Conserved C-Terminus of the Phosphatase CheZ Is a Binding Domain for the Chemotactic Response Regulator CheY†

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ABSTRACT: CheZ is the phosphatase of the chemotactic response regulator, CheY. There are three conserved domains on CheZ. Here we determined the function of the C-terminal domain (residues 202−214). A truncated form of CheZ, missing residues 202−214, hardly bound to the phosphorylated form of CheY. Conversely, a peptide composed of the last 19 amino acid residues of CheZ (residues 196−214), generated by tryptic digestion, bound specifically to the phosphorylated form of CheY. This was demonstrated by both fluorescence depolarization of the peptide (labeled with fluorescein) and cross-linking. It is concluded that the conserved C-terminus of CheZ functions as a CheY-binding domain.

CheY, the signal molecule of bacterial chemotaxis, is a response regulator which belongs to the prevalent two-component family [see Parkinson (1993), Parkinson and Kofoid (1992), and Swanson et al., (1994) for recent reviews]. CheY functions as a molecular switch at the end of the signal transduction cascade of bacterial chemotaxis [for reviews, see Barak and Eisenbach (1996), Bourret *et al.* (1991), Eisenbach (1991), Hazelbauer *et al.* (1993), Lukat and Stock (1993), and Stock *et al.* (1991)]. CheY is turned on by phosphorylation (Hess *et al.*, 1988; Wylie *et al.*, 1988). This enables the protein to interact with the switch/motor complex at the base of each flagellum with a resultant clockwise rotation of the motor (Barak & Eisenbach, 1992; Welch *et al.*, 1993).

The biochemical activities of CheZ—the phosphatase of CheY—were recently shown to be tightly dependent on the phosphorylation level of CheY. Thus, CheZ binds to the phosphorylated form of CheY (CheY∼P)[1] two orders of magnitude better than to the nonphosphorylated form (Blat & Eisenbach, 1994). Upon binding to phosphorylated CheY, CheZ undergoes oligomerization which seems to increase its phosphatase activity (Blat & Eisenbach, 1996a,b).

The amino acid sequence of CheZ from three organisms, *Escherichia coli, Salmonella typhimurium*, and *Pseudomonas aeruginosa*, is known (Masduki *et al.*, 1995; Mutoh & Simon, 1986; Stock & Stock, 1987). The sequences of CheZ from *E. coli* and *S. typhimurium* are almost identical. Alignment of these sequences with the sequence of CheZ from the more distantly related *P. aeruginosa* reveals that there are three highly conserved regions in this protein: residues 50−62, 138−148, and 202−214 (Figure 1). Replacement of arginine 54 by cysteine leads to an increased phosphatase activity of CheZ (Huang & Stewart, 1993). Mutations within residues 138−148 prevent the oligomerization of CheZ and result in defective phosphatase activity, but they do not affect the binding of CheZ to CheY (Blat & Eisenbach, 1996b). However, no mutations in the conserved region between residues 202 and 214 (the C-terminus of CheZ) have been reported in the literature, and no function has been assigned to this region. Assuming that the CheZ domain which binds CheY∼P is one of the three highly-conserved regions, we examined in this study whether the binding domain is the conserved region at the C-terminus.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. *E. coli* strain RP3098 [RP437Δ*(flhC-flhA)* (Smith & Parkinson, 1980)] carrying pEWW7 (Blat & Eisenbach, 1996b) or pEWC1 (Blat & Eisenbach, 1996a) was used for the overexpression of *S. typhimurium* CheZ or CheZ214FC, respectively. *E. coli* strain RP1091 [RP437Δ*(cheA-cheZ)* (Parkinson & Houts, 1982)] carrying pEWY5 (Blat & Eisenbach, 1996a) was used for the overexpression of *S. typhimurium* CheY. For the purification of His-tagged CheY we used strain BL21 (Studier *et al.*, 1990) carrying pREP4 (Qiagen) and pQE12-CheY-His-tag (received from R. V. Swanson and M. I. Simon). The plasmid pEWD202, used for the overproduction of $CheZ_{1-201}$, was constructed by cloning a PCR-generated fragment into pBTac, essentially as described earlier for other CheZ overproducers (Blat & Eisenbach, 1996a). The deletion at the C-terminus was generated by replacing the *cheZ* 3′ primer with the primer 5′-CCGGATC-CTTAACTGGCAACGACGCCAGC-3′. Overproduction of $CheZ_{1-201}$ was carried out in BW3 (Blat & Eisenbach, 1996b).

