

# Alteration of a Nonconserved Active Site Residue in the Chemotaxis Response Regulator CheY Affects Phosphorylation and Interaction with CheZ\*

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**CheY is a response regulator in the well studied two-component system that mediates bacterial chemotaxis. Phosphorylation of CheY at Asp<sup>57</sup> enhances its interaction with the flagellar motor. Asn<sup>59</sup> is located near the phosphorylation site, and possible roles this residue may play in CheY function were explored by mutagenesis. Cells containing CheY59NR or CheY59NH exhibited hyperactive phenotypes (clockwise flagellar rotation), and CheY59NR was characterized biochemically. A continuous enzyme-linked spectroscopic assay that monitors P<sub>i</sub> concentration was the primary method for kinetic analysis of phosphorylation and dephosphorylation. CheY59NR autodephosphorylated at the same rate as wild-type CheY and phosphorylated similarly to wild type with acetyl phosphate and faster (4–14×) with phosphoramidate and monophosphoimidazole. CheY59NR was extremely resistant to CheZ, requiring at least 250 times more CheZ than wild-type CheY to achieve the same dephosphorylation rate enhancement, whereas CheY59NA was CheZ-sensitive. However, several independent approaches demonstrated that CheY59NR bound tightly to CheZ. A submicromolar K<sub>d</sub> for CheZ binding to CheY59NR-P or CheY-BeF<sub>3</sub><sup>-</sup> was inferred from fluorescence anisotropy measurements of fluoresceinated-CheZ. A complex between CheY59NR-P and CheZ was isolated by analytical gel filtration, and the elution position from the column was indistinguishable from that of the CheZ dimer. Therefore, we were not able to detect large CheY-P-CheZ complexes that have been inferred using other methods. Possible structural explanations for the specific inhibition of CheZ activity as a result of the arginyl substitution at CheY position 59 are discussed.**

results in smooth swimming) and clockwise (CW) rotation (which causes the bacterium to tumble). Chemical information outside of the cell is transmitted to the flagella by a network of chemotaxis proteins. Ligand binding to the periplasmic portion of transmembrane receptors controls the autophosphorylation of CheA, a kinase bound to the cytoplasmic portion of the receptors. The phosphoryl group is then transferred from a histidyl residue on CheA to an aspartyl residue on the freely diffusible protein CheY. Phosphorylated CheY (CheY-P) binds to the FliM protein in the flagellar switch, which increases the likelihood of CW rotation (see Refs. 1–4 for recent reviews). CheY exhibits an intrinsic autodephosphorylation activity, but this reaction occurs slowly relative to the time scale of changes in *in vivo* behavior (5), and the primary means of dephosphorylation of CheY in the cell is via an auxiliary protein, CheZ. The proteins controlling chemotaxis are an example of a two-component regulatory system, a large family of systems employed in many organisms to mediate sensory processes. An essential biochemical feature of these systems is phosphotransfer from a histidyl residue on a sensor kinase (*e.g.* CheA) to an aspartyl residue on a response regulator (*e.g.* CheY) and subsequent hydrolysis of the phosphoryl group from the response regulator (6).

A series of studies have combined to suggest basic mechanisms for the phosphorylation and autodephosphorylation reactions of CheY, which are likely applicable to other response regulators. CheY can receive a phosphoryl group from small molecules such as acetyl phosphate (7) as well as from CheA, evidence that the fundamental machinery for phosphorylation resides on CheY. Both phosphorylation and autodephosphorylation reactions are centered around a Mg<sup>2+</sup> ion, and both structural (8) and mechanistic studies using small molecules as models of the reaction chemistry (9, 10) are consistent with direct substitution at the phosphoryl phosphorous via a bipyramidal transition state for both reactions. In contrast, the mechanism of CheZ-dependent CheY dephosphorylation is not known. CheZ activity is also Mg<sup>2+</sup>-dependent (11), and CheZ does not catalyze the hydrolysis of acetyl phosphate (7). These features are consistent with the possibility that CheZ may act as a positive allosteric modulator of CheY autodephosphorylation. Alternatively, CheZ may contribute its own residues to catalysis as does a conventional phosphatase. Possible regulation of CheZ activity by other chemotaxis proteins could help explain the ultrasensitivity of chemotactic sensing in *E. coli* (12–14) or provide an additional mechanism for chemotactic adaptation (15). The interaction of CheZ and CheY-P has been reported to result in large oligomeric complexes (16), which may be involved in CheZ regulation (15, 17).

The strict conservation among response regulators of essential active site residues (18) as well as the relative orientation

Flagellated bacteria such as *Escherichia coli* and *Salmonella typhimurium* move toward chemical attractants and away from repellants by regulating the frequency with which their flagella switch between counterclockwise (CCW)<sup>1</sup> rotation (which re-

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<sup>1</sup> The abbreviations used are: CCW, counterclockwise; CW, clockwise; MPI, monophosphoimidazole; PAM, phosphoramidate; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside; CheY-P, phosphorylated CheY.

of these residues in the folded proteins (19) reinforces the notion that the basic phosphorylation reaction mechanisms for two-component systems are conserved. Despite this consistency, the rates of the autodephosphorylation reactions can differ over several orders of magnitude for different systems (20). Therefore, it is plausible that nonconserved residues in the vicinity of the active sites of response regulators have evolved to regulate the rates of the phosphorylation reactions in a way that meets the individual needs of a system. Asn<sup>59</sup>, a nonconserved residue, has a central location in the active site of *E. coli* CheY as does the analogous residue in other CheY proteins and response regulators. The backbone carbonyl of Asn<sup>59</sup> directly chelates the active site Mg<sup>2+</sup> in both the inactive (8, 21) and active (22) conformations, and the backbone amide directly interacts with a fluorine atom in the activated CheY·BeF<sub>3</sub><sup>-</sup> structure (22). The side chain of Asn<sup>59</sup> is oriented toward the active site. In this study, we explored the effect of various amino acid substitutions at position 59 of CheY on the activity of this protein. Characterization of a set of mutant proteins, in particular CheY59NR, gave information regarding CheY-CheZ interactions as well as the role Asn<sup>59</sup> may play in CheY phosphorylation and autodephosphorylation reactions.

