JOURNAL OF BACTERIOLOGY, July 1996, p. 4208–4215 Vol. 178, No. 14
0021-9193/96/$04.00+0

Tyrosine 106 of CheY Plays an Important Role in Chemotaxis Signal Transduction in *Escherichia coli*

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Received 28 February 1996/Accepted 1 May 1996

CheY is the response regulator in the signal transduction pathway of bacterial chemotaxis. Position 106 of CheY is occupied by a conserved aromatic residue (tyrosine or phenylalanine) in the response regulator superfamily. A number of substitutions at position 106 have been made and characterized by both behavioral and biochemical studies. On the basis of the behavioral studies, the phenotypes of the mutants at position 106 can be divided into three categories: (i) hyperactivity, with a tyrosine-to-tryptophan mutation (Y106W) causing increased tumble signaling but impairing chemotaxis; (ii) low-level activity, with a tyrosine-to-phenylalanine change (Y106F) resulting in decreased tumble signaling and chemotaxis; and (iii) no activity, with substitutions such as Y106L, Y106I, Y106V, Y106G, and Y106C resulting in no chemotaxis and a smooth-swimming phenotype. All three types of mutants can be phosphorylated by CheA-phosphate in vitro to a level similar to that of wild-type CheY. Autodephosphorylation rates are similar for all categories of mutants. All mutant proteins displayed less than twofold increased rates compared with wild-type CheY. Binding of the mutant proteins to FliM was similar to that of the wild-type CheY in the CheY-FliM binding assays. The combined results from in vivo behavioral and in vitro biochemical studies suggest that the diverse phenotypes of the Y106 mutants are not due to a variation in phosphorylation or dephosphorylation ability nor in affinity for the switch. With reference to the structures of wild-type CheY and the T87I CheY mutant, our results suggest that rearrangements of the orientation of the tyrosine side chain at position 106 are involved in the signal transduction of CheY. These data also suggest that the binding of phosphoryl-CheY to the flagellar motor is a necessary, but not sufficient, event for signal transduction.

Two-component regulatory systems play major roles in allowing bacteria to respond to changes in their environments (24). The sensor component is an autophosphorylating protein kinase. The second component, the response regulator, becomes activated after receiving the phosphoryl group from the sensor, and it then interacts with its target (either DNA or proteins) to induce a physiological or transcriptional response (3, 36).

Chemotaxis in *Escherichia coli* is a response to microenvironmental heterogeneity. Bacteria swim toward chemical attractants and away from repellents. The flow of information through the chemotactic signal transduction pathway has been characterized in detail (reviewed in reference 3). Briefly, the sensor, CheA, receives signals from transmembrane chemoreceptors with the help of CheW (4, 9, 18, 21) and then transfers the signal to the response regulator, CheY, by protein phosphorylation (10, 11). Phosphoryl-CheY (CheY-P) is dephosphorylated by its autophosphatase activity (10, 11). Dephosphorylation is also enhanced by CheZ. CheY-P interacts with the motor switch complex and causes a reversal in flagellar rotation from counterclockwise (counter-CW) to CW (16, 17, 20, 25, 26). Counter-CW rotation causes cells to move forward through the medium in a motion called "smooth swimming." When some proportion of the flagella reverse to CW rotation, the cell stops swimming and the cell body rotates into a new, random orientation, which is called a "tumble." An alternation of tumbles and smooth swimming results in normal chemotaxis (15). Both smooth-swimming (5, 8) and tumbly CheY mutants (5) impair the normal chemotactic function of bacteria.

CheY is the only member of the response regulator superfamily for which a detailed three-dimensional structure is known (34, 36, 37). The highly conserved residues D-12, D-13, D-57, T-87, and K-109 in the primary structure of CheY are configured in the three-dimensional structure to form an active site. D-57 is the site of phosphorylation (6, 29), and D-12 and D-13 coordinate the Mg^{2+} ion, which is essential for the phosphorylation and dephosphorylation reactions (33). K-109 forms a hydrogen bond with D-57 in the apo- form of CheY (19, 37). T-87 participates in the hydrogen-bonding network of the solvent molecules within the active site through its γ-hydroxyl group. Mutagenesis studies indicate that a substitution of any one of these residues causes functional defects in CheY and results in a change in cell behavior (5, 7, 8).

Position 106 of CheY is a conserved aromatic residue (tyrosine or phenylalanine) in the response regulator superfamily (36). In the three-dimensional structure of wild-type apo-CheY, tyrosine 106 is located on the putative switch binding surface of CheY (26, 37) and is a rotamer whose side chain can occupy both the inside (solvent-inaccessible) position and the outside (solvent-exposed) position. Figure 1 shows the solvent-accessible surface of the wild-type CheY with the side chain of Y-106 removed. There is a cavity in which the Y-106 side chain can freely rotate between its inside position and outside position. When the Y-106 side chain is inside, the hydroxyl group forms a hydrogen bond with the O_γ of T-87 through a solvent molecule. One CheY mutant, with a threonine-to-isoleucine substitution at position 87 (T87I), causes a smooth-swimming nonchemotactic phenotype (8). In the T87I mutant CheY,

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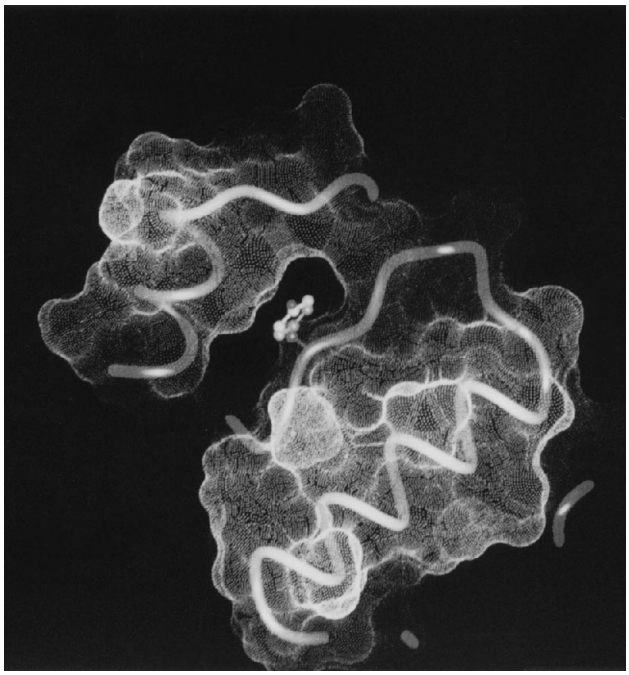

FIG. 1. Solvent-accessible surface of the wild-type CheY (Mg^{2+} free form) with the side chain of Y106 removed. There is a cavity where the Y106 side chain can rotate freely between the inside and outside. Here, the Y106 side chain is shown in the outside position.

residue 106 exclusively occupies the outside, solvent position (8). These observations suggest that tyrosine 106 is involved in the CheY activation pathway.