Protein Purification. CheY and CheZ from *S. typhimurium* were purified as described earlier (Blat & Eisenbach, 1996a). His-tagged CheY was purified as follows: Cells were grown at 35 °C in Luria broth containing 100 μg/mL ampicillin and 50 μg/mL kanamycin. Overexpression of CheY was induced at $OD_{590} = 0.5$ by 3 h incubation with aeration at 35 °C in the presence of isopropyl β-D-thiogalactopyranoside (0.5 mM). His-tagged-CheY was purified on His·Bind resin (Novagen), according to the manufacturer's instructions, from a soluble extract of the cells obtained by sonication.

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[1] Abbreviations: AcP, acetyl phosphate; CheY∼P, phosphorylated CheY.

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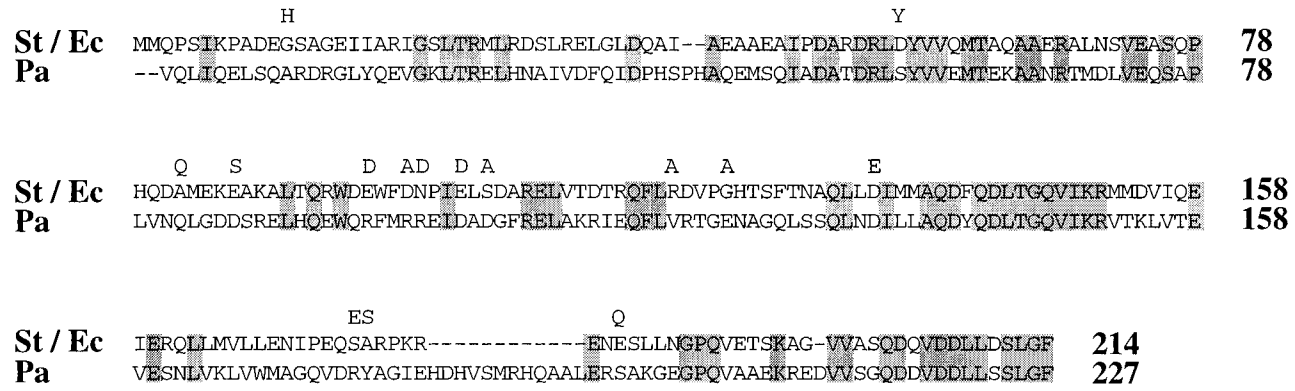

FIGURE 1: Conserved regions in CheZ. Identical residues between *S. typhimurium* (St), *E. coli* (Ec), and *P. aeruginosa* (Pa) are shadowed. Since the CheZ sequences from *E. coli* and *S. typhimurium* are almost identical, only the sequence of *S. typhimurium* is shown. Whenever different, the *E. coli* amino acid is indicated above the *S. typhimurium* amino acid.

Radiolabeling of CheZ. Radiolabeling of CheZ and $CheZ_{1-201}$ by [^{3}H]$NaBH_4$ was carried out as described earlier (Blat & Eisenbach, 1996b).

Labeling and Trypsin Digestion of CheZ214FC. CheZ214FC was labeled by fluorescein maleimide as previously described (Blat & Eisenbach, 1996a), except that the concentrations of fluorescein maleimide and CheZ214FC were 1 mM and 200 μM, respectively. Fluorescein-labeled CheZ214FC (157 μM) was incubated with trypsin (6 μg/mL) in Tris-HCl (50 mM, pH 7.9) at 25 °C. After 10, 20, and 30 min of incubation, 200 μL samples were removed, added to tubes containing 50 μL of soy bean trypsin inhibitor (1 mg/mL), and immersed immediately in liquid nitrogen.

