Activation of the Phosphosignaling Protein CheY

II. ANALYSIS OF ACTIVATED MUTANTS BY ^{19}F NMR AND PROTEIN ENGINEERING*

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The *Escherichia coli* CheY protein is activated by phosphorylation, and in turn alters flagellar rotation. To investigate the molecular mechanism of activation, an extensive collection of mutant CheY proteins was analyzed by behavioral assays, *in vitro* phosphorylation, and ^{19}F NMR chemical shift measurements. Substitution of a positively charged residue (Arg or Lys) in place of Asp[13] in the CheY activation site results in activation, even for mutants which cannot be phosphorylated. Thus phosphorylation plays an indirect role in the activation mechanism. Lys[109], a residue proposed to act as a conformational "switch" in the activation site, is required for activation of CheY by either phosphorylation or mutation.

The ^{19}F NMR chemical shift assay described in the preceding article (Drake, S. K., Bourret, R. B., Luck, L. A., Simon, M. I., and Falke, J. J. (1993) *J. Biol Chem.* 268, 13081–13088) was again used to monitor six phenylalanine positions in CheY, including one position which probed the vicinity of Lys[109]. Mutations which activate CheY were observed to perturb the Lys[109] probe, providing further evidence that Lys[109] is directly involved in the activating conformational change. Two striking contrasts were observed between activation by mutation and phosphorylation. (i) Each activating mutation generates a relatively localized perturbation in the activation site region, whereas phosphorylation triggers a global structural change. (ii) The perturbation of the Lys[109] region observed for activating mutations is not detected in the phosphorylated protein. These results are consistent with a two-step model of activated CheY docking to the flagellar switch.

The ability to detect properties of interest in the external environment and respond appropriately is a fundamental function for most cells, whether of unicellular or multicellular organisms. One universal mechanism for signal transduction is the regulation of protein phosphorylation cascades, which in some cases can be constitutively activated by specific mutations in a critical component, leading to oncogenesis (1–

4). The preceding paper (5) examined the phosphorylation-triggered activation of CheY, the terminal protein in the *Escherichia coli* chemotaxis phosphorylation cascade (reviewed in Refs. 6–9). Here we investigate activation and inactivation by specific mutations adjacent to the phosphorylation site of CheY. This study makes use of an experimentally important feature of the *E. coli* chemotaxis system: the signaling state of CheY can be assayed by its effect on motor rotation, since activated CheY is needed to switch the motor from counterclockwise to clockwise (CW)[1] rotation.

Two classes of mutant CheY proteins have been generated for the purpose of analyzing the activation mechanism. (i) *Activated mutants*, CheY mutants that mimic the phosphorylated form have been sought, in order to augment the one known mutant of this type (10). Activated mutants are particularly useful because the short half-life of phospho-CheY (11, 12) precludes direct high resolution structural analysis. (ii) *Docking site mutants*, mutations which alter residues involved in the motor docking event have been pursued, and are identified as surface muations those which do not affect CheY phosphorylation or grossly affect CheY structure, yet block the CW signal normally transmitted by phospho-CheY. Such mutations help ascertain the location of the docking surface in the known structure of CheY (13, 31).

As in the preceding study (5), ^{19}F NMR has been used to physically characterize the conformational effects of activation. ^{19}F NMR is sensitive to perturbations in the local environment of fluorine labels and can be used to map out the regions of a protein affected by an activating mutation. Briefly, 4-fluorophenylalanine (4F-Phe) reporter groups were incorporated at each of the six Phe positions, chosen due to their useful locations in the molecule. Phe[14] is adjacent to the highly conserved activation site carboxylate cluster (Fig. 1A). The cluster is composed of Asp[57], the site of phosphorylation (14), which together with Asp[12] and Asp[13] forms the binding site for the essential Mg(II) ion in the phosphorylation and dephosphorylation reactions (15, 16). Phe[111] is in van der Waals contact with the Lys[109] residue, which in the crystal structure of the apoprotein forms a salt bridge with Asp[57] (13) (Fig. 1A). Such a salt bridge could in principle act as a conformational "switch" in activation (12, 13). The other four phenylalanines (Phe[8], Phe[30], Phe[53], and Phe[124]) are on the opposite side of CheY from the activation site where they can monitor long range movements of eight of the ten secondary structural elements (Ref. 5, Fig. 1).

Activation of CheY (*i.e.* generation of CW flagellar rotation) begins with phosphorylation, then the phosphoprotein interacts with the flagellar switch. Phosphorylation of wild-

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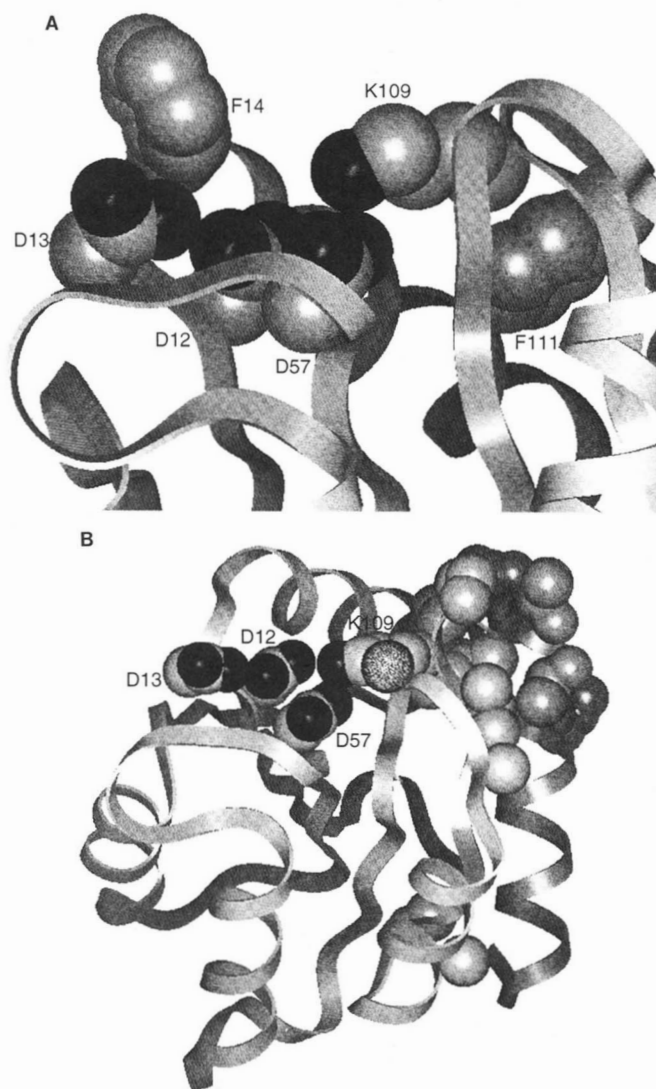‖ To whom correspondence should be addressed. Tel.: 303-492-3503; Fax: 303-492-5894.