Several substitutions at position 106 have been made by site-directed mutagenesis. The resulting mutant proteins were characterized by both behavioral and biochemical studies. The results demonstrated that Y106 is not required for phosphorylation or increased affinity for the motor but suggest that Y106 participates in an activation step after CheY-P binds to the motor itself.

MATERIALS AND METHODS

Reagents. Restriction enzymes, T4 DNA polymerase, and T4 DNA ligase were purchased from Gibco-BRL and used as suggested by the manufacturer. The T7 Sequenase 2.0 kit was purchased from U.S. Biochemical (Cleveland, Ohio), and [γ-^{32}P]ATP was from Amersham Corp. (Arlington Heights, Ill.). Culture media components were from Difco Laboratories (Detroit, Mich.). Caged aspartate was purchased from Molecular Probes (Eugene, Oreg.).

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used and their sources are listed in Table 1. L broth and L agar were used for routine culturing, with antibiotics added as required, as previously described (26). For behavioral studies, all cells were grown in 50 ml of tryptone broth in 300-ml flasks at 30°C (2).

Site-directed mutagenesis. Point mutations in *cheY* were generated by using the M13 system, in which a unique *Hin*dIII-*Pvu*II DNA fragment containing *cheY* was cut from pRL22ΔZ and inserted into the *Hin*dIII and *Hin*cII sites of M13mp18. Single-stranded uracil-substituted template DNA of phage M13mp18cheY was prepared from strain CJ236 (28). Primers containing the designed changes for substitutions were obtained from Operon Technologies, Inc. (Alameda, Calif.). The template was mixed with a mutagenic primer at 65°C and slowly cooled to room temperature. Extension and ligation of the primed template were done as described elsewhere (28). After the mutation was created in *cheY*, it was confirmed by DNA sequencing, and a *Hin*dIII-*Sma*I fragment containing the mutation was moved back to the *Hin*dIII and *Pvu*II sites of pRL22ΔZ.

Transfer of plasmid-borne *cheY* mutations to the host chromosome. The plasmid-borne *cheY* mutants generated by the above methods were transferred by gene conversion into a host chromosome to obtain a single-copy mutant *cheY* gene on the chromosome in *E. coli* (27). The *Eco*RI-*Sal*I fragments of pXYZ20 (Y106W), pXYZ21 (Y106F), pXYZ22 (Y106L), and pRL22ΔZ (wild-type *cheY*), which contain *cheB′* and one third of *cheY*, were replaced with the pMC100 *Eco*RI-*Sal*I fragment extending upstream of *cheY* to *cheA*. The resulting plasmids pXYZ100W (containing Y106W), pXYZ110F (containing Y106F), pXYZ111L (containing Y106L), and pXYZ98 (containing the wild-type *cheY*), which carry *′cheAW tar tap cheRBYZ′*, were used as donors to cross the *cheY* alleles into the chromosome of a recipient strain, D345 (*cheB*::*rpsl*::Kan^r::*cheY377*), to yield strains XYZ3, XYZ4, XYZ5, and XYZ6, which correspond to Y106W, Y106F, Y106L, and wild-type *cheY*, respectively.

Bacterial chemotaxis assay. A spectrophotometric assay for chemotactic behavior was based on methods described by Zhulin et al. (42). Because of the thermotactic response, temperature is critical in this assay, so all steps were performed at 30°C. Briefly, cells were grown in T broth to early postexponential phase. Three milliliters of the cells was harvested by gentle centrifugation. The cell pellets were resuspended in a chemotaxis buffer (10 mM potassium phosphate [pH 7.0], 0.1 mM EDTA, and 0.1 mM L-methionine), incubated at 30°C for 5 min, and then transferred into a cuvette with a piece of agarose gel, containing 5 mM serine, on the bottom of the cuvette. The cuvette was jacketed by 30°C water to maintain a constant temperature. The cell-density changes at the interface between the gel and the medium were determined over time, by using a spectrophotometer.

Quantitation of tumble frequency. Experiments were performed as described by Amsler et al. (2). Briefly, cells were grown in tryptone broth at 30°C to early postexponential phase. Swimming cells were visualized at room temperature (≈21°C) with the aid of a light microscope and phase-contrast optics. Videotaped images of unstimulated, swimming cells were processed with a Motion Analysis Corporation VP 110 video processor. Cell tumble frequency was quantitated by computer motion analysis programs developed with Motion Analysis Corporation CellTrak software and Lotus 123 version 3.1 (1).

Flash photolysis. Flash photolysis, using UV epi-illumination, was done as described previously (14) except that the buffer contained only 2.5 mM dithiothreitol and the cells were imaged with an Olympus BX60 microscope. Before observation the cells were maintained at 30°C in either an incubator or a water bath, and during observation the cells were maintained at 28°C, by using a Physitemp TS-4 thermal stage (Physitemp Instruments Inc., Clifton, N.J.). Photorelease of L-aspartic acid, α-(4,5-dimethoxy-2-nitrobenzyl)ester, hydrochloride [α-(DMNB-caged) L-aspartate] was activated by a 100-ms flash of UV light. The concentration of caged aspartate in the buffer was 500 μM, and on the basis of a release efficiency of 30% (14) this would result in 150 μM aspartate being released per flash.