Purification of CheZ Proteolytic Fragments. The fragments of fluorescein-labeled CheZ214FC, generated by tryptic digestion, were resolved on an HPLC gel filtration column (TSK125, Bio-Rad; flow rate 1 mL/min), preequilibrated with a solution of Tris-HCl (50 mM, pH 7.9), KCl (100 mM), and $MgCl_2$ (5 mM). The fractions (0.5 min each) corresponding to peak no. 2 in Figure 2 were pooled together and further purified on an HPLC C_{18} reversed-phase column (Vydac; flow rate 0.6 mL/min) which had been preequilibrated with 0.05% trifluoroacetic acid. After loading the sample, the column was washed for 3 min by a 0−25% gradient of acetonitrile followed by a 25−80% gradient for 40 min (at this stage 15 s fractions were collected). The major peak, identified as a peptide composed of residues 196−214 of CheZ, was eluted between 23.25 and 24.00 min. The fractions were pooled, lyophilized, resuspended in Tris-HCl (50 mM, pH 7.9), and stored at −20 °C.

CheY Binding to CheZ Fragments Measured by Fluorescence Depolarization. Purified fluorescein-labeled $CheZ_{196-214}$ and fractions containing fluorescent CheZ fragments were diluted into a solution containing Tris-HCl (50 mM, pH 7.9), $MgCl_2$ (5 mM), sucrose (5%), and—where indicated—acetyl phosphate (AcP, 18 mM). CheY was then added from a stock solution of 500 μM in the same solution to form the indicated concentration. Fluorescence depolarization was measured as described earlier (Blat & Eisenbach, 1996a).

Cross-Linking of CheY and $CheZ_{196-214}$. The reaction mixture (40 μL) contained Tris-HCl (50 mM, pH 8.2), KCl (25 mM), His-tagged-CheY (10 μM), and fluorescein-labeled $CheZ_{196-214}$ (9.6 μM). $MgCl_2$ (5 mM) and AcP (18 mM) were added as appropriate. The reaction was initiated by the addition of the cross-linker dimethylsuberimidate (3 mg/mL). After 3 h at 25 °C, 200 μL of His·Bind resin, preloaded with $NiSO_4$, were added. Following a 2 min incubation at 0 °C, the resin was washed twice with 400 μL of binding buffer [NaCl (0.5 M), Tris-HCl (20 mM, pH 7.9), and imidazole (8 mM)] and once with 400 μL of wash buffer [NaCl (0.5 M), Tris-HCl (20 mM, pH 7.9), and imidazole (60 mM)] to remove non-cross-linked fluorescein-labeled $CheZ_{196-214}$. His-tagged-CheY was then eluted from the resin by agitation in 500 μL of strip buffer [NaCl (0.5 M), EDTA (100 mM), and Tris-HCl (20 mM, pH 7.9)] for 10 min. The resin was then removed by centrifugation and the amount of $CheZ_{196-214}$, labeled with fluorescein, was determined according to fluorescence intensity. In order to determine the nonspecific binding of labeled $CheZ_{196-214}$ to His·Bind resin, a similar reaction, but in the absence of His-tagged-CheY, was carried out in parallel. The extent of CheY-$CheZ_{196-214}$ cross-linking was calculated from the difference between the amounts of $CheZ_{196-214}$ measured in the presence and absence of His-tagged-CheY.