#### EXPERIMENTAL PROCEDURES

**Chemicals**—The calcium salt of monophosphoimidazole (MPI) (23) and the potassium salt of phosphoramidate (PAM) (24) were synthesized as described. Stock solutions of MPI were made fresh and centrifuged before each use to remove small amounts of insoluble calcium phosphate. Acetyl phosphate was from Aldrich.

**Proteins**—The mutant genes *cheY59NR*, *59NA*, *59NK*, *59ND*, and *59NE* were made by *dut<sup>-</sup> ung<sup>-</sup>* mutagenesis (25) of the *cheYZ* plasmid pRBB40 (26), and *cheY23ND* was found in a random mutant search as previously described (27). Wild-type and mutant CheY proteins were purified from the K0641*recA*/pRBB40 strain (26) according to published procedures (28). CheZ was overexpressed from strain K0642*recA*/pKCB1.134KE, which was made by site-directed mutagenesis of pRBB40 to correct an inadvertent mutation at position 134 (29). CheZ was purified using a published protocol (28), except for the following two changes. First, a 5-ml Hi-trap Q-Sepharose Column (Amersham Pharmacia Biotech) was used in place of a MonoQ column (Amersham Pharmacia Biotech) to allow for larger scale preparations. Second, after gel filtration on Superose 12, a portion of the CheZ pool (2–3 mg) was chromatographed on a 1.0-ml MonoQ column. This step removed a contaminating enzymatic activity for MPI hydrolysis that was evident with the phosphate assay (see below) as well as a small amount of the CheZ proteolytic product CheZ<sub>1–181</sub>, which sometimes forms during purification (29). The concentrations of CheY and CheZ were determined by absorbance at 280 nm using extinction coefficients of 10,200 M<sup>-1</sup> cm<sup>-1</sup> (CheY) and 16,700 M<sup>-1</sup> cm<sup>-1</sup> (CheZ). The extinction coefficients were determined empirically by parallel measurements of absorbance and protein concentration by quantitative amino acid analysis (The Protein Chemistry Laboratory of University of North Carolina, Chapel Hill, NC).

**Chemotaxis Behavior**—All chemotaxis assays were performed on K0641*recA*/pRBB40 strains. The plasmid pRBB40 carries both *cheY* and *cheZ* so that any effect of possible CheY overexpression is counteracted by the presence of a proportional amount of CheZ (30). Rates of bacterial swarming on semi-solid agar plates were carried out at 30 °C as described (31). The rotational behavior of the bacterial flagella was determined by tethering analysis (13) and the Hobson Tracker system was used to analyze rotating cells (33).

**Kinetics of Phosphate Release**—The steady state rates of release of inorganic phosphate from reactions containing CheY, MPI, and CheZ were measured using an enzyme-linked spectroscopic assay (Enzchek P<sub>i</sub> Kit, Molecular Probes). In this assay, the reactions of interest were carried out in the presence of purine nucleoside phosphorylase and a guanine analog substrate, 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG). Purine nucleoside phosphorylase catalyzes the reaction of phosphate with MESG to rapidly form a product that absorbs at 360 nm (34). In our experiments, buffer (final concentration of 100 mM Hepes, pH 7.0, 20 mM MgCl<sub>2</sub>), MESG (final concentration of 200 μM), and MPI were mixed in a cuvette and placed into the spectrophotometer (Beckman DU7500 diode array), and the absorbance at 360 nm was continuously monitored. Purine nucleoside phosphorylase (5 μl; 0.5

units) was added, and an absorbance increase (typically 0.2–0.4 units) resulted due to the presence of inorganic phosphate in the MPI preparations. If appropriate, CheZ was then added. The addition of CheZ did not affect the absorbance of the sample. After 3 min in the cuvette chamber to ensure temperature equilibration, CheY was added to initiate the reaction. After a short lag period (<10 s), a linear increase in absorbance was observed due to release of P<sub>i</sub> from CheY-P. Linearity continued until an absorbance of about 1.6, whereupon the slope decreased gradually, as is expected at high absorbance. The time course was monitored for 2–4 min, and the slope of the absorbance change was determined by instrument software. The total volume of the reaction was 450 μl, the temperature of the cuvette was kept at 25 °C with electronic temperature control, and mixing was done manually. The slopes were converted to μM P/s by using an empirically determined extinction coefficient at 360 nm of 0.0091 μM<sup>-1</sup> cm<sup>-1</sup> at pH 7.0. This is slightly less than a published extinction of 0.011 μM<sup>-1</sup> cm<sup>-1</sup> measured at pH 7.6, as expected (34). There was no increase in slope if larger amounts of purine nucleoside phosphorylase were added, indicating that the observed rate was not limited by the linked enzymatic reaction. PAM and acetyl phosphate could not be used as phosphodonors in the enzyme-linked phosphate assay because preparations of these compounds contained levels of contaminating phosphate that gave off-scale absorbances at the concentrations required for efficient phosphorylation.

**Fluorescence**—All fluorescence measurements were carried out using a PerkinElmer LS-50B spectrofluorimeter with a circulating water bath for temperature control. Time courses for phosphorylation of CheY were monitored by tryptophan fluorescence using a stopped flow apparatus (Applied Photophysics RX2000) for rapid mixing of the reactants. Fluorescence was measured at an excitation wavelength of 292 nm and an emission wavelength of 346 nm, and all reactions were carried out at 25 °C. Data were fitted to an exponential decay, which yielded a first order rate constant (*k*<sub>obs</sub>), which is a function of both the rates of phosphorylation and dephosphorylation of CheY, as follows: *k*<sub>obs</sub> = *k*<sub>phosph</sub> [phosphodonor]/*K*<sub>s</sub> + *k*<sub>dephosph</sub> (33). The fluorescence anisotropy of fluoresceinated CheZ was monitored as a function of CheY concentration as described (16, 35).

