

Isolation and *in Vitro* Characterization of CheZ Suppressors for the *Escherichia coli* Chemotactic Response Regulator Mutant CheYN23D*

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The phosphorylated form of the response regulator CheY promotes the tumble signal in *Escherichia coli* chemotaxis. Phospho-CheY is thought to interact with the switch at the base of the flagellar motor and cause reversal of flagellar rotation from counterclockwise to clockwise changing the swimming direction. Thus the level of phospho-CheY controls the direction of flagellar rotation. The decay of the tumble signal is caused by dephosphorylation of CheY. CheY has an intrinsic autophosphatase activity; however, this reaction is greatly accelerated by the presence of the CheZ protein.

We have shown previously that mutations at residues Asn-23 and Lys-26 in CheY confer resistance to the dephosphorylation activity of CheZ (Sanna, M. G., Swanson, R. V., Bourret, R. B., and Simon, M. I. (1995) *Mol. Microbiol.* 15, 1069–1079). Here we show that mutant CheY(N23D) is impaired in binding to CheZ, which provides a possible explanation for its resistance to the dephosphorylation activity of CheZ. Moreover, we isolated CheZ second-site suppressors of CheY(N23D), which restore both dephosphorylation and binding activity in a CheY(N23D) background. When the CheZ suppressor mutations are mapped, they are found in two clusters at the N and C termini of the CheZ protein which could define two regions of interaction with CheY. Furthermore, these regions may generate a surface in the folded three-dimensional structure of CheZ required for interaction with CheY.

The ability of *Escherichia coli* to survive in different environments may be the result of its ability to swim toward favorable conditions using the rotation of 6–8 flagella. This process is called bacterial chemotaxis and has been extensively studied in the past 30 years. When flagella rotate counterclockwise, they promote swimming in one direction for an extensive period of time (smooth swimming); reverse rotation (clockwise) causes tumbling of the cell, thus reorienting the swimming direction. The signal transduction pathway that regulates bacterial chemotaxis consists of the transmembrane receptor proteins Tar, Tsr, Trg, and Tap; the two-component system CheA/CheY; and the CheW and CheZ proteins (for reviews see Refs. 1–4). The external changes detected by the receptor are “communicated” to the histidine kinase CheA, which is able to modulate its autophosphorylation capacity (5, 6). Phosphorylated CheA

transfers the phosphate group to the response regulator CheY (7, 8), which in the phosphorylated form is able to interact with the flagellar switch and modify the sense of flagellar rotation from counterclockwise to clockwise, promoting tumble motion (9–13). The CheZ protein accelerates the dephosphorylation of CheY, thus restoring smooth swimming (7, 14, 15).

The mechanism of interaction between CheY and CheZ has not been elucidated, although some observations suggest an allosteric effect of CheZ on CheY instead of canonical phosphatase activity (16). We isolated a mutant CheY(N23D) that is resistant to the dephosphorylating activity of CheZ but is not impaired in phosphorylation or autodephosphorylation (16), which suggests that the mutant CheY may have a wild-type conformation but could be impaired in its ability to interact with CheZ.

We now report direct evidence that the CheY(N23D) mutant has lower binding affinity for CheZ compared to the wild-type CheY protein. Also, in order to identify possible regions of CheZ that interact with CheY, we used the background CheY(N23D) to isolate CheZ suppressor mutants with restored dephosphorylation activity. The CheZ suppressor mutations we have isolated cluster in two regions that may define binding sites for the CheY protein.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—The *E. coli* bacterial strains used in this study are: RP437*recA* (wild-type for chemotaxis; Ref. 9); KO641*recA* (Δ *cheY* strain; Ref. 9); MGS138 (KO641*recA*/pREP4*cheY*N23D); MGS141 (Δ *cheY*pREP4*cheY* wild-type/pQE12*cheZ* wild-type); MGS143 (Δ *cheY*pREP4*cheY*N23D/pQE12*cheZ* wild-type) and the library of *cheZ* mutant suppressors in strain MGS138. Construction of pQE12*cheZ* wild-type has already been described (strain MGS55; Ref. 16). Strain MGS118 (M15 pREP4/pQE12*cheY*N23D with C-terminal His₆ tag) was used for purification of His-tagged CheY(N23D), and strain RVS1.6, kindly provided by Ron Swanson (Recombinant BioCatalysis, Inc., Sharon Hill, PA), for wild-type His-tagged CheY.

pREP4 (Qiagen) carrying the wild-type or N23D *cheY* gene was obtained by subcloning the *cheY* genes from plasmids pRBB40 and pRBB40.N23D (16), respectively.

