Action at a Distance: Amino Acid Substitutions That Affect Binding of the Phosphorylated CheY Response Regulator and Catalysis of Dephosphorylation Can Be Far from the CheZ Phosphatase Active Site

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Transient protein phosphorylation is a common means to accomplish signal transduction. Phosphorylation-mediated signaling in microorganisms often involves the detection of a stimulus by a sensor kinase, followed by the transfer of a phosphoryl group to a response regulator protein. In bacterial chemotaxis, one of the best-studied examples of such a two-component regulatory system (36, 37), extracellular stimuli control the autophosphorylation of the CheA sensor kinase with subsequent phosphotransfer to the cytoplasmic response regulator CheY. The phosphorylation state of CheY then dictates the direction or duration of flagellar rotation and thus swimming behavior.

The concentration of phosphorylated CheY (CheYp) at any given time is a function of the rates of both phosphorylation and dephosphorylation. The kinetics of phosphoryl group addition and removal set an upper bound on how quickly a cell can respond to a stimulus. During chemotaxis, bacteria integrate information about their chemical environment and make split-second responses that determine whether to continue on their current course or change direction (29). Accordingly, CheY proteins from various species have among the fastest known rates of self-catalyzed dephosphorylation for response regulators (39). Nevertheless, in many bacteria CheY autodephosphorylation is too slow to support chemotaxis, so phosphatases of the CheZ or CheC/CheX/FliY families further stimulate the rate of removal of the phosphoryl group from CheYp. CheZ and CheX exhibit little similarity in structure or amino acid sequence but use the same mechanism to accelerate CheYp dephosphorylation (22, 24, 30, 41). The present study explores amino acid substitutions that perturb CheZ activity and potentially provide insight into phosphatase regulation.

The structure of Escherichia coli CheZ cocrystallized with CheY and the stable phosphoryl group analog BeF$_3^-$ (41) revealed basic features of CheZ architecture and the mechanism of CheZ-mediated CheYp dephosphorylation. CheZ is a highly helical, homodimeric protein that binds two CheYp molecules. The CheZ dimer forms a long (>100-Å) four-helix bundle consisting of amphipathic helices that fold into a hairpin structure (Fig. 1). Two additional alpha helices are present in each CheZ monomer. One helix at the N terminus of CheZ (N helix; residues 1 to 34) connects directly to the four-helix bundle, whereas the other helix, located at the C terminus of CheZ (C helix; residues 199 to 214), is tethered to the bundle by a 32-residue highly flexible linker (31, 41).
Each CheYp interacts with CheZ at two distinct sites: the C helix (2, 11, 12, 20, 41) and the CheZ active-site region near the center of the four-helix bundle (41). In the latter interaction, the essential catalytic residue Gln147 inserts into the CheY active site (Fig. 1). In a current model of the CheZ-mediated dephosphorylation of CheYp, CheYp is believed to initially bind to the C helix of CheZ (31, 32). Following this initial interaction, CheYp is tethered to the four-helix bundle via the flexible linker, which brings the CheYp active site in closer proximity to the CheZ catalytic residue Gln147 and thus leads to CheYp dephosphorylation (31). The rate of the CheZ-mediated dephosphorylation of CheYp exhibits positive cooperativity (sigmoidal curve) with respect to CheYp concentration (3, 32). The positive cooperativity is consistent with a model whereby there is a slower association of CheYp with uncomplexed CheZ relative to the rate of association of CheYp with CheZ-CheYp. Positive cooperativity could serve as a mechanism to limit CheZ activity when the CheYp concentration is low (32).

CheZ residues outside the active site and sites of CheYp interaction also impact phosphatase activity. In particular, amino acid substitutions that increase phosphatase activity (15, 25, 26, 35) cluster along the N helix and the nearby region of the four-helix bundle (Fig. 1) (41). One such gain-of-function (GOF) mutation, cheZ21IT, has been the subject of significant study. Although CheZ21IT exhibits a $k_{cat}$ similar to that of wild-type CheZ (32), CheZ21IT does not support chemotaxis (26). A loss of the positive cooperativity observed for wild-type CheZ means that CheZ21IT has increased phosphatase activity at low CheYp concentrations and reaches half-maximal activity at a 4-fold lower CheYp concentration than wild-type CheZ (32). Moreover, CheZ21IT has a rate constant of association with CheY that is 6-fold faster than that for wild-type CheZ and a rate constant of dissociation from CheYp that is 1.7-fold slower than that for wild-type CheZ, and hence it binds CheYp with 10-fold greater affinity ($K_d$ of 0.68 nM) than wild-type CheZ ($K_d$ of 7.1 nM) (32).