**Autodephosphorylation Rates**—Rates of autodephosphorylation of CheY-P at room temperature were determined by electrophoretic analysis of <sup>32</sup>P]CheY-P as described (36). The buffer was 100 mM Tris, pH 7.5, containing 10 mM MgCl<sub>2</sub>. Multiple (two to four) independent trials were carried out for each CheY.

**Analytical Gel Filtration**—Chromatography was carried out on a high resolution Superose 12 column (1 × 30 cm) (Amersham Pharmacia Biotech) using fast protein liquid chromatography with a flow rate of 0.8 ml/min. CheY and CheZ were mixed in a 1:1 molar ratio (final mix concentrations of 0.60 mg/ml CheY and 1.0 mg/ml CheZ) in 50 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>. For phosphorylating conditions, acetyl phosphate (final concentration of 20 mM) was added to the CheY/CheZ mixtures, allowed to incubate for 3 min to allow for maximal phosphorylation, and then chromatographed on the column that had been equilibrated with freshly prepared 50 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, 20 mM acetyl phosphate immediately before application of the sample. Elution was monitored by ultraviolet detection, and fractions (0.40 ml) were collected. The fractions that corresponded to the observed peaks were pooled, concentrated by Centricon 10 (Amicon/Millipore), and electrophoresed. Molecular weight standards (Bio-Rad gel filtration standards kit and other individual proteins from Sigma) were independently chromatographed on the same column for estimation of the molecular weight of the species eluting from the column.

#### RESULTS

**Phenotypic Effects of Various Substitutions at Position 59 of CheY**—Using site-directed mutagenesis, we made six substitutions at position 59 (arginine, lysine, histidine, aspartate, glutamate, and alanine). The effects of the mutations on chemotaxis were assessed by measurement of the swarm rates and flagellar rotational biases of strains containing the mutant genes (Table I). All the mutant strains except that containing CheY59NA swarmed at a significantly slower rate than the wild-type strain. Decreased swarm rates can result from either an increase or decrease in CCW bias (37). Strains containing CheY59NK, CheY59ND, and CheY59NE displayed rotational biases more CCW than wild type, with CheY59ND displaying the most extreme phenotype, with nearly exclusively CCW

TABLE I  
Phenotypes of K0641recA/pRBB40 strains containing CheY with substitutions at position 59

Amino acid at position 59	Swarm rate <sup>a</sup>	Rotational Bias $\pm$ S.E. <sup>b</sup>
Asn (wild type)	1	0.71 $\pm$ 0.07
Arg	0.20	0.01 $\pm$ 0.00
Lys	0.22	0.92 $\pm$ 0.04
His	0.20	0.05 $\pm$ 0.01
Asp	0.03 <sup>c</sup>	0.98 $\pm$ 0.01
Glu	0.23	0.93 $\pm$ 0.02
Ala	0.80	0.91 $\pm$ 0.04

<sup>a</sup> Ratio of swarm rate to that of wild-type control. The reported value is an average of triplicate measurements. The deviation between individual measurements was less than 10%.

<sup>b</sup> The fraction of time bacteria rotated in the CCW direction using the tethering assay. 20–30 cells were analyzed for each strain, and the average value is reported.

<sup>c</sup> The swarm rate was not significantly different from that of the parent strain K0641recA, which is deleted for CheY.

behavior and inability to swarm. In contrast, cells that contained CheY59NR or CheY59NH displayed extreme CW biases relative to wild-type strains. Transformation of plasmids carrying *cheY59NR* or *cheY59NH* into a CheA-deficient host resulted in fully CCW behavior (data not shown), indicating that the CW activity was dependent on CheY phosphorylation by CheA. Strong CW behavior could be due to an increased rate of phosphorylation or decreased rate of dephosphorylation, both of which would result in increased CheY-P levels or enhanced binding of CheY-P to the flagellar switch. Because we believed that any of these explanations would be informative as to the role of position 59 in CheY function, we chose to focus our biochemical studies on the basis for the CW behavior of the CheY59NR protein.

**Phosphorylation Properties of CheY Using Phosphate Release Assay**—To assess the phosphorylation, autodephosphorylation, and CheZ-dependent phosphorylation properties of CheY59NR (and CheY59NA and wild-type CheY for comparison), we used a commercially available enzyme-linked spectroscopic assay that continuously measures phosphate concentration. The steady state rate of release of phosphate for the reaction of CheY with MPI is a function of both the phosphorylation and dephosphorylation rates. The data in Fig. 1A for wild-type CheY show that as the concentration of MPI was increased, the rate of phosphate release increased and eventually saturated. The sensitivity of the P<sub>i</sub> release rate to MPI concentrations indicates that the observed rate is at least partially dependent on the phosphorylation rate. Saturation occurs when autodephosphorylation becomes rate-limiting. That saturation is due to limiting autodephosphorylation is evident because a similar titration carried out in the presence of excess CheZ, which will greatly increase the dephosphorylation rate, gave higher phosphate release rates, which continued to increase well past 1 mM MPI (Fig. 1A). The autodephosphorylation rate constant ( $k_{\text{dephosph}}$ ) can be estimated from the saturating rate by dividing the saturating rate by the concentration of CheY. This gives a  $k_{\text{dephosph}}$  value of 0.035 s<sup>-1</sup>, which compares well with other determinations (36, 38).