Protein phosphorylation assays. Purified CheA was phosphorylated by [γ-^{32}P]ATP as described elsewhere (10), and ^{32}P-CheA was purified by Sephadex G-75 to remove ATP and other small radiolabeled components. The phosphotransfer reaction mixtures contained 1.5 pmol of ^{32}P-CheA and 1.5 pmol of either the wild-type CheY or mutant CheY in a buffer with 50 mM Tris (pH 7.5),

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or characteristic	Source or reference
Strains		
RP437	Che^{+a}	J. S. Parkinson
RP4079	*cheY216 recA*	J. S. Parkinson
CJ236	Ung$^-$ Dut$^-$	B. J. Bachmann
594	F$^-$ ΔLac *galK2 galT22 epsL179*	F. W. Dahlquist
D345	*recD1903 cheB*::*rpsl*::Kan^r::*cheY377*	C. Russell
XYZ3	*recD1903*, Y106W	This study
XYZ4	*recD1903*, Y106F	This study
XYZ5	*recD1903*, Y106L	This study
XYZ6	*recD1903*, Y106Y^a	This study
Plasmids		
pRL22ΔZ	*cheZ* deletion, CheY^{+a}	S. J. Roman
pXYZ20	Y106W	This study
pXYZ21	T106F	This study
pXYZ22	Y106L	This study
pXYZ23	Y106I	This study
pXYZ24	Y106V	This study
pXYZ25	Y106G	This study
pXYZ26	Y106C	This study
pMC100	*′cheAW tar tap cheRBYZ′*	S. J. Roman
pXYZ100W	*′cheAW tar tap cheRBY*(Y106W)*Z′*	This study
pXYZ110F	*′cheAW tar tap cheRBY*(Y106F)*Z′*	This study
pXYZ111L	*′cheAW tar tap cheRBY*(Y106L)*Z′*	This study
pXYZ98	*′cheAW tar tap cheRBYZ′*	This study

[a] Wild type.

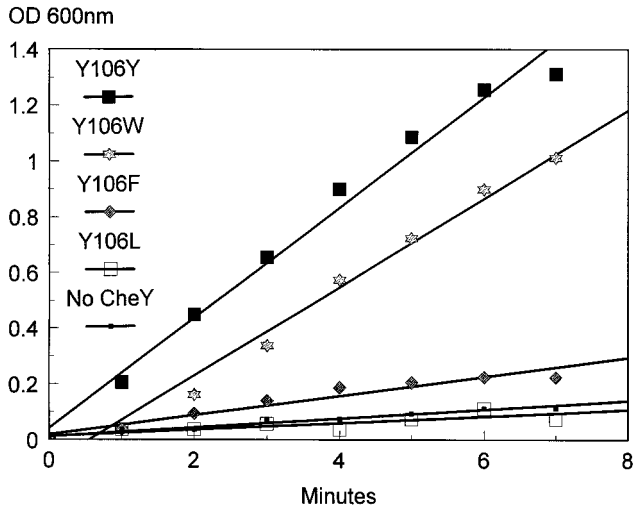

FIG. 2. Chemotactic abilities of *cheY* mutants versus the wild-type *cheY* strain based on spectrophotometric assay. Y106Y (wild type) is strain XYZ6. CheY mutations: Y106W, in strain XYZ3; Y106F, in strain XYZ4; and Y106L, in strain XYZ5. The *cheY* gene is not present in strain D345. Slopes were compared by analysis of covariance (SAS statistical software; SAS Institute, Cary N.C.). Significant differences ($P < 0.0001$) were detected in comparisons of Y106Y with Y106F, Y106W with Y106F, and Y106F with Y106L. Y106W was significantly different from the wild type ($P < 0.0017$), even when compared only from 1 to 6 min, where the slopes are most similar. There was no significant difference between Y106L and D345 ($P < 0.2493$). The data are means of three individual experiments. OD, optical density.

3 mM $MgCl_2$, and 50 mM KCl. The reaction was carried out at 4°C. Reaction products were analyzed as described by Morrison and Parkinson (23) except that they were quantitated by using an Ambis β-scanning system.

The stability of phosphorylated CheY was analyzed as previously described (38). CheA was coupled to Sepharose beads and was phosphorylated by [γ-^{32}P]ATP. CheY was then added to the CheA-coupled beads to allow the phosphoryl group transfer reaction to occur. The phosphorylated CheA reaction mixture contained 3 μl of 10× phosphorylation buffer, 1.5 μl of [γ-^{32}P]ATP (specific activity, 0.75 μCi/nmol), and 0.5 μl of unlabeled ATP (15 mM) in a total volume of 30 μl, with approximately 1 μg of CheA per μl of beads. The reaction was carried out at room temperature by rotating the tube containing the beads for 30 min and was stopped by washing the beads with excess phosphorylation buffer. One hundred microliters of purified CheY (0.25 mg/ml in 50 mM Tris, pH 7.5) was added to the phosphorylated CheA beads, and phosphate transfer was allowed to progress for 30 s while the reaction tubes were rotated at room temperature. Phosphorylated CheY was removed with a Hamilton syringe and immediately transferred into a buffer with 0.5 mM Mg^{2+} and 50 mM Tris (pH 7.5) at room temperature. Samples of phosphorylated CheY were removed at various times, and the autodephosphorylation reaction was quenched by 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Samples were loaded directly for SDS-PAGE (15% polyacrylamide). Radiolabeled proteins were visualized by autoradiography, and the radioactivity of the protein bands was determined with an Ambis β-scanning system.

CheY phosphorylation by acetyl phosphate. Acetyl [^{32}P]phosphate was synthesized as described by Welch et al. (40). Aliquots of different amounts of wild-type or mutant CheY were mixed with different amounts of acetyl [^{32}P]phosphate in a buffer with 5 mM $MgCl_2$, 2 mM dithiothreitol and 50 mM Tris-HCl (pH 7.9), in a total volume of 20 μl. The reactions were allowed to stand at room temperature (22°C) for 10 min before being quenched by 2× SDS-PAGE loading buffer. Ten-microliter samples of reaction products were analyzed by SDS–15% polyacrylamide gels followed by autoradiography.

CheY-FliM binding assays. The assay for binding of CheY to FliM was carried out as previously described (39, 40), with some modifications. CheY-coupled beads were suspended to homogeneity in 50 mM Tris-HCl, pH 7.9, and dispensed in 50-μl aliquots into Eppendorf tubes. Forty microliters of stabilization buffer (consisting of 3.4 M glycerol and 12.5 mM $MgCl_2$) was added. Purified FliM (150 μM, more than 80% pure) was added to each tube. Acetyl phosphate was added, when needed, from a 1 M stock solution to the concentrations indicated. The final volume of each reaction mixture was adjusted to 250 μl with 50 mM Tris-HCl. The reactions were incubated at room temperature for 30 min. The beads were washed twice with 1 ml of cold Tris buffer containing 2 mM $MgCl_2$ (acetyl phosphate was added for some reactions). Sixty microliters of 2× SDS-PAGE loading buffer was added and mixed at room temperature for 5 min. The beads were boiled for 2 min in order to remove the bound FliM from the CheY beads. Two microliters of the supernatant was loaded for SDS–15% PAGE. Gels were stained with Coomassie brilliant blue dye. FliM bands were scanned by SigmaGel Gel Analysis software. Bovine serum albumin (BSA)-coupled beads served as controls.

