Phosphorylation-Dependent Binding of the Chemotaxis Signal Molecule CheY to Its Phosphatase, CheZ†

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ABSTRACT: Bacterial chemotaxis is accomplished by regulating the direction of flagellar rotation. The primary target of the control appears to be CheY, a diffusible clockwise-signal molecule which interacts with the switch at the base of the flagellar motor and causes clockwise rotation. The regulatory mechanism appears to be phosphorylation/dephosphorylation of CheY. Here we demonstrate that CheZ, which accelerates the dephosphorylation of CheY, binds to CheY (immobilized on CNBr-activated Sepharose beads), that the binding to phosphorylated CheY is higher by over 2 orders of magnitude than the binding to nonphosphorylated CheY, and that the binding to both the phosphorylated and nonphosphorylated forms of CheY is significantly higher in the presence of Mg^{2+}. We also show that the mutant proteins CheY13DK, CheY57DE, and CheY109KR bind CheZ to the same extent as wild-type CheY. The extent of the binding of these mutant proteins was not, however, increased in the presence of acetyl phosphate, the phosphorylating agent. The results indicate that neither a conformation which has a clockwise-causing activity *in vivo* nor phosphorylation is sufficient, alone, for maximal binding of CheZ to CheY and that Mg^{2+} is required for the binding of these proteins as well as for the phosphorylation and dephosphorylation of CheY.

Bacteria such as *Escherichia coli* and *Salmonella typhimurium* respond to chemical changes in the environment by modulating the direction of rotation of their flagella and thereby their mode of swimming. Attractants cause the flagella to rotate counterclockwise, leading to smooth swimming of the bacteria toward the attractant source. Repellents cause clockwise-biased rotation, giving rise to tumbling and reorientation of the bacteria. Thus, the regulation of the direction of flagellar rotation is the basis of bacterial chemotaxis. [See Adler (1987), Eisenbach (1991), Koshland (1980), Macnab (1987), Parkinson and Hazelbauer (1983), and Stewart and Dahlquist (1987) for reviews.]

Attractants and repellents are sensed by the chemotaxis membrane receptors, each of which has its own set of specific ligands (Adler, 1987; Eisenbach, 1991; Koshland, 1980; Macnab, 1987). The chemotaxis receptors, which are localized at one of the poles of the bacterial cell (Gegner *et al.*, 1992; Maddock & Shapiro, 1993), are complexed with two other chemotaxis proteins, CheA and CheW. This complex communicates with the flagella *via* the chemotaxis signal protein CheY, which is believed to freely diffuse within the cytoplasm (Eisenbach *et al.*, 1985; Segall *et al.*, 1985). In the absence of CheY, the motor rotates in the default direction of rotation, counterclockwise (Eisenbach & Adler, 1981; Eisenbach *et al.*, 1990; Parkinson & Houts, 1982; Ravid & Eisenbach, 1984; Wolfe *et al.*, 1987). CheY can interact with the switch–motor complex at the base of the flagellum and enables the motor to rotate clockwise (Clegg & Koshland, 1984; Parkinson *et al.*, 1983; Ravid *et al.*, 1986; Wolfe *et al.*, 1987; Yamaguchi *et al.*, 1986).

Studies with CheY-containing envelopes implied that CheY may be in active and nonactive forms, activity being defined as the ability to interact with the switch–motor complex and cause clockwise rotation [Eisenbach & Matsumura, 1988; Ravid *et al.*, 1986; see Eisenbach (1991) for review]. One of the modes by which CheY can be activated is phosphorylation. Using semi-envelopes of bacteria, it was shown that phosphorylation increases the activity of CheY at the switch–motor complex by 2 orders of magnitude (Barak & Eisenbach, 1992). Furthermore, it was demonstrated that the level and rate of CheY phosphorylation can be modulated by chemotactic stimuli *in vitro* in a system consisting of receptor-containing vesicles and the chemotaxis proteins CheY, CheA, and CheW (Borkovich *et al.*, 1989; Ninfa *et al.*, 1991). *In vivo* the presumed sequence of events is as follows. Attractant-free receptor, along with CheW, stimulates the phosphorylation of CheA, and phosphorylated CheA transfers its phosphate group to CheY. Attractant-bound receptor inhibits the phosphorylation of CheA and therefore also of CheY. CheY dephosphorylates spontaneously; this dephosphorylation is enhanced by the chemotaxis protein CheZ, with a resultant deactivation of CheY [See Bourret *et al.* (1991), Eisenbach (1991), and Stock *et al.* (1992) for reviews].