Similar titrations of CheY59NR and CheY59NA reveal several properties of the phosphorylation reactions for these proteins (Fig. 1B). First, the phosphate release rates for the three CheY proteins all saturate at similar rates, implying that these proteins have similar autodephosphorylation rate constants ( $k_{\text{dephosph}} \sim 0.030\text{--}0.040$  s<sup>-1</sup>). This conclusion was confirmed by direct determination of autodephosphorylation rates by following the decomposition of [<sup>32</sup>P]CheY-P by gel electrophoresis and phosphorimaging analysis. This analysis gave  $k_{\text{dephosph}}$  values of 0.036  $\pm$  .006 (1 S.D.) s<sup>-1</sup> (wild-type CheY), 0.035  $\pm$

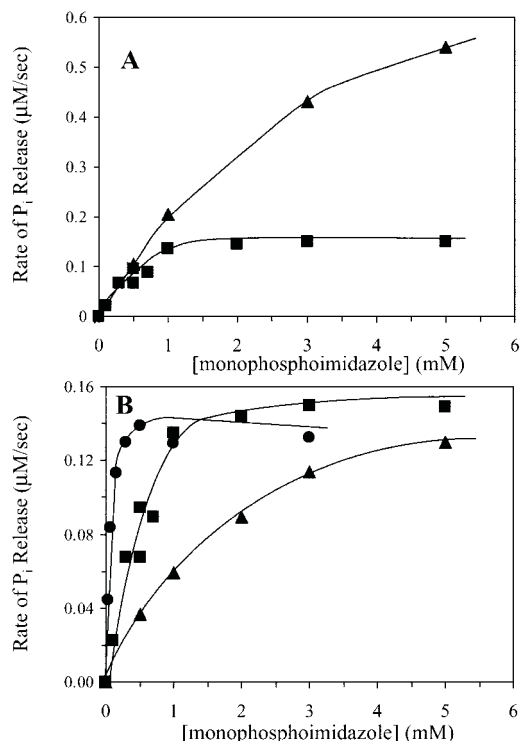


FIG. 1. The rate of release of inorganic phosphate as a function of MPI concentration. Reactions were carried out as described under "Experimental Procedures." A, wild-type CheY (4.5  $\mu$ M) in the presence (▲) and absence (■) of 1  $\mu$ M CheZ. B, comparison of MPI titrations of wild-type CheY (■), CheY59NR (●), and CheY59NA (▲). The ionic strength of the reactions were kept constant by the addition of sodium chloride so that the final ionic strength was 40 mM.

0.005 s<sup>-1</sup> (CheY59NR), and 0.037  $\pm$  0.008 s<sup>-1</sup> (CheY59NA). Second, the shift of the titration curve to a lower MPI concentration for CheY59NR indicates that the phosphorylation rate at a given MPI concentration is faster for this mutant than for wild-type CheY. Similarly, the shift of the curve to the right for CheY59NA indicates slower autophosphorylation at a given MPI concentration. These conclusions were supported by fluorescence measurements of rates of CheY-P formation. With MPI as phosphodonor, the observed rate constant for accumulation of CheY-P ( $k_{\text{obs}}$ ) was more than 10-fold higher for CheY59NR compared with wild-type CheY, whereas the rate for CheY59NA was about 2-fold lower than wild-type CheY (Table II). Because the autodephosphorylation rates are similar for the proteins (above), these differences in  $k_{\text{obs}}$  reflect differences in phosphorylation rates ( $k_{\text{phosph}}/K_s$ ). The accelerated phosphorylation rate for CheY59NR was also observed with PAM as phosphodonor, as CheY59NR had a  $k_{\text{obs}}$  about four times higher than wild-type CheY. However, with acetyl phosphate, CheY59NR had a slightly slower  $k_{\text{obs}}$  than wild-type CheY. Therefore the accelerated rate of phosphorylation for CheY59NR occurred only with the nitrogen-phosphorous phosphodonors. Possible mechanistic implications are discussed below.

**CheZ Sensitivities**—Titrations of the phosphate release rate with CheZ were carried out at 3 mM MPI, a concentration where the phosphate release rates for all three proteins were predominantly limited by autodephosphorylation (Fig. 1B). For wild-type CheY, the rate of phosphate release increased with CheZ and saturated by 150 nM, with 40–60 nM CheZ required for half saturation (Fig. 2). Saturation is expected when autophosphorylation becomes rate-limiting, and the rate at which saturation occurs should correlate with the phosphorylation rate. Like wild-type CheY, the phosphate release rate for

TABLE II  
Observed rate constants for CheY phosphorylation with various phosphodonors measured by fluorescence

CheY	$k_{\text{obs}}$		
	Acetyl phosphate (50 mM)	PAM (50 mM)	MPI (12.5 mM)
Wild type	0.335	0.611	0.153
59NR	0.233	2.55	2.09
59NA	0.083	0.585	0.070

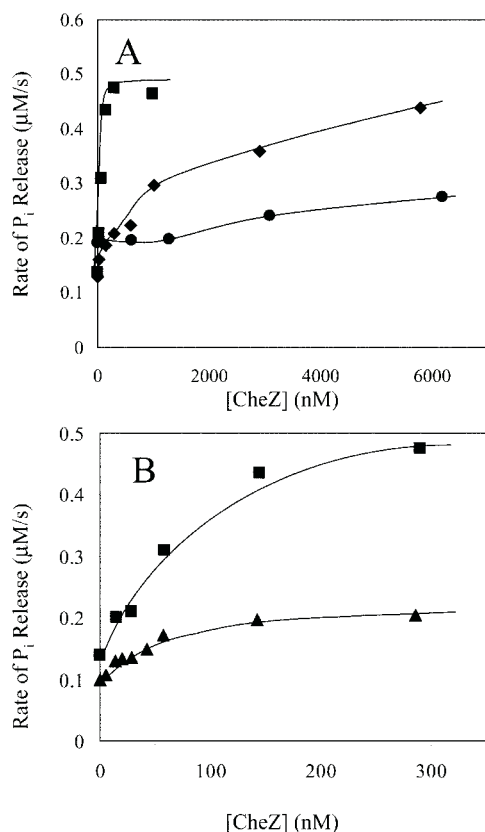


FIG. 2. **CheZ sensitivities of wild-type and mutant CheY assessed by the rate of phosphate release.** The rate of release of phosphate was measured for wild-type CheY (■), CheY59NR (●), CheY59NA (▲), and CheY23ND (◆) in the presence of various concentrations of CheZ. Wild-type CheY, CheY59NA, and CheY23ND were present at 4.5 µM; CheY59NR concentration was 4.9 µM. Note the different CheZ concentration scales for panels A and B.