RESULTS

Substitutions at position 106 in CheY. Tyrosine at position 106 was replaced with various amino acids. These substitutions can be divided into three different groups according to the size of the side chain: the Y106W mutant has a larger aromatic side chain than the wild-type CheY; the Y106F mutant has an aromatic side chain similar to that of wild-type CheY and lacks only a hydroxyl group; and the Y106L, Y106I, Y106V, Y106G, and Y106C mutants have nonaromatic residues with nonpolar smaller side chains relative to the tyrosine of wild-type CheY.

Since the amount of CheY in a cell affects swimming behavior (16, 17), the plasmid-borne *cheY* mutations were moved onto the chromosome to ensure wild-type levels of expression. Strain XYZ6 had the same genotype as the *cheY* mutant strains and served as a control for wild-type chemotaxis.

Chemotactic activity. A spectrophotometric assay was used to analyze chemotaxis. Strain XYZ6 had the same chemotactic response as RP437, which is wild type for chemotaxis (data not shown). Strain XYZ3 (Y106W) showed about 80% chemotaxis relative to the wild type, strain XYZ4 (Y106F) had much less activity (about 10 to 20% of the wild-type response), whereas strain XYZ5 (Y106L) showed no chemotactic activity (Fig. 2). Mutations Y106I, Y106V, Y106C, and Y106G produced the same phenotype as Y106L (data not shown). These results indicate that mutations at position 106 affect CheY activity and that the level of chemotactic activity varies for different substitutions.

Cell tumble frequency. The wild-type level of chemotactic activity depends on the control of cell tumbling and smooth swimming. Chemotactic ability should be decreased by either excessive tumbling or smooth swimming. Mutations in *cheY* can cause cells to be dominant tumbly or dominant smooth-swimming. Mutation D13K causes a tumbly phenotype (5), while mutant K109R results in an exclusively smooth-swimming phenotype (5, 19, 40), and both impair chemotaxis. To determine if different substitutions at position 106 cause tumbly or smooth-swimming phenotypes, tumble frequencies were quantified (1). In the absence of a chemical gradient, the tumble frequency of strain XYZ6 (wild-type *cheY* in the D345 background) is statistically indistinguishable from that of RP437, which is the most widely used control strain for wild-type chemotaxis (data not shown). Strain XYZ3 (Y106W) had a significantly higher, and strain XYZ4 (Y106F) had a significantly lower, tumble frequency than strain XYZ6, although the tumble frequency of XYZ4 was slightly higher than that of the *cheY* deletion strain D345 (Fig. 3). The tumble frequency of strain XYZ5 (Y106L) was statistically indistinguishable from that of strain strain D345 (Fig. 3). The Y106I, Y106V, Y106G, and Y106C mutant strains were exclusively smooth-swimming, like the Y106L strain (data not shown). These results suggest that changes in swimming behavior result from the substitutions at position 106 of CheY.

Flash photolysis. The spectrophotometric assay described above measures the response to attractants of a cell population. We utilized flash photolysis to determine the time course of the response of individual cells to attractants by measuring the rate of change of direction (RCD) before and after the release of caged aspartate. Strains expressing wild-type, Y106W, or Y106F CheY all exhibited statistically significant responses to aspartate pulses (Fig. 4), and there were no apparent differences in response time; all developed maximal

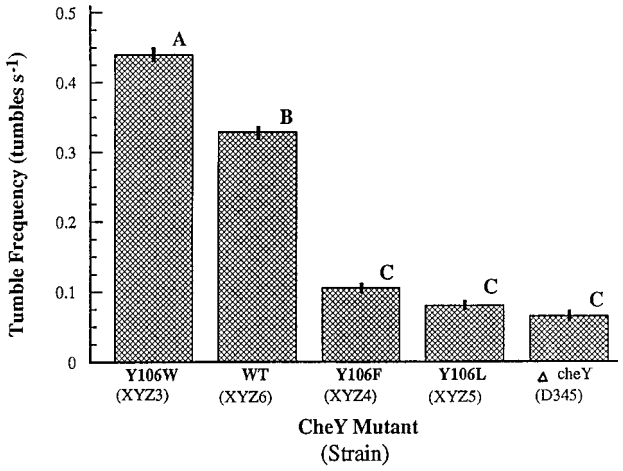

FIG. 3. Tumble frequencies (means with standard errors) as a function of CheY mutations. All measurements were at room temperature (21 ± 1°C). Sample size was from 500 to 1,700 cells per strain. Statistical analyses were performed as described by Amsler et al. (2). One-way analysis of variance indicated a significant effect of the CheY strain on tumble frequency ($F_{4,5295}$ = 272.25; $P < 0.0001$). Letters above the bars indicate the results of a multiple-comparison test (REGWQ). Bars with different letters are significantly different ($P = 0.005$) (i.e., the Y106W mutant is significantly different from all other strains; the wild type (WT) is significantly different from all other strains; the Y106F, Y106L, and Δ*cheY* mutants are not significantly different from one another but are significantly different from Y106W and the wild type). The difference between Y106F (strain XYZ4) and the *cheY* deletion (strain D345) could be considered marginally significant ($P = 0.0537$).

response within 0.2 s. However, the amplitude of the response to aspartate release was different for each strain. Mean prerelease and postrelease RCD levels were, respectively, 833 and 573 for the wild type, 908 and 625 for the Y106W mutant, and 598 and 534 for the Y106F mutant. The Y106W mutant showed almost the same amplitude as the wild-type strain but with a significantly higher RCD both prerelease and postrelease. The Y106F mutant exhibited significantly less amplitude than the wild-type strain. The Y106L strain did not respond to the chemoattractant (RCD levels, 521 and 528), an observation which is consistent with its lack of response in the spectrophotometric assay. Prerelease RCD levels were all significantly different from each other (Fig. 4), with relative levels corresponding to the levels in unstimulated tumble frequency as described above (Fig. 3).

In vitro phosphorylation biochemistry. Phosphorylation is the primary event in the activation of CheY. Wild-type CheY can be phosphorylated by CheA and dephosphorylated by its autophosphatase activity and by CheZ in vitro. The concentration of CheY-P determines whether cells are tumbly or smooth-swimming. A substitution of D-57 in CheY, the phosphorylation site, results in CheY losing its ability to be phosphorylated and causes cells to be smooth-swimming and nonchemotactic. To explore whether the different swimming phenotypes of Y106 mutants are due to defects in the phosphorylation ability of CheY, an in vitro phosphotransfer reaction from CheA to CheY was performed. Under the described conditions, the CheA kinase was phosphorylated and the phosphoryl group was transferred to the CheY protein. As shown in Fig. 5, all three mutants (Y106W, Y106F, and Y106L) were found to be phosphorylated in vitro and to have transfer rates similar to that of the wild-type CheY.