The observation that the CheZ21IT substitution located on the N helix more than 40 Å from the active site does not support chemotaxis and abolishes the positive cooperativity of phosphatase activity without affecting $k_{cat}$ (32) is consistent with the possibility that there are as-yet unidentified interactions within CheZ that influence the dephosphorylation of CheYp. The means by which residues so distant from the CheZ active site and sites of CheYp interaction affect phosphatase activity might involve structural changes that propagate along the four-helix bundle to alter the properties of the active site region. Alternatively, perturbed packing interactions between the N helix, four-helix bundle, flexible linker, and/or C helix of CheZ could influence CheYp binding to the C helix. Here, we identified and characterized intragenic suppressors of cheZ21IT to investigate the mechanism by which distant residues alter phosphatase activity and disrupt positive cooperativity. Taken together, the results of this and other studies suggest that the binding of CheYp to the CheZ active site is the rate-limiting step in association with CheYp and the source of CheZ cooperativity.

**MATERIALS AND METHODS**

**Site-directed mutagenesis.** Site-specific mutations were generated using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The GOF mutation cheZ21IT was generated in plasmid pRS3 (31). Like its predecessor, the ampicillin-resistant pBR322 derivative pRB40 (5), pRS3 contains a 2.6-kb BamHI-HindIII fragment encoding ‘cheZY fbbB’; however, the cheZ134E.K mutation present in pRB40 (4) has been corrected in pRS3. The mutagenic primer used to generate cheZ21IT contained changes at two of the nucleotides encoding residue 21 to eliminate the possibility that the reversion of the GOF mutation could occur after a single base change, thus favoring the isolation of second-site suppressors rather than revertants in the screen for the restoration of chemotaxis. Plasmids carrying only the suppressor mutation were constructed by correcting the cheZ21IT mutation back to the wild type in plasmids containing both cheZ21IT and a suppressor mutation (described below). To assess the allele specificity of suppression, the gain-of-function mutation cheZ24LP subsequently was introduced into pRS3 and plasmids containing only a suppressor mutation. Plasmids potentially carrying cheZ21IT, cheZ24LP, and/or cheZ suppressor mutations from the constructions described above were isolated from ampicillin-resistant transformants, and the cheYZ genes were sequenced. Plasmids containing wild-type cheY and the desired cheZ mutations were transformed into the cheB12 strain RP5231, and the resultant strains were characterized with a motility plate assay (described below). RP5231, derived from the wild-type chemotaxis strain RP437 (23), carries ΔcheY9431 (18) and was a gift from J. S. Parkinson.

**Suppressor generation and isolation.** To generate suppressor mutations of cheZ21IT, pRS3 containing cheZ21IT was transformed into NR9458 (27) cells rendered chemically competent following growth in minimal media as described previously (4). NR9458 carries the muid5 allele and exhibits increased mutation frequencies (50 to 100 times higher than that of the wild type) when grown in minimal medium (9). Transformation cultures of the mutagenic NR9458 strain were grown overnight, and the resulting library of plasmids was isolated and subsequently transformed into the motility assay strain RP5231. A 100-μl sample of the RP5231 transformation mix was diluted into 5 ml of LB broth with

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**FIG. 1.** Ribbon diagram of the CheY · CheZ cocrystal structure (Protein Data Bank accession number 1KMI). The two chains composing the CheZ dimer are in cyan and gold, and the two CheY molecules are in blue and gray. The locations of the CheZ21IT GOF substitution as well as suppressor substitutions are drawn as sticks on the ribbon diagram and listed in matching colors. Suppressor substitutions chosen for biochemical analysis are underlined. BeF3 suggests that the binding of CheYp to the CheZ active site is the rate-limiting step in association with CheYp and the source of CheZ cooperativity.
ampicillin and again grown overnight. To screen for suppressors, 20 μl of the overnight culture of RP5231 carrying mutagenized pRS3.cheZ22ITT was streaked across the center of motility agar (1% [wt/vol] tryptone, 0.5% [wt/vol] NaCl, 0.3% [wt/vol] BactoAgar) plates and incubated overnight at 30°C. Transformants were screened for the normal chemotactic behavior of radial growth pattern from the inoculation site accompanied by swarm ring formation, in contrast to the CheZ GOF mutant nonchemotactic phenotype. Potential suppressor mutants were picked from the swarm rings, single-colony purified, and assayed in a motility plate assay (described below) to confirm swarm phenotypes. Plasmid DNA from candidate suppressor mutants was isolated, and the cheYZ genes were sequenced. Plasmids containing only cheZ point mutations in addition to cheZ22ITT were retransformed into RP5231 to confirm that the swarm phenotypes were linked to the plasmid and not the result of spontaneous chromosomal mutations. To confirm that the swarm phenotypes were due to mutations in cheZ, the cheZ gene from plasmids containing cheZ double (cheZ22ITT and suppressor) mutations was subcloned into nonmutagenized PR33. Plasmids were double digested with PvuII and Bsa36I, which both cleave within cheZ, and the fragments were separated by agarose gel electrophoresis. Because PvuII cleaves at +40 bp and Bsa36I cleaves at −80 bp from the 5′ and 3′ ends of cheZ, respectively, the 0.5-kb DNA fragment generated following double digestion includes the codon for position 21 and all of the suppressor mutations. This 0.5-kb fragment was gel purified and ligated into the PvuII/Bsa36I sites of pRS3 that had been treated with calf intestinal alkaline phosphatase (Promega) using the rapid DNA ligation kit (Roche) according to the manufacturer’s instructions. Ten-μl samples of the ligation reactions were transformed into Max Efficiency DH5α-cells competent (Invitrogen). Plasmids were isolated from ampicillin-resistant transforms, the cheYZ genes were sequenced from plasmids of the correct size (∼4.8 kb), and those containing cheZ double mutations and wild-type cheY were transformed into RP5231. The resulting strains were subjected to a motility plate assay (described below).