CheY59NA increased throughout the 0–100 nM CheZ range but gave a lower saturation rate, implying a slower phosphorylation rate (Fig. 2B), consistent with the results described above (Fig. 1B, Table II). In contrast, CheY59NR demonstrated extreme CheZ resistance. There was no detectable change in phosphate release rate for CheZ concentrations up to 1 µM, showing that CheZ had no effect, either negative or positive, on the rate of dephosphorylation of CheY59NR. A CheZ concentration of 12 µM (the concentration of CheY was 3.3 µM) was required to increase the rate 68% relative to the rate in the absence of CheZ (data not shown). Saturation of the rate was not achieved and would be expected to occur at a rate much higher than that of wild-type CheY (Table II). Given the rate increase by the highest amount of CheZ, we estimate that CheY59NR requires a minimum of 250 times more CheZ to get the same rate enhancement as wild-type CheY and CheY59NA. The exceptional CheZ resistance of CheY59NR would result in high concentrations of CheY-P in the cell, thus accounting for the CW phenotype of cells containing this protein. Finally, for

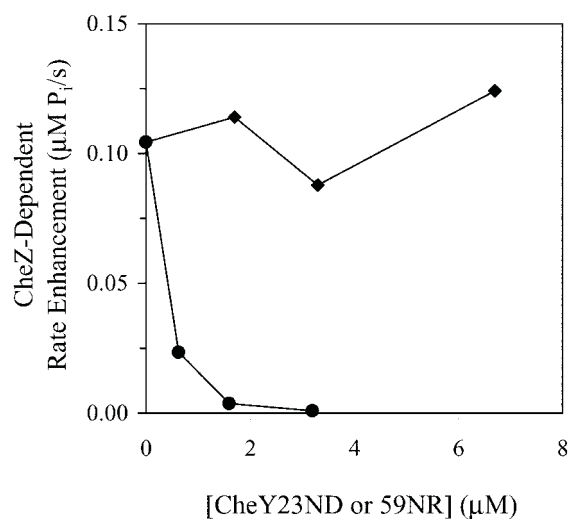


FIG. 3. **Competition of mutant CheZ-resistant CheY with wild-type CheY for CheZ.** The ordinate represents the CheZ-dependent increase in the rate of phosphate release for wild-type CheY in the absence (*y* intercept) and presence of various concentrations of CheY 23ND (◆) or CheY 59NR (●), two CheZ-resistant CheY proteins. The concentration of CheZ was 33 nM and that of wild-type CheY was 3.3 µM.

comparison, the CheZ sensitivity of a previously characterized CheZ-resistant mutant, CheY23ND (27), was assessed using this assay. Asn<sup>23</sup> is a surface residue located on  $\alpha$  helix 1, about 20 Å from the active site Asp<sup>57</sup>. CheY23ND required 800–1000 nM CheZ to reach half of the possible rate acceleration (Fig. 2A), about 15–20 times more than wild-type CheY. This value is in reasonable agreement with a previous observation that CheY23ND required about 50-fold more CheZ than wild-type CheY for a similar rate acceleration (27).

**Competition of CheY23ND and CheY59NR with Wild-type CheY for CheZ**—The biochemical basis for the extreme CheZ resistance of CheY59NR was explored further by assessment of the ability of the mutant protein to bind to CheZ. The abilities of the CheZ-resistant proteins CheY23ND and CheY59NR to affect CheZ phosphatase activity toward wild-type CheY was assessed by the phosphate release assay. The catalytic effect of a small amount of CheZ (33 nM) for dephosphorylation of wild-type CheY was determined by measuring the difference in phosphate release rates in the presence and absence of CheZ (Fig. 3; data point on ordinate). This concentration of CheZ had no impact on the phosphate release rate of CheY23ND or CheY59NR (Fig. 2A). The effect of this amount of CheZ was then determined in the presence of increasing concentrations of competing CheY23ND or CheY59NR. For CheY23ND the difference in rates stayed the same despite the presence of an extraneous CheZ-resistant CheY (Fig. 3). This result is consistent with the previously established defect in CheZ binding of CheY23ND (27). In contrast, the presence of CheY59NR inhibited CheZ activity toward wild-type CheY (Fig. 3). When equimolar quantities of CheY59NR and wild-type CheY were present, there was no detectable CheZ-dependent increase in rate, implying that binding of CheZ to CheY59NR was at least as tight as to wild-type CheY. Therefore CheY59NR acts as a competitive inhibitor for CheZ activity toward wild-type CheY.

