOT177. It was prepared by ammonium sulfate (25% of saturation) precipitation, followed by dialysis to remove excess ammonium sulfate, and stored at −20°C. FliG and FliM were found in both the soluble and particulate fractions of cells containing pKOT113 and pKOT179, respectively. Since the particulate fraction (containing membranes and inclusion bodies) afforded the highest degree of enrichment and yield, this was used for further purification. The particulate fractions containing FliG and FliM/N were suspended in 18–20 ml of 7 M urea and centrifuged again. The supernatant was dialyzed twice (first for ≈6 hr and then overnight) against 2 liters of 50 mM Tris·HCl, pH 7.9/10 mM dithiothreitol/0.1 mM EDTA. In the case of pKOT113, FliG was obtained in the precipitate of the 18–54% ammonium sulfate fraction. In the case of pKOT179, we used 16% ammonium sulfate, which preferentially precipitated FliM, leaving FliN (and some FliM) in the supernatant. The ammonium sulfate precipitates of FliM and FliG were dissolved in 7 M urea to a low protein concentration (30 μg/ml for each) and refolded by dialysis against 50 mM Tris·HCl, pH 7.9/10 mM dithiothreitol/0.1 mM EDTA. The proteins were concentrated by ultrafiltration to a concentration of 1–2 mg/ml and stored at −20°C. The proteins obtained in this way were not pure but were specifically labeled and constituted 20–40% (according to batch) of the total protein as estimated by scanning densitometry of Coomassie-stained SDS/polyacrylamide gels.

Determination of the Extent of CheY Phosphorylation. CheY (5 nmol) in 50 mM Tris·HCl (pH 7.9) was aliquoted into tubes and [^{32}P]acetyl phosphate was added to give a final concentration of 0.1–26.5 mM. $MgCl_2$ was added to give a final concentration of 2 mM and the volume was adjusted to 20 μl with Tris buffer. The mixture was incubated for 12.5 min at 24°C and quenched by addition of 5 μl of 5× SDS sample buffer. Samples (15 μl) were immediately electrophoresed in an SDS/15% polyacrylamide gel. The gel was stained briefly with Coomassie blue G250 at 4°C to resolve the CheY bands, then destained at the same temperature to remove background ^{32}P contamination. The CheY bands were excised from the wet gel and dissolved in 30% H_2O_2 overnight at 80°C. The resultant clear solution was then counted in a scintillation counter to assay the amount of CheY phosphorylation. Phosphorylation was calibrated by a titration curve of free [^{32}P]acetyl phosphate in the presence of dissolved gel slices. We were unable to estimate phosphorylation of immobilized CheY directly on the beads due to the relatively high background binding of [^{32}P]acetyl phosphate.

RESULTS

To determine whether or not CheY binds to the switch proteins, we immobilized purified CheY onto a solid support (CNBr-activated Sepharose beads) and added a mixture of FliM, FliN, and FliG, specifically labeled with [^{14}C]leucine. As a control for nonspecific binding, we used BSA beads, prepared in parallel to the CheY beads. As shown in Fig. 1, binding of the switch proteins to CheY was significantly higher than to the control, indicating that CheY has some affinity for the switch. The net binding (CheY beads minus BSA beads) is shown in the *Inset* of Fig. 1. To examine the effect of phosphorylation of CheY, we included in the reaction mixture acetyl phosphate, which has been shown to act as a low-molecular-weight phosphate donor to CheY (25). This resulted in a 4-fold increase in binding (Fig. 1 *Inset*). Resolution of the bound proteins by SDS/polyacrylamide gel electrophoresis demonstrated that when all three switch proteins were added together to the CheY beads (Fig. 2, lane 1), the main component(s) bound was FliM and/or FliG (lane 2). In the presence of acetyl phosphate, the binding of FliM and/or FliG increased significantly (lane 3). Binding of FliN was not detected (compare lane 2 or 3 with lane 1). Due to the close proximity of the FliM and FliG bands on the gel and the difficulty in distinguishing which of them increases upon phosphorylation, we examined the binding of the same mixture in the absence of FliG (lane 4) and of FliG alone (lane 7). FliM was indeed bound to the CheY beads (lane 5), and the binding was higher in the presence of acetyl phosphate (lane 6). FliG binding was weakly detected (lane 8), but its magnitude did not change in the presence of acetyl phosphate (lane 9) and its intensity was similar to that of FliG bound to BSA beads (as a control; not shown). Thus, of the three switch proteins, only FliM appears to specifically bind CheY. This binding is further increased in the presence of acetyl phosphate.

