

Conserved aspartate residues and phosphorylation in signal transduction by the chemotaxis protein CheY

(bacterial chemotaxis/two-component regulatory systems/protein phosphorylation/*Escherichia coli*)

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ABSTRACT The CheY protein is phosphorylated by CheA and dephosphorylated by CheZ as part of the chemotactic signal transduction pathway in *Escherichia coli*. Phosphorylation of CheY has been proposed to occur on an aspartate residue. Each of the eight aspartate residues of CheY was replaced by using site-directed mutagenesis. Substitutions at Asp-12, Asp-13, or Asp-57 resulted in loss of chemotaxis. Most of the mutant CheY proteins were still phosphorylated by CheA but exhibited modified biochemical properties, including reduced ability to accept phosphate from CheA, altered phosphate group stability, and/or resistance to CheZ-mediated dephosphorylation. The properties of CheY proteins bearing a substitution at position 57 were most aberrant, consistent with the hypothesis that Asp-57 is the normal site of acyl phosphate formation. Evidence for an alternate site of phosphorylation in the Asp-57 mutants is presented. Phosphorylated CheY is believed to cause tumbling behavior. However, a dominant mutant CheY protein that was not phosphorylated *in vitro* caused tumbling *in vivo* in the absence of CheA. This phenotype suggests that the role of phosphorylation in the wild-type CheY protein is to stabilize a transient conformational change that can generate tumbling behavior.

A large family of signal transducing systems, referred to as "two-component regulatory systems," allows bacteria to sense and respond to numerous changes in their environment (1–3). This family was originally defined on the basis of protein sequence similarities between pairs of "sensor" and "regulator" proteins. Further study has provided increasing evidence for a common mechanism of action. To date, the information transduction pathways that underlie bacterial chemotaxis (4–11), nitrogen assimilation (12–14), outer membrane porin expression (15–19), and phosphate assimilation (K. Makino *et al.*, as cited in ref. 20) have been reported to involve regulated phosphate group transfer between proteins. Furthermore, the best understood systems are all known to include the following components: (i) a sensory protein to detect environmental changes, (ii) an autophosphorylating protein kinase triggered by the sensor, (iii) a phosphorylated regulator protein, (iv) a target of regulator action, and (v) a protein phosphatase that restores the regulator protein to its unphosphorylated state. These functional domains are organized in a variety of ways within the proteins comprising each signaling system.

Escherichia coli governs its swimming behavior in response to environmental changes by controlling the frequency with which flagellar rotation is reversed (reviewed in refs. 21 and 22). Numerous proteins participate in the chemotactic signal transduction network: (i) Transmembrane receptor proteins (e.g., Tar) detect attractant and repellent substances. (ii) The cytoplasmic CheA protein is an auto-

phosphorylating protein kinase that can transfer phosphate to the CheY protein (4, 5, 7). (iii) The CheW protein couples the rate of CheY-phosphate formation to the occupancy of the receptors' ligand binding sites (9). (iv) CheY-phosphate is unstable and spontaneously autodephosphorylates (10). (v) The CheZ protein accelerates the removal of phosphate from CheY (5). (vi) CheY-phosphate is believed to interact with proteins at the base of the flagellar apparatus (23, 24) to promote clockwise flagellar rotation, which leads to tumbling of the bacteria. Smooth swimming results from counterclockwise flagellar rotation. Thus, mutants that cannot make CheY-phosphate (*cheW*⁻, *cheA*⁻, or *cheY*⁻) are typically smooth swimming, whereas those that have elevated levels of CheY-phosphate (*cheZ*⁻) tumble (25).

The site of phosphorylation in CheA is His-48 (8), whereas CheY is probably phosphorylated on an acidic (aspartate or glutamate) residue (11). The chemistry of protein phosphorylation in other two-component regulatory systems appears to be quite similar (refs. 12–18; K. Makino *et al.*, as cited in ref. 20). The data are consistent with a general scheme in which the sensor proteins autophosphorylate a histidine residue and transfer the phosphate to an aspartate residue of their cognate regulator proteins.