Construction of CheZ Mutant Library—Mutagenic PCR¹ was performed on the 5' or 3' halves of the *cheZ* gene in separate reactions. Primers with the following sequences 5'-TCACACAGAATTCATTAAGAGGAGAAATTAACATGATG-3' (Z16 sense) and 5'-GTGTATCTGTACTAGTTCACG-3' (Z12 antisense) were used for amplifying *cheZ* base pairs 1–343; 5'-CCCCTGAAGTACTAGTACAGATAC-3' (Z3 sense) and 5'-GGAGTCCAAGCTCAGCTAAT-3' (Z8 antisense) for amplifying *cheZ* base pairs 320–645. In addition, base pair 330 (underlined in Z12 and Z3) was changed from T to C and from A to G, respectively, in order to create a unique *SpeI* restriction site which would facilitate subcloning of double mutations located on separate halves of the *cheZ* gene. Two separate mutagenic PCR reactions were performed in the presence

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¹ The abbreviations used are: PCR, polymerase chain reaction; AcPO₄, acetyl phosphate; PAGE, polyacrylamide gel electrophoresis; NTA, nitrilotriacetic acid.

of 0.05 mM MnCl₂ (17) or with nucleotides mixed in different concentrations (0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, 1 mM dTTP; Ref. 18) to induce nucleotide misincorporation by *Taq* polymerase. Each mutated CheZ half was ligated performing a bridge PCR with a non-mutagenized CheZ half. Full-length PCR products were double-digested with *Eco*RI and *Bam*HI, cloned into vector pQE12, and electroporated into a $\Delta cheY$ /pREP4/*cheY*N23D background. Plasmid DNA was prepared from each of 20 different suppressor strains, and the *cheZ* gene was sequenced manually or using *Taq* cycle sequencing by the Caltech automated sequencing facility (Applied Biosystems model 373A sequencer) to determine the nature of the mutation.

In Vivo Assays—The chemotactic capacity of the analyzed strains was measured by means of swarm and swimming assays. Swarm and swimming assays were performed as described previously (16). Miniswarm assays were performed essentially as swarm assays, except that 1 μ l of electroporated bacteria was mixed with 3 ml of motility agar (melted and kept at 40 °C) and immediately poured onto motility plates. Plates were incubated overnight at 30 °C and swarming colonies selected.

Protein Purification and Dephosphorylation Assays—Wild-type CheZ, wild-type CheY, and CheZ mutant suppressor proteins were purified as described previously (16, 19). His-tagged CheY and His-tagged CheY(N23D) were purified on a Ni²⁺-NTA column as described by Qiagen. The phosphorylation and autodephosphorylation of wild-type CheY-His and CheY(N23D)-His, as well as the dephosphorylation mediated by wild-type CheZ, were found to be the same as the untagged CheY and CheY(N23D) proteins. The CheZ and CheY proteins were ~95% pure when analyzed by SDS-PAGE and Coomassie Blue staining.

Single time point assays and kinetics of dephosphorylation of CheY were performed as described previously (16).

Binding Assays—300 pmol of wild-type His-tagged CheY or His-tagged CheY(N23D) were incubated in a microcentrifuge tube containing 20 μ l of Ni²⁺-NTA beads previously washed with 1 \times phosphorylation buffer (25 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂) containing 0.005% Nonidet P-40. Beads were centrifuged (5 s at 14,000 rpm) and washed once with 1 ml of the same buffer to remove unbound CheY. To the washed beads, 25 mM cold AcPO₄ (acetyl phosphate) was added (where indicated) for 2 min, 6 pmol of CheZ were added to the reaction for 30 s, and beads were washed and centrifuged twice and resuspended in 15 μ l of 5 \times Laemmli buffer containing 250 mM imidazole. Reaction products were separated by SDS-PAGE. CheZ was detected by Western blot using a polyclonal antibody, whereas CheY was stained with Coomassie Blue or Ponceau Red. The CheZ polyclonal antibody was generated using gel-purified CheZ protein (Cocalico).