Binding of $CheZ_{1-201}$ to Immobilized CheY. CheY was immobilized as follows. His·Bind resin (400 μL) was washed twice with water (600 μL each time), incubated with 800 μL of $NiSO_4$ (50 mM) for 5 min, washed twice with 600 μL of binding buffer, incubated for 10 min at 0 °C with 400 μL of binding buffer containing His-tagged-CheY (49 μM), washed twice with 0.5 mL of Tris-HCl (50 mM, pH 7.9), and resuspended in 350 μL of the same buffer. For binding $CheZ_{1-201}$ and, as a control, wild-type CheZ to the CheY resin, we used the following reaction mixture in a total volume of 200 μL: immobilized CheY (100 μL), Tris-HCl (50 mM, pH 7.9), NaCl (100 mM), $MgCl_2$ (5 mM), imidazole (5 mM), BSA (2 mg/mL), and, as appropriate, AcP (18 mM). The reaction was initiated by the addition of [^{3}H]CheZ (0.2 μM, 30 000 cpm) and rapid mixing. After 1 min of incubation at 23 °C, the resin was spun down, the supernatant was removed, and the resin was washed. For the wash we used 450 μL of wash buffer containing, in addition, $MgCl_2$ (5 mM) and—when included also in the binding assay—AcP (18 mM). CheZ bound to the washed resin was solubilized by incubation with 300 μL of elute buffer [Tris-HCl (20 mM, pH 7.9), imidazole (1 M), and NaCl (0.5 M)] for 10 min. The resin was spun down, and the amount of CheZ in the supernatant was determined by scintillation counting. As a control for nonspecific binding, we carried out a similar procedure, only that instead of CheY-bound resin we used CheY-free resin (precharged with $NiSO_4$).

Measurement of $CheZ_{196-214}$ Phosphatase Activity. The phosphatase activity of $CheZ_{196-214}$ was measured by monitoring the steady-state level of CheY phosphorylation in the

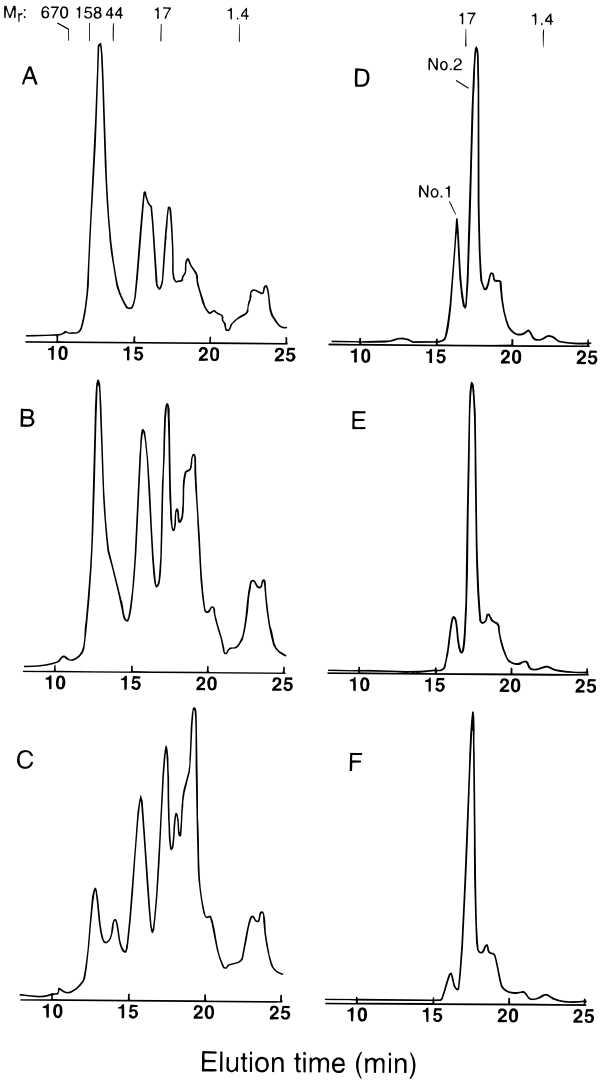

FIGURE 2: Size-exclusion chromatography of CheZ pre-digested by trypsin. CheZ was digested by trypsin, and 25 μL samples of the digestion product were fractionated on an HPLC size-exclusion column. The resulting fractions were detected both at 215 nm (panels A−C) and at 490 nm (panels D−F) after 10 (panels A and D), 20 (panels B and E), and 30 min (panels C and F) of incubation with trypsin. Peak nos. 1 and 2 were eluted at positions corresponding to spherical proteins of 17 and 10 kDa, respectively.

presence of [^{32}P]AcP (11 mM, 300−420 cpm/pmol) and varying concentrations of $CheZ_{196-214}$, as described earlier (Blat & Eisenbach, 1996a).