The crystal structure of one regulator protein, CheY, is known (26). Three of the aspartate residues found in CheY are conserved in the amino acid sequences of other regulator proteins (1, 27). In the CheY crystal structure these aspartates cluster together to form an "acid pocket" on one face of the protein, and it has been suggested that they might be involved in regulating CheY function (26). To further understand the roles of aspartate residues and phosphorylation in CheY function, we systematically mutagenized all of the aspartate residues of CheY, individually and in combination. Genetic and biochemical characterization of the resultant mutant proteins is reported here.

MATERIALS AND METHODS

Strains. KO641*recA* and RP437*recA* were made by transducing *recA*Δ(*Sst* II–*Eco*RI) *srl*::Tn10 from KO685 (4) into the *cheY*Δm60–21 strain RP5232 (28) or the *che*⁺ strain RP437 (29) with P1. RBB382 was made by transducing *cheA*Δm102–11 *zec*::Tn10 from RP1788 (30) and *recA*Δ(*Sst* II–*Eco*RI) into RP437.

The ampicillin-resistant pBR322 derivative pRL22 carries *cheY* and *cheZ* under the control of the *Serratia marcescens* *trp* promoter (31). The plasmid pRBB40 was made from pRL22 by standard techniques and carries '*cheBYZflhB*' on a unique *Bam*HI–*Hind*III fragment, which was inserted into M13mp19 for site-specific mutagenesis (32).

Mutations created in *cheY* were confirmed by DNA sequencing, moved to pRBB40 on the *Bam*HI–*Hind*III frag-

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Abbreviations: Che⁺, chemotactic; Che⁻, nonchemotactic.

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ment, and transformed into KO641*recA* for analysis. Mutations are named in the text with the amino acid number followed by the standard single-letter abbreviations for the wild-type and mutant amino acids (D, aspartate; E, glutamate; K, lysine; N, asparagine).

Chemotaxis Assays. Colonies of KO641*recA*/pRBB40 carrying *cheY* mutations were stabbed into motility plates (1% tryptone/0.5% NaCl/0.3% Bactoagar) and incubated at 30°C for 10–12 hr. Swarm diameter was measured periodically to determine swarming rate.

To determine swimming behavior, cultures were grown overnight in T broth (1% tryptone/0.5% NaCl) plus 50 µg of ampicillin per ml at 30°C, diluted 1000-fold into fresh medium, coded to reduce observer bias, and grown 1 hr at 30°C. Approximately 20 individual cells in each sample were examined under the microscope and classified as smooth swimming, reversing, or tumbling.

Protein Purification. CheA, CheW, CheY, CheZ, and membranes containing Tar or no Tar were purified as described (5, 9).

Biochemical Assays of CheY Activity. To measure phosphorylation of CheY by CheA, 15 pmol of CheA, 60 pmol of CheY, and 15 pmol of CheZ (where indicated) were incubated at room temperature with 50 mM KCl and 5 mM MgCl₂ in TEDG buffer (5) in a total volume of 10–12 µl. Reactions were initiated by the addition of 0.5 mM [γ -³²P]ATP (\approx 7000 cpm/pmol) and stopped after 2.5 min by the addition of NaDodSO₄ sample buffer (4). Samples were subjected to NaDodSO₄/PAGE, after which the gel was stained with Coomassie blue, destained, and dried. After autoradiography, the bands containing CheA and CheY were excised from the gel and their radioactivity was quantitated by liquid scintillation spectrometry.

Reactions measuring phosphorylation of CheY by CheA in the presence of receptor-containing membranes were performed exactly as described above, except that 1.5 µl of membranes, 1 pmol of CheA, 20–40 pmol of CheW, 60 pmol of CheY, and no CheZ were used, and the reaction time was 5 sec.

Reactions measuring phosphate transfer from CheA-phosphate to CheY contained 40 pmol of CheA-phosphate, 4 pmol of CheY, and 4 pmol of CheZ (where indicated) and were performed as described (5). For the CheY57DE and CheY57DN mutant proteins, 40 pmol of CheY was used.