Computer Secondary Structure Analysis of CheZ—The secondary structure prediction was performed using the algorithms in the GCG package (University of Wisconsin). The distribution of the mutated residues on the hypothetical α -helices was done using the Helicalwheel program (GCG package).

RESULTS

CheY(N23D) Is Impaired in CheZ Binding—The resistance of CheY(N23D) to the dephosphorylation activity of CheZ (16) could be explained by at least two mechanisms. (i) The CheY(N23D) mutant protein is impaired in its interaction with CheZ, thus affecting the ability of CheZ to bind and dephosphorylate it; or (ii) the binding of CheY(N23D) to CheZ is stronger compared to wild-type CheY, so that most of the CheZ present in the reaction would be “sequestered” leaving residual CheY(N23D), which would be dephosphorylated only by spontaneous hydrolysis. Both mechanisms would result in impaired ability of CheZ to dephosphorylate CheY(N23D) at the wild-type rate.

In order to compare the affinity of mutant CheY(N23D) and wild-type CheY for CheZ, His-tagged CheY and His-tagged CheY(N23D) were immobilized on Ni²⁺-NTA beads and CheZ was added in the presence or absence of acetyl phosphate (AcPO₄), which is a small phosphodonor molecule able to phosphorylate CheY in the absence of CheA (20). The amount of CheZ bound to wild-type CheY and CheY(N23D) was detected by Western blot using polyclonal antibody against CheZ. In the presence of AcPO₄, the amount of CheZ bound by CheY(N23D) is about 7-fold lower compared to the amount bound by wild-type CheY as quantified by densitometry (Fig. 1); since we

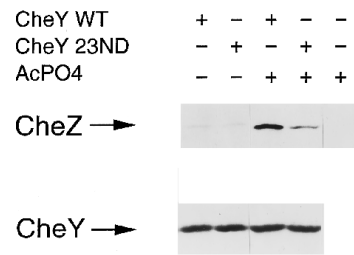


FIG. 1. Binding of CheZ to wild-type CheY and CheY(N23D). Wild-type CheY or CheY(N23D) were incubated in the presence (+) or absence (-) of phosphorylating agent (AcPO₄). Then wild-type CheZ was added to the reaction. The amount of CheZ bound to CheY or CheY(N23D) was detected by Western blot. CheY was detected with Coomassie and Ponceau Red staining.

found that CheY and CheY(N23D) are equally well phosphorylated, this suggests an impaired capacity of phosphorylated CheY(N23D) to interact with CheZ. It is also evident in this experiment that the amount of CheZ bound to CheY is much higher in the presence of AcPO₄ compared to the reactions in which the phosphate donor is omitted. This result suggests that CheZ has a higher affinity to the phosphorylated form of CheY compared to the unphosphorylated form and is consistent with recently published results by Blat and Eisenbach (21).

In Vivo Screening for CheZ Suppressors of CheY(N23D)—In order to find CheZ mutants that could suppress the *cheY*N23D mutation, we constructed a library of random mutations in *cheZ* using mutagenic PCR (see “Materials and Methods”). Plasmids carrying mutant *cheZ* genes were electroporated into a $\Delta cheY$ strain containing the plasmid pREP4/*cheY*N23D.

Approximately 300 plates were screened by miniswarm assay looking for bacteria with restored swarming capacity (Fig. 2, A and B). The negative control strain MGS143, which carries *cheY*N23D and wild-type *cheZ* genes, is impaired in chemotaxis (Fig. 2, A, C (right), and D (top)), because it constantly tumbles, thus forming small colonies when assayed for swarm capacity on motility plates.

Single colonies were isolated from 440 selected swarming strains, assayed on motility agar, and tethered on glass coverslips to confirm their phenotype. All the potential suppressor strains were found to be better swimmers than the negative control (Fig. 2D; control swarming strains are shown in Fig. 2C), and they showed either wild-type or counterclockwise bias of flagellar rotation (data not shown). Among the 440 selected clones, 20 strains were chosen for further analysis because they showed different capacities for restoring chemotaxis.