**Complex Formation between CheY59NR and CheZ**—The competition experiment implied that CheY59NR, but not CheY23ND, was capable of binding to CheZ with a sufficiently tight affinity to prevent interaction with wild-type CheY. This suggested the possibility that CheY59NR was capable of binding to CheZ but that the CheZ phosphatase activity was disabled in the complex. To more directly assess binding, mixtures of CheY and CheZ were analyzed by analytical gel filtration

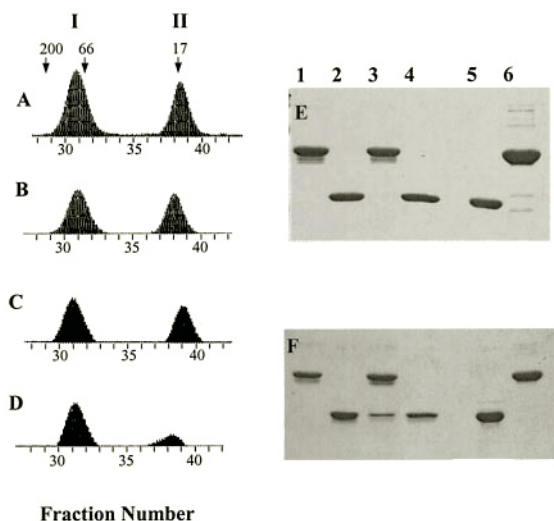


FIG. 4. Analytical gel filtration of CheY/CheZ mixtures. Shown are absorbance elution profiles of mixtures containing wild-type CheY (A and B) and CheY59NR (C and D) after chromatography on Superose 12 under nonphosphorylating (A and C) and phosphorylating (B and D) conditions. The profiles are aligned with each other according to fraction number. The arrows in A show the mobility of molecular mass standards, with mass expressed in kDa. Each profile showed two peaks: I, the early peak; II, the late peak. Fractions composing each peak were pooled, concentrated, and run on SDS gels for wild-type CheY (E) and CheY59NR (F). For each gel, the lanes are peak I - P (1), peak II - P (2), peak I + P (3), peak II + P (4), CheY standard (5), and CheZ standard (6).

under both nonphosphorylating and phosphorylating conditions. Gel filtration chromatography of mixtures of wild-type CheY and CheZ (1 CheY chain:1 CheZ chain) resulted in clean separation of the two proteins, both in the presence and absence of acetyl phosphate (Fig. 4, A, B, and E), as has been previously observed (16). Based on the calibration of the column by molecular mass standards, CheZ eluted at an apparent molecular mass of 87 kDa (the molecular mass of CheZ<sub>2</sub> is 48 kDa), consistent with previous observations (39) that have implicated an elongated structure for CheZ<sub>2</sub>. CheY (molecular mass 14 kDa) eluted at an apparent molecular mass of 17 kDa. Similar mixtures of CheY59NR and CheZ showed the presence of two peaks with the same mobilities as seen with wild-type CheY but a reduction of the intensity of the CheY peak under phosphorylating conditions (Fig. 4, C and D). Gel electrophoresis showed that this was due to the co-elution of a portion of the CheY (about one-third to one-half) with the CheZ (Fig. 4F). The elution position of the peak that contained both CheY and CheZ was identical to that of CheZ alone. Therefore, CheY59NR and CheZ formed a phosphorylation-dependent complex that stays associated through gel filtration chromatography. The complex has a size that was indistinguishable, within the sensitivity of this method, from that of the CheZ dimer. The inability to detect a wild-type CheY-CheZ complex under the conditions used for gel filtration is likely because there is very little phosphorylated CheY present due to the phosphatase activity of CheZ.

CheY-CheZ complexes have been reported to form higher associative states under phosphorylating conditions (15–17). One of the methods used to detect the oligomers is fluorescence anisotropy measurements on fluoresceinated CheZ (16). Because the gel filtration experiment suggested that the CheY59NR-CheZ complex was similar in size to CheZ<sub>2</sub>, it was of interest to assess the size of the CheY59NR-CheZ complex using fluorescence anisotropy. When CheY59NR was added to fluoresceinated CheZ under phosphorylating conditions, the anisotropy increased and saturated at a similar anisotropy value as observed for wild-type CheY (Fig. 5), indicating that

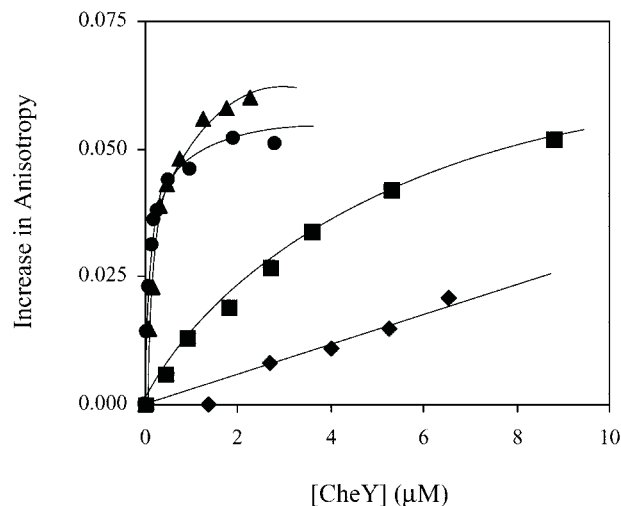


FIG. 5. Fluorescence anisotropy of fluoresceinated CheZ in the presence of various CheY proteins. Aliquots of CheY were added sequentially to a solution of fluoresceinated CheZ (0.20  $\mu\text{M}$ ), and the fluorescence anisotropy was recorded after each addition. The conditions were wild-type CheY + 20 mM acetyl phosphate (■), wild-type CheY + 0.1 mM  $\text{BeF}_3^-$  (●), CheY59NR + 20 mM acetyl phosphate (▲), and CheY 23ND + 20 mM acetyl phosphate (◆). When indicated, acetyl phosphate was added to CheZ before the addition of CheY.  $\text{BeF}_3^-$  was made *in situ* by the addition of 0.1 mM  $\text{BeCl}_2$  and 10 mM NaF directly to the CheZ before the addition of CheY.

the anisotropy measurements were monitoring the same event with both proteins and that CheZ forms a similarly sized complex with CheY59NR-P as with wild-type CheY-P.