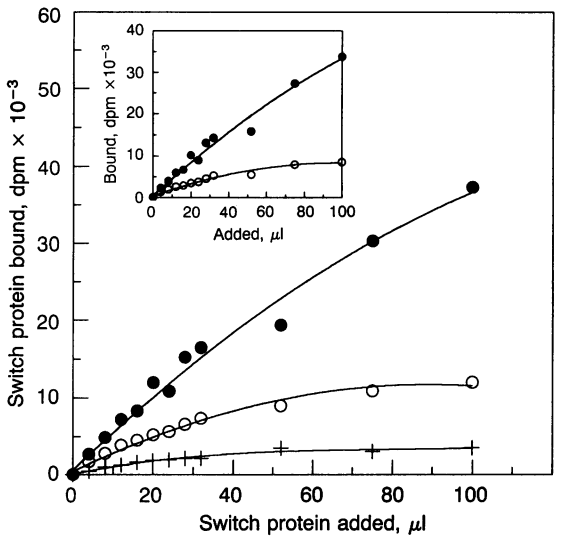

FIG. 1. Binding of switch proteins to CheY beads. Portions (200 μl each) of CheY-bead or BSA-bead suspensions in 50 mM Tris·HCl (pH 7.9) were placed in Eppendorf tubes and mixed with 80 μl of stabilizer, consisting of BSA (2 mg/ml), $MgCl_2$ (25 mM), glycerol (3.4 M) supplemented with [^{3}H]glycerol (2.5 Ci/mmol, 4.2 μCi per ml stabilizer mix) in 50 mM Tris·HCl (pH 7.9). A mixture of ^{14}C-labeled switch proteins (in a molar ratio of 2 FliM:1 FliG:1 FliN) was titrated in, and the volume of each tube was adjusted to 400 μl with Tris·HCl buffer. Where indicated, acetyl phosphate (9 mM) was added. Binding was allowed to proceed for 30 min at room temperature (24°C). The beads were pelleted by centrifugation at 4°C and the supernatant was removed by carefully soaking up with a tissue. The beads were then suspended in 1 ml of cold washing buffer (Tris·HCl buffer with 5 mM $MgCl_2$ and with or without 9 mM acetyl phosphate as appropriate), incubated at 4°C for 10 min, and then pelleted and washed once again as before. SDS (400 μl of a 10% solution) was added to the bead pellet to solubilize the bound protein. The beads were pelleted by centrifugation and aliquots of the supernatant were assayed for ^{14}C and ^{3}H by scintillation counting. The ^{3}H counts allowed us to correct for ^{14}C counts resulting from switch proteins trapped in the interstices of the beads. +, BSA beads; ○, CheY beads; ●, CheY beads in the presence of acetyl phosphate. (*Inset*) Net binding obtained by subtraction of the background (BSA beads).

To quantify the binding, we titrated FliM alone against the CheY beads. Very little binding was observed in the absence of phosphorylation [Fig. 3; in this figure and the subsequent one, only the net binding (CheY beads minus BSA beads) is shown]. This situation changed markedly in the presence of acetyl phosphate. The binding of FliM increased by a factor of 7. Since we found that only 35% of the CheY molecules were phosphorylated under these conditions, this means that CheY phosphorylation increased the amount of FliM bound by a factor of 20 relative to the nonphosphorylated protein. Acetyl phosphate did not affect the binding of FliM to the