Suppressor Mutations Define Two Clusters on the CheZ Protein—Sequencing of the suppressors revealed that among the 20 selected strains, 10 carried a single point mutation in *cheZ*; two mutants, L28P and N170D, were isolated twice (Fig. 3). The remaining strains had double and triple mutations. Double mutations were subcloned as single point mutations. Three of the six resulting strains showed enhanced swarming capacity when compared with strain MGS143 and were included in the list of the suppressors. The plasmids carrying the suppressor *cheZ* genes were reintroduced into a $\Delta cheY$ /*cheY*N23D background to ensure that the phenotypes observed were not due to reversion of *cheY*N23D and/or mutations elsewhere in the chromosome.

Interestingly, the suppressor mutations are distributed on CheZ in two regions that seem to define separate clusters (Fig. 3). We selected five mutants for *in vitro* analysis, considering that they were candidates from both regions and that they showed different degrees of suppression *in vivo*. The *in vivo* suppression capacity of the CheZ mutants was quantified by measuring the diameter of the swarming colonies (Fig. 3).

CheZ Suppressor Proteins Dephosphorylate CheY(N23D)—The dephosphorylation activity of the CheZ mutant suppressors with CheY(N23D) as substrate was investigated using purified mutant CheZ proteins L24P, L28P, R29C, V166E, and N170D. The CheZ mutants were assayed for their capacity to dephosphorylate the CheY(N23D) protein as described previously (16). The amount of residual CheY phosphorylated (Fig. 4, A and B) and the relative rates of dephosphorylation (Fig. 4C) are shown. All of the five CheZ mutant proteins

dephosphorylate phospho-CheY(N23D) more efficiently than wild-type CheZ. The dephosphorylation activities of CheZ mutants L28P and L24P were found to be stronger compared to that of the other CheZ mutants, while CheZ(R29C) appears to be the least active of the mutants that we tested.

Based on cold-chase experiments the estimated $t_{1/2}$ for phosphorylated CheY(N23D) in the presence of CheZ(L24P) and CheZ(L28P) is ~ 10 and ~ 40 s for CheZ(V166E), CheZ(N170D), and CheZ(R29C) compared to ~ 100 s for wild-type CheZ (Fig. 4C). The results of *in vitro* experiments also parallel the results obtained *in vivo*; for example CheZ(L24P) and CheZ(L28P) were found to be among the strongest suppressors *in vivo* (see Fig. 3), and these proteins exhibit the highest dephosphorylation activities on phospho-CheY(N23D) *in vitro*.

When the suppressors were assayed on wild-type phospho-CheY, four of them showed enhanced dephosphorylation capacity compared to wild-type CheZ (Table I). Similar results were found when suppressors were incubated with mutant CheY(K26E), which was previously found to be partially resistant to the dephosphorylation of wild-type CheZ (Ref. 16 and Table I). Only mutant R29C showed weaker activity on wild-type CheY as well on CheY(K26E) when compared to wild-type CheZ.

CheZ Suppressors Restore Binding to CheY(N23D)—As the CheZ suppressor mutations were found to suppress the CheY(N23D) phenotype, we investigated whether the CheZ mutants have restored interaction with CheY(N23D). Similar amounts of each of the CheZ mutants L24P, L28P, R29C, V166E, and N170D were bound to CheY(N23D). These amounts were comparable to the amounts of wild-type CheZ bound to CheY (Fig. 5). This suggests that the mutant CheZ proteins have restored affinity for CheY(N23D).