[1] The abbreviations used are: CW, clockwise; 4F-Phe, 4-fluorophenylalanine.

FIG. 1. **Structure of the CheY activation site and location of selected residues.** *A*, the activation site includes Asp[12], Asp[13] (site of activating mutations), Asp[57] (site of phosphorylation), and Lys[109] (putative conformational switch). 4F-Phe reporter group locations for the carboxylate cluster (Phe[14]) and Lys[109] (Phe[111]) in ¹⁹F NMR experiments are also indicated. *B*, the face of CheY that binds to the flagellar switch has been proposed to include residues Leu[24], Glu[27], Ala[90], Ala[99], Ser[104], Val[108], Pro[110], Phe[111], Thr[112], Ala[113], Thr[115], and Glu[117] (unlabeled side chains) (23, 32). In addition, Ala[88] (*stippled*) and Lys[109], which are contiguous with this surface, are implicated by the present study.

type CheY triggers an allosteric conformational change extending from the activation site over much of the molecule, but has little effect on the 4F-Phe[111] probe of the Lys[109] region (5). Constitutively activated CheY mutants that function in the absence of phosphorylation offer an alternative window into the conformation of CheY that docks to the flagellar motor and induces CW rotation. The ¹⁹F NMR results described here suggest that perturbation of the Lys[109] region is in fact a key feature of CheY activation by mutation. Furthermore, mutational activation triggers structural changes which are largely confined to the vicinity of the activation site. Together the ¹⁹F NMR studies suggest a multistep model for CheY activation.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Mutant Construction—The *E. coli* strains RP437 (wild-type for chemotaxis), KO641*recA* (Δ*cheY*

Δ*recA*), and RBB382 (Δ*cheA* Δ*recA*) have been described (10, 17). RBB455 was made by transducing *aux::Tn10 #43A phe* from NK5138 (a gift from Nancy Kleckner) into HCB440 (a gift from Alan Wolfe) with P1. The complete genotype of RBB455 is Δ*tsr7021 Δ(cheA-cheY)1590::XhoI(Tn5) aux::Tn10#43A phe thr(am)1 leuB6 his4 metF(am)159 eda50 rpsL136 thi1 ara14 lacY1 mtl1 xyl5 tonA31 tsx78*. RBB455 is thus a phenylalanine auxotroph lacking all cytoplasmic chemotaxis proteins and all chemotaxis transducer proteins except Trg. RBB455 was used as the host strain to produce 4F-Phe-labeled CheY protein for ¹⁹F NMR. RBB838 was made by transducing *cheB::rpsL::Kan*ᴿ*::cheY377* from D345 (a gift from Steve Roman) (18) into RP437 with P1.

pRBB40, which expresses *cheYZ* under the control of p_trp, has been described (10). pMC100, a Kanᴿ derivative of pLC1–28 (19), was a gift from Steve Roman. The *Eco*RI-*Bst*XI fragment of pRBB40 containing p_trp *cheB'* was replaced with pMC100 fragments extending from the *Eco*RI site in *cheA* to the *Bst*XI site in *cheB* using standard techniques. The resulting plasmid, pRBB42, carries *'cheAW tar tap cheRBYZ flhB'* and was used as a donor to cross *cheY* alleles into the chromosome of RBB838 as described (18).

Single mutations in *cheY*, and double mutations at adjacent codons, were constructed by oligonucleotide-directed site-specific mutagenesis as previously described (10). Other double and triple *cheY* mutants were created by recombining appropriate restriction fragments of pRBB40 *in vitro* using standard techniques. All mutations were confirmed by DNA sequencing of the final plasmid.

Behavioral Assays—The ability of KO641*recA*/pRBB40 derivatives expressing various mutant CheY proteins to form chemotactic swarms on soft agar plates was assayed as previously described (10). The rotational behavior of tethered cells was assayed as previously described (20).

Protein Phosphorylation Assays—*E. coli* CheA and CheY proteins were purified to ≥95% homogeneity as described (21), except that the final gel filtration step was performed on a Superdex 75 FPLC column (Pharmacia LKB Biotechnology Inc.) equilibrated in TEDG buffer (5) with 0.75 M NaCl. Protein concentrations were determined using the BCA protein assay with bovine serum albumin as a standard (22).

Phosphorylation reactions, carried out as previously described (21), consisted of 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 0.1 mM [γ-³²P]ATP (4,000–8,000 cpm/pmol), 1.2 μM CheA, and 12 μM CheY in a volume of 35–40 μl. Those reactions involving CheYD13K, CheYD13R, or the four double mutant proteins including these substitutions contained 8 μM CheA and 24 μM CheY, in order to favor formation of phospho-CheY and detect any phosphotransfer between CheA and these mutant CheY proteins. All reactions were performed at room temperature and were quenched at various times between 10 s and 2 h by adding 2 volumes of 2 × SDS-polyacrylamide gel electrophoresis sample buffer containing 25 mM EDTA. Samples were then analyzed by SDS-polyacrylamide gel electrophoresis and the level of phospho-CheY was determined by phosphorimaging (Molecular Dynamics).

¹⁹F NMR Procedures—CheY protein samples were prepared and ¹⁹F NMR spectra obtained as described in the preceding paper (5).