**Chemotaxis assay.** The standard motility plate assay was performed as described previously (1). Briefly, single bacterial colonies were stabbed into motility agar plates and incubated at 30°C for approximately 8 h. Swarm diameters were measured throughout the growth period, and growth rates (in mm/h) after the lag phase were determined and compared to those of swarms generated by chemotactic (RP5321/pRS3) and nonchemotactic (RP5323) control strains on the same plate.

**Protein purification.** Wild-type and mutant CheZ proteins and CheY113AP were overexpressed and purified previously published procedures (14, 32, 33). Briefly, following the overexpression of CheY and CheZ proteins encoded on plasmids in K06421/3 (ΔcheZ6725) (6), RP5321 (ΔcheZ24313), or K064114/5 (ΔcheY6021) (5), crude lysates were applied to an Affi-Gel blue gel (Bio-Rad) column matrix, which binds CheY. CheZ does not bind Affi-Gel blue gel and was collected in the flowthrough, precipitated with (NH₄)₂SO₄, resuspended in 50 mM TzP, pH 7.5, 0.5 mM EDTA, 0.1% (vol/vol) glycerol, and chromatographed on a 5% Hi-Trap Q Sepharose FF column (GE Healthcare). CheZ-containing fractions were pooled and concentrated to ≤5 ml, chromatographed on a Superose-12 (GE Healthcare) fast-protein liquid chromatography (FPLC) gel filtration column, and finally concentrated and stored at −70°C. Each purified CheZ protein yielded a gel filtration peak corresponding to a dimer; no CheZ monomers were observed. For CheY113AP purification, CheY was eluted from the Affi-Gel column using a high-salt buffer, dialyzed, applied to a DEAE cellulose (DE52, Whatman) anion exchange column, and again eluted with a high-salt buffer. CheY-containing fractions were pooled and concentrated to ≤5 ml, chromatographed on a Superose-12 (GE Healthcare) FPLC gel filtration column, and finally concentrated and stored at −20°C. Prior to storage, the concentrations of CheY and CheZ were determined by measuring the absorbance at 280 nm and using empirically determined extinction coefficients of 0.73 (mg/ml)-¹ cm⁻¹ and 0.70 (mg/ml)-¹ cm⁻³ (33), respectively.

**Western blot analysis.** Strains were grown to exponential phase (optical density at 600 nm of ~0.6 to 0.7) in LB, and 1-ml samples were harvested by centrifugation. Pellets were resuspended in 1 ml of wash buffer (50 mM Tris-HCl, 0.5 mM EDTA, 2 mM dithiothreitol, 10% [vol/vol] glycerol, pH 7.5) and centrifuged again. The supernatant was removed and pellets were stored at −20°C for later use. To prepare crude lysates for SDS-PAGE analysis, pellets were thawed, resuspended in 100 to 150 μl of 1% (wt/vol) SDS, and boiled for 6 min. The total protein concentration was measured using the Micro-Bicinchoninic acid (BCA) protein assay kit (Pierce) according to the manufacturer’s microplate procedure with the following changes: 5 to 15 μl of crude lysate was diluted to a final volume of 150 μl in sterile deionized H₂O in microcentrifuge tubes; following the addition of the BCA kit reagent, reaction mixtures were incubated for 1 h at 30°C and then transferred to a 96-well microtiter plate for the measure-
appears that uncertainties arising from the extensive processing of P<sub>r</sub> release assay data could be responsible for errors in the determination of Hill coefficients. The apparent Hill coefficient is particularly sensitive to variation in the ~10 points that comprise the data set for each CheZ mutant. Removing a single point, or altering the value of a point within the range of its error bars, can be sufficient to substantially change the Hill coefficient calculated for fitting that data set. Therefore, the values we derive for k<sub>on</sub>, k<sub>off</sub>, and particularly n must be considered approximate. As a result, the categorization of various mutants as exhibiting cooperative or noncooperative kinetics is not made objectively based on the value of the apparent Hill coefficient but rather subjectively based on the shape of the best-fit curve.