DISCUSSION

The CheY(N23D) mutant shows a specific lack of interaction with CheZ. It is resistant to the dephosphorylating activity of CheZ without being impaired in its phosphorylation or autodephosphorylation activity, suggesting that a highly localized change in structure is responsible for loss of activity. This is consistent with two-dimensional NMR analysis in which the CheY(N23D) mutant does not appear to have an altered overall conformation compared to the wild-type CheY protein.² We have found that the CheY(N23D) mutant is impaired in CheZ binding. In order to study the contribution of CheZ to the CheY-CheZ interaction, we have isolated CheZ suppressors of the CheY(N23D) protein. The suppressors restore chemotactic behavior *in vivo* as well as *in vitro* dephosphorylation and

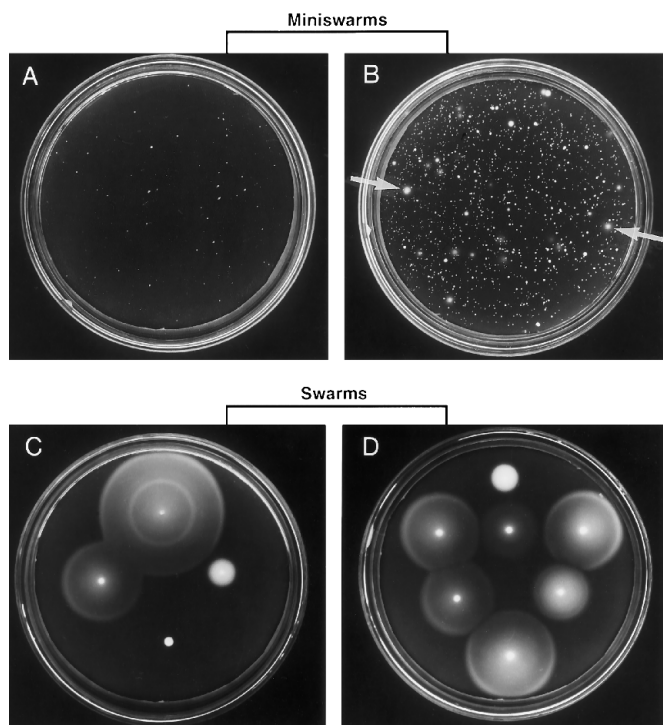
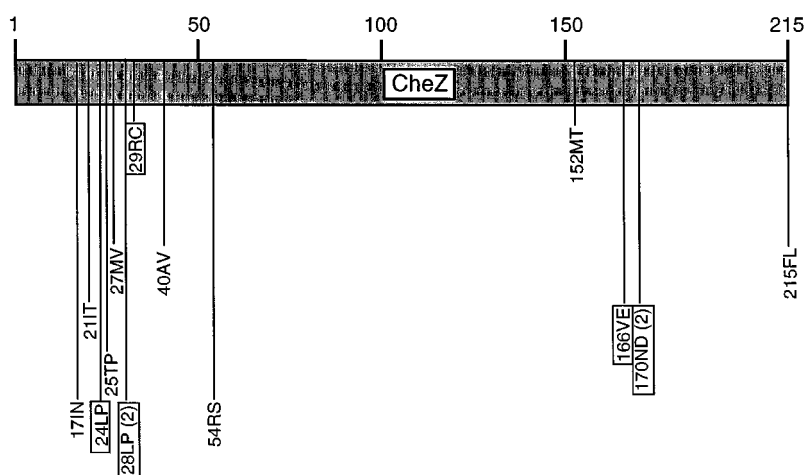


FIG. 2. Screening for *cheZ* suppressors of *cheY(N23D)*. A, miniswarm assay of control strain MGS143 ($\Delta cheY/pREP4cheY(N23D)/pQE12cheZ$ wild-type), which is non-chemotactic (unable to swarm) and has tumble (clockwise) bias swimming behavior. B, miniswarm assay of $\Delta cheY/pREP4cheY(N23D)/pQE12cheZ$ mutant library; swarming colonies of *cheZ* suppressor mutants were isolated (examples are indicated by the arrows). C, swarm assay of control strains; top, RP437*recA* (wild-type for chemotaxis); bottom, KO641*recA* ($\Delta cheY$ strain; this strain is non-chemotactic with smooth (counterclockwise) bias swimming behavior); left, MGS141 ($\Delta cheY/pREP4cheY$ wild-type/ $pQE12cheZ$ wild-type; this strain is partially chemotactic with slightly smooth bias swimming behavior); right, MGS143 ($\Delta cheY/pREP4cheY(N23D)/pQE12cheZ$ wild-type; this strain is dramatically impaired for chemotaxis with strong tumble bias swimming behavior). D, top, MGS143 (as in C (right)); other panels, MGS138/ $pQE12cheZ$ mutant suppressors.