RESULTS

Mutagenesis of the Aspartate Residues of CheY. CheY contains eight aspartate residues (27, 31), three of which (Asp-12, Asp-13, and Asp-57) are conserved among the five regulator proteins in *E. coli* that have been reported to be phosphorylated. Substitution of the other aspartate residues in CheY, Asp-3 (with K or N), Asp-38 (with K or N), Asp-41 (with N), Asp-64 (with K or N), or Asp-75 (with E or N), resulted in a Che⁺ (chemotactic) phenotype. Bacterial colonies bearing each of these mutations swarmed at \geq 50% of the wild-type rate on motility plates (data not shown). Therefore, the “nonconserved” aspartate residues do not appear to be critical to CheY function.

The three conserved aspartates in CheY were each changed, in order to assess the effect of a nonphosphorylatable residue (asparagine), a larger acidic residue (glutamate), or a basic (lysine) residue at these positions. Among the nine single mutants thus constructed, only *cheY12DE* had a Che⁺ swarm phenotype. Seven of the single mutants (12DK, 12DN, 13DE, 13DN, 57DE, 57DK, 57DN) did not swarm on motility plates and, when observed by light microscopy, were found to swim smoothly. This behavior was the same as the null phenotype exhibited by a strain (KO641*recA*) in which the *cheY* gene is deleted. The ninth mutant, *cheY13DK*, was also Che⁻ (nonchemotactic) and will be discussed in more detail later. In addition, all possible multiple combinations of

the aspartate to asparagine or aspartate to glutamate substitutions at these three positions were made. The eight mutants thus constructed (12DE13DE, 12DN13DN, 12DE57DE, 12DN57DN, 13DE57DE, 13DN57DN, 12DE13DE57DE, 12DN13DN57DN) were each Che⁻ and smooth swimming.

The CheY protein participates in multiple reactions: it accepts phosphate from CheA (5, 7), autodephosphorylates (10), acts as a substrate for dephosphorylation by CheZ (5), and interacts with the flagellar switch proteins (23, 24). A mutation in *cheY* could conceivably affect any or all of these activities. To further investigate the effect of the various *cheY* mutations on CheY function, purification of the 15 mutant CheY proteins involving single or double substitutions at Asp-12, Asp-13, and Asp-57 was attempted. All except for CheY12DK and CheY12DN57DN were successfully purified, suggesting that in most cases the Che⁻ phenotype observed was not the result of degradation of an unstable mutant CheY protein. The *in vitro* biochemical activities of the purified CheY proteins were examined using three distinct assays: (i) phosphorylation by CheA, (ii) phosphorylation by CheA in the presence of receptor-containing membranes, and (iii) phosphate transfer from CheA-phosphate. These reactions permit examination of different aspects of CheY function due to their differing conditions and will be described in turn.

Phosphorylation of CheY by CheA. In a reaction containing [γ -³²P]ATP, CheA, and CheY, the radioactive phosphate label flowed from ATP through CheA and CheY to P_i. The reaction was allowed to proceed through many cycles in the presence of excess ATP and is a relatively sensitive assay for phosphorylation of CheY. CheA and CheY were separated by NaDodSO₄/PAGE, which permitted simultaneous monitoring of the relative rates of phosphate transfer through CheA and CheY. All 11 of the purified mutant CheY proteins bearing asparagine or glutamate substitutions at positions 12, 13, or 57 were detectably phosphorylated, except for CheY12DE57DE (Fig. 1). However, the mutant proteins all displayed phosphorylation properties different from wild-type CheY (Fig. 1).

The presence of CheZ reduced the amount of CheY-phosphate formed with wild-type and most of the mutant CheY proteins (Fig. 1). However, all of the mutants with a substitution at position 57 that were phosphorylated (CheY57DE, CheY57DN, CheY12DE57DE) appeared to be resistant to the action of CheZ in this assay, except for CheY13DN57DN (Fig. 1).