RESULTS

Intragenic suppressor substitutions of cheZ21IT spanned the CheZ four-helix bundle. To investigate the mechanism by which residues distant from the active site affect CheZ activity, intragenic suppressors of the cheZ21IT GOF phenotype were generated in a DNA repair-deficient strain. Potential mutants were screened phenotypically for the restoration of chemotaxis (Table 1), and plasmids were isolated from chemotactic mutants and sequenced. From the 18 chemotactic mutants isolated, 10 intragenic suppressor mutations were identified (cheZ58VA, cheZ65AT, cheZ72SN, cheZ74EK, cheZ79HY, cheZ83MV, cheZ134EG, cheZ144LF, cheZ159IN, and cheZ163LS). Four of the 10 mutations were isolated multiple times. All the suppressor mutations caused amino acid substitutions that were located along the CheZ four-helix bundle (Fig. 1). However, none were near the original GOF substitution site. The suppressor substitutions could be put into four groups based on their locations. The first group included substitutions 83MV and 79HY, which are located closest to the hairpin turn (or furthest from the original GOF substitution site) (Fig. 1). Although separated from each other by one alpha-helical turn, the side chains are long enough that Met83 points toward the interior of the four-helix bundle, whereas His79 points outward into solution. The next group contained substitutions 74EK, 72SN, and 134EG located in very close proximity to each other. These residues point in completely different directions: Glu74 points toward the nearby CheYp, Ser72 points inward into the four-helix bundle, and Glu134 points out into solution. A third group was close to the active site and included substitutions 65AT, 58VA, and 144LF. Both Ala65 and Val58, which are separated from each other by two alpha-helical turns, point toward the center of the four-helix bundle. Leu144, which is one alpha-helical turn from the CheZ catalytic residue Gln147, points outward toward bound CheYp. Substitution 144LF is adjacent to a CheZ-CheYp stabilizing residue, Asp143, which interacts directly with CheYp (41). The final group, at the linker-proximal end of the four-helix bundle, included substitutions 159IN and 163LS, which are separated from each other by one alpha-helical turn. Both Ile159 and Leu163 point into the center of the four-helix bundle.

Suppressor substitutions supported a range of chemotaxis phenotypes in the absence of CheZ21IT. Following the phenotypic screening and identification of the suppressor substitutions (in the cheZ21IT background), suppressor mutations were generated in the wild-type cheZ background and assessed for their effect on chemotaxis. Mutants expressing the suppressor mutations alone demonstrated a wide range of swarm phenotypes, including wild type (Table 1). Substitutions 79HY, 83MV, 159IN, and 163LS, located furthest from the active site, were phenotypically silent and exhibited no significant swarming defects. Substitutions 72SN, 74EK, and 134EG, located proximal to each other and to sites of CheYp interaction, had strikingly different effects on swarm rates that ranged from 5 to 79% of wild-type rates. Suppressor substitutions 58VA, 65AT, and 144LF, near the catalytic residue Gln147, also supported swarm rates ranging from nonchemotactic to nearly wild type (8 to 75%). To determine if these swarm rates were due to altered intracellular CheZ concentrations, crude lysates of mutants chosen for further analysis (see below) were subjected to Western blot analysis to quantify the amount of CheZ present. CheZ expression by the five suppressor mutant strains assayed was similar to that of the cheZ21IT GOF strain (data not shown), suggesting that the swarm rate effects were due to changes in CheZ function rather than concentration.

**Suppressor substitutions were not allele specific.** Interestingly, 9 of the 10 suppressor substitutions also successfully suppressed the swarm phenotype of a different GOF mutation, cheZ24LP (26) (Table 1), based on nearly 100% wild-type
swarm rates for the double mutants. The remaining substitution, 72SN, significantly enhanced the swarm rate while not fully restoring chemotaxis. The lack of allele specificity demonstrated by the suppressor substitutions suggested that the mechanism of suppression did not involve a direct interaction between the original GOF and suppressor substitutions, which is consistent with the disparate locations of the substitutions along the four-helix bundle (Fig. 1). Furthermore, the ability of each suppressor to suppress two different GOF substitutions suggests that these GOF substitutions activate CheZ via a similar mechanism.

Suppressor mutants exhibited various phosphatase activity profiles. The direction of rotation of the Escherichia coli flagellar motor, and hence the swimming behavior of the cell, exhibits a cooperative dependence on CheYp concentration with a Hill coefficient of about 10 and therefore is exquisitely sensitive to changes in CheYp (8). Previous studies identified GOF and loss-of-function mutations in cheZ that increase or decrease phosphatase activity, respectively, either of which results in the loss of chemotaxis (4, 15, 25, 26, 35). In addition, a reduction in CheZ phosphatase activity at low CheYp levels appears to be required for successful chemotaxis (32). Thus, the increased phosphatase activity demonstrated by CheZ21IT at (physiologically) low CheYp concentrations (32) likely precludes chemotaxis. If the suppression of cheZ21IT was achieved by lowering the activity of CheZ21IT, especially at low concentrations of CheYp, then several different mechanisms of suppression (see Fig. 3A) are possible alone or in combination, including (i) the reduced binding of CheYp substrate, (ii) the reduced catalysis of the phosphatase reaction, and/or (iii) the restoration of the positive cooperativity of phosphatase activity exhibited by wild-type CheZ. To distinguish between these possibilities, five suppressor mutants that demonstrated a range of chemotaxis phenotypes (Table 1) and locations along the CheZ four-helix bundle (Fig. 1) were chosen for further analysis. Proteins containing the cheZ21IT suppressor substitution 72SN, 74EK, 134EG, 144LF, or 163LS expressed either alone in the wild-type cheZ background (single mutants) or in the cheZ21IT (double mutant) background were purified and assayed for phosphatase activity. The single-mutant data are described first.