Fluorescence Measurement. The fluorescence was measured by a Perkin-Elmer LS 50 B luminescence spectrometer. The excitation and emission wavelengths were 490 and 520 nm, respectively (5 nm slit width).

RESULTS

Generation and Isolation of Proteolytic Fragments of the C-Terminus of CheZ. In order to determine whether or not the C-terminus of CheZ contains a CheY-binding domain, we generated C-terminus-containing fragments of CheZ by limited trypsin digestion, known to cleave CheZ preferentially near the C-terminusl (Stock & Stock, 1987). According to the sequence of CheZ, the shortest tryptic fragment that should contain the conserved 202−214 region is the fragment 196−214. Fragments of CheZ, labeled with fluorescein at residue 214 (Blat & Eisenbach, 1996a), were resolved on an HPLC size-exclusion column (Figure 2). While several

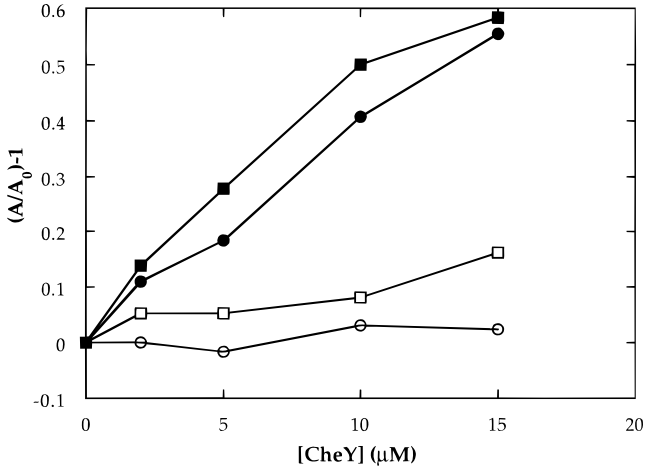

FIGURE 3: Binding of CheY to CheZ fragments in fractions 1 and 2, measured by fluorescence depolarization. Fractions containing peak 1 after 10 min incubation with trypsin and peak 2 after 30 min incubation with trypsin (Figure 2) were diluted 25- and 50-fold, respectively, to yield final fragment concentration of 0.3−0.5 μM. CheY was added in the presence (closed symbols) or absence (open symbols) of AcP, and then the fluorescence depolarization anisotropy [$A = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$ (Cantor & Schimmel, 1980)] was measured. Squares, peak 1; circles, peak 2. The initial anisotropy values (A_0) in the absence and presence of AcP were 0.037 and 0.036 for peak 1 and 0.032 and 0.027 for peak 2, respectively.

fragments with different molecular sizes were observed at 215 nm (panels A−C), only a few of them had absorbance at 490 nm (the absorbance peak of fluorescein; panels D−F), indicative of C-terminus fragments. The area under peak no. 1 significantly decreased during the incubation with trypsin (Figure 2D−F), suggesting that the fragment is a product of partial degradation of CheZ which still contains at least one trypsin cleavage site. The area under the other C-terminus-containing fragments did not change with time, implying that the initial cleavage site is readily accessible to trypsin and that the resulting fragments do not contain trypsin cleavage sites. In the rest of the study we concentrated on peaks nos. 1 and 2, the largest two peaks. Peaks 1 and 2 were eluted at positions corresponding to spherical proteins of 17 and 10 kDa, respectively. However, the actual sizes of the fragments are probably much smaller, because small proteolytic fragments rarely fold into globular structures and the molecular sizes of such structures are often over-estimated several fold by size-exclusion chromatography (Giddings *et al.*, 1968; Long & Weis, 1992a,b; Westwood & Wu, 1993).