CheY interacts with three proteins: CheA (which phosphorylates it) (McNally & Matsumura, 1991), CheZ (which dephosphorylates it) (McNally & Matsumura, 1991), and FliM (the component of the switch–motor complex to which it binds) (Welch *et al.*, 1993). In this work we studied the interaction between CheY and CheZ. We demonstrate biochemically that CheZ binds directly to CheY, that phosphorylation increases this binding by more than 2 orders of magnitude, and that the binding of CheZ to both phosphorylated and nonphosphorylated CheY is dependent on the presence of Mg^{2+}.

EXPERIMENTAL PROCEDURES

Chemicals. [^{32}P]Phosphoric acid (9.4 mCi/nmol) was obtained from Nuclear Research Centre—Negev. L-[U-^{14}C]-Leucine (300 mCi/mmol) and [1-^{14}C]acetic anhydride (100

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mCi/mmol) were obtained from Amersham. Acetyl phosphate was obtained from Sigma. [^{32}P]Acetyl phosphate was synthesized from $^{32}P_i$ and acetic anhydride according to Stadtman (1957). [^{14}C]Acetyl phosphate was similarly synthesized, except that [1-^{14}C]acetic anhydride (one-half of the concentration used for the synthesis of [^{32}P]acetyl phosphate) and nonlabeled P_i were used. Phosphoramidate was synthesized as described (Sheridan *et al.*, 1971). Other chemicals were of analytical grade.

Bacterial Strains and Plasmids. All bacterial strains used were *E. coli* derivatives. The plasmid pRL22 (Matsumura *et al.*, 1984), carrying CheZ and CheY, was used for the overproduction of CheZ in strain RP437, wild type for chemotaxis (Parkinson, 1978). The plasmid pRL22(Δ*PvuII*), carrying CheY without CheZ, was used for the overproduction of CheY in strain EW30, which is a *tet*^{r} derivative of RP1091 [Δ(*cheA-cheZ*) (Parkinson & Houts, 1982)]. The plasmids pRL22 and pRL22(Δ*PvuII*) were received from P. Matsumura. The plasmids pRBB40 13DK, pRBB40 57DE, and pRBB40 109KR, received from R. B. Bourret and M. I. Simon, were used for the overproduction of CheY13DK, CheY57DE, and CheY109KR, respectively, in KO641 *recA* (Bourret *et al.*, 1990).

Protein Purification. CheY was purified as described (Barak & Eisenbach, 1992) except that the protein was concentrated by ultrafiltration through a membrane with a cutoff of 5000 Da, using an Amicon chamber (Model 52). CheZ was purified as described by Margolin (1987) with a few modifications. Essentially, the soluble fraction of an extract of the bacterial strain mentioned above was prepared as described for the production of CheY. The extract was loaded onto a Cibacron column (90 mL), and CheZ was eluted by washing the column with 150 mL of Tris-HCl (50 mM, pH 7.9). CheZ was concentrated by ammonium sulfate precipitation (100% saturation), resuspended in 25 mL of HEPES[1] (20 mM, pH 7.4), dialyzed twice against the same buffer, and loaded onto a 40-mL Sepharose CL-6B column preequilibrated with HEPES. The column was washed with 250 mL of HEPES, followed by 250 mL of HEPES containing 225 mM NaCl. CheZ was then eluted from the column by 200 mL of HEPES containing 275 mM NaCl in 6-mL fractions. The fractions were first dialyzed against HEPES and concentrated by ultrafiltration as described for the purification of CheY.