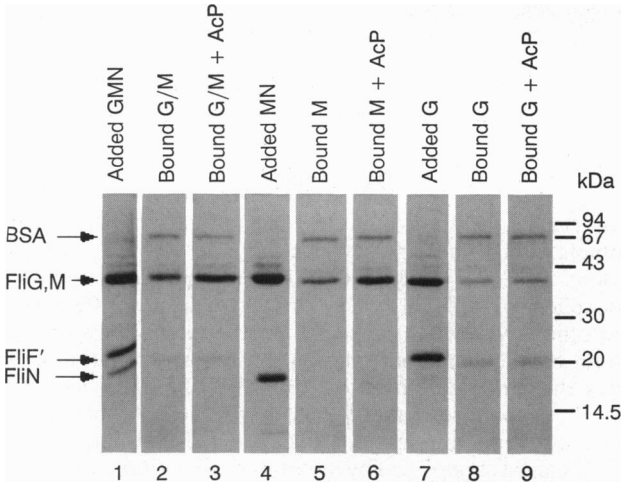

FIG. 2. Resolution of the switch proteins that bind to CheY. Switch proteins (0.1 mg), either singly or mixed together, were bound to CheY beads (carrying 23 μg of CheY) as described in the legend to Fig. 1, except that 40 μl of CheY beads was used instead of 200 μl and that the bound proteins were solubilized in 20 μl of sample buffer for gel electrophoresis instead of 400 μl of 10% SDS. An aliquot of 10 μl was loaded in each lane. The proteins were then resolved by SDS/15% polyacrylamide gel electrophoresis and stained with Coomassie blue. The added switch proteins (13 μg per lane) are also shown for reference. Lane 1, a mixture of FliG, FliM, and FliN applied to the CheY beads; lanes 2 and 3, the profile of proteins bound to the beads in the absence and presence of acetyl phosphate (AcP, 8 mM), respectively; lanes 4–6, as lanes 1–3 but without FliG in the mixture; lanes 7–9, as lanes 1–3 but with FliG alone instead of the mixture. FliF' denotes a partially deleted FliF (K.O., T. Ueno, and S.-I.A., unpublished work).

control (BSA) beads, as expected. No effect on the binding of [^{14}C]FliM to CheY beads was observed when unlabeled

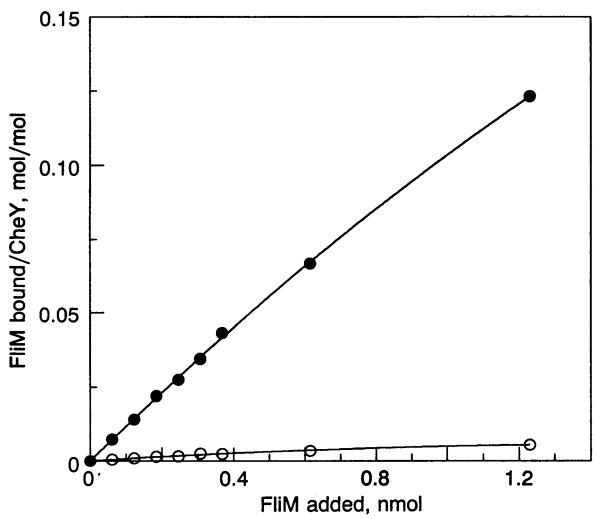

FIG. 3. FliM binding to CheY beads. Aliquots (100 μl) of a suspension of CheY beads (containing 0.2 mg of CheY; 25 μg of CheY per mg of dry weight of beads) or BSA beads and 40 μl of stabilizer mix (detailed in the legend to Fig. 1) were added to Eppendorf tubes. [^{14}C]FliM (46 dpm/pmol) was added to each tube as indicated and the volume was adjusted to 250 μl with 50 mM Tris·HCl (pH 7.9). Acetyl phosphate was added (18 mM) where appropriate. The beads were then treated as described in the legend to Fig. 1 and bound protein was estimated by scintillation counting. Maximally 49% of the added label was bound under phosphorylating conditions. The data are the average values of two experiments. ○, Net binding of FliM to CheY beads; ●, net binding of FliM to phosphorylated CheY.