The results produced by the P_i release assay are noisy, because the concentration of the substrate CheY113APp cannot be directly measured but instead is inferred, and multiple corrections and calculations are necessary to convert the experimental data into an interpretable form (see Materials and Methods). One indicator of this noise is the derivation of Hill coefficients that in some cases exceed the maximum value of 2 expected for a dimeric enzyme. Nevertheless, three distinct profiles of phosphatase activity could be distinguished among single suppressor mutants compared to wild-type CheZ. (i) Mutants CheZ74EK and CheZ144LF exhibited minimal (if any) phosphatase activity under the conditions tested (Fig. 2A) and did not support chemotaxis (Table 1). Because of very low activity, it was not possible to accurately determine kinetic values from the data available for these mutants. (ii) CheZ72SN and CheZ134EG exhibited apparent positive cooperativity (sigmoidal curves) as observed for wild-type CheZ (Fig. 2A), but their activities were shifted to higher concentrations of CheYp than that of wild-type CheZ, suggesting a reduction in CheYp binding. The maximal reaction rates (V_max), and thus k_cat for CheZ72SN and CheZ134EG, were nearly equivalent and reduced by about 2-fold compared to that of the wild-type CheZ (Table 2). (iii) Like CheZ21IT, CheZ163LS demonstrated apparently noncooperative phosphatase activity that was increased at low concentrations of CheYp compared to that of wild-type CheZ (Fig. 2A). Unlike CheZ21IT, however, CheZ163LS demonstrated wild-type swarming (Table 1), perhaps as a result of a 2-fold reduction in k_cat relative to that of CheZ21IT (Fig. 2A and Table 2). The phosphatase activities resulting from the suppressor substitutions correlated fairly well with the swarm rates of the single suppressor mutants: swarm rates increased as phosphatase activity increased (Fig. 2A and Table 1). This observation is consistent with successful chemotaxis requiring a finite but modest amount of phosphatase activity at low CheYp concentrations (32).

Decreased catalysis, reduced substrate binding, and restoration of positive cooperativity can contribute to suppression of cheZ21IT. All of the suppressor substitutions reduced CheZ21IT activity at low CheYp concentrations (0 to 0.2 μM) to similar levels that were at or near that of wild-type CheZ (Fig. 2B, circled area). However, the phosphatase activities of double mutants bearing suppressor substitutions in the cheZ21IT background varied at higher CheYp concentrations (Fig. 2B), which suggested that suppression was accomplished via different mechanisms (Fig. 3A).

Because CheZ21IT has an alteration that results in an increased binding affinity for CheYp (32), mutants might achieve the suppression of CheZ21IT phosphatase activity by impeding binding to CheYp. Suppression by this mechanism should shift the phosphatase activity curve to higher CheYp concentrations (Fig. 3A, hypothesis i). cheZ74EK appeared to suppress cheZ21IT in just this manner and reduced phosphatase activity to nearly wild-type levels for a range of CheYp concentrations (Fig. 3B and Table 2), but this activity appeared to be noncooperative. Moreover, in the wild-type background, CheZ74EK demonstrated low phosphatase activity, but its activity was rescued when expressed in the cheZ21IT background (Fig. 3B and Table 2). This further suggests that CheZ74EK has a substantial CheYp binding defect that is consistent with the location of the 74EK substitution as a surface residue in the central portion of the CheZ four-helix bundle (Fig. 1). No interaction between CheZ Glu74 and CheY was observed in the CheZ·CheY cocrystal structure; however, the electron density observed for much of CheY was poor (41).

The suppression of cheZ21IT also might occur by slowing the catalysis of the phosphatase reaction, which would lower the maximum rate of P_i release achieved (Fig. 3A, hypothesis ii) and would affect activity at low concentrations of CheYp. cheZ144LF and cheZ163LS suppressed CheZ21IT phosphatase activity to levels well below that of wild-type CheZ (Fig. 3C and Table 2) except at very low CheYp concentrations. Moreover, double-mutant combinations that include CheZ21IT provided only minimal enhancement of phosphatase activity compared to the CheZ144LF single mutant and substantially reduced phosphatase activity compared to the CheZ163LS single mutant (Fig. 3C). These results suggest that unlike CheZ74EK, the dominant defects of CheZ144LF and CheZ163LS were reduced catalysis of the phosphatase reac-
tion, and impaired binding to CheYp did not make important contributions to the suppression of cheZ21IT. In the case of CheZ21IT163LS, reduced phosphatase activity at low CheYp concentrations also appears to be due to the restoration of cooperativity (hypothesis iii). CheZ163LS is the only one of the five suppressor substitution mutants measured for which the addition of the 21IT GOF substitution increased the estimated Hill coefficient (Table 2) and reduced rather than enhanced phosphatase activity at low CheYp concentrations.