Radiolabeling of CheZ. RP437 carrying the plasmid pRL22 was grown at 35 °C in 15 mL of H1 medium (Kaiser & Hogness, 1960) supplemented with histidine, methionine, and threonine (1 mM each), leucine (200 μM), thiamin (5 μg/mL), ampicillin (125 μg/mL), and glucose (0.3%, w/w). The overproduction of CheZ was induced at OD_{590nm} = 0.5 by addition of indoleacrylic acid (20 μg/mL final concentration). [^{14}C]Leucine (2 μCi/mL final concentration) was added together with the indoleacrylic acid. The cells were grown for an additional 4 h and then harvested by centrifugation. Further purification steps were as for nonlabeled CheZ, except that minicolumns (4-mL volume) were used instead of the regular columns. The fractions pooled from these columns were concentrated with a Centricon 10 microconcentrator (Amicon). Pure CheZ was concentrated to 1000 dpm/μL and stored at −20 °C.

Immobilization of CheY and BSA. Two tubes containing 1 g dry weight of CNBr-activated Sepharose beads (Pharmacia) were washed in 1 mM HCl followed by coupling buffer (0.1 M Na_2HCO_3, 0.5 M NaCl, pH 8.3). CheY (6.5–16 mg), predialyzed against coupling buffer, was added to one tube, and an equal volume of coupling buffer was added to the other tube. Both tubes were mixed end over end at 4 °C overnight or for 2 h at room temperature. Most (95–100%) of the CheY became covalently immobilized in this way. BSA (20 mg), predialyzed against coupling buffer, was then added to both tubes, and the tubes were mixed for a further 2 h at room temperature or for 5–10 h at 4 °C. Uncoupled protein was removed by washing with coupling buffer, and the remaining reactive groups on the beads were blocked by a 2-h incubation with Tris-HCl (0.1 M, pH 8.0) at room temperature. The beads were then washed in 50 mM Tris-HCl (pH 7.9) and stored at 4 °C. Immediately before use, the beads were washed once again in 50 mM Tris-HCl.

CheZ Binding to CheY Beads. Portions (100 μL each) of CheY–bead or BSA–bead suspensions in Tris-HCl (50 mM, pH 7.9) were placed into Eppendorf tubes and mixed with 40 μL of stabilizer, consisting of BSA (17 mg/mL), $MgCl_2$ (17 mM), glycerol (33%, v/v), and Tris-HCl (50 mM, pH 7.9). CheZ (5000–40 000 dpm) was added to a final concentration of 0.08–100 μM, and the volume of each tube was adjusted to 250 μL with Tris-HCl buffer. Where indicated, acetyl phosphate or phosphoramidate (18 mM final concentration each) 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 carefully soaked up with a tissue. The beads were then resuspended in 0.5 mL of cold washing buffer (Tris-HCl + 5 mM $MgCl_2$ ± 18 mM acetyl phosphate or phosphoramidate as appropriate) and then pelleted and washed once again as before. Sodium dodecyl sulfate (SDS; 400 μL of 10%) was added to the bead pellet to solubilize the bound protein. After a 1-h incubation at room temperature with mixing, the beads were pelleted by centrifugation and aliquots of the supernatant were assayed for ^{14}C by scintillation counting.

Measurement of CheY Phosphorylation. [^{32}P]Acetyl phosphate (1.4–1.8 × 10^8 dpm; 18 mM final concentration) was incubated with 56 μM CheY and the indicated concentration of CheZ under conditions identical to those in the binding assays in a volume of 20 μL. The reaction was quenched by the addition of 100 μL of 10% ice-cold trichloroacetic acid (TCA), and the proteins were precipitated by centrifugation and washed once again with 100 μL of 10% TCA at 0 °C. (TCA was used as the quenching agent in order to get rid of the radiolabeled acetyl phosphate and thus to get higher signal-to-noise ratios on the gel. This was especially important because of the low phosphorylation level in the presence of CheZ. We verified that TCA does not hydrolyze phosphorylated CheY under our experimental conditions.) The precipitate was then solubilized in 30 μL of 2-fold-concentrated SDS sample buffer containing Tris-HCl (100 mM, pH 7.9). Aliquots (24 μL each) were subjected to SDS–polyacrylamide gel electrophoresis, after which the gels were stained with Coomassie blue in an ice-cold water bath and destained. The CheY band was sliced and solubilized by incubation with 800 μL of 30% H_2O_2 for 12 h at 80 °C, and the radioactivity was measured by a β-counter.