RESULTS

Genetic and Behavioral Characterization of Mutations Which Activate CheY—Replacement of Asp[13] with Lys results in activation of *E. coli* CheY, even in the absence of the CheA kinase (10). To further explore the nature of the activation by the *cheYD13K* mutation, we constructed a series of additional amino acid substitutions at position 13. Among 15 amino acids tested to date, only Arg and Lys caused activation, whereas other large side chains such as Gln, Met, or Tyr did not (10, 14) (Table I). These results strongly suggest that a positive charge at position 13 is critical for CheY activation.

A series of substitutions at positions 57 and 109 were next constructed to determine whether these activation site positions could generate additional activating mutations. No substitutions among a total of eight tested at position 57 were sufficient for activation, and each abolished chemotaxis (10, 14) (Table I). It follows that Asp[57] is critical for CheY function, consistent with its role as the site of phosphorylation (14). Similarly, all 14 substitutions tested at position 109 abolished activity (12) (Table I), strongly suggesting that

TABLE I

Amino acid substitutions in the CheY activation site

Each of the 37 different amino acid substitutions tested at CheY positions 13, 57, or 109 abolish chemotaxis.

Wild-type amino acid	Species[a]	Mutant amino acid(s)	Ref.
Activated mutants[b]			
Asp[13]	E. coli	Lys	10
		Arg	This work
Inactivated mutants[c]			
Asp[13]	E. coli	Asn, Glu	10
		Ala, Cys, Gln, Gly, His, Leu, Phe, Tyr	This work
	S. typhimurium	Asn, Met, Ser, Thr	14
Asp[57]	E. coli	Asn, Glu, Lys	10
		Ala, Gln, Leu, Ser	This work
	S. typhimurium	Asn, Tyr	14
Lys[109]	E. coli	Arg, Cys, Gln, Glu, Gly, His, Leu, Met, Phe, Pro, Ser, Thr, Tyr, Val	This work
	S. typhimurium	Arg	12

[a] The *E. coli* and *S. typhimurium* CheY proteins differ by only 3 out of 128 amino acids (19, 26) and are functionally interchangeable (36).

[b] Activated mutant CheY proteins cause extensive CW flagellar rotation when expressed from pRBB40 in either the Δ*cheY* strain KO641*recA* or the Δ*cheA* strain RBB382.

[c] Expression of inactivated mutant *E. coli* CheY proteins from pRBB40 in the Δ*cheY* strain KO641 *recA* resulted in exclusively CCW flagellar rotation, except for the D13Q substitution, which retained some CheA-dependent CW-generating activity. The related D13N substitution similarly retained slight activity in *S. typhimurium* (14). Expression of inactivated mutant *S. typhimurium* CheY proteins resulted in exclusively smooth swimming behavior (12, 14); thus these substitutions presumably also fail to support CW flagellar rotation.

Lys[109] is essential for wild-type CheY function.

Thus, the known CheY activating mutations each introduce a positive charge at position 13. A key question regarding these mutants is whether phosphorylation is required for their activity. The observation that the *cheYD13K* and *cheYD13R* mutations cause tumbling behavior in a Δ*cheA* host suggests that phosphorylation of CheY may not be necessary for CW flagellar rotation. However, CheA can phosphorylate the CheYD13K and CheYD13R proteins *in vitro*, although at a dramatically reduced rate in comparison to wild-type CheY (Ref. 23; see also Table III below). Furthermore, it is conceivable that a kinase related to CheA, or a small molecule phosphodonor, could phosphorylate these mutant CheY proteins *in vivo* (9, 24). To determine if phosphorylation is necessary for CheYD13K or CheYD13R function, we constructed a series of double mutants containing an activating mutation at position 13 and a second mutation at position 57, the site of phosphorylation (Table II). The CheYD13K and CheYD13R mutants retained activity even upon replacement of Asp[57] with either Ala, which cannot be phosphorylated, or Glu, which is phosphorylated very slowly (10) (Tables II and III). The CheYD13K D57E, CheYD13K D57A, CheYD13R D57E, and CheYD13R D57A proteins were not detectably phosphorylated by CheA *in vitro* (Table III), and mutation of Asp[57] has previously been shown to eliminate phosphorylation by small molecule phosphodonors (5, 24), ruling out the possibility of phosphorylation occurring at a site other than Asp[57]. Thus CheY phosphorylation is not essential for CW flagellar rotation, and the phosphoryl group must play an indirect role in the activation process. It follows that phosphorylation is likely to activate wild-type CheY by a conformational change; a change in multimeric state was ruled out previously (10, 23).

It is worth noting that although Ala or Glu substitutions at position 57 were functionally tolerated in the activated mutants, Asn or Gln substitutions were not (Table II). Furthermore, the Ala substitution at position 57 slightly reduced CW flagellar rotation caused by the *cheYD13K* mutation and actually enhanced activity of the *cheYD13R* mutation (data not shown). These observations suggest that the precise struc-

TABLE II

Dependence of CheY activated mutants on Asp[57] and Lys[109]

cheY Mutant	Bias in Δ*cheA* Host[a]
Wild-type	CCW
Activated Asp[13] single mutants	
D13K	CW
D13R	CW
Asp[13]/Asp[57] double mutants	
D13K D57A	CW
D13K D57E	CW
D13K D57N	Mostly CCW
D13K D57Q	CCW
D13R D57A	CW
D13R D57E	CW
D13R D57N	CCW
D13R D57Q	CCW
Asp[13]/Lys[109] double mutants	
D13K K109E	CCW
D13K K109L	CCW
D13K K109Q	CCW
D13K K109R	CCW
D13R K109E	CCW
D13R K109L	CCW
D13R K109Q	CCW
D13R K109R	CCW

[a] Rotational behavior of tethered RBB382/pRBB40 derivatives carrying the various *cheY* mutations. "CCW" denotes exclusively counterclockwise flagellar rotation unless otherwise noted, whereas "CW" denotes varying degrees of predominantly clockwise flagellar rotation. Note that each of the single CheY mutants at positions 57 and 109 (D57A, D57E, D57N, D57Q, K109E, K109L, K109Q, K109R) are CCW by themselves.

ture of the activation site region is important for CheY activation.