The stabilities of the phosphorylated Y106W, Y106F, and Y106L mutants were compared with that of the wild-type CheY (8). The phosphorylated Y106W and Y106L mutants were both about twice as stable as phosphorylated wild-type CheY (Fig. 6), whereas the phosphorylated Y106F mutant was 1.5 times as stable. These results suggest that the mutants have a slight defect in the autophosphatase activity of the mutants. A mutant CheY (T87I) was used as a control to ensure that the assay reliably detected the defects in the autophosphatase activity in CheY. The results showed that the stability of phosphorylated T87I was the same as previously determined (8) and about six times more stable than that of wild-type CheY. To test whether dephosphorylation can be enhanced by CheZ, the mutant forms of CheY were incubated with various amounts of purified CheZ. The reactions were carried out at 4°C with a reduced Mg^{2+} concentration (0.1 mM) in order to slow the rate of dephosphorylation. All three mutants showed similar sensitivities to CheZ, and all were slightly less sensitive than wild-type CheY (data not shown). We conclude that the three mutants (Y106W, Y106F, and Y106L) have similar phosphorylation and dephosphorylation properties.

CheY-FliM binding. As a response regulator, CheY receives signals from CheA and transfers signals to the motor switch complex. Mutations in CheY that abolish binding between CheY and switch proteins could affect signal transduction and result in a loss of chemotactic ability (26, 40). To explore whether these Y106 mutants interfere with this binding, the binding affinity between the wild-type or mutant CheY and FliM was measured by a previously published method (40). FliM has been demonstrated to interact with CheY directly (39). As shown in Fig. 7, in the absence of acetyl phosphate, all three mutants were able to bind to FliM as well as wild-type CheY. The amount of FliM binding to CheY increased as the FliM concentration increased, and it eventually reached saturation. The control beads (BSA-coupled beads) showed much less binding to FliM, and the binding ability did not increase as the FliM concentration increased (data not shown). To measure the ability of the CheY-P form to bind FliM, both wild-type and mutant CheY proteins were phosphorylated with acetyl [^{32}P]phosphate. Surprisingly, all three mutant CheY's showed 10 times less phosphorylation by acetyl phosphate than wild-type CheY. However, the labeling of mutant CheY in 200 mM acetyl [^{32}P]phosphate was equivalent to the labeling of wild-type CheY in 20 mM acetyl [^{32}P]phosphate (data not shown). With the same amount of CheY-P, both the wild-type and the mutant CheY proteins exhibited a three- to fourfold increases in FliM binding (Fig. 7). This result indicates that the mutant CheY proteins have the same affinity for FliM as wild-type CheY.

DISCUSSION

An aromatic residue at position 106 is required for CheY activation. Our behavioral studies indicate that residue Y106 is critical for CheY activation. Changes at position 106 can have three different effects: (i) hyperactivity, with a tyrosine-to-tryptophan mutation (Y106W) causing increased tumble signaling but less chemotactic ability; (ii) low-level activity, with a tyrosine-to-phenylalanine change (Y106F) resulting in decreased tumble signaling and less chemotactic ability; and (iii) no activity, with substitutions such as Y106L, Y106I, Y106V, Y106G, and Y106C eliminating all chemotaxis and producing a smooth-swimming phenotype. Since both the Y106W and the Y106F CheY proteins are partially functional, and even though they create opposing signaling biases, we believe that an aromatic residue at position 106 is required for CheY activation. Indeed, this position is occupied by an aromatic residue in more than 80% of the known response regulators (36). It has also been reported that a tyrosine-to-cysteine substitu-