RESULTS

Binding of CheZ to CheY. We immobilized CheY on a solid support (CNBr-activated Sepharose beads). [^{14}C]CheZ was added to the immobilized CheY, and the binding was

[1] Abbreviations: BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

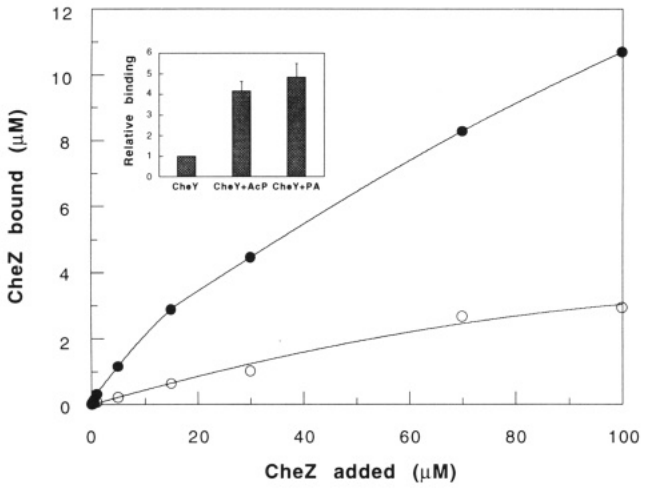

FIGURE 1: Binding of CheZ to immobilized CheY. The final concentration of CheY was 56 μM. The experimental points represent the net specific binding of [^{14}C]CheZ to CheY beads (i.e., the binding to CheY beads minus the binding to BSA beads) in the absence (○) and presence (●) of acetyl phosphate (18 mM). Inset: Binding of CheZ (0.7 or 1 μM) to CheY (30 or 56 μM) in the presence of acetyl phosphate (AcP) or phosphoramidate (PA, 18 mM) ±SD, relative to the binding in the absence of phosphate donors.

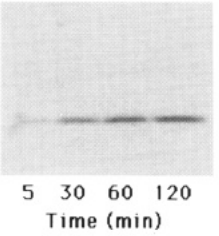

FIGURE 2: Time course of CheY labeling by [^{14}C]acetyl phosphate. CheY (170 μM) was incubated with [1-^{14}C]acetyl phosphate (7.3 mM; 5 mCi/mmol) in Tris-HCl (50 mM, pH 7.5), KCl (50 mM), and $MgCl_2$ (5 mM). Samples of 20 μL were quenched at the indicated time points by addition of 10 μL of 5-fold-concentrated sample buffer. Samples of 25 μL were subjected to SDS–polyacrylamide gel electrophoresis, after which the gel was dried and autoradiographed. Two percent of the CheY molecules were found by using densitometry to be labeled after a 2-h incubation.

determined by measuring the radioactivity associated with the Sepharose beads after removal of the unbound CheZ. Nonspecific binding [amounting to 13 ± 8% (±SD) of the binding to immobilized CheY] was determined by carrying out the same assay using immobilized BSA. Net specific binding was determined by subtracting the amount of CheZ bound to BSA beads from the amount of CheZ bound to the CheY beads. The results of a typical binding assay are shown in Figure 1, indicating that CheZ does indeed bind to CheY.

Effect of Phosphorylation on the Binding of CheZ to CheY. Acetyl phosphate, which was recently shown to phosphorylate CheY in the absence of the chemotaxis proteins and ATP (Lukat *et al.*, 1992), increased the binding of CheZ to immobilized CheY 3–5-fold (Figure 1).

In addition to phosphorylating CheY, acetyl phosphate can apparently also acetylate it (Figure 2). To verify that the increase in binding of CheZ to CheY is due to phosphorylation, we carried out a similar experiment with phosphoramidate (which cannot acetylate CheY) substituting for acetyl phosphate as a phosphodonor (Lukat *et al.*, 1992). Phosphoramidate caused an increase in binding similar to that of acetyl phosphate (Figure 1, inset), indicating that the increased CheZ–CheY binding is due to CheY phosphorylation. In all subsequent experiments we used acetyl phosphate as the phosphorylating agent.