The behavioral assays described so far were performed using cells that expressed the mutant CheY proteins from a multicopy plasmid. To determine if the activated mutant proteins retained function when expressed at single copy levels, the *cheYD13K*, *cheYD13K D57A*, *cheYD13R*, and *cheYD13R D57A* alleles were crossed into the chromosome of an otherwise wild-type host. Each mutant retained the ability to generate CW rotation, although it was significantly less pro-

TABLE III

Characterization of selected CheY mutants

CheY mutant	Swarm[a]	Bias in ΔcheA host[b]	Phe[111] shift	In vitro phosphorylation[c]
			ppm[d]	
Wild-type	Che+	CCW	0	++
Salt bridge mutants (see Fig. 2)				
D57N	Che−	CCW	−0.3	+
D57Q	Che−	CCW	−0.3	0
D57A	Che−	CCW	−0.5	0
K109Q	Che−	CCW	−0.6	++
K109R	Che−	CCW	−0.9	++
Activation site mutants (see Fig. 3)				
D13N	Che−	CCW	−0.1	++
F14Y	Che+	CCW	0	ND[e]
N59Q	Che+, Sm	CCW	0	++
A88S	Che−, Lg	CCW	−0.1	++
D57E	Che−	CCW	−0.1	+
Activated mutants (see Fig. 5)				
D13K	Che−, Lg	CW	−0.3	+
D13K D57E	Che−, Lg	CW	−0.4	0
D13K D57A	Che−, Lg	CW	−0.7	0
D13R	Che−, Lg	CW	−0.4	+
D13R D57E	Che−, Lg	CW	−0.5	0
D13R D57A	Che−, Lg	CW	−0.9	0
Phe cluster mutants (see Fig 6)				
K4R	Che+, Sm	CCW	−0.1	ND
L6C	Che+	CCW	0	ND
L84I	Che+	CCW	−0.1	ND
N121L	Che+	CCW	0	ND
A113P	Che−	Reversing	−0.1	++

[a] Swarms of KO641 *recA*/pRBB40 derivatives carrying the various mutations are classified according to their rate of growth in comparison to wild-type: ≥50% of wild-type = Che+; 25–50% of wild-type = Che+, Sm; 10–25% of wild type = Che−, Lg; ≤10% of wild-type = Che−. In addition, rings are observed on swarms in the two "Che+" classifications, but not on the "Che−" swarms.

[b] Rotational behavior of tethered RBB382/pRBB40 derivatives carrying the various *cheY* mutations. "CCW" denotes exclusively counter-clockwise flagellar rotation, whereas "CW" denotes varying degrees of predominantly clockwise flagellar rotation.

[c] *In vitro* phosphorylation of CheY by phosphotransfer from CheA. Relative amount of phospho-CheY observed after a 30-min reaction: 25–100% of wild-type = ++; 1–25% of wild-type = +, not detectably phosphorylated = 0.

[d] The error in determining resonance frequency inherent in the ^{19}F NMR technique is ~0.1 ppm; thus only frequency shifts larger than this are significant (5).

[e] ND, not determined.

nounced than in the multicopy case (data not shown). Wild-type phospho-CheY (in a ΔcheZ host) was more efficient at generating CW rotation than any of the four mutants tested.

To examine the role of Lys[109] in the activated *cheY* mutants, double mutants containing Arg or Lys activating substitutions at position 13 and Arg (basic), Gln (polar), Glu (acidic), or Leu (hydrophobic) substitutions at position 109 were constructed. All eight combinations completely abolished CW rotation in a ΔcheA strain (Table II). This indicates that Lys[109] is essential for the CW signaling activity of the activated mutants, just as it is for wild-type phospho-CheY.

Mutations Suggested by Other Response Regulator Proteins—The chemotaxis signal transduction network belongs to a diverse family of bacterial two-component regulatory systems defined by amino acid sequence similarity (8, 25–27). The residues corresponding to Asp[13], Asp[57], and Lys[109] of CheY are almost universally conserved among the response regulator proteins (9, 25). Two exceptions are *Caulobacter crescentus* FlbD and *Myxococcus xanthus* FrzG, which carry Lys at position 13 and Arg or Leu at position 109 (28, 29). The unusual activation site residues found in FlbD or FrzG might provide insight into the mechanism of CheY activation and thus were explored further. The combinations of Lys at position 13 with Arg or Leu at position 109, which are presumably active in FlbD and FrzG, were not functional in CheY (Table II). However, FlbD and FrzG also contain Gly at position 12, rather than a very highly conserved acidic (Asp or Glu) residue (25, 28, 29). The analysis was therefore ex-

tended to examine the activity of *cheYD12G*, *cheYD12G D13K*, *cheYD12G D13K K109L*, and *cheYD12G D13K K109R* mutations. All four mutations eliminated CW rotation in KO641*recA*/pRBB40 (data not shown). It follows that the Gly[12] substitution does not compensate for the absence of Lys[109].

