CheZ is the phosphatase of CheY, the response regulator in bacterial chemotaxis. The mechanism by which the activity of CheZ is regulated is not known. We used cheZ mutants of Salmonella typhimurium, which had been isolated by Sockett et al. (Sockett, H., Yamaguchi, S., Kihara, M., Irikura, V. M., and Macnab, R. M. (1992) J. Bacteriol. 174, 793–806), for cloning the mutant cheZ genes, overexpressing and purifying their products. We then measured the phosphatase activity, binding to CheY and to phosphorylated CheY (CheY–P), and CheY–P-dependent oligomerization of the mutant CheZ proteins. While all the mutant proteins were defective in their phosphatase activity, they bound to CheY and CheY–P as well as wild-type CheZ. However, unlike wild-type CheZ, all the four mutant proteins failed to oligomerize upon interaction with CheY–P. On the basis of these and earlier results it is suggested that (i) oligomerization is required for the phosphatase activity of CheZ, (ii) the region defined by residues 141–145 plays an important role in mediating CheZ oligomerization and CheY–P dephosphorylation but is not necessary for the binding to CheY–P, (iii) the oligomerization and hence the phosphatase activity are regulated by the level of CheY–P, and (iv) this regulation plays a role in the adaptation to chemotactic stimuli.

Regulation of bacterial chemotaxis is essentially regulation of the direction of flagellar rotation (1). This is done by phosphotransfer reactions ending up with the phosphorylation of CheY, the signal protein in bacterial chemotaxis (for recent reviews see, Refs. 2–6). Phosphorylated CheY (CheY–P) interacts with the switch at the base of the flagellar motor (7–12) (see Refs. 6 and 13 for recent reviews on the switch) with a resultant clockwise (CW) rotation (14). The default direction of rotation is counterclockwise (CCW) (8, 9, 15–17). CheY is phosphorylated by an autophosphorylatable kinase, CheA (18–20). This activity is regulated by chemotactic stimuli (21–23). CheY–P is dephosphorylated by CheZ (19, 20). However, unlike the kinase, no regulation of the phosphatase activity of CheZ has been found, even though a computer simulation of signal transduction (24) implicated the occurrence of such a regulation mechanism.

In addition to its phosphatase activity, CheZ was found to be involved in two other processes: binding to CheY (25, 26) (mainly to its phosphorylated form (26)), and oligomerization upon binding to CheY–P (27). With the goal of finding out whether the oligomerization is involved in CheZ regulation, this study examines, by biochemical analysis of mutant CheZ proteins, the inter-relationship between the different functions of CheZ.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—The strains used in this study are listed in Table I. Cloning of Mutant cheZ Genes onto an Overexpressing Vector—For the overexpression of mutant CheZ proteins we used a collection of cheZ mutants generated and characterized by Sackett et al. (28). The cheZ mutant alleles were amplified from total DNA of the respective Salmonella typhimurium strain by polymerase chain reaction using the primers 5′-CCGAATTCATGATGCAACCATCTATCAAGCC-3′ and 5′-CCGGATCCCTTAACAGCCAAGACTGTCCAGCA-3′, which contained added EcoRI and BamHI sites, respectively, at their 5′ end. The amplified cheZ-containing fragments were digested with EcoRI and BamHI, and ligated with pBTac1 (Boehringer Mannheim) predigested with EcoRI and BamHI (Fig. 1). The resultant plasmids (Table I) overexpressed the wild-type and mutant CheZ proteins under the control of the tac promoter. The existence of the mutations in the cloned cheZ genes and the lack of additional mutations that might be caused by polymerase chain reaction were confirmed by DNA sequencing.

Overexpression and Purification of CheZ—Wild-type CheZ and the mutant proteins CheZ141IF and CheZ143DE were overexpressed in RP3098. CheZ110LP was overexpressed in RP1616, and CheZ145TM in BW3. The purification of CheZ was carried out as described in the preceding paper (27) except that, in the case of CheZ110LP and CheZ145TM, buffer A used for washing the Sepharose CL-6B column, contained 225 mM NaCl (instead of 275 mM), and the elution of CheZ was carried out by a gradient of 225–450 mM NaCl (instead of 275–450 mM). The overexpression and purification of CheY from S. typhimurium was described in the preceding paper (27).

**Sensitivity of CheZ to Proteolysis**—CheZ (83 μM) in Tris-HCl (50 mM, pH 7.9) and MgCl2 (5 mM) were incubated at room temperature (22°C) with trypsin (Sigma T-8642, 4 μg/ml). Samples of 10 μl of this mixture were removed at the indicated time points, quenched by addition of 3 μl of 5× concentrated sample buffer and 10 min boiling, and analyzed by 15% SDS-polyacrylamide gel electrophoresis.