Phosphorylation of CheY by CheA in the Presence of Receptor-Containing Membranes. The assay that most completely reconstructs the chemotaxis signaling pathway tests the ability of membranes containing Tar to stimulate formation of CheY-phosphate from [γ -³²P]ATP and CheA in the presence of CheW. This reaction primarily measures the efficiency of phosphate transfer from CheA to CheY, because of the short time course of the reaction and the relatively low amount of CheA present (\approx 1 CheA:60 CheY).

Under the conditions used, the amount of CheY-phosphate formed from wild-type CheY increased about 30-fold in the presence of the Tar receptor (Table 1). The enhancement with CheY12DE was somewhat greater, and with CheY12DN somewhat less, than with wild-type CheY (Table 1). This modest difference between CheY12DE and CheY12DN may not be sufficient to explain the observation that *cheY12DE* is Che⁺, whereas *cheY12DN* yields a Che⁻ phenotype. The mutant CheY proteins with single substitutions at position 13 or 57 were clearly deficient in phosphorylation by CheA in the presence of membranes, as were all double mutants tested (Table 1). This behavior may account for the Che⁻ phenotype of most of the *cheY* mutations.

Phosphate Transfer from CheA-Phosphate to CheY. The phosphate transfer reaction contained CheA-phosphate,

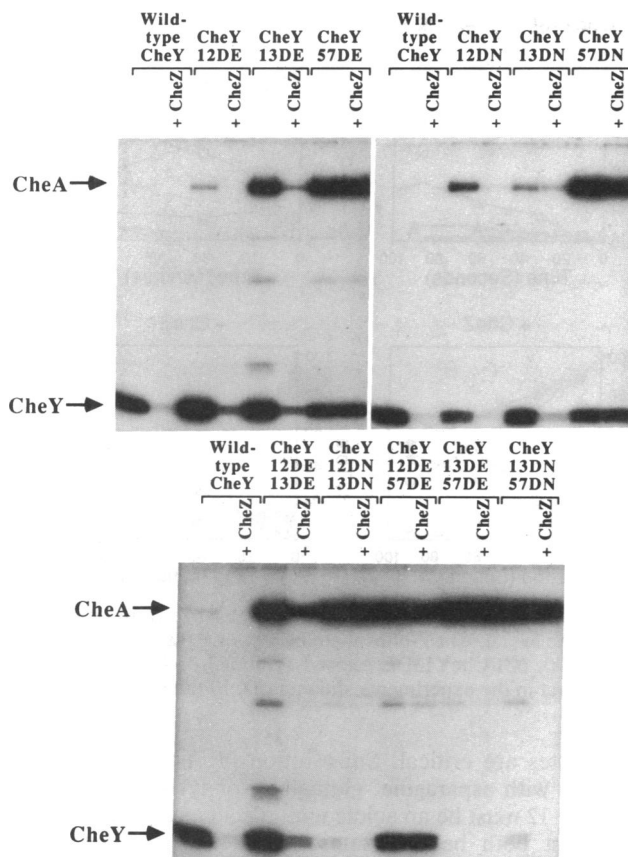


FIG. 1. Autoradiograms of the products of the phosphorylation reaction performed with various mutant CheY proteins. The positions of CheA and CheY are indicated. Additional bands are degradation products of CheA.

CheY, and no ATP. Because the reaction contained a molar ratio of ≈ 10 CheA:1 CheY, dephosphorylation of CheY was rate limiting. This can be seen by examining the reaction with wild-type CheY (Fig. 2A); when CheZ was present to accelerate CheY dephosphorylation, dephosphorylation of CheA was also markedly accelerated.