^{19}F NMR Analysis of the Activated Conformation: An Assay for Perturbations of Lys[109]—The effect of activation on Lys[109] is of particular interest due to its putative function as a conformational switch (12, 13). Thus, protein engineering was employed to determine the effect of perturbations in the Lys[109] region on the ^{19}F NMR resonances of 4F-Phe-labeled CheY. ^{19}F NMR spectra were obtained for mutant CheY proteins bearing substitutions at positions 57 or 109 that would be expected to prevent formation of the Asp[57]-Lys[109] salt bridge observed in the crystal structure of apo-CheY (13) (CheYD57N, CheYD57Q, CheYD57A, CheYK109Q). A key focus was the effect of such substitutions on the chemical shift of the 4F-Phe[111] resonance, since this residue is in van der Waals contact with the Lys[109] side chain and is located in the same β5-α5 loop as Lys[109]. An upfield frequency shift (0.3–0.6 ppm) of the 4F-Phe[111] resonance was observed for each of the indicated electrostatic substitutions (Fig. 2); these shifts are significantly larger than the 0.1 ppm measurement error (see "Materials and Methods" of Ref. 5). A complementary test used a steric substitution at the 109 position (K109R) which, due to the critical dependence of salt bridges on favorable steric interactions (30), might also prevent salt bridge for-
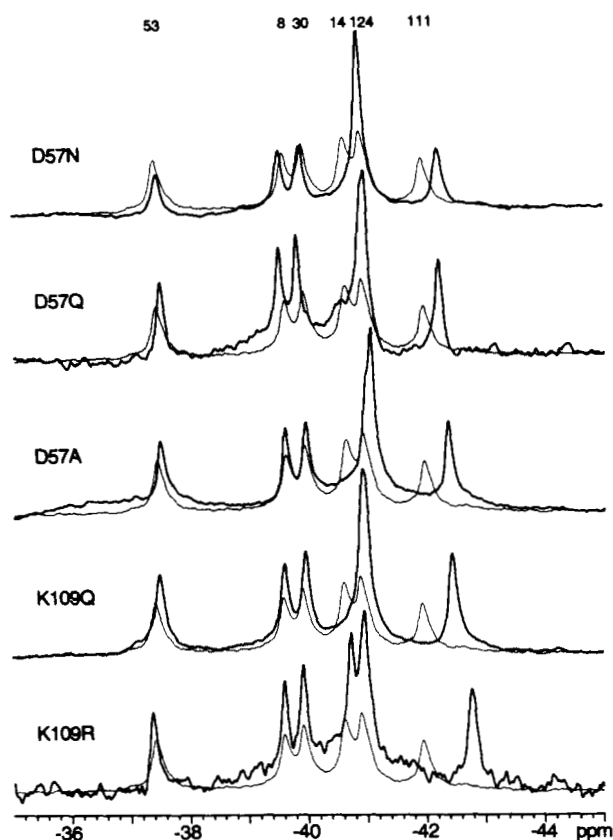
FIG. 2. **Effect of mutations that perturb the Asp[57]-Lys[109] interaction on the ¹⁹F NMR spectra of 4F-Phe-labeled CheY.** Each spectral pair consists of the spectrum from wild-type CheY (*fine line*) overlaid on the spectrum from the indicated mutant (*bold line*). Sample parameters were: 2 mM CheY, 2 mM MgCl₂, 50 mM KCl, 50 mM NaCl, 50 mM Tris-HCl, pH 7.0, 10% D₂O, 50 μM 5-fluorotryptophan as internal frequency standard, 25 °C. Assignments are from the preceding paper (Ref. 5, Fig. 3).

FIG. 3. **Effect of control activation site mutations on the ¹⁹F NMR spectra of 4F-Phe-labeled CheY.** Each spectral pair consists of the spectrum from wild-type CheY (*fine line*) overlaid on the spectrum from the indicated mutant (*bold line*). Sample parameters were as described in the legend to Fig. 2.

mation. A large upfield frequency shift (0.9 ppm) was observed for this mutant (Fig. 2, Table III), even larger than the shift observed for the electrostatic substitutions. In contrast, alteration of other activation site residues that are not expected to be directly coupled to the Lys[109] region (CheYD13N, CheYF14Y, CheYN59Q, and CheYA88S) did not perturb the 4F-Phe[111] resonance (Fig. 3, Table III). Therefore, the chemical shift of the 4F-Phe[111] resonance can be utilized as a specific spectroscopic signature to monitor perturbations of the Lys[109] region in the activated CheY mutants.

Assignment of ¹⁹F NMR Resonances in Activated CheY Mutants—In order to interpret ¹⁹F NMR spectra of the various activated mutant CheY proteins, it is necessary to eliminate ambiguities in the resonance assignments. The six 4F-Phe resonances in the ¹⁹F NMR spectrum of wild-type CheY were assigned as described in the preceding paper (5). Most resonances in mutant CheY proteins can be straightforwardly assigned by comparison with the wild-type resonances. However, it was important to directly assign the 4F-Phe[14] resonance, since the activated mutants all contain an altered residue at the adjacent position 13, which could yield dramatic frequency shifts. The 4F-Phe[14] residue provides the most solvent exposed probe side chain in CheY (5, 13, 31), such that the 4F-Phe[14] resonance is significantly broadened by collision with the water-soluble paramagnetic probe Gd(III)·EDTA, via a magnetic dipole-dipole interaction between the ¹⁹F nucleus and the Gd(III) unpaired electrons (S = 7/2) (5). Spectra of each of the six activated mutant CheY proteins
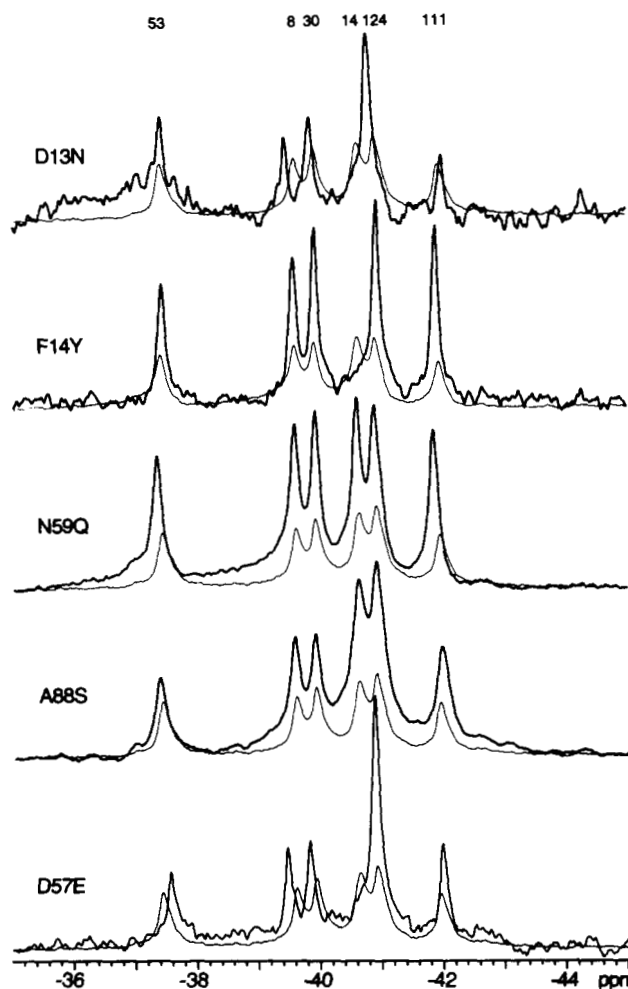
were thus obtained in the presence and absence of Gd(III)·EDTA, enabling resolution of the overlapping 4F-Phe[14] and 4F-Phe[124] resonances. The most broadened 4F-Phe[14] resonance was found to be upfield of the less broadened 4F-Phe[124] resonance in the CheYD13K mutant, rather than downfield as in wild-type CheY (Fig. 4). The relative positions of the 4F-Phe[14] and 4F-Phe[124] resonances were similarly reversed in the CheYD13R and CheYD13R D57E spectra (Fig. 5).