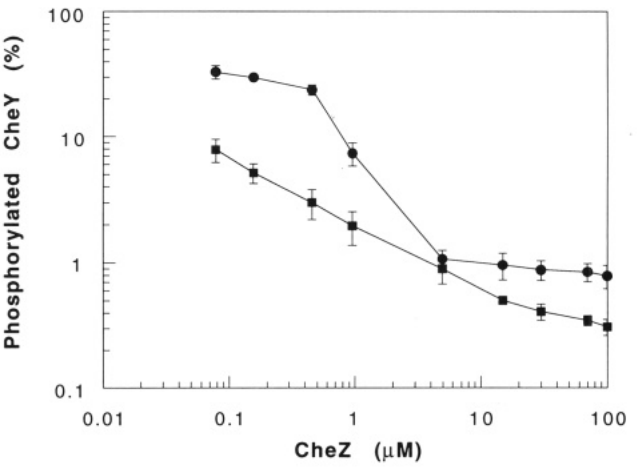

FIGURE 3: Phosphorylation of CheY by [^{32}P]acetyl phosphate in the presence of CheZ. The concentration of CheY was 56 μM. The circles and squares stand for the phosphorylation in the presence and absence of Mg^{2+}, respectively. The following modifications were carried out in order to examine the level of phosphorylation subsequent to the removal of Mg^{2+} by EDTA. CheY was phosphorylated in the presence of $MgCl_2$ (2.7 mM) for 15 min; then the Mg^{2+} was removed by addition of EDTA (20 mM final concentration). The reaction mixture was then incubated for an additional 30 min at room temperature.

Since CheZ dephosphorylates CheY (Hess *et al.*, 1988), we had to determine the amount of phosphorylated CheY at each concentration of CheZ added. The determination was carried out under conditions identical to those used for the binding assays. As shown in Figure 3, the phosphorylation level of CheY was inversely dependent on the concentration of CheZ. At CheZ concentrations above 5 μM, not more then 1% of the CheY molecules were phosphorylated. The fact that the phosphorylation-dependent increase in binding was large even though only a very small fraction of the CheY molecules were phosphorylated indicates that the actual effect of phosphorylation of a single CheY molecule was very high (e.g., at 5 μM CheZ there was a ~500-fold increase in the amount bound upon phosphorylation).

Effect of Magnesium on the Binding of CheZ to Phosphorylated and Nonphosphorylated CheY. Divalent metal ions are known to bind CheY and to be required for both the phosphorylation and the dephosphorylation of CheY (Lukat *et al.*, 1990). To determine the effect of divalent metal ions on the binding of CheZ to phosphorylated and nonphosphorylated CheY, we omitted Mg^{2+} from the binding assay. The fraction of nonphosphorylated CheY bound to CheZ was ~6-fold lower in the absence of Mg^{2+} (Figure 4A). For calculating the fraction of phosphorylated CheY bound to CheZ, we first determined the phosphorylation level of CheY at each concentration of CheZ following the removal of Mg^{2+} by EDTA (squares in Figure 3) under conditions identical to those of the binding assay. As in the case of nonphosphorylated CheY, the fraction of phosphorylated CheY bound to CheZ was 2.4–3.0-fold lower in the absence of Mg^{2+} (Figure 4B; note that the scales of the ordinates in panels A and B of Figures 4 are different). Thus the binding of both the phosphorylated and the nonphosphorylated form of CheY to CheZ is Mg^{2+}-dependent.

Binding of CheZ to Mutant CheY Proteins. In order to identify sites on CheY that are important for the interaction with CheZ, we examined the binding of CheZ to several CheY mutant proteins: CheY13DK, which is constitutively active *in vivo* (i.e., the mutant is clockwise-biased) but cannot be phosphorylated (Bourret *et al.*, 1990); CheY57DE, which is inactive *in vivo* and is defective at the site of phosphorylation