Table 1. Phosphorylation of mutant CheY proteins by CheA in the presence of receptor-containing membranes and CheW

| CheY protein | cpm incorporated* | | | | Stimulation† |
|--------------|-------------------|--------|----------|--------|--------------|
| | Without Tar | | With Tar | | |
| | CheA-P | CheY-P | CheA-P | CheY-P | |
| Wild-type | 350 | 1170 | 300 | 39,600 | 30 |
| 12DE | 210 | 1380 | 320 | 55,700 | 40 |
| 12DN | 290 | 710 | 1300 | 15,000 | 20 |
| 13DE | 310 | 1180 | 920 | 7,530 | 6 |
| 13DN | 260 | 450 | 380 | 1,920 | 4 |
| 57DE | 800 | 270 | 1250 | 280 | 1 |
| 57DN | 930 | 170 | 1390 | 220 | 1 |
| 12DE13DE | 560 | 570 | 1200 | 1,990 | 3 |
| 12DN13DN | 970 | 490 | 2040 | 430 | 1 |
| 12DE57DE | 1010 | 540 | 1540 | 530 | 1 |
| 13DN57DN‡ | 710 | 380 | 960 | 240 | 1 |

*Each line gives the results of a single experiment. Because the specific activity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ varied between experiments, comparison of raw data can be made only within a line.

†Stimulation is the approximate ratio of CheY-P formed in the presence versus the absence of Tar. Background (typically ≈ 200 cpm) was not subtracted prior to calculation.

‡This reaction was 10 sec.

In the presence of CheY12DE or CheY13DE, CheY-phosphate formed in amounts similar to wild-type CheY, but dephosphorylation of CheA was slow unless CheZ was also present (Fig. 2B and C). This suggests that the phosphate group is more stable on these mutant CheY proteins than on wild-type CheY. The reaction of CheY12DE in the presence of CheZ was similar to that of wild-type CheY. The presence of CheY13DE led to dephosphorylation of CheA-phosphate at rates that were significantly slower than wild-type CheY, even with CheZ present. This could be due to a defect in transfer from CheA-phosphate and/or reduced susceptibility of CheY-phosphate to CheZ. Addition of CheZ accelerated phosphate transfer reactions with CheY12DN and CheY-13DN, but not nearly as much as those with wild-type CheY or CheY12DE (Fig. 2A and B bottom). Since CheZ presumably recognizes a specific conformation of its CheY-phosphate substrate, the differences in reactivity with CheZ may reflect changes in the three-dimensional structures of the mutant CheY proteins. These changes could also account for their defects in signaling.

CheY proteins bearing substitutions at position 57 were very poor substrates for the phosphate transfer reaction. In order to observe the interactions of CheY57DE or CheY-57DN with CheA-phosphate, it was necessary to allow the reaction to proceed for much longer times and to increase the amount of CheY 10-fold (to ≈ 1 CheA:1 CheY). Phosphate was transferred very slowly from CheA to CheY57DE or CheY57DN (Fig. 2D). Substantial amounts of CheY-phosphate eventually accumulated, however, and release of P_i was markedly delayed (Fig. 2D), clearly demonstrating the resistance to hydrolysis of the phosphate group on the CheY57DE or CheY57DN proteins.

Characterization of the Phosphate Group in CheY57DN.

The various mutant CheY proteins bearing substitutions at position 57 all exhibited phosphorylation properties very different from wild-type and the other mutant CheY proteins examined (Figs. 1 and 2; Table 1). These results suggest that Asp-57 may be the usual site of phosphorylation in CheY, and when it is changed to asparagine or glutamate, phosphorylation occurs at an alternate site.

To investigate the nature of the putative second phosphorylation site, a phosphate transfer reaction with ^{32}P -labeled CheA and CheY57DN was allowed to proceed for 1 hr and CheY57DN-phosphate was purified by gel filtration. The ^{32}P -labeled CheY57DN thus prepared was subjected to a variety of chemical treatments and stability was determined by separating the products (CheY-phosphate and P_i) on TLC (4). Proteins can be phosphorylated on acidic (aspartate, glutamate), basic (arginine, histidine, lysine), hydroxy (serine, threonine, tyrosine), or cysteine residues (33). CheY does not contain any cysteine or histidine residues (27, 31). Treatment with acid (1 M HCl, 15 min, 37°C), base (1 M NaOH, 15 min, 37°C), or hydroxylamine (0.16 M NH_2OH , pH 7, 15 min, 37°C), which released all the phosphate from wild-type CheY, did not dephosphorylate CheY57DN detectably (data not shown). These characteristics suggest that the site of phosphorylation in CheY57DN is not aspartate or glutamate (33). CheY57DN-phosphate was not detectably hydrolyzed by treatment with acid (1 M HCl, 15 min, 37°C) or hydroxylamine (0.4 M NH_2OH , pH 7, 70 min, 37°C), which released all the phosphate from CheA, or with pyridine (0.1 M pyridine, 70 min, 37°C), which released $\approx 40\%$ of the phosphate from CheA (data not shown). These results imply that the site of phosphorylation in CheY57DN is not arginine or lysine (33). Together, these experiments suggest (by process of elimination) that CheY57DN may be phosphorylated on a serine, threonine, or tyrosine residue.