FIG. 3. Distribution of *cheZ* mutations isolated as suppressors of *cheY(N23D)* mutation. The amino acid substitutions encoded by *cheZ* suppressor mutations are clustered primarily in two regions of the CheZ protein. Mutations are named with the residue number followed by the *single-letter abbreviation* for the wild-type amino acid and the mutant amino acid. The length of the vertical lines is proportional to the *in vivo* suppression capacity of each mutant, which was calculated as their capacity to restore swarming in the *cheY(N23D)* background (i.e. L24P strong suppression activity, diameter of the swarming colony ~ 2.7 cm; R29C weak suppression activity diameter of the swarming colony ~ 1.0 cm; diameter of the MGS143 swarming colony ~ 0.7 cm). The suppressors chosen for *in vitro* analysis are boxed.



² F. W. Dahlquist, personal communication.

FIG. 4. Dephosphorylation of phospho-CheY(N23D) by CheZ suppressors. A, image of phosphorylated CheY(N23D) after incubation with 4 pmol of purified wild-type or suppressor CheZ proteins. Phosphorylated CheY(N23D) in the absence of CheZ is also shown for comparison as well as wild-type phospho-CheY with or without wild-type CheZ. B, graphic representation of different amounts of phosphorylated wild-type or CheY(N23D) as quantified with a PhosphorImager (Molecular Dynamics). Amounts are expressed as PhosphorImager units and represent the mean of three separate experiments. The standard deviation was <8%. C, cold chase dephosphorylation kinetics of phospho-CheY(N23D) mediated by CheZ mutant suppressors. 0.5 pmol of wild-type or mutant CheZ were used in the assays. ●, no CheZ; ⊕, wild-type CheZ; ○, CheZ(V166E); ◇, CheZ(R29C); ■, CheZ(N170D); △, CheZ(L28P); ◆, CheZ(L24P).