Binding of CheZ Proteolytic Fragments to CheY. Fractions containing peaks 1 and 2 were tested for CheY binding by fluorescence depolarization. Binding of CheY to labeled proteolytic fragments of CheZ in these fractions is expected to result in an increased molecular volume of the fragments. This should be reflected in slower rotational diffusion, reduced fluorescence depolarization, and higher anisotropy (Cantor & Schimmel, 1980; Weber, 1953). As shown in Figure 3, only a slight increase in anisotropy was observed at increasing concentrations of nonphosphorylated CheY. However, in the presence of AcP, a phosphate donor of CheY (Lukat *et al.*, 1992), a significant increase in anisotropy was observed at increasing concentrations of CheY. This indicates that the C-terminus-containing fragments of CheZ bind CheY and that the fragments retain the binding preference of the intact protein toward the phosphorylated form of CheY. The binding of both fractions to CheY∼P did not

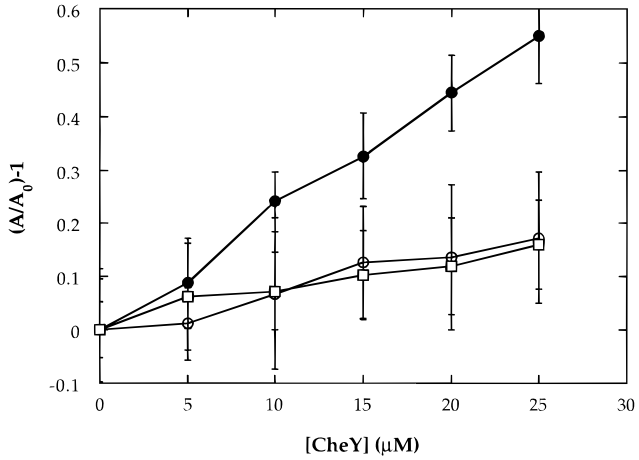

FIGURE 4: Binding of purified $CheZ_{196-214}$ to CheY, measured by fluorescence depolarization. The figure shows the mean ± SD of three independent experiments. The concentration of fluorescein-labeled $CheZ_{196-214}$ was 0.4 μM. Open squares, $-Mg^{2+}$ +AcP; open circles, $+Mg^{2+}$ −AcP; closed circles, $+Mg^{2+}$ +AcP. The A_0 values were 0.030 ± 0.003, 0.032 ± 0.002, and 0.034 ± 0.004, respectively.

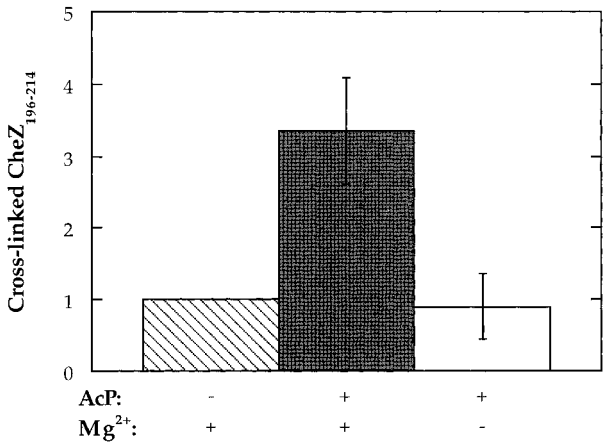

FIGURE 5: Cross-linking of His-tagged-CheY and fluorescent $CheZ_{196-214}$. The amount of $CheZ_{196-214}$ cross-linked to CheY is expressed relative to the binding in the absence of AcP (0.9−2.9% of added fluorescent $CheZ_{196-214}$) and considered as 1. The results are the mean ± SD of three independent experiments.