Analysis of the *cheY13DK* Mutation. Bacteria bearing the *cheY13DK* mutation form Che⁻ swarms that have diffuse edges and are larger than those of a Δ *cheY* control. These

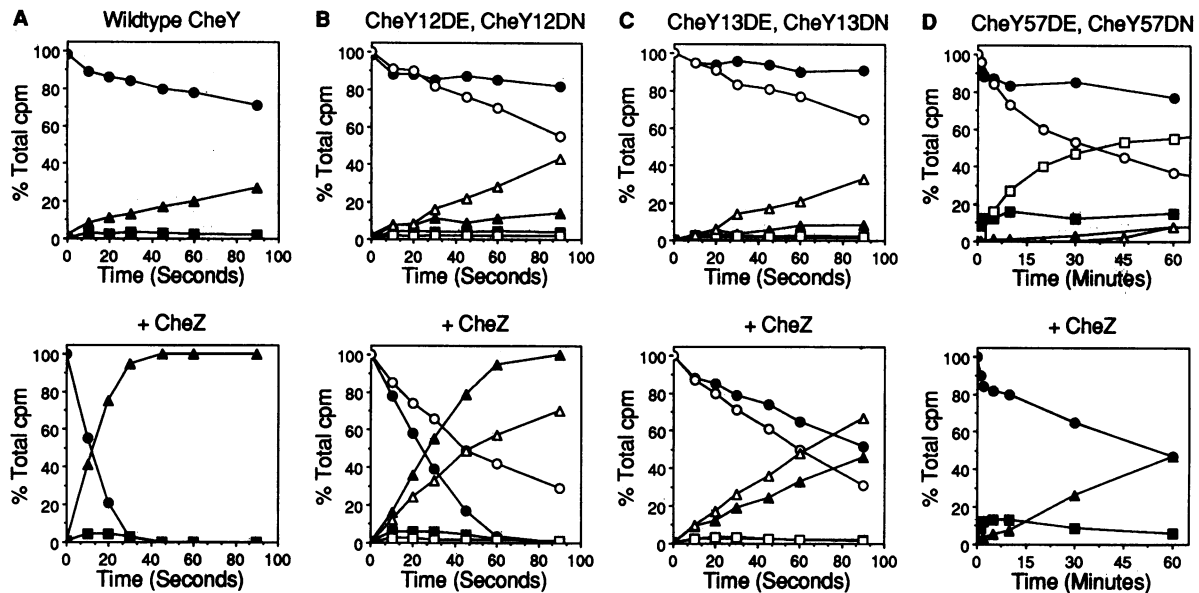


FIG. 2. Time course of the phosphate transfer reaction performed with various mutant CheY proteins in the absence (top) or presence (bottom) of CheZ. The percentage of total radioactivity present in CheA-phosphate (●, ○), CheY-phosphate (■, □), or P_i (▲, △) is shown. (A) Wild-type CheY. (B) CheY12DE (closed symbols) or CheY12DN (open symbols). (C) CheY13DE (closed symbols) or CheY13DN (open symbols). (D) CheY57DE (closed symbols) or CheY57DN (open symbols). Note that in the experiments shown in D, 10 times more CheY was used and the time scale is different.