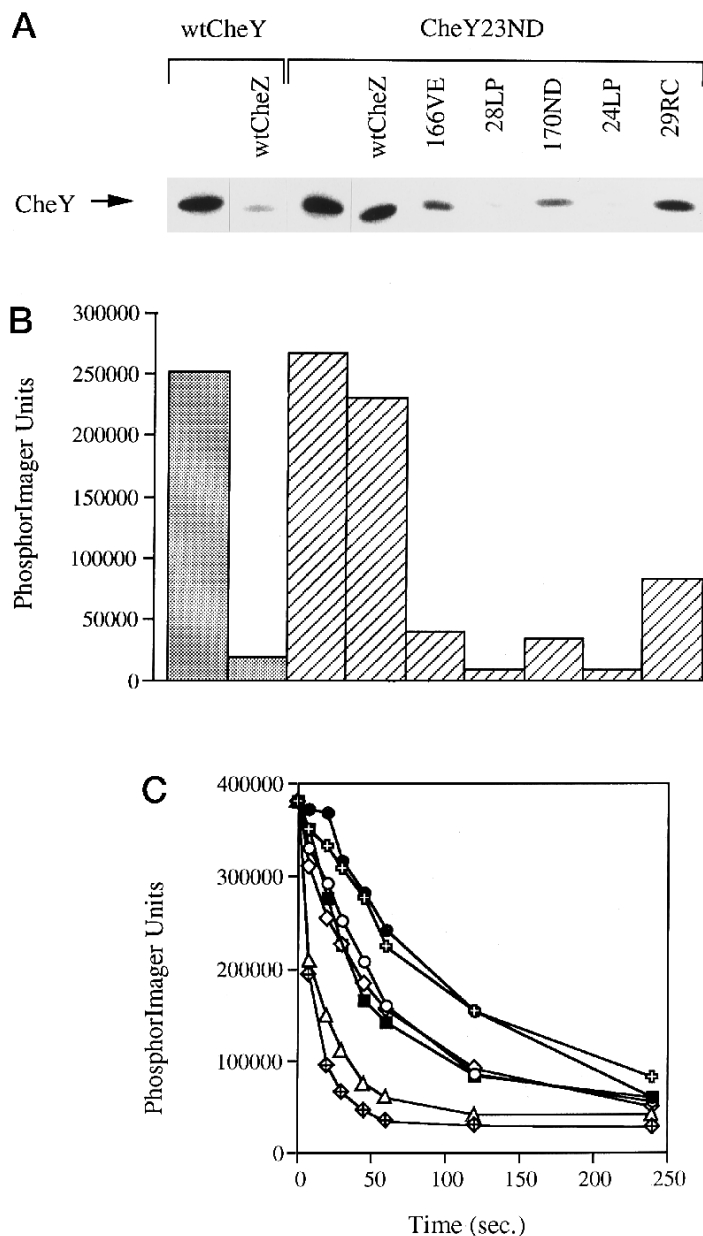


TABLE I
Activity of CheZ suppressors

The activity of the CheZ suppressors is relative to the activity that wild-type CheZ has on the listed CheY protein. Values are calculated considering the wild-type CheZ as 1.0 (*i.e.* CheZ(R29C) is 2.7 times more active on CheY(N23D) than wild-type CheZ). Values are calculated as a mean of three experiments.

CheZ mutants	Dephosphorylation of CheY(N23D)	Dephosphorylation of wild-type CheY	Dephosphorylation of CheY(K26E)
CheZ(R29C)	2.7	0.7	0.8
CheZ(V166E)	5.8	1.5	3.5
CheZ(N170D)	6.7	2.0	4.8
CheZ(L28P)	28.0	2.2	15.3
CheZ(L24P)	29.0	4.6	16.5

binding activity between CheZ and CheY(N23D).

Interestingly, two of the isolated CheZ suppressors of CheY(N23D) carried amino acid substitutions of residues 54R and 166V. Changes at these residues, CheZ(R54C) and CheZ(V166G), were previously reported by Huang and Stewart (22) as *Salmonella typhimurium* CheZ mutants with enhanced dephosphorylation activity on *E. coli* CheY. Although the iden-

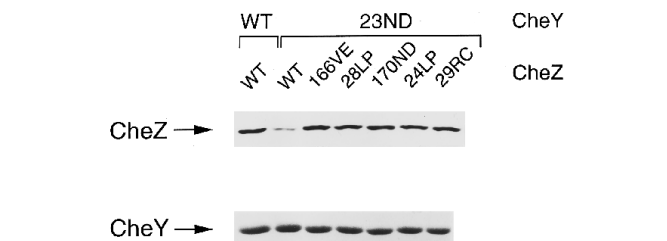


FIG. 5. Binding of CheZ mutant suppressors to CheY(N23D). The binding experiments were performed as described under "Materials and Methods." All the reactions were done in the presence of AcPO_4 . Bands representing wild-type CheZ bound to wild-type CheY or to CheY(N23D) are also shown (*first two left lanes*).

tity of the specific amino acid changes were different, we also isolated mutants at residues 54 and 166 as suppressors of CheY(N23D) (Fig. 3). These mutants showed suppression activity *in vivo*, and mutant CheZ(V166E) was found to dephosphorylate the *E. coli* wild-type CheY at a rate slightly higher than that of wild-type CheZ (Table I).