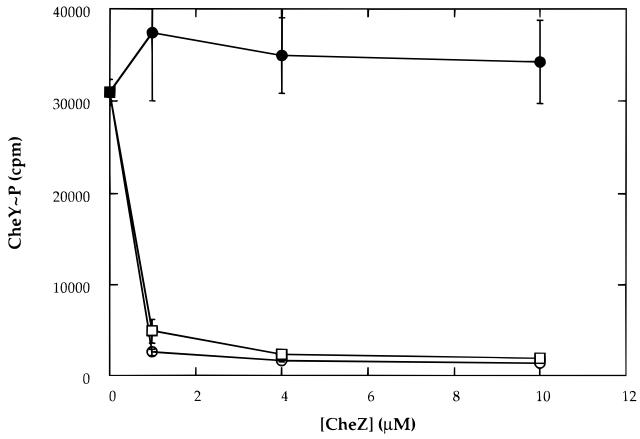

FIGURE 6: Phosphatase activity of $CheZ_{196-214}$. The activity is reflected in the steady-state level of CheY∼P in the presence of increasing concentrations of the fragment or, as a control, of intact CheZ. Closed circles, $CheZ_{196-214}$ labeled with fluorescein; open circles, wild-type (non-labeled) CheZ; open squares, CheZ214FC labeled with fluorescein. The results are the mean ± SD of three independent experiments.

reach saturation up to CheY concentration of 25 μM (not shown). Since no significant differences were observed between the binding of the two fractions, further studies were carried out only with the fraction of lower molecular size.

Purification and Identification of the CheY-Binding Fragment. To identify the smaller CheY-binding fragment, it was further purified on a reversed-phase HPLC column and then subjected to N-terminal amino acid sequence analysis. The sequence obtained for the first five amino acids, AGVVA, was of a single peptide and identical to the sequence of residues 196−200 of CheZ. Evidently, the fragment was composed of residues 196−214, since it contained also the C-terminal fluorescent label. Hereafter, this fragment will be termed $CheZ_{196-214}$.

Binding of Purified $CheZ_{196-214}$ to CheY. To confirm that $CheZ_{196-214}$ binds to CheY, we used again fluorescence depolarization [note that the complex between CheZ and CheY∼P readily dissociates and its detection requires a technique that allows interaction between CheZ and CheY∼P throughout the measurement (Blat & Eisenbach, 1996a)]. As shown in Figure 4, the anisotropy slightly increased with the CheY concentration, indicative of $CheZ_{196-214}$ binding to CheY. The binding further increased in the presence of AcP, indicating that the preference of CheZ toward the phosphorylated form of CheY is maintained also in purified $CheZ_{196-214}$. In the presence of AcP, omission of Mg^{2+}, known to be essential for CheY phosphorylation (Lukat *et al.*, 1990), reduced the level of binding to that in the absence of AcP. This result ensured that the observed effect of AcP on CheY−$CheZ_{196-214}$ binding was the result of CheY phosphorylation. We failed to detect binding of purified $CheZ_{196-214}$ to immobilized CheY, an approach which successfully measured the binding of intact CheZ to CheY (Blat & Eisenbach, 1994). This suggests that the affinity of the fragment to CheY is lower than that of intact CheZ (see Discussion).

In view of the apparent low CheY-$CheZ_{196-214}$ binding, we investigated the binding by cross-linking as well. Fluorescein-labeled $CheZ_{196-214}$ was cross-linked to His-tagged CheY by dimethylsuberimidate and then separated from the unreacted peptide by nickel-charged His·Bind resin. The extent of CheY−$CheZ_{196-214}$ cross-linking was quantified by measuring the fluorescence of the C-terminal fluorescein label. In accordance with the fluorescence depolarization approach, phosphorylation-dependent binding (requiring the presence of both AcP and Mg^{2+}) was observed (Figure 5).

Phosphatase Activity of $CheZ_{196-214}$. As might be expected, $CheZ_{196-214}$ did not retain the phosphatase activity of intact CheZ: the fragment did not catalyze dephosphorylation of CheY∼P, unlike fluorescein-labeled CheZ214FC (from which $CheZ_{196-214}$ had been derived) which retained the phosphatase activity of wild-type CheZ (Figure 6).

Binding of $CheZ_{1-201}$ to CheY. Since $CheZ_{196-214}$ binds CheY∼P with an apparently low affinity, we examined the importance of the conserved C-terminus for CheY binding in the context of the CheZ molecule as a whole. To this end, we have constructed a plasmid, pEWD202, which overexpresses a truncated form of CheZ lacking the 13 conserved amino acid residues at the C-terminus. This truncated protein ($CheZ_{1-201}$) was purified, radiolabeled, and then examined for CheY binding. As shown in Figure 7, the binding of $CheZ_{1-201}$ (unlike intact CheZ) to CheY∼P was marginal, indicating that the conserved region of residues 202−214 is essential for CheZ−CheY∼P binding.