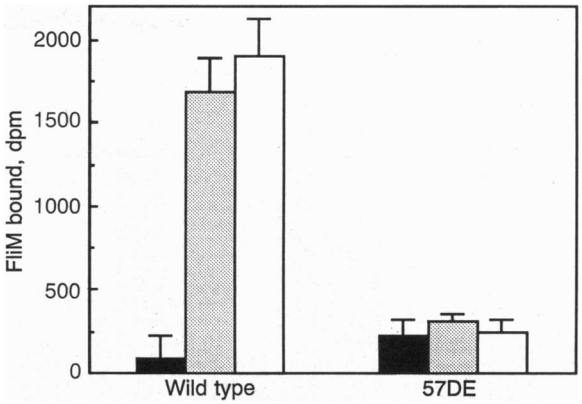

FIG. 4. Effects of acetyl phosphate and phosphoramidate on the extent of binding of FliM to beads of wild-type CheY and of CheY57DE (mutant with aspartate-57 → glutamate). Aliquots (75 μl) of CheY-bead suspension (27 μg of wild-type CheY or CheY57DE; 3.5 μg per mg of bead dry weight) were mixed with 3 nmol of [^{14}C]FliM (specific activity, 10.8 dpm/pmol) and 40 μl of stabilizer mix. Acetyl phosphate or phosphoramidate (18 mM) or 50 mM Tris·HCl buffer (pH 7.9) was added as indicated below. The volume was adjusted to 250 μl, and the mixture was incubated at 25°C for 15 min. Each sample was then washed twice in ice-cold Tris·HCl buffer supplemented with $MgCl_2$ (2 mM) and acetyl phosphate or phosphoramidate (18 mM) as appropriate. Bound protein was estimated as in Fig. 1. Filled bars, no additions (buffer only); stippled bars, acetyl phosphate; open bars, phosphoramidate.

FliG or FliN was titrated into the binding mixture, indicating that the binding of FliM to CheY was independent of the other switch proteins. We also examined ATP (10 mM), ADP (10 mM), fumarate[§] (10 mM), and Ca^{2+} (1 mM) as potential binding regulators; none of them affected the binding. Various buffers with pK_a values in the range 7.5–8.5 supported binding equally well.

Acetyl phosphate has, in addition to its phosphorylating potential, an ability to acetylate CheY, albeit to a very low extent (Y. Blat and M.E., unpublished observations). Since acetylation apparently activates CheY (20), we wished to establish by which mechanism, phosphorylation or acetylation, acetyl phosphate acts. We therefore used another phosphate donor, phosphoramidate (25), which cannot acetylate CheY. Phosphoramidate affected the binding of FliM to CheY beads in a comparable manner to acetyl phosphate (Fig. 4), indicating that CheY phosphorylation was responsible for the increase in binding. To verify this conclusion, we repeated the experiment with a CheY protein in which the phosphorylation site, aspartate-57, was replaced by glutamate. The level of binding of this protein to FliM was low and was unaffected by either phosphate donor (Fig. 4). In all subsequent experiments we used acetyl phosphate as the phosphorylating agent. [We found that CheA (3.5 mg/ml) and ATP (20 mM) in the presence of $MgCl_2$ (2 mM) were unable to phosphorylate CheY bound to beads, presumably because of steric hindrance. Free CheY is readily phosphorylated under these conditions (8, 9, 11).]

As shown in Fig. 5, the binding of FliM was stable to washing as long as the conditions favored phosphorylation of CheY and/or prevented its dephosphorylation. [Note that both acetyl phosphate and Mg^{2+} are required for CheY phosphorylation, but only Mg^{2+} is required for its dephosphorylation (28).] Similar results were obtained with phosphoramidate as a phosphate donor (not shown).

[§]Fumarate has been identified as a factor facilitating counterclockwise-to-clockwise transitions of flagellar rotation and vice versa (26, 27).

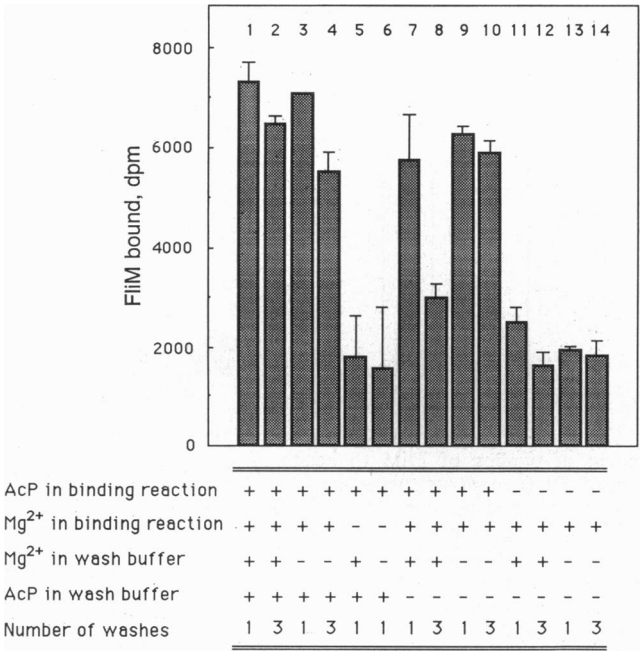

FIG. 5. Stability of CheY–FliM binding to washing. The experiment was carried out as in Fig. 4, except that 30 μl of stabilizer mix was used and the amount of CheY on the beads was greater (8.7 μg of CheY per mg of bead dry weight). The final reaction volume was adjusted to 188 μl. The reaction mixtures of tubes 5 and 6 also contained EDTA (20 mM). The samples were washed once or three times (as indicated) in 50 mM Tris·HCl (pH 7.9) containing the indicated combinations of acetyl phosphate (AcP, 18 mM), $MgCl_2$ (2 mM), or EDTA (20 mM; added whenever Mg^{2+} was omitted).