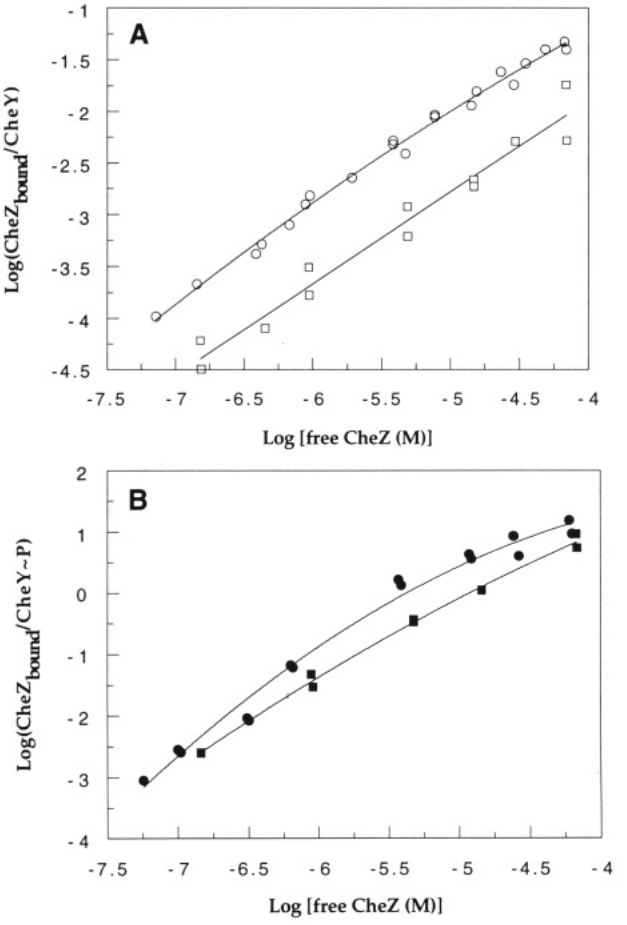

FIGURE 4: Effect of Mg^{2+} on the binding of CheZ to CheY. The effect was measured under phosphorylating and nonphosphorylating conditions (A and B, respectively). The circles and squares stand for CheZ–CheY binding in the presence and absence of $MgCl_2$ (2.7 mM), respectively. The CheY concentration was 56 μM. Under nonphosphorylating conditions, absence of Mg^{2+} was achieved by omitting $MgCl_2$ and adding EDTA (2 mM final concentration). [The affinity of Mg^{2+} to EDTA (Welcher, 1958) is over 5 orders of magnitude higher than to CheY (Lukat *et al.*, 1990); EDTA should therefore efficiently remove not only the free Mg^{2+} ions but also those bound to CheY.] Under phosphorylating conditions, CheY was first incubated with acetyl phosphate (18 mM) and $MgCl_2$ (2.7 mM) for 15 min to allow phosphorylation, and only then was Mg^{2+} removed, by addition of EDTA (20 mM final concentration), and CheZ added. The binding of CheZ to phosphorylated CheY (CheY~P) was calculated by the difference between the amount of CheZ bound to CheY in the presence and absence of acetyl phosphate, divided by the concentration of CheY~P (taken from Figure 3).

and therefore cannot be phosphorylated (Bourret *et al.*, 1990); and CheY109KR, which is inactive *in vivo* even though it is phosphorylatable (albeit defective in both spontaneous and CheZ-mediated dephosphorylation) (Lukat *et al.*, 1991). In parallel, we confirmed that while CheY109KR was phosphorylated under our experimental conditions (i.e., by acetyl phosphate), CheY13DK and CheY57DE were not phosphorylated. As shown in Figure 5, all of these mutant proteins could bind CheZ to the same extent as wild-type CheY, but unlike wild-type CheY, the extent of the binding remained essentially unchanged in the presence of acetyl phosphate.

DISCUSSION

In this study we have shown that CheZ binds to CheY, that the binding to phosphorylated CheY is higher by over 2 orders of magnitude than to nonphosphorylated CheY, that the phosphorylation-dependent increase in binding does not occur in key CheY mutant proteins, and that the binding is enhanced