Effect of cheY-activating Mutations on the Asp[57]-Lys[109] Salt Bridge—The ¹⁹F NMR spectra of each of the six available activated CheY mutants are displayed in Fig. 5. As expected, the position of the 4F-Phe[14] resonance, adjacent to the activating mutation at position 13, was in each case shifted relative to its position in the spectrum of wild-type CheY. However, the most striking difference between the spectra of the mutant and wild-type proteins was that the 4F-Phe[111] resonance exhibited large upfield frequency shifts of 0.3–0.9 ppm in each of the activated mutants (Table III). These were similar to the shifts observed for 4F-Phe[111] when Lys[109] was perturbed by disrupting the Asp[57]-Lys[109] interaction (Fig. 2). Such results strongly suggest that the activating mutations generate a conformational change in the vicinity of Lys[109]. Moreover, the activated mutants CheYD13K D57A and CheYD13R D57A provide further evidence that the electrostatic interaction between the 57 and 109 positions is not required in the activated state, since these proteins are able
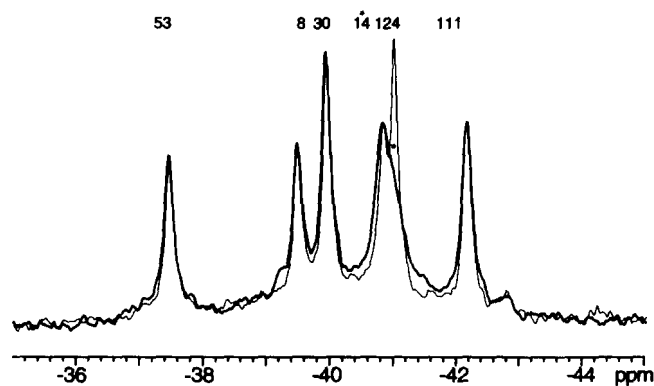
FIG. 4. **Use of Gd(III)·EDTA to assign the 4F-Phe[14] ¹⁹F NMR resonance from 4F-Phe-labeled CheYD13K.** Spectra taken in the absence (*fine line*) or presence (*bold line*) of the aqueous paramagnetic probe Gd(III)·EDTA are displayed. Sample parameters were as described in the legend to Fig. 2, except MgCl₂ was omitted, and additional divalent-free KCl was substituted for the NaCl.
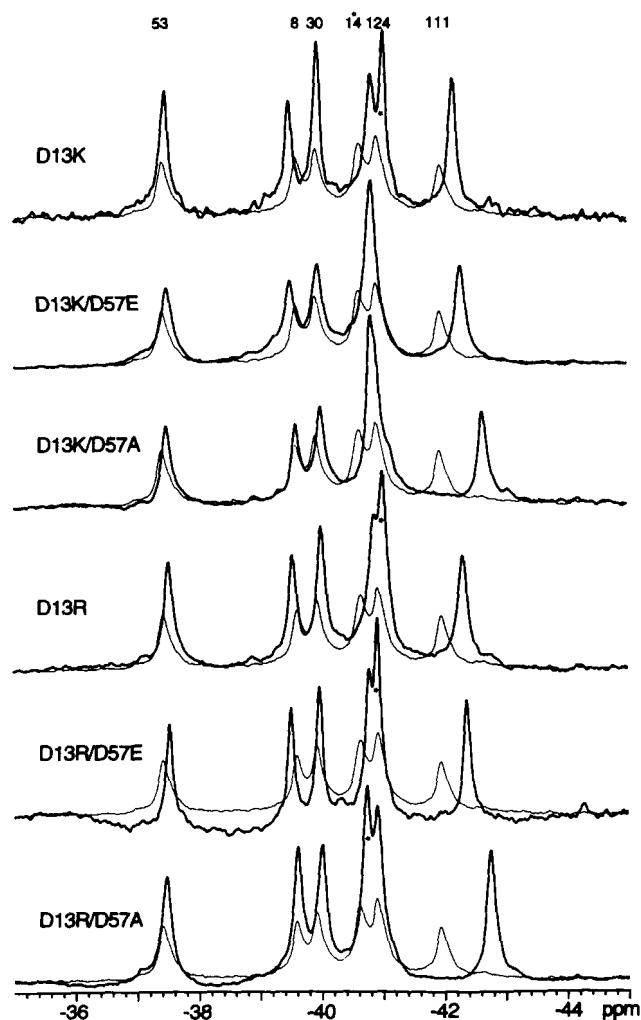

FIG. 5. **Effect of activating mutations on the ¹⁹F NMR spectra of 4F-Phe-labeled CheY.** Each spectral pair consists of the spectrum from wild-type CheY (*fine line*) overlaid with the spectrum from the indicated mutant (*bold line*). Assignment of the 4F-Phe14 resonance, determined with Gd(III)·EDTA, is indicated by the *asterisk*. Sample parameters were as described in the legend to Fig. 2.

to regulate the motor without any possibility of a Lys[109] salt bridge to the 57 position (Table III). Together these results are consistent with the proposal that the electrostatic interaction between the 57 and 109 positions is weakened or broken in the activated state.