The anisotropy results also showed that the titration curve for CheY59NR was shifted to lower CheY concentrations than for wild-type CheY (Fig. 5). Notably, the titration curve for CheY59NR was superimposable, within experimental error, with that for wild-type CheY in the presence of  $\text{BeF}_3^-$ . CheY binds  $\text{BeF}_3^-$  to form a stable complex that is an excellent analogue of CheY-P (40, 41). Assuming that the anisotropy measurements reflect the extent of binding between CheZ and CheY with saturation of the signal occurring when all of the CheZ is bound, the results imply that binding of CheY59NR-P or CheY- $\text{BeF}_3^-$  to CheZ occurs with extremely high affinity. Under the conditions of this assay (0.20  $\mu\text{M}$  CheZ), two independent titrations for both CheY59NR-P and CheY- $\text{BeF}_3^-$  showed that at CheY concentrations of 0.10  $\mu\text{M}$ , between 50 and 90% of added CheY is bound to the CheZ. Taking this nearly quantitative binding into account as well as results obtained from fitting the anisotropy data to a simple binding equation, we estimate  $K_d$  values of between 50 and 250 nM for CheZ binding to either CheY59NR-P or CheY- $\text{BeF}_3^-$ . Much higher wild-type CheY concentrations (Fig. 5) are necessary to achieve maximal anisotropy values, likely because the phosphatase activity of CheZ lowers the concentration of CheY-P in solution. CheY23ND, which does not bind well to CheZ (27) (Fig. 3), showed a modest increase in anisotropy.

#### DISCUSSION

**CheY-CheZ Interactions**—CheY59NR was extremely resistant to CheZ (>250 $\times$  wild-type, Fig. 2A) despite binding to CheZ at least as well as wild-type CheY (Figs. 3–5). The CW behavior of cells containing this mutant protein (Table I) was evidence that CheY59NR was capable of interacting effectively with the switch and that the amino acid substitution had no obvious deleterious effects on CheY conformation. In keeping with this, CheY59NR had the same autodephosphorylation rate as wild-type CheY and the same or better (discussed below) rates of autophosphorylation (Table II), both demonstrations that the

active site geometry was intact. Therefore, the behavior of CheY59NR appeared to be due to specific inhibition of CheZ activity and not a conformational defect, which would be expected to affect many activities. The fact that CheY59NA was sensitive to CheZ (Fig. 2B) implied that it is not the asparagine at position 59 that is necessary for CheZ sensitivity but rather that the arginine at this position inhibited CheZ activity. This combination of properties is unique; a previously characterized CheZ-resistant CheY mutant, CheY23ND (Ref. 27 and this study) was defective in CheZ binding. Likewise, several CheY mutants showed varying degrees of CheZ resistance (5–50×) despite binding to CheZ but were incapable of conferring CW behavior (42). The strong CheZ resistance of CheY59NR can explain the CW phenotype of cells containing this protein as higher than normal concentrations of intracellular CheY-P would result.

The result that an arginine substitution at position 59 allows binding but not catalysis by CheZ implies that the presence of the arginine prevents the critical residue(s) from achieving optimal positions for catalysis. We used computer modeling (Insight II and Swiss Protein Data Bank Viewer) using the coordinates for the CheY·BeF<sub>3</sub><sup>-</sup>·FliM peptide structure (22)<sup>2</sup> to assess possible structural explanations for the observations. The analysis pointed to two general possibilities. First, modeling predicted that substitution of an arginine at position 59 could result in a new interaction between the side chains of Arg<sup>59</sup> and Glu<sup>89</sup>, located on the flexible loop between β4 and α4 of CheY. This interaction was not predicted to occur in inactive CheY due to a different conformation of the β4/α4 loop. Precedent for such an interaction comes from the structure of the response regulator FixJ, which has an arginine at position 56 (analogous to position 59 in *E. coli* CheY) that interacts directly with Asp<sup>86</sup> (analogous to CheY position 89) (43). There are also attractive interactions between Asn<sup>61</sup> (analogous to position 59) and two residues on the β4/α4 loop in the inactive structure of NarL (44). Therefore, it is possible that CheZ binding to activated CheY induces further movement of the flexible “nineties” loop, which puts the necessary residue(s) (on CheY) into position to aid catalysis, as proposed by Zhu *et al.* (42). It is conceivable that Glu<sup>89</sup> itself has a catalytic role in CheZ-dependent dephosphorylation. An arginine at position 59 could prevent this event due to interactions with the loop. A search for CheZ proteins using the Entrez search engine (NCBI) yielded CheZ proteins from 7 bacterial species. It is noteworthy that the CheY proteins of all 7 species contain an asparagine at the position corresponding to position 59 of *E. coli* CheY as well as a glutamate at position 89. In CheY proteins from other bacterial species there are a variety of amino acids at these two positions.

An alternative explanation for the behavior of CheY59NR is that the arginine side chain at position 59 could adopt a conformation, perhaps by interaction with the phosphoryl group, that sterically prohibits access to the active site. This could prevent potential catalytic residues, likely from CheZ, from physical access to the active site. Current studies are directed at differentiation between these two models by analysis of CheY proteins with single site substitutions in the β4/α4 loop.

Of the five other substitutions that were made at position 59 of CheY, only CheY59NH gave the same strong CW rotational bias as CheY59NR (Table I). Because the histidyl side chain can be positively charged and is capable of participating in hydrogen bonding, it follows that substitution at this position could result in similar interactions as the arginyl substitution, which led to CheZ resistance. For example, the plausibility of

an interaction between the His<sup>59</sup> and Glu<sup>89</sup> is also supported by computer modeling. However, the correlation between CW rotational bias and positive charge did not extend to the lysine substitution (Table I). Further studies are necessary to determine if the CCW rotational bias of CheY59NK, in fact, reflects CheZ sensitivity or is instead due to another effect of the substitution such as defective interaction with the flagellar switch.

The inability of the arginine or alanine substitutions at position 59 in CheY to affect the rate of autodephosphorylation contrasts with results from the response regulator Spo0F, where changing the analogous residue had a large impact on the autodephosphorylation rate (20). Spo0F with the native lysine residue has a slow rate, whereas substitution with an asparagine increases the rate. Because we did not observe any change in autodephosphorylation rates for similar mutations in CheY, we conclude that the trends observed with Spo0F are not generally applicable to all response regulators.