**Phosphatase Activity of CheZ**—The phosphatase activity of CheZ was assayed by monitoring the steady-state level of CheY phosphorylation in the presence of [32P]pyrophosphate (AcP) as described earlier (27).

CheZ Radio labeling—CheZ was radio labeled by methylation of the e-amino of its lysine residues with formaldehyde and NaBH4; a mixture (100 μl) of CheZ (100 μM), H2BO3-NaOH (0.2 M, pH 9.0), formaldehyde (5 mM), and NaBH4 (175 μM, 24 Ci/mmol, obtained from Amersham) was incubated on ice for 25 min, and then the reaction was terminated by the addition of 100 μl of Tris-HCl (50 mM, pH 7.5). The radio labeled CheZ (1200–1500 cpm/μmol) was separated from the unreacted NaBH4 by a brief spin at 480 × g in a 0.8-ml G-50 mini-column followed by dialysis against Tris-HCl containing 0.2 mM phenylmethylsulfonyl fluoride, and stored at –20°C.

Binding of CheZ to CheY—Binding of CheZ to CheY immobilized on CNBr-activated Sepharose beads was measured as follows. Immobi-
The Phosphatase Activity of Mutant CheZ Proteins—

In order to determine whether any of the functions of CheZ (binding to CheY, phosphatase activity, and oligomerization) are correlated, we chose to analyze these functions in several mutant CheZ proteins. To this end we used S. typhimurium cheZ point mutants isolated and characterized by Sackett et al. (28) (Table I). Flagellar rotation in all these mutants is CW biased, indicating that these cheZ alleles code for CheZ proteins with impaired ability to antagonize the CW causing activity of CheY in vivo. To produce the proteins, we amplified the mutant genes by polymerase chain reaction directly from the chromosomal DNA of the mutant strains, and cloned the amplified genes into the expression vector pBTac (Fig. 1 and Table I). The plasmids containing the cloned genes indeed overexpressed the mutant CheZ proteins, thus enabling us to purify the proteins to near homogeneity (Fig. 2).

The Phosphatase Activity of Mutant CheZ Proteins—The phosphatase activity of each of the mutant CheZ proteins was determined by measuring its effect on the level of CheY phosphorylation under steady-state conditions. All the mutant proteins had a significantly lower phosphatase activity than wild-type CheZ (Fig. 3). For example, in the presence of 2 μM CheZ, the level of CheY-P under steady-state conditions was 7% in the wild type versus 75, 76, 62, or 37% in CheZ110LP, CheZ141FI, CheZ143DE, or CheZ145TM, respectively (100% is the steady-state level of CheY-P in the absence of CheZ). This means that CheZ110LP and CheZ141FI had over 10-fold lower phosphatase activity than the wild type, and CheZ 143DE and CheZ145TM had 9- and 5-fold lower activities, respectively.

Binding of Mutant CheZ Proteins to CheY—The apparent defects in the phosphatase activity of the mutant CheZ proteins could result from one of the following possibilities: (i) a reduced binding to CheY-P; (ii) an increased binding to non-phosphorylated CheY, as a result of which the CheZ-CheY complex could not dissociate and CheZ could not become available to other CheY-P molecules (CheZ was used in catalytic concentrations); or (iii) a reduced catalytic activity. To distinguish between these possibilities, we measured the binding of the mutant CheZ proteins to both phosphorylated and non-phosphorylated CheY. For this purpose the binding of [3H]CheZ to CheY immobilized onto CNBr-activated Sepharose beads was carried out by a mixture of 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride and N-hydroxysuccinimide as described in the preceding paper (27).

RESULTS

Cloning of Mutant cheZ Genes, and Overexpression and Purification of Their Products—In order to determine whether any of the functions of CheZ (binding to CheY, phosphatase activity, and oligomerization) are correlated, we chose to analyze these functions in several mutant CheZ proteins. To this end we used S. typhimurium cheZ point mutants isolated and characterized by Sackett et al. (28) (Table I). Flagellar rotation in all these mutants is CW biased, indicating that these cheZ alleles code for CheZ proteins with impaired ability to antagonize the CW causing activity of CheY in vivo. To produce the proteins, we amplified the mutant genes by polymerase chain reaction directly from the chromosomal DNA of the mutant strains, and cloned the amplified genes into the expression vector pBTac (Fig. 1 and Table I). The plasmids containing the cloned genes indeed overexpressed the mutant CheZ proteins, thus enabling us to purify the proteins to near homogeneity (Fig. 2).