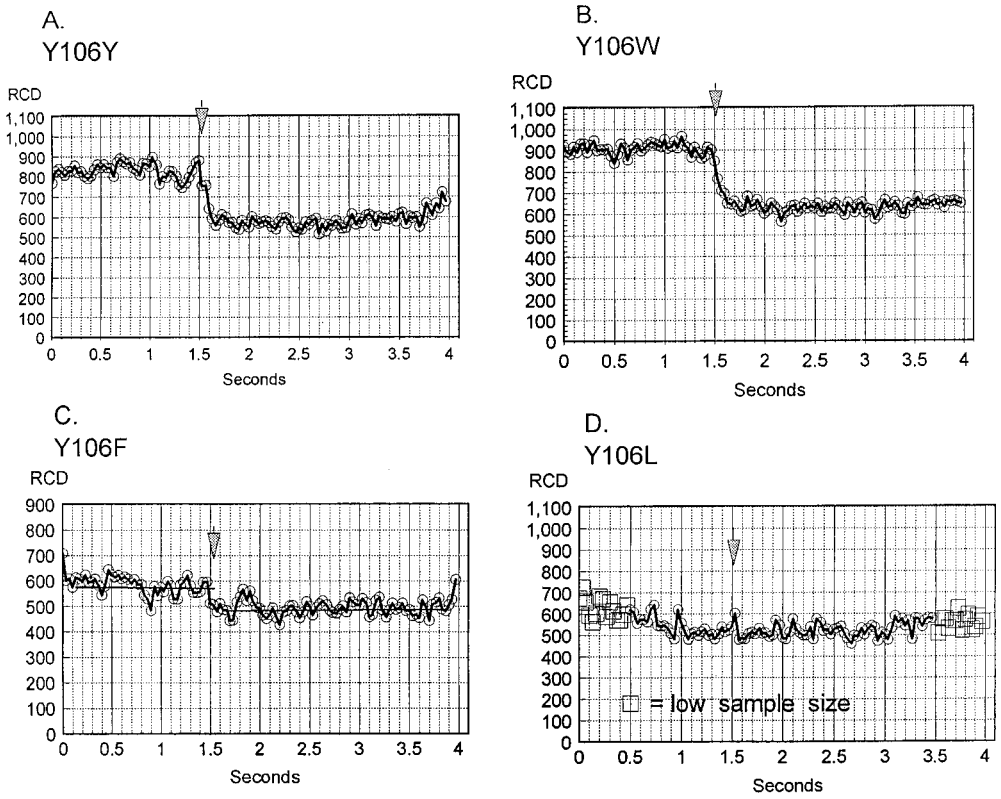

FIG. 4. Behavioral responses of the wild type and CheY106 mutants to the release of caged aspartate in flash photolysis assays. RCD was measured in degrees per second. Arrows indicate the release of aspartate at 1.5 s. *T* tests comparing prerelease (0.5 to 1.5 s in the Y106Y, Y106W, and Y106F strains and 0.75 to 1.5 s in the Y106L mutant) and postrelease (2.0 to 3.5 s in all strains) RCD levels revealed significant differences ($P < 0.0001$) between pre- and postrelease RCD levels in the Y106Y (260-U decrease), Y106W (283-U decrease), and Y106F (55-U decrease) strains. The change in the Y106L strain (6-U increase) was not statistically significant ($P = 0.4779$). Pre- and postrelease RCD levels were compared between strains by one-way analysis of variance and a multiple-comparison test (REGWQ) as described in reference 2. Analysis of variance indicated significant differences in both pre- and postrelease rates ($F_{3,123} = 690.11$ [$P < 0.0001$] and $F_{3,183} = 132.02$ [$P < 0.0001$], respectively). Multiple-comparison tests indicated that the prerelease RCD level of each strain was significantly different from that of each of the others ($P = 0.005$). The postrelease RCD levels of Y106Y and Y106W were significantly different from each other and from each of the other strains ($P = 0.005$). The postrelease levels of the Y106F and Y106L mutants were not significantly different ($P > 0.05$).

tion at position 102 of OmpR, which corresponds to Y106 in CheY, results in a constitutive activation (13).

Cell tumble frequency correlates to cell chemotactic activity. Variation in cell tumble frequency is the biological response to chemical gradients. Population RCD is the parameter directly measured by computer-assisted video analysis during flash photolysis experiments. This parameter is a derivative of and is correlated to cell tumble frequency (14). Our data also demonstrate that prestimulus (before aspartate release) population RCD correlates with tumble frequency. The tumble frequencies of each strain as measured in our steady-state assays had the same rank order as the prestimulus RCD levels measured during flash photolysis experiments. The specific rank order in both types of assays was Y106W > wild type > Y106F > Y106L = ΔCheY.

The flash photolysis assay allowed us to measure the kinetic response to attractants. We consider the response amplitude to be a measure of the "strength" of the chemotactic signal produced in response to the aspartate attractant. However, the lowest possible poststimulus RCD level is that of a completely smooth-swimming cell, and so it is possible for the response amplitude to be constrained by this lower smooth-swimming limit. Both the pre- and the poststimulus RCD levels were significantly higher in the Y106W mutant than in the wild type, but neither showed a poststimulus RCD level as low as that recorded from the completely smooth-swimming Y106L mutant. Since neither of the poststimulus levels was constrained by the smooth-swimming minimum, we can safely conclude that roughly the same amplitude signal is being transmitted by the two proteins and the significant pre- and poststimulus RCD difference reflects the tumbly offset bias of Y106W. For the Y106F mutant, the significant difference between pre- and poststimulus RCD levels indicates that the protein is able to transmit a signal. However, the poststimulus level is the same as the steady-state level of the completely smooth-swimming Y106L. Since this represents the minimum possible RCD, it is impossible to know whether the smaller amplitude relative to wild-type CheY is the result of a reduced ability to transmit a signal or of the reduced prerelease (steady-state) tumble frequency of the Y106F mutant cells.

Our behavioral studies also indicate that the tumble frequency is related to chemotactic performance. Presumably, the wild-type strain XYZ6 has an optimal tumble frequency for chemotaxis. Mutations that increase or decrease tumble frequency interfere with chemotaxis. XYZ3 (Y106W) has a slightly increased tumble frequency of the cells, which correlates with the slightly reduced chemotaxis shown by this mutant. On the other hand, the Y106F mutation in strain XYZ4 dramatically decreases the tumble frequency, which correlates with the very poor chemotaxis in this strain. XYZ5 (Y106L) is exclusively smooth-swimming, like the *cheY* deletion strain