The remaining suppressor substitutions did not fall clearly into a single category but instead affected both substrate binding (hypothesis i) and catalysis (hypothesis ii). CheZ72SN and CheZ134EG suppressed CheZ21IT phosphatase activity to levels below that of wild-type CheZ (Fig. 3D) and also reduced CheYp binding affinity to near the wild-type value (Table 2). Reductions in substrate binding and catalysis also were observed in the CheZ72SN and CheZ134EG single suppressor mutants. Interestingly, the mechanism of cheZ21IT suppression was not the restoration of cooperativity (hypothesis iii),

TABLE 2. Phosphatase activity kinetic constants for cheZ suppressors of 21IT as single mutants or as double mutants with 21IT

<table>
<thead>
<tr>
<th>CheZ type</th>
<th>Kinetic constant$^a$</th>
<th>$n$, apparent Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$K_{1/2}$ (µM)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>2.7 ± 0.2</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>21IT</td>
<td>3.8 ± 0.6</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>74EK</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>74EK-21IT</td>
<td>2.8 ± 0.9</td>
<td>0.30 ± 0.2</td>
</tr>
<tr>
<td>144LF</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>144LF-21IT</td>
<td>1.0 ± 1</td>
<td>0.71 ± 0.3</td>
</tr>
<tr>
<td>163LS</td>
<td>1.9 ± 0.5</td>
<td>0.17 ± 0.07</td>
</tr>
<tr>
<td>163LS-21IT</td>
<td>0.5 ± 0.1</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>72SN</td>
<td>1.6 ± 0.4</td>
<td>0.72 ± 0.2</td>
</tr>
<tr>
<td>72SN-21IT</td>
<td>1.5 ± 0.4</td>
<td>0.21 ± 0.1</td>
</tr>
<tr>
<td>134EG</td>
<td>1.7 ± 0.2</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td>134EG-21IT</td>
<td>0.9 ± 0.3</td>
<td>0.25 ± 0.2</td>
</tr>
</tbody>
</table>

$^a$ The values listed are ± standard deviations from a nonlinear regression fit of the data.

$^b$ ND indicates that values could not be accurately determined due to extremely low phosphatase activity under the experimental conditions employed.
even though CheZ72SN and CheZ134EG were the only proteins among the five single suppressor mutants tested that retained cooperativity.

Effects of suppressor and gain-of-function substitutions on CheZ activity are not due to altered dimerization. One potential explanation for how amino acid substitutions far from the active site could affect CheZ activity is that CheZ monomers are inactive and the substitutions altered the CheZ monomer/dimer equilibrium. Such a hypothesis is consistent with the location of many substitutions at the dimer interface. However, the available data are inconsistent with the hypothesis that diminished CheZ activity is the result of a decrease in the fraction of dimers in the CheZ population. First, in preliminary experiments before choosing the assay conditions used in this study, the rate of Pi release from a saturating (6 μM) concentration of CheY was measured as a function of CheZ concentration (50 to 760 nM) for wild-type CheZ, the gain-of-function mutants CheZ21IT and CheZ24LP, the suppressor mutants CheZ72SN, CheZ74EK, CheZ134EG, and CheZ144LF, and double mutants containing the 21IT substitution with each of the four listed suppressor mutants. In each case, the rate of Pi release per unit of concentration of CheZ (i.e., specific activity) was constant from 50 nM to at least 150 nM CheZ (and in some cases much higher concentrations) (data not shown). In every case, the specific activity appears to decrease at high CheZ concentrations, as would be expected when CheY auto-phosphorylation becomes limiting, and the increase in specific activity at higher CheZ concentrations predicted by the monomer/dimer hypothesis was never observed. Second, the phosphatase activities of the various CheZ proteins (Table 2) correlated well with the chemotactic swarm rates of cells expressing the mutants (Table 1). The in vitro measurements were made at ~50 nM CheZ, whereas the in vivo concentration of CheZ depends on growth conditions but is ~1 to 30 μM (16, 17, 19, 28). The consistent properties exhibited by CheZ at very different protein concentrations directly contradict the monomer/dimer hypothesis.