Long Range Conformational Effects in CheY Mutants—In contrast to the 4F-Phe[14] and 4F-Phe[111] resonances, the 4F-Phe[8], 4F-Phe[30], 4F-Phe[53], and 4F-Phe[124] resonances in the spectra of the activated mutants exhibited small or undetectable frequency shifts (Fig. 5). In general, the shifts were in the same direction (upfield for 4F-Phe[30] and 4F-Phe[53]; downfield for 4F-Phe[8] and 4F-Phe[124]) as observed for phospho-CheY, but smaller in magnitude (5). However, the failure to consistently observe these resonance shifts in all six mutants suggests the structural changes that cause them are not essential for activation. For example, the 4F-Phe[8] resonance did not shift in CheYD13K D57A and CheYD13R D57A, but shifted downfield in the other four mutants.

The long range conformational change triggered by phosphorylation (5) indicates that it is possible to couple structural changes in the activation site of CheY to changes on the opposite side of the molecule. However, the mechanism by which phosphorylation affects the global structure of CheY appears to be highly specific, since structural changes are not easily transmitted within the molecule. Specifically, the resonances of the Phe cluster were not significantly perturbed by 10 of 16 mutations in the activation site (Figs. 2, 3, and 5). Similarly, amino acid substitutions in the van der Waals environment of the Phe cluster (CheYK4R, CheYL6C, CheYL84I, CheYN121L) resulted in large frequency shifts for the resonances from the cluster, but had little effect on the activation site probes 4F-Phe[14] and 4F-Phe[111] (Fig. 6).

Ala[88] May Interact with the Flagellar Switch—Identification of the CheY docking surface that interacts with the flagellar switch is necessary for a complete understanding of the mechanism of activation. Progress has been made in this regard from analysis of second site suppressor mutations that restore interaction between CheY and the switch proteins (23, 32) (Fig. 1B). A complementary approach is to identify residues whose alteration specifically reduces or abolishes the CheY/switch interaction. Ala[88] appears to have these properties. The *cheYA88S* mutation has a minimal effect on CheY structure, but a large effect on signaling function. The ¹⁹F NMR spectrum of the CheYA88S mutant is indistinguishable from that of wild-type CheY (Fig. 3), and spectra of the two proteins obtained during phosphorylation reactions were also the same (data not shown). However, the CheYA88S mutant has a reduced ability to generate CW flagellar rotation. Virtually all rotating tethered KO641recA/pRBB40 cells expressing wild-type CheY exhibited reversing flagellar rotation with no strong rotational bias (data not shown). In contrast, half of the rotating tethered cells expressing CheYA88S exhibited exclusively counterclockwise rotation. These observations are consistent with the proposal that Ala[88] interacts with the flagellar switch.

DISCUSSION

Structural Features of CheY Activation

We have undertaken a combined physical, genetic, and biochemical investigation of the CheY activation mechanism. The following key conclusions are suggested by the results described here and in the accompanying paper (5).

The Inactive Conformation—(a) Mg(II) binding to the activation site of CheY does not perturb the Lys[109] residue proposed to be involved in the activation switch (Ref. 5, Fig.
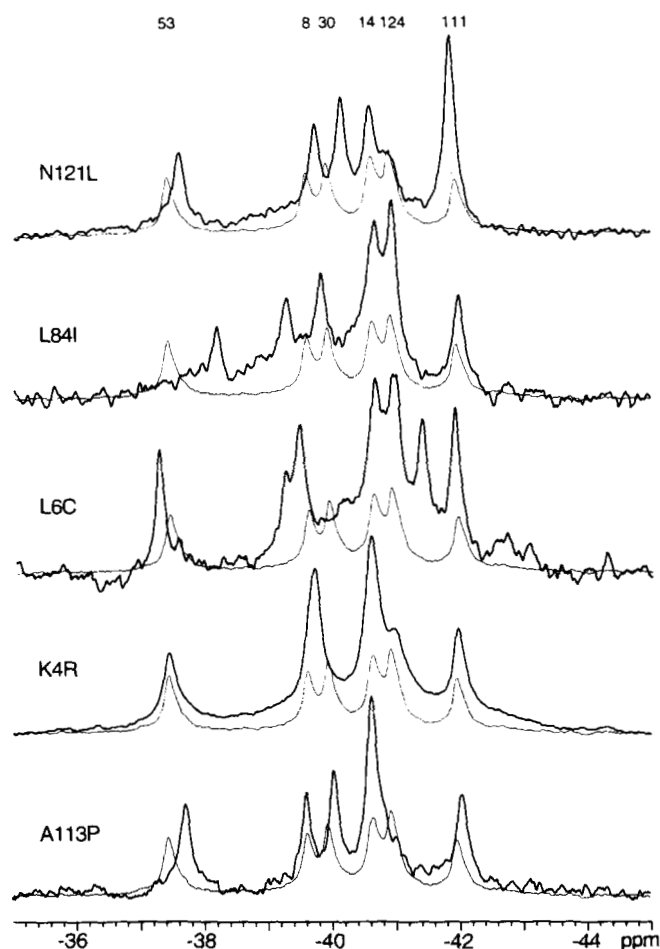
FIG. 6. **Effect of mutations perturbing the Phe cluster on the ^{19}F NMR spectra of 4F-Phe-labeled CheY.** Each spectral pair consists of the spectrum from wild-type CheY (*fine line*) overlaid with the spectrum from the indicated mutant (*bold line*). Sample parameters were as described in the legend to Fig. 2.

5). (b) *Larger divalent cations* (Ca(II), Sr(II), Ba(II)) *do perturb* Lys^{109} (Ref. 5, Fig. 5). (c) *Divalent cation binding generates a conformational change localized predominantly in the activation site region* (Ref. 5, Fig. 5).

The Phosphorylated Conformation—(a) Phosphorylation of Asp^{57} does not perturb Lys^{109} (Ref. 5, Fig. 8). (b) Phosphorylation triggers an allosteric conformational change detected up to 20 Å from Asp^{57} (Ref. 5, Fig. 8).