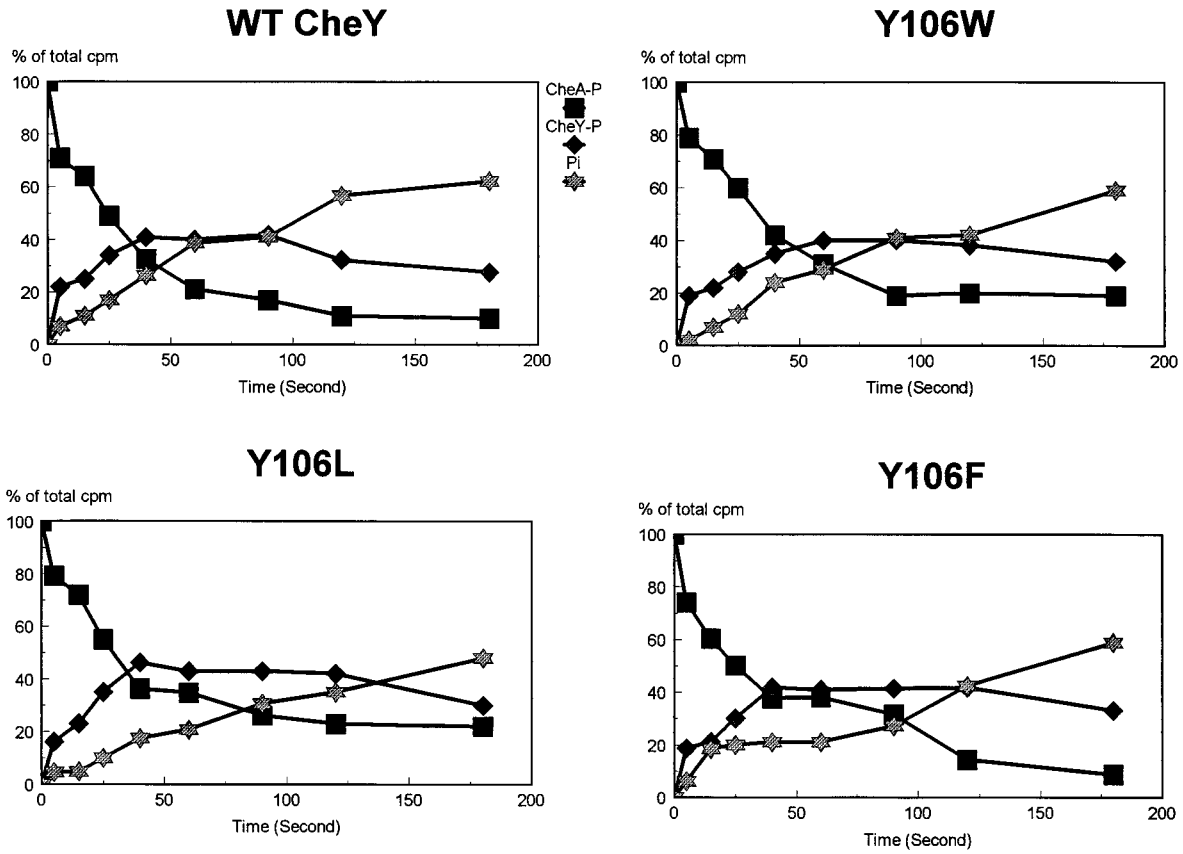

FIG. 5. Phosphotransfer reactions of the wild-type (WT) and mutant CheY proteins. The phosphotransfer reactions were performed in 100 μl with 1.5 pmol of ^{32}P-CheA and 1.5 pmol of CheY at 4°C. Ten microliters of the reaction mixture was added to 10 μl of 2× SDS loading buffer to quench the reaction. Ten-microliter samples of the reaction products were loaded for SDS–16.5% PAGE. Each labeled band was scanned and quantified. Each experiment was repeated three times, and similar results were obtained.

D345, a characteristic which correlates with the loss of chemotaxis in this strain.

Three mutants have similar phosphorylation properties but differ from wild-type CheY in their abilities to hydrolyze acetyl phosphate. Under our conditions, all three kinds of mutants were found to be phosphorylated with kinetics similar to wild-type CheY (Fig. 5). The phosphotransfer from CheA to CheY in vivo might take place in milliseconds, which is beyond the temporal resolution of our manually operated experiment. However, at low protein concentration and 4°C, the reaction was slow enough to be monitored. All of the mutant CheY-P's showed similar stabilities and sensitivities to CheZ and within twofold of those of the wild-type CheY. Surprisingly, all three mutants showed 10 times less phosphorylation by acetyl phosphate than wild-type CheY. This is the only obvious biochemical difference we can detect between wild-type CheY and these mutant proteins. The K109R and T87I mutant CheY proteins are also much less able to be phosphorylated by acetyl phosphate than is wild-type CheY (7, 41), even though both can be phosphorylated by CheA-P. It has been suggested that the transfer reaction from acetyl phosphate might involve a proton donor in CheY (35), and Lys-109 or Thr-87 may take part in this reaction. Our data demonstrate that the hydroxyl group of tyrosine 106 promotes phosphorylation by acetyl phosphate. Interestingly, these three residues are structurally related and physically interact. On one side T-87 is connected with D-57 through two solvent molecules, and on another side T-87 is hydrogen bonded to the hydroxyl group of Y-106

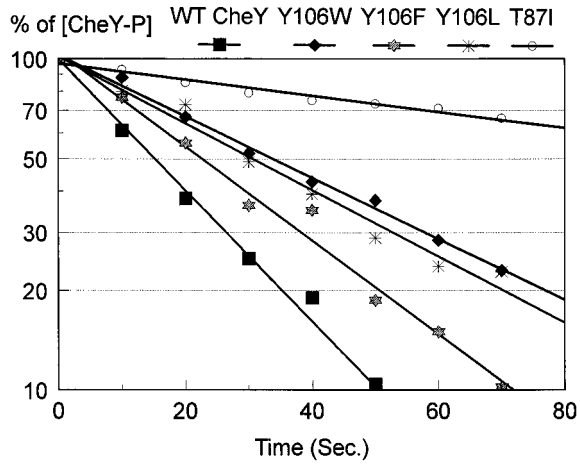

FIG. 6. Stability of phosphorylated wild-type (WT) and mutant CheY proteins. Reactions were carried out with 0.5 mM Mg^{2+} at room temperature; the intensity of each band was determined with an Ambis β-scanner, and percent CheY-P concentration was plotted versus time. The half-lives of CheY-P calculated as ln 2/slope, were as follows: wild-type CheY, 15.5 s; Y106W, 33 s; Y106F, 23 s; Y106L, 30 s; T87I, 102 s.