**DISCUSSION**

Bacterial chemotaxis depends on the appropriate modulation of CheYp concentration, which in turn depends on the stimulation of CheYp dephosphorylation by CheZ to an appropriate extent (not too much or too little). The \(\text{cheZ}^{21\text{IT}}\) gain-of-function mutant lacks cooperativity, is hyperactive at low CheYp concentrations, and hence is nonchemotactic. Ten...
suppressor substitutions that restore chemotaxis to cheZ21IT were located along the length of the CheZ four-helix bundle (Fig. 1). Nine of the substitutions also could suppress a different GOF mutation, cheZ24LP (Table 1). In the absence of a GOF substitution, the suppressor substitutions supported a range of chemotaxis phenotypes (Table 1). The suppressor substitutions altered CheZ phosphatase activity at low CheYp concentrations by reducing CheYp binding, reducing $k_{\text{cat}}$, and/or by increasing cooperativity (Table 2 and Fig. 2 and 3). To interpret the CheZ GOF suppressor mutant data reported here, it is useful to first provide the appropriate context by summarizing previously published data on the binding of CheYp to CheZ, cooperativity of CheZ activity, and CheZ GOF mutants.

Cooperativity and binding of CheYp to CheZ. One CheZ$_2$ dimer can bind and stimulate the dephosphorylation of two molecules of CheYp. The rate of release of phosphoryl groups from CheYp is a sigmoidal (cooperative) function of CheYp concentration when stimulated by wild-type CheZ but is a hyperbolic (noncooperative) function of CheYp concentration when stimulated by a CheZ GOF mutant (3, 32). A computational model makes an excellent fit to the experimental data by assuming (i) the association rate of the second CheYp to wild-type CheZ is about 40 times faster than the rate of the association of the first CheYp, and (ii) the rate of association for the second CheYp to wild-type CheZ and the rates of association for the first and second CheYp molecules to the CheZ21IT GOF mutant are all the same (32). Thus, the model suggests that the rate of the binding of the first CheYp to wild-type CheZ is somehow diminished, and this inhibition can be relieved either by binding CheYp or by GOF substitutions. In this view, the GOF substitutions actually result in the gain of CheZ function via the loss of the inhibition of CheYp binding.

Each of the two CheYp molecules binds directly to two distinct regions of CheZ, the C helix and the active-site region of the four-helix bundle (41). CheYp can bind to a peptide comprising the CheZ C helix alone with micromolar affinity (2, 20) but does not detectably associate with a truncated version of CheZ lacking the C helix (2, 41). The large difference in affinities of the two regions of CheZ for CheYp strongly suggests that CheYp binds first to the C helix of intact CheZ, and the increased local concentration of CheYp resulting from this tethering then facilitates the binding of CheYp to the CheZ active site. However, the data described do not reveal which interaction (with the C helix or the active site) is rate limiting for the association of CheYp with CheZ and hence is responsible for cooperativity.

Cooperativity and CheZ gain-of-function mutants. CheZ GOF mutants have been isolated using three different genetic screens: (i) the restoration of chemotaxis in bacteria bearing flagellar switches biased in the direction of rotation that is caused by high concentrations of CheYp identified CheZ GOF mutants with enhanced phosphatase activity (15, 35) and hence presumably reduced CheYp concentration; (ii) the restoration of chemotaxis in bacteria bearing a mutant CheY with reduced binding affinity for CheZ identified CheZ GOF mutants with enhanced binding to CheY (26); and (iii) additional CheZ GOF mutants were identified in bacteria that rotated their flagella in the direction caused by low CheYp concentrations (25). Altogether, 22 CheZ GOF substitutions were found in 18 different positions, and position 166 was hit in all three screens. Remarkably, only one GOF substitution (at the C terminus) could plausibly interact directly with CheYp. All of the other GOF substitutions are clustered together on the CheZ structure either in the N helix (at positions 17 to 29) or the nearby portions of the four-helix bundle (in positions 37 to 54 or 152 to 170). Although only two CheZ GOF mutants (CheZ21IT in the N helix and CheZ54RC in the four-helix bundle) have been shown to lack cooperativity, the observations that (i) all GOF mutants that have been tested lack cooperativity, (ii) GOF substitutions isolated in three different screens on the basis of enhanced phosphatase activity and/or enhanced CheY binding cluster in the same regions of the CheZ structure, and (iii) nine different substitutions that all suppress the nonchemotactic phenotype of one noncooperative GOF mutation also all restore chemotaxis to another GOF mutant (Table 1) are consistent with the simplifying assumption that all reported CheZ GOF substitutions (except 214FL at the C terminus) enhance CheZ activity by the same mechanism of relieving the inhibition of the binding of the first CheYp to CheZ$_2$ and eliminating cooperativity.

Genetic and biophysical evidence suggests that cooperativity arises at the CheZ active site. How then might GOF substitutions distant from the active site relieve the inhibition of binding of the first CheYp to CheZ$_2$? Several hypotheses can be considered.

If the C helices or the adjacent linkers of CheZ interact with the N helix or nearby portions of the four-helix bundle, then the C helices might have diminished availability for CheYp binding. In this scenario, the GOF substitutions would release the C helices from such interactions. However, fluorescence anisotropy measurements indicate that the C helices of CheZ are fully mobile in the absence of CheYp (31), contrary to the predictions of this hypothesis. A related scheme that preserves the mobility of the CheZ C terminus would be if the two linkers or C helices within a CheZ dimer interacted with one another. The binding of CheYp to one C helix then would free the other C helix for enhanced binding to a second CheYp. However, this hypothesis predicts that GOF substitutions should occur in the C helix or linker, where they could directly disrupt the postulated self interaction, whereas almost all GOF substitutions actually are located in the N helix and nearby portions of the four-helix bundle. If the rate-limiting aspect of the first CheYp binding to CheZ (and hence the source of cooperativity) is association with the C helix, then it is difficult to envision how the known GOF substitutions could influence CheYp binding.