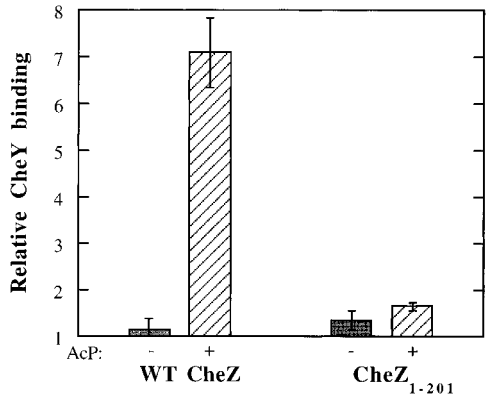

FIGURE 7: Binding of [^{3}H]CheZ$_{1-201}$ to immobilized CheY. The binding is expressed relatively to the nonspecific binding (2.2 ± 0.2% of added CheZ, measured as described under Experimental Procedures; this value of nonspecific binding was considered as 1). The results are the mean ± SD of three independent experiments.

DISCUSSION

The results of this study suggest that the CheY-binding domain of CheZ is located at the C-terminus of the protein (residues 202−214). This is based on the observations that the CheZ$_{196-214}$ fragment binds to CheY∼P (Figures 4 and 5) and that a truncated CheZ (CheZ$_{1-201}$) is unable to bind CheY∼P (Figure 7).

The binding of the CheZ$_{196-214}$ fragment to CheY appears to be specific in the sense that, in spite of being negatively charged, it binds to the phosphorylated form of CheY much better than to the nonphosphorylated form (Figure 4). Nevertheless, its affinity to CheY appears to be low, as binding to immobilized CheY could not be detected. The notion of low affinity is supported by the observation that the binding, as reflected in fluorescence depolarization experiments, did not approach saturation even when 25 μM CheY were present (i.e., over 60-fold excess of CheY). The presumed low affinity of the CheZ$_{196-214}$ fragment to CheY could be a consequence of any of the following possibilities. (i) The CheY-binding domain in CheZ is likely to acquire the correct binding conformation only when it is a part of the whole protein. It is known that peptides composed of less than 20 residues are unstructured and may therefore exhibit an affinity lower than that of a constrained peptide or of the intact protein (Ladner, 1995). (ii) CheZ may have additional amino acid residues, outside the 196−214 domain, that participate in the binding to CheY. (iii) Since CheZ is a dimer (Blat & Eisenbach, 1996a), it is possible that its binding to CheY requires the binding domains at both C-termini, positioned in the dimer at a specific orientation.

It was recently shown by Sanna *et al*. (1995) that alterations in CheY which introduce a negative charge or eliminate a positive charge (a glutamate for lysine replacement at position 26 or an aspartate for asparagine replacement at position 23) reduce CheY dephosphorylation by CheZ. CheZ$_{196-214}$ contains three adjacent conserved aspartate residues at positions 203, 206, and 207. Therefore, it is possible that the interactions between CheZ and CheY are stabilized by the electrostatic interactions between Lys26 of CheY and one or more of the conserved aspartate residues at the C-terminus of CheZ.

Even though CheZ regulates the activity of the response regulator CheY, it has only a very limited homology to any of the other known members of the two-component family (Kofoid & Parkinson, 1988). This lack of homology restricts our ability to assign specific functions to different regions of CheZ according to sequence homology. However, on the basis of functional studies [this study and Blat and Eisenbach (1996b)], it is now possible to assign functions to some of the conserved domains of CheZ (Figure 8).

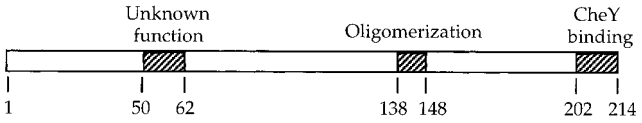

FIGURE 8: Functions proposed for conserved domains of CheZ. The conserved domains are indicated by hatched areas.

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