characteristics are typical of frequently tumbling bacteria (34). Examination of the swimming behavior of *cheY13DK* bacteria confirmed that they tumble frequently, but *in vitro* phosphorylation of CheY13DK by CheA could not be demonstrated. The properties of the *cheY13DK* mutation were further examined by transferring it into other bacterial hosts. A *cheY⁺ ΔcheA* strain swims smoothly, presumably because it is defective in CheY-phosphate formation. Introduction of the *cheY13DK*, but not the *cheY13DN*, allele into such a strain caused it to tumble (Table 2). Therefore, CheY13DK can apparently cause tumbling in the absence of phosphorylation by CheA. Furthermore, this behavior is apparently dominant, since introduction of the *cheY13DK* allele on a multicopy plasmid into a wild-type strain caused it to tumble (Table 2). The simplest explanation for these observations, consistent with the evidence that CheY-phosphate has a role in generating tumbles, is that phosphorylation of wild-type CheY stabilizes a form of the protein that causes tumbling and that the *cheY13DK* mutation has the same effect—i.e., it stabilizes the active (tumble generating) form of CheY.

DISCUSSION

Role of Aspartate Residues in CheY Function. The five aspartates in CheY that are not conserved among the regulator proteins of two-component regulatory systems are not essential for CheY function, whereas the three conserved

Table 2. Behavior of bacteria bearing the *cheY13DK* allele

| Plasmid | $\Delta cheY$ host | | $\Delta cheA$ host | | <i>che⁺</i> host | |
|--------------------|-----------------------|------|-----------------------|------|-----------------------------|------|
| | Swarm | Swim | Swarm | Swim | Swarm | Swim |
| None | Che ⁻ | Sm | Che ⁻ | Sm | Che ⁺ | Rev |
| pBR322 | Che ⁻ | Sm | Che ⁻ | Sm | Che ⁺ | Rev |
| pcheY ⁺ | Che ⁺ | Rev | Che ⁻ | Sm | Che ⁺ | Rev |
| pcheY13DK | dif. Che ⁻ | Tum | dif. Che ⁻ | Tum | * | Tum |
| pcheY13DN | Che ⁻ | Sm | Che ⁻ | Sm | Che ⁺ | Rev |

Control plasmids or pRB40 carrying the indicated *cheY* allele were introduced into $\Delta cheY$ (KO641*recA*), $\Delta cheA$ (RBB382), and *che⁺* (RP437*recA*) strains. dif, Diffuse edge characteristic of tumbly mutants; Sm, smooth; Rev, reversing; Tum, tumbling.

*An apparently Che⁺ swarm developed after a delay of ≈ 2 hr, in either the presence or absence of 500 μ g of ampicillin per ml.

aspartates are critical. Substitution of Asp-12, Asp-13, or Asp-57 with asparagine, glutamate, or lysine suggests that residue 12 must be an acidic amino acid and residues 13 and 57 must both be aspartates in order to maintain CheY function *in vivo*. The importance of the "conserved" aspartates is emphasized by the result that changing these three residues generated a set of mutant proteins in which each of the known activities of CheY was altered. Mutants were obtained that exhibited: (i) changed ability to accept phosphate from CheA, (ii) altered autodephosphorylation properties, (iii) differing ability to act as a substrate for CheZ-mediated dephosphorylation, or (iv) possibly modified interaction with the flagellar switch.

All the nonchemotactic CheY aspartate mutants are defective to one degree or another in phosphate group transfer from CheA to CheY (Table 1). This result supports a role for the aspartate acid pocket of CheY in removing phosphate from CheA. Transfer defects appear to be sufficient to account for the Che⁻ phenotype of all of the CheY mutants tested except CheY12DN. It is not immediately evident why *cheY12DE* bacteria are Che⁺ and *cheY12DN* bacteria are Che⁻, when the respective CheY proteins are so similar to wild type in the magnitude of phosphorylation observed with membranes present (Table 1). We believe this assay most closely mimics the *in vivo* situation. The only obvious change found in CheY12DE was increased phosphate group stability, but CheZ can partially compensate for this change (Fig. 2B). CheY12DN apparently is impaired both in transfer from CheA (Table 1; Fig. 2B) and in phosphate group stability (Fig. 2B). These changes might prevent formation of adequate CheY-phosphate in the cell to cause tumbling. Alternatively, the *cheY12DN* mutation might distort the shape of the CheY protein sufficiently to prevent it from acting as a tumble signal even when phosphorylated.