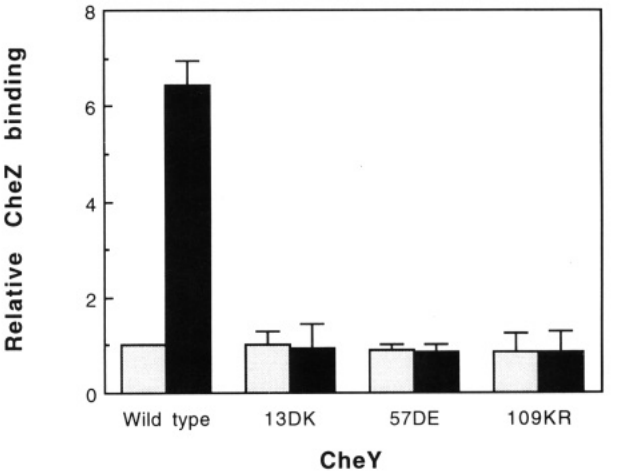

FIGURE 5: Binding of CheY mutant proteins to CheZ. The CheY (bead-bound) concentration was 23 or 107 μM. The CheZ concentration was 0.22 or 0.27 μM. The results are the mean ±SD of three experiments, normalized to the binding to nonphosphorylated wild-type CheY. The gray and black columns stand for the absence and presence of acetyl phosphate (18 mM), respectively.

by Mg^{2+}. This study thus endorses the findings of McNally *et al.* (1991) that CheZ binds to CheY, and it provides evidence that the binding is direct. The significance of these results is discussed below.

One of the major observations of this study is that CheZ binds orders of magnitude better to phosphorylated CheY than to nonphosphorylated CheY. CheZ is thus the second protein shown to bind to CheY and to bind much better to its phosphorylated form. [The first protein is the switch protein, FliM (Welch *et al.*, 1993).] At this stage, it is not yet clear whether CheZ and FliM compete *in vivo* with each other on CheY, or whether they interact with CheY at different sites. Also, it has not yet been established whether immobilized CheY is identical to CheY free in solution with respect to its CheZ-binding properties. The phosphorylated form of CheY is considered to be an active form in the sense that its interaction with the switch is high (Welch *et al.*, 1993) and that it has a high ability to cause clockwise rotation (provided that other cytoplasmic constituents are present) (Barak & Eisenbach, 1992). Therefore, the preference of CheZ for phosphorylated CheY seems to be important for its function as a deactivator of CheY: it allows CheZ to bind preferentially to phosphorylated CheY, to deactivate it by dephosphorylation, and eventually to dissociate and be available for the deactivation of another CheY molecule. Interestingly, under optimal conditions (phosphorylation in the presence of Mg^{2+}) as many as 12 molecules of CheZ were bound to a single phosphorylated CheY molecule (Figure 4B). This may reflect the polymeric nature of CheZ (Stock & Stock, 1987).

Divalent metal ions are required for the phosphorylation of CheY as well as for the autodephosphorylation or CheZ-mediated dephosphorylation of CheY (Lukat *et al.*, 1990). The results of this study indicate that, in addition, the presence of Mg^{2+} increases the actual binding of CheZ to CheY. This is true for both the phosphorylated and nonphosphorylated forms of CheY (Figure 4). Our study thus suggests that the involvement of Mg^{2+} in the regulation of CheY activity extends beyond its role in promoting phosphorylation and dephosphorylation of CheY.

The binding of the mutant CheY proteins to CheZ sheds some light on the requirements for optimal interaction of CheZ with CheY (Figure 5). These data indicate that neither a conformation which has a clockwise-causing activity *in vivo*

(as in the case of CheY13DK) nor phosphorylation by itself (as in the case of CheY109KR in the presence of acetyl phosphate) is sufficient for maximal binding. A common feature in all three mutants is that none of them can undergo the long-range conformational change that occurs upon phosphorylation of wild-type CheY (Bourret *et al.*, 1993). This may indicate that this conformational change is required for CheZ binding. The fact that CheY13DK, which is constitutively active (Bourret *et al.*, 1990), does not bind CheZ better than wild-type CheY (Figure 5) may be explained by the following possibilities: (a) the phosphate group in CheY is an integral part of the CheZ-recognition site; or (b) the conformation of CheY13DK, in spite of being active at the switch *in vivo* [but not *in vitro* (Barak & Eisenbach, 1992; M. Welch and M. Eisenbach, unpublished)], is different from the conformation of phosphorylated wild-type CheY (Bourret *et al.*, 1993). The fact that phosphorylation does not facilitate binding of CheZ to CheY109KR (Figure 5) is well in line with the observation that CheY109KR is not dephosphorylated by CheZ (Lukat *et al.*, 1991).

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