The assayed CheZ mutants showed different degrees of de-

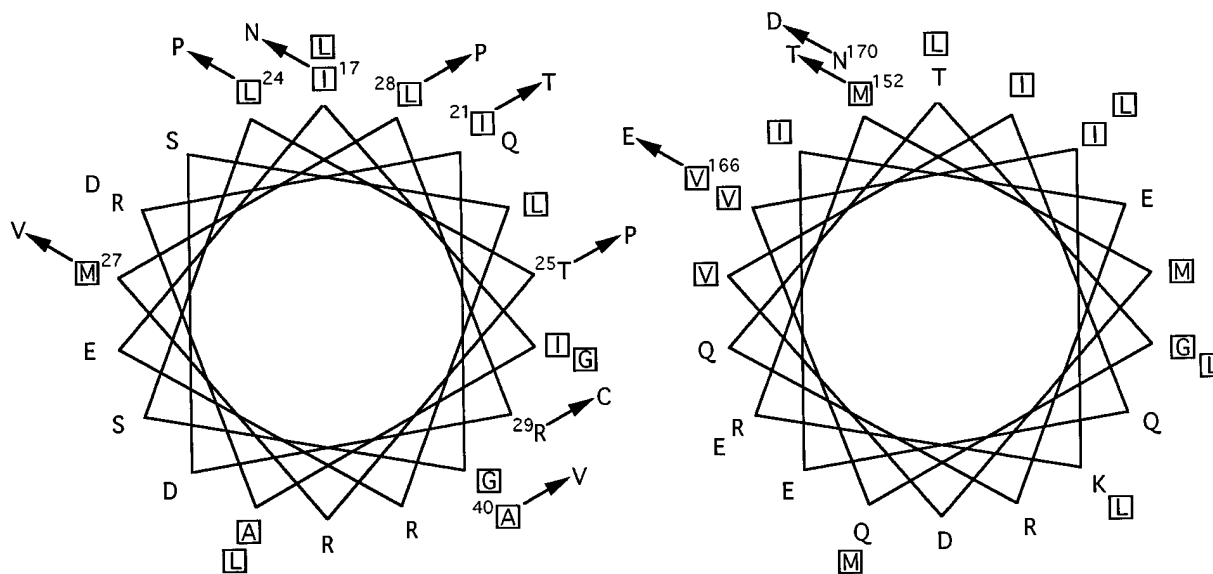


FIG. 6. **Distribution of mutated residues on hypothetical α -helical CheZ structures.** The CheZ regions defined by the suppressor mutations were predicted to be α -helical secondary structures by the Chou-Fasman and Garnier-Robson algorithms (GCG package). The Helicalwheel plot shows how the mutated residues (arrows) would be distributed defining a face on the hypothetical α -helix 1 from Ile-17 to Ala-40 (left), which represent the cluster of mutations at the N terminus of the CheZ protein or α -helix 2 from Thr-145 to Asn-170 (right, mutants at the C terminus) defining a potential surface of interaction with CheY. Hydrophobic residues are boxed.

phosphorylation of wild-type CheY ranging from 0.7 to 4.6 times the wild-type CheZ dephosphorylation rate. In particular, CheZ mutants V166E, N170D, L28P, and L24P were found to dephosphorylate wild-type CheY and CheY(K26E) better than wild-type CheZ, whereas CheZ(R29C) was partially impaired in its dephosphorylation activity (Table I). These observations suggest that mutants V166E, N170D, L28P, and L24P are also gain-of-function CheZ mutants. Moreover these results suggest that suppressors V166E, N170D, L28P, and L24P are not allele specific for the N23D mutation, whereas R29C, which dephosphorylates CheY(N23D) better than wild-type CheZ but is less active on wild-type CheY or CheY(K26E), seems to show specificity for the N23D mutation.

There are at least three possible models to explain the suppression of the CheY(N23D) tumbling phenotype by the CheZ mutants. (i) the mutations are located on the CheZ protein in regions that are directly involved in the interaction with CheY; (ii) the mutations change the conformation of CheZ enhancing its binding to CheY; (iii) the oligomerization capacity is enhanced in the CheZ mutant suppressors. Each of the three proposed mechanisms would alter the rate of dephosphorylation and/or the substrate specificity. It has been recently shown (23) that CheZ forms oligomers in the presence of phosphorylated CheY. This property seems to enhance the dephosphorylating activity of CheZ, and it could represent a mechanism of CheZ regulation.

The suppressor mutations map approximately on the CheZ protein in two regions rich in leucine and isoleucine residues. It is known that leucine-rich sequences can be involved in protein-protein interaction. The results of a secondary structure prediction indicate that the suppressor sites may be clustered in two α -helices (Fig. 6). On the hypothetical α -helices, the mutated residues would be located on one face of each helix and overlapping with the leucine-rich and hydrophobic region, which could be expected to be involved in the interaction with

other protein(s) (Fig. 6). If this is the case, these regions of the α -helices may represent CheZ binding sites for CheY, or form a continuous surface of interaction with CheY in the folded three-dimensional structure of CheZ; an alternate hypothesis is that they could be CheZ oligomerization sites.

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