To examine whether or not immobilized CheY is equivalent to free CheY in its FliM-binding properties, we titrated soluble CheY against immobilized CheY and a fixed amount of FliM in the presence of 18 mM acetyl phosphate. The FliM partitioned between the soluble and immobilized CheY (Fig. 6, experimental points) in accordance with the theoretical curve calculated by assuming equal binding capacity (Fig. 6, solid line). This indicates that immobilized CheY and free CheY are indeed equivalent.

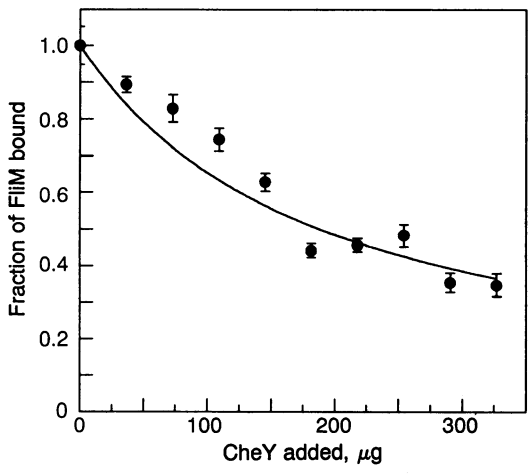

FIG. 6. Comparison of the binding activity of free and immobilized CheY. CheY beads (50 μl, containing 190 μg of CheY; 45 μg of CheY per mg of bead dry weight) were aliquoted into Eppendorf tubes containing 20 μl of stabilizer mix and acetyl phosphate (25 mM). Soluble CheY was added in the amounts indicated and the volume adjusted to 125 μl with 50 mM Tris·HCl (pH 7.9). [^{14}C]FliM (30 μl) was added to each tube and incubated for 30 min. Each sample was washed in 1 ml of ice-cold Tris·HCl buffer containing acetyl phosphate (25 mM). The amount of bound FliM was determined as in Fig. 3. The experimental points are the mean values of triplicates ± SD. The theoretical values of the fraction of FliM bound were calculated according to 1 − [free CheY/total (immobilized + free) CheY], i.e., by assuming identical activity of the immobilized and free CheY.

DISCUSSION

Our results demonstrate that CheY binds to the switch complex, that the primary docking site is FliM, that the extent of CheY binding to FliM is dependent upon the phosphorylation level of CheY, and that it is unaffected by the other two switch proteins. Furthermore, they provide direct biochemical confirmation that phosphorylation regulates the binding activity of the diffusible signal molecule CheY. These results are consistent with those obtained by genetic reversion analysis, which showed that mutations in CheY can be suppressed by mutations in FliM (and to a lesser extent FliG) and vice versa (10, 16–18). They are also strongly consistent with recent studies using partially lysed cells (semi-envelopes), in which phosphorylation of CheY increased its clockwise-causing activity at the switch ≈100-fold (11). The observation that phosphorylated CheY was unable to cause clockwise rotation in envelopes totally devoid of cytoplasm (11), taken together with the observation that phosphorylation increases the binding of CheY to the switch (Figs. 1–3), suggests that binding of phosphorylated CheY is necessary but not sufficient to cause clockwise rotation. An additional (yet unidentified) cytoplasmic component(s) is probably required (11). The difference between the binding results of this study and the results obtained in semi-envelopes, on the one hand, and those obtained in envelopes, on the other hand, supports the multistate model for switching proposed by Kuo and Koshland (29). Accordingly, in the first step phosphorylated CheY binds to the switch without altering the switch bias (we assume that the number of nonphosphorylated CheY molecules bound is negligible under physiological conditions). Only in a subsequent step, probably involving an additional cytoplasmic factor, clockwise rotation is generated. Since *fliG*, like *fliM*, is the locus of many mutations which affect switching (17), an economical hypothesis is that FliM binds phosphorylated CheY and then communicates with FliG, causing the latter to undergo a conformational change leading to a switching event.

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