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monomer is 23.9 kDa (30)); in the presence of CheY but absence of AcP, higher molecular size products (up to ~70 kDa) were also observed (lanes 3, 6, 9, 12, and 15). However, under phosphorylating conditions there was a marked difference between the wild-type and the mutants: all the mutant proteins (lanes 7, 10, 13, and 16), unlike the wild-type protein (lane 4), did not form the high molecular size oligomer with CheY. (In the case of CheZ110LP, other forms of CheZ, higher than the dimer but lower than the CheZ-CheY−P oligomer, were also observed, possibly because of its global structure defects (see below.).) These results demonstrate that the mutant CheZ proteins are unable to oligomerize. When taken together with the conclusion reached earlier that all the mutants have a reduced catalytic activity, the results further suggest that the oligomerization is involved in the phosphatase activity of CheZ.

The Effect of the Mutations on the Global Structure of CheZ—To determine whether the strong effects of the point mutations on the oligomerization and the phosphatase activity of CheZ were the consequence of local effects or global perturbation of CheZ structure, we measured the susceptibility of the proteins to limited trypsin proteolysis. The rationale behind this approach was that perturbation in the structure of CheZ is expected to make the protein less compact and thereby to expose more sites to trypsin action, with a consequent faster proteolysis. As shown in Fig. 6, the mutant proteins CheZ141FI, CheZ143DE, and CheZ145TM exhibited similar proteolysis patterns as wild-type CheZ, indicative of no major structural changes in these three mutant proteins. These patterns are similar to those observed earlier in wild-type CheZ by Stock and Stock (30). In contrast, CheZ110LP was significantly less resistant to trypsin proteolysis and was almost completely degraded to small fragments already within 1.5 min of incubation with trypsin; the wild-type protein and the other mutant proteins remained almost intact during this time period. This result suggests that the 110LP mutation causes global perturbation in the structure of CheZ.

DISCUSSION

In this study we demonstrated that the phosphatase activity of CheZ is correlated with its ability to oligomerize upon interaction with CheY−P. The study also confirmed the correlation between the phosphatase activity of CheZ and the CCW bias of flagellar rotation, and it provided an insight into the involvement of specific CheZ residues in the functions of the protein. These issues are discussed below.

Relation between CheZ-CheY−P Binding, Direction of Flagellar Rotation, and Phosphatase Activity—We studied four mutant CheZ proteins that in vivo are unable to antagonize the CW causing activity of CheY−P (28). We observed that all these mutant proteins were, on the one hand, severely impaired in their ability to dephosphorylate CheY (Fig. 3) but, on the other hand, apparently normal in their ability to bind to CheY (Fig. 4). This indicates that CheZ-CheY−P binding is not sufficient for CheY−P dephosphorylation. The observation that CW biased cheZ mutants are defective in the phosphatase activity of CheZ (this study) taken together with the observation that CCW biased cheZ mutants have phosphatase activity
higher than wild-type CheZ (31) indicates that the direction of flagellar rotation is tightly dependent on the phosphatase activity of CheZ.

Involvement of Specific CheZ Residues in the Functions of the Protein—Three of the four mutant alleles of CheZ were clustered between residues 141 and 145. These mutant proteins were normal in binding CheY-P (Fig. 4) but were severely impaired in their ability to oligomerize in its presence (Fig. 5). Furthermore, even conservative substitutions such as Phe → Ile (at position 141) or Asp → Glu (at position 143) were sufficient for CheZ inactivation. This suggests that the region defined by residues 141–145 plays an important role in mediating CheZ oligomerization and CheY-P dephosphorylation, but is not necessary for the binding to CheY-P. Indeed, this region is part of a conserved domain in CheZ of E. coli, S. typhimurium, and Pseudomonas aeruginosa (30, 32, 33). The substitution Leu → Pro (at position 110) in the fourth mutant was also a substitution of a conserved residue. This mutation had apparently a global effect on the structure of CheZ, as evident from the increased susceptibility of the protein to proteolysis (Fig. 6). Since proline residues are known to break helices, it is possible that Leu110 is in an α-helix crucial for the structure of CheZ. In accordance with a global structural effect, the mutation in residue 110, unlike the mutations in residues 141–145, did not have an “all or none” effect on the oligomeric state (Fig. 5). This may be attributed to the indirect effect of this mutation on the domain responsible for the oligomerization.

Correlation between the Phosphatase Activity and the Oligomerization of CheZ—All the mutant CheZ proteins that we studied were defective in both the phosphatase activity and the oligomerization (Figs. 4 and 5), suggesting that oligomerization is required for the expression of the phosphatase activity. This suggests that the phosphatase activity of CheZ, like the oligomerization of CheZ (27), is dependent on the level of CheY phosphorylation. This is in line with an earlier observation (which was not understood at the time) that, when the level of CheY phosphorylation is very low (~1%), it is essentially inde-

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2 The adaptation defect observed in these cheZ mutants is not likely to be due to the increased life span of CheY–P. The increased life span is expected to affect only the latency of the response (i.e. the excitation process) (35, 38–40).
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