Another class of possibilities is that the rate-limiting step in the binding of CheYp to CheZ is association with the CheZ active site, even if CheYp first binds to the C helix of CheZ in a rapid equilibrium. It has been suggested that in the absence of CheYp, the N helices of CheZ fold along the four-helix bundle and occlude the active site (41). In this circumstance, the GOF substitutions would disrupt interactions between the N helices and the four-helix bundle, thus permitting access to the active site. Similarly, in such a model CheYp binding to the CheZ active site would displace both N helices. However, fluorescence anisotropy measurements indicate that the mobility of the N helices is not substantially different in the pres-
ence or absence of CheYp (31), contrary to the predictions of this hypothesis.

The hypothesis most consistent with available evidence is that the binding of CheYp to the CheZ active site is indeed the rate-limiting step for association, and that cooperativity results because the binding of the first CheYp changes the conformation of the other CheZ active site on the opposite side of the four-helix bundle. Consistent with this model, a kink in the CheZ four-helix bundle in the vicinity of the bound CheYp would be a consequence of CheYp binding. In this model for positive cooperativity, the CheZ GOF substitutions such as 21IT would be predicted to result in structural or dynamic effects that propagate along the four-helix bundle and change the features of the active sites that slow down the binding of the first CheYp to wild-type CheZ. The CheZ21IT suppressor substitutions dampen this enhanced activity, either by directly or indirectly reducing CheYp binding and/or catalysis. Indirect effects of suppressors located far from the active site could occur via a similar mechanism of transmission through the four-helix bundle. Several observations support this proposed mechanism for the GOF mutants and their suppressors. First, there is direct nuclear magnetic resonance evidence for the propagation of structural changes along the CheZ four-helix bundle upon the binding of the CheA protein to the hairpin end (7, 13). Furthermore, CheA metabolic activity, either by directly or indirectly reducing CheYp binding and/or catalysis. Indirect effects of suppressors located far from the active site could occur via a similar mechanism of transmission through the four-helix bundle. Several observations support this proposed mechanism for the GOF mutants and their suppressors. First, there is direct nuclear magnetic resonance evidence for the propagation of structural changes along the CheZ four-helix bundle upon the binding of the CheA protein to the hairpin end (7, 13). Furthermore, CheA has been reported to enhance CheZ activity about 2-fold (21, 40), an enhancement similar in magnitude to that exhibited by GOF mutants, thus raising the possibility that structural changes in the bundle affect CheZ activity. However, it is not known whether CheA binding relieves CheZ cooperativity. The effect of CheA was observed using assay conditions (4°C, 0.1 mM Mg2+, 4 nM CheZ, and 1 nM CheA) under which CheZ stimulated the rate of CheYp dephosphorylation by 40% (21, 40). Under the assay conditions used in this work (room temperature, 10 mM Mg2+, and 50 nM CheZ), CheA stimulates CheYp dephosphorylation 100-fold (32). Under very similar conditions (100 instead of 50 nM CheZ), the addition of CheA was equimolar to CheZ had no further effect (E. Schilling and R. E. Silversmith, unpublished results). Second, all 10 of the suppressor substitutions reported in this study are found on the CheZ four-helix bundle on either side of the active site, and most have side chains that insert into the interior of the four-helix bundle. The suppressors thus are positioned either within the active site or at positions that could cause perturbations to propagate from their location to the active site. The complete absence of suppressor substitutions in the C helix or adjacent linker further suggests that the impact of the original GOF substitution is not on the C helix, and therefore that the key step for cooperativity is binding to the active site. Finally, before the present study, some GOF substitutions were known to affect CheYp binding at a distance (26). However, the alteration of CheYp binding cannot be unambiguously interpreted, because two different regions of CheZ interact with CheYp. Those GOF mutants known to increase CheZ phosphatase activity were not characterized in sufficient detail to distinguish whether CheYp binding or the catalysis of the dephosphorylation reaction was affected. However, the present work shows that several suppressor substitutions affect the catalysis of dephosphorylation (Table 2) and therefore almost certainly affect the active site from a distance.

In summary, the preponderance of available evidence suggests that CheYp first binds to the C helix of CheZ, the second step of CheYp binding (to the CheZ active site) is rate limiting, and the cooperativity of CheZ phosphatase activity arises from properties of the CheZ active site. Furthermore, CheZ GOF and suppressor substitution mutants can influence active-site properties (CheYp binding and the catalysis of dephosphorylation) from a distance, possibly via structural or dynamic changes that propagate through the CheZ structure from the site of substitution to the active site.

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