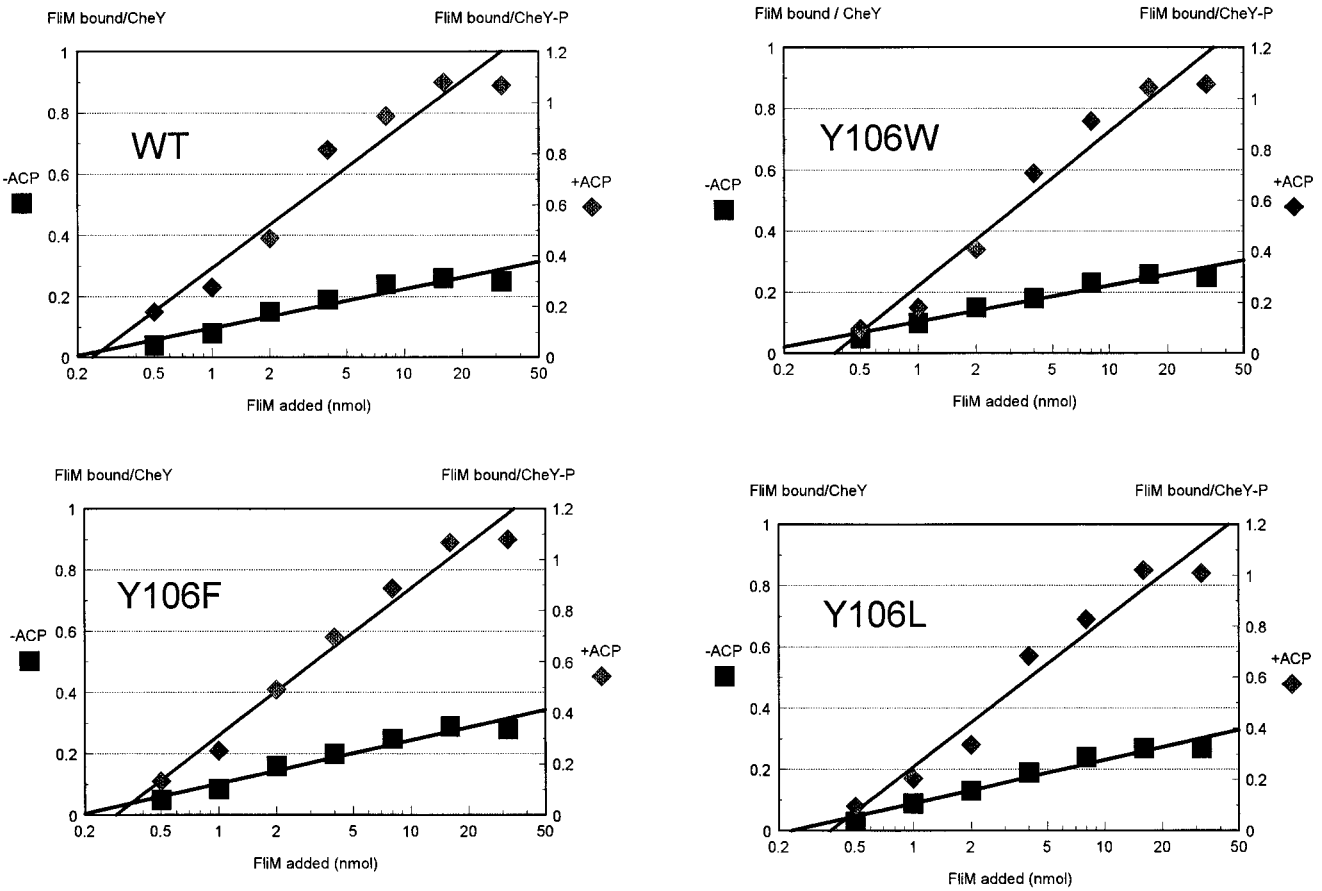

FIG. 7. Binding of FliM to wild-type (WT) or mutant CheY protein. Identical amounts of CheY beads (50 μl of CheY beads containing 8 nmol of CheY) were incubated for 30 min with increasing amounts of purified FliM (0.5 to 16 nmol, from a 150 μM FliM stock solution) in the binding buffer at room temperature. Acetyl phosphate (ACP) was added to 20 mM for wild-type CheY and 200 mM for the mutant proteins to ensure that the amounts of wild-type CheY-P and mutant CheY-P were the same (the levels of CheY-P under these conditions were determined separately by using acetyl [^{32}P]phosphate). The data show the net amount of FliM bound to the CheY beads (in moles per mole) after subtraction of the amount of FliM bound to the BSA control beads.

through one solvent molecule when the side chain of Y-106 is in its inside position. K-109 is also hydrogen bonded to D-57 in the apo- form of CheY (37). However, the significance of CheY phosphorylation by acetyl phosphate in relation to CheY activation needs further investigation.

The diverse phenotypes of the Y106 mutants are not due to variation in phosphorylation or dephosphorylation ability nor to changes in binding affinity for the switch. CheY receives the signal from CheA by phosphotransfer and then transduces the signal to the switch through CheY-FliM interaction. Since all three mutant CheY proteins studied here have similar phosphorylation and dephosphorylation abilities, the different activities of these mutants could not be explained by differences in phosphorylation or dephosphorylation. Unlike D13K, an activated mutant in which phosphorylation and activation are unlinked (5, 8), the activation of mutant Y106W is phosphorylation dependent. The Y106W mutant shows a smooth-swimming phenotype when it is expressed in a *cheA* deletion background or when a second mutation, D57C (altered for the phosphorylation site in CheY), is combined with Y106W (41).

Y106 is located in the β-5 strand of CheY, which is away from the phosphorylation site but close to the signaling surface defined by suppressor mutations (26, 31, 37). The diverse phenotypes of these mutants might result from a change in their binding affinities for the switch, but our results from CheY-FliM binding assays showed that the three mutants had similar binding affinities for FliM in both the unphosphorylated form and the phosphorylated form, which demonstrated that the different phenotypes of the mutants were not due to variations in their binding affinities for FliM. Therefore, tyrosine 106 is probably not involved in switch binding. This conclusion is supported by the observation that no suppressors of either *fliG* or *fliM* mutations map to position 106 in CheY (26, 32). A previous report (40) showed that the D13K mutant CheY did not increase its binding affinity for FliM, although it did cause cells to be more tumbly than wild-type. Y106L could be phosphorylated by CheA and bind to FliM as well as wild-type CheY, but Y106L failed to generate a tumbly signal. Therefore, CheY binding to the switch is a necessary, but not sufficient, step in generating a tumble signal.

A similar observation has been reported for Ras (30). Mutants with the Y32F, K42A, and L53A mutations in Ras retained their GTP binding abilities, caused conformational changes, and were able to bind Raf-1. However, these mutations eliminated the transforming activity of oncogenic Ras. Our data support the suggestion of Welch et al. (40) that the binding of CheY to the switch and the generation of tumble signals are separable processes. Kuo and Koshland also have indicated that the counter-CW-to-CW switching involves two or more kinetically resolvable steps (17).

CheY has a cavity that allows the Y106 side chain to rotate freely between the inside position and the outside position

(Fig. 1) (37). A preliminary measurement of this cavity suggested that it could not accommodate the bulk of a tryptophan ring. However, we have recently determined the crystal structure of the Y106W CheY mutant and found that the side chain of tryptophan unambiguously occupies the inside of the cavity, and this conformation is apparently stabilized by hydrophobic forces and assisted by local rearrangements (41). In the structure of the T87I mutant, this cavity is partially occupied by the side chain of isoleucine, which forces the side chain of Y106 to be exclusively outside (8). The fact that the Y106W mutation causes cells to be more tumbly while the T87I mutation results in smooth swimming, is consistent with this structural information (8, 41).

These observations suggest that the side chain position of Y106 may correlate with the signaling state of CheY. Such positional rearrangements of the tyrosine side chains are known to be important in other systems, such as antigen-antibody recognition (12, 22). It has been suggested that the activation of CheY might be a multistep event (5, 8). Our data also support the multistep activation model. It appears likely that tyrosine 106 is involved in the propagation of the chemotactic signal within the CheY molecule.

ACKNOWLEDGMENTS

We thank H. Wang for help in the in vitro phosphorylation experiments; A. VanMeurs and R. Keogh for providing the purified wild-type CheY and FliM proteins; and J. S. Parkinson, F. W. Dahlquist, and B. J. Bachmann for generously providing strains.

This work was supported by National Institute of Health grants GM47522 (K.V.) and AI18985 (P.M.) and a UAB Faculty Research Grant Program award (C.D.A.).

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