CheZ-Mediated Dephosphorylation. The mutant CheY proteins that have substitutions at position 12 or 13 are substrates for accelerated dephosphorylation by CheZ (Fig. 1). This finding is consistent with phosphorylation of these mutant proteins occurring at the normal site—i.e., Asp-57. The precise mechanism of CheZ action is not known. Although wild-type CheY-phosphate is an optimal substrate, CheZ was previously shown to slowly dephosphorylate CheA-

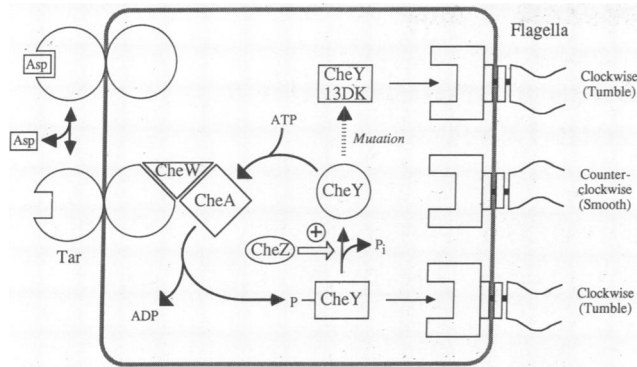


FIG. 3. Model of the excitation pathway of bacterial chemotaxis. Detection of a negative chemotactic signal (removal of attractant or addition of repellent) by a receptor protein results in increased formation of CheY-phosphate by CheA. CheW links information from the receptor to CheA; the nature of the interactions between receptor, CheW, and CheA is unknown. Phosphorylated CheY stimulates clockwise flagellar rotation. CheZ accelerates dephosphorylation of CheY. The *cheY13DK* mutation apparently alters the form of CheY to mimic that of CheY-phosphate.

phosphate as well (5). CheZ can also very slowly dephosphorylate CheY57DE-phosphate (Fig. 2D), which is presumably phosphorylated at a secondary site. (This reaction occurs so slowly that it is not visible under the experimental conditions used for Fig. 1.) Apparently, CheY13DN57DN is phosphorylated so slowly that even the slow rate of CheZ-mediated removal of secondary site phosphate groups from CheY is sufficient to destroy CheY13DN57DN-phosphate as fast as it is formed (Fig. 1).

Site(s) of Phosphorylation in CheY. Asp-53 has been (indirectly) reported to be the site of phosphorylation in the PhoB protein (K. Makino *et al.*, as cited in ref. 20), which is related to CheY. The results described here are consistent with the hypothesis that Asp-57 is the normal site of phosphorylation in CheY. When Asp-57 is changed to asparagine or glutamate, the phosphorylation properties of the mutant CheY proteins are dramatically altered (Figs. 1 and 2; Table 1). When Asp-57 is changed to another amino acid, CheY is phosphorylated at a site that apparently is not an acidic residue. It is not known if this secondary site is phosphorylated in the wild-type protein or if it has any functional role.

Mechanism of CheY Action. The CheY13DK mutant protein indicates that the presence of a phosphate group may not be necessary for CheY to generate tumbling behavior. Thus, phosphorylation of CheY may produce tumbling by stabilizing or converting CheY to an alternate form, rather than by the phosphate group on CheY interacting with or being transferred to another member of the signal transduction pathway. The putative change in CheY following phosphorylation could be a conformational change or a change in multimeric state. The CheY13DK protein elutes from a gel filtration column at a position indistinguishable from wild-type CheY, which argues against the latter possibility (data not shown). Our current view of the transduction pathway for the excitation (tumble) signal in chemotaxis is summarized in Fig. 3.

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