*Associative State of the CheY-P·CheZ Complex*—Complexes of CheY-P and CheZ, like any enzyme-substrate complex, are inherently difficult to study using wild-type proteins because the rate of dephosphorylation of CheY is so rapid that very little or no CheY-P is present at steady state. Thus, gel filtration chromatography of mixtures of wild-type CheY and CheZ in the presence of phosphodonor resulted in separate elution of the two proteins (this study and Ref. 16). In contrast, we were able to isolate a CheY59NR·CheZ complex by gel filtration (Fig. 4), presumably because the rate of dephosphorylation is slow enough (approximately equal to the autodephosphorylation rate) that there is appreciable CheY-P in solution. However, it was surprising that this complex had an elution position indistinguishable from that of CheZ<sub>2</sub>. In our experiments, CheZ<sub>2</sub> (molecular mass 48 kDa) eluted at a position that corresponded to an apparent molecular mass of 87 kDa, consistent with previous observations (39) that implicate an elongated structure for CheZ<sub>2</sub>. The elution position of the CheY59NR-P·CheZ complex is consistent with a CheZ<sub>2</sub>·CheY composition because such a complex would not be expected to show any change in mobility by this method (CheZ<sub>2</sub>·CheY<sub>2</sub> is improbable due to the surplus of CheZ in the eluted peak). Any higher aggregation of CheZ<sub>2</sub>·CheY, which retained the elongated shape, would result in earlier elution from the column, based on the mobility of molecular weight standards, and this was not detected. However, we cannot rule out the possibility that CheZ<sub>4</sub>·CheY<sub>2</sub> (molecular mass of 124 kDa) could coelute with CheZ<sub>2</sub> if the shape of the former complex was near spherical. The indistinguishable elution positions of the CheY59NR-P·CheZ complex and CheZ<sub>2</sub> is in apparent contradiction to the observation that CheY59NR-P caused an increase in the anisotropy of fluoresceinated-CheZ, a change that has been ascribed to an oligomerization process with the formation of species of about 200 kDa (16). It is conceivable that higher oligomers dissociated during the chromatography process. However, protein concentrations were 100-fold higher than in the fluorescence anisotropy experiment where stable oligomers were observed, so this seems unlikely. Therefore, despite isolation of a CheY-P·CheZ complex using gel filtration, we were not able to confirm the higher aggregation of this complex by this method.

The titration curves showing the effect of CheY concentration on the anisotropy of CheZ for CheY59NR-P and CheY·BeF<sub>3</sub><sup>-</sup> were shifted to lower CheY concentrations compared with a similar titration with wild-type CheY (Fig. 5). Assuming that anisotropy was an indicator of CheY/CheZ binding (an assumption that is strengthened by the observation that the curve for CheY23ND, a protein known to be defective in its CheZ binding, was shifted to much higher CheY concen-

<sup>2</sup> D. Wemmer, personal communication.

trations),  $K_d$  values for CheY59NR-P and CheY-BeF<sub>3</sub><sup>-</sup> binding to CheZ were estimated to be submicromolar (50–250 nM) and likely represent the true binding of activated CheY to CheZ. This value is much smaller than the measured binding constant of a C-terminal peptide of CheZ to CheY-P ( $K_d$  of 26 μM (45)). This peptide (32) exhibits enhanced binding to CheY under phosphorylating conditions. Therefore our results agree with the conclusion of McEvoy *et al.* (45) that the interactions with the peptide represent a small portion of the overall binding between CheY and CheZ. Wild-type CheY-P shows a weaker apparent binding interaction in this experiment because there is a lower concentration of activated CheY-P in solution due to the CheZ phosphatase activity.

**Mechanism of Autophosphorylation**—Whereas wild-type CheY and CheY59NR had similar  $k_{\text{obs}}$  values for phosphorylation with acetyl phosphate, CheY59NR had significantly enhanced rates with PAM and MPI. Because the autodephosphorylation rates ( $k_{\text{dephosph}}$ ) were the same, this difference reflected differences in the rate of phosphorylation ( $k_{\text{phosph}}/K_s$ ). A notable difference between the phosphodonors with an N-P linkage and those with an O-P is the charge on the leaving group. Phosphoramidates only react with CheY when they are positively charged (36) and will give a cationic leaving group, whereas the acetate leaving group is negatively charged. Therefore, the positive charge at position 59, through charge/charge repulsion, may increase the probability that the PAM or MPI substrate is oriented favorably for reaction.

**The P<sub>i</sub> Kinetic Assay Gives New Information about Phosphorylation Kinetics**—In this study we demonstrate the application of a commercially available enzyme-linked assay to kinetically assess phosphotransfer reactions involving CheY and/or CheZ. Favorable comparison of rate constants obtained by this assay to parallel experiments using fluorescence to monitor the rate of accumulation of CheY-P and <sup>32</sup>P measurement to directly measure loss of the phosphoryl group established the validity of the P<sub>i</sub> release assay. The P<sub>i</sub> assay provides information unavailable from other assays. For example tryptophan fluorescence, which monitors levels of CheY-P, is not able to assess CheZ-dependent dephosphorylation of CheY-P when either CheZ or CheY is defective in the reaction. In this situation, the fluorescence of CheZ becomes large compared with the increase in fluorescence due to dephosphorylation, and large errors occur in subtraction of this background signal. In addition, the assay can provide information about phosphorylation in systems where fluorescence cannot be used due to lack of a fluorophore near the phosphorylation site or where phosphorylated response regulator does not accumulate. Inability to accumulate phosphorylated response regulator could be due to either the absence of phosphorylation or to slow phosphorylation relative to dephosphorylation. These two scenarios can be distinguished using the P<sub>i</sub> assay, because P<sub>i</sub> would be released due to continuous turnover in the latter case.

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