The Mutation-activated Conformation—(a) Activation of CheY can occur in the absence of phosphorylation (Tables II and III). (b) Positively charged Arg or Lys side chains at position 13 activate CheY (Table I). (c) The Lys^{109} region is significantly perturbed in the activated mutant CheY proteins (Fig. 5). (d) Activation of CheY by mutation results in structural changes that appear to be primarily localized to the activation site (Fig. 5).

Docking to the Motor—(a) Activating mutations give rise to a different conformation than phosphorylation, indicating that one or both conformations must change upon docking to the motor (Fig. 5; and Ref. 5, Fig. 8). (b) Lys^{109} is essential for activation of CheY either by phosphorylation or mutation (Tables I and II). (c) An electrostatic interaction between Lys^{109} and the 57 position is unnecessary for docking, since the CheYD13K D57A and CheYD13R D57A mutants can signal (Table II). However, breaking the Asp^{57}-Lys^{109} salt bridge is not sufficient for activation (Table III). (d) Ala^{88}

appears to lie on the motor docking surface (Figs. 1B and 3; Table III).

Together these observations may be synthesized into a multistep model of CheY activation in which phosphorylation and the activating mutations have similar structural consequences when docked to the motor, but not in solution.

Two-step Working Model of CheY Activation

The following working model of CheY activation is the simplest model consistent with all available information (Fig. 7). In the absence of a divalent metal ion, there is a salt bridge between Asp^{57} and Lys^{109} (13).

In the *initial inactive state*, Asp^{57} coordinates Mg(II) bound in the activation site (16). The lack of a 4F-Phe^{111} frequency shift upon Mg(II) binding (Ref. 5, Fig. 5) indicates that the position of Lys^{109} is not significantly perturbed. Thus, either the Asp^{57} carboxylate group bridges the Lys and Mg(II) charges, as has been observed in other phospho-chemistry enzymes (33–35), or the electrostatic interaction between Asp^{57} and Lys^{109} is broken, but Lys^{109} does not relocate.

The observation that diverse substitutions at the 57 and 109 positions cause similar 4F-Phe^{111} frequency shifts in the inactive state (Fig. 2) can be explained in two ways. The simplest hypothesis is that the salt bridge between Asp^{57} and Lys^{109} in the apo-site remains intact when Mg(II) binds, but is broken by specific mutations at these positions, thereby yielding similar perturbations of the 4F-Phe^{111} probe. Alternatively, if the Asp^{57}-Lys^{109} salt bridge is already broken in the Mg(II)-occupied conformer, one must propose that the indicated substitutions generate structural perturbations which fortuitously yield similar 4F-Phe^{111} frequency shifts. Additional studies are needed to characterize the status of the salt bridge in the solution structure of CheY.

In the *first activation step*, Asp^{57} is phosphorylated. The Lys^{109} side chain repositions only slightly (Fig. 7A), thereby explaining the lack of perturbation of the 4F-Phe^{111} resonance. In this conformation Lys^{109} might form a salt bridge with the phosphoryl group. An analogous lack of perturbation is observed for this resonance in the D57E mutant (Fig. 3) which, like phosphorylation, is proposed to move the negative charge of the 57 position to a more distal location on its side chain. Phosphorylation of Asp^{57} causes a long range conformational change in CheY (5); however, this global change does not occur in the activated CheY mutants (Figs. 5 and 7B), and thus must be of secondary importance for generating CW flagellar rotation.

The *second activation step* occurs when phospho-CheY docks to the flagellar switch complex. A Mg(II) ion is proposed to bind to the phosphoryl group and thus break any remaining Lys^{109}-phosphoryl group electrostatic interaction (Fig. 7A). It is unclear whether the original Mg(II) ion is repositioned or a second Mg(II) ion enters the activation site. The essential Lys^{109} side chain and Mg(II) ion are proposed to interact directly with the flagellar switch to cause CW flagellar rotation. This second activation step has not been observed experimentally, but is postulated in order to account for the required (i) Lys^{13} or Arg^{13} and (ii) Lys^{109} positive charges in the activated mutants.

This model can account for the phenotypes of numerous *cheY* mutations. The positively charged Arg or Lys side chain at position 13 in the activated mutants is proposed to mimic the Mg(II) that interacts with the switch in phospho-CheY (Fig. 7B). The presence of the basic residue at position 13 is also proposed to disrupt the Asp^{57}-Lys^{109} electrostatic interaction, allowing Lys^{109} to bind to the switch. Evidence consistent with this proposed disruption of the Asp^{57}-Lys^{109} in-
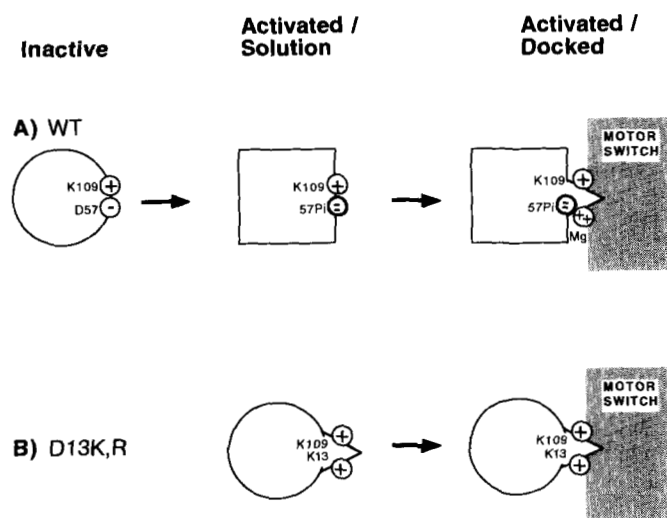
FIG. 7. **Two-step working model of CheY activation.** The indicated schematic model accounts for observations described in this and the preceding paper (5). See text for detailed discussion. A, activation by phosphorylation. Shown are the Asp[57] and Lys[109] side chain charges in the activation site of wild-type CheY. Upon phosphorylation of Asp[57], the Lys[109] side chain retains its original conformation, perhaps forming a salt bridge with the phosphoryl group. When phospho-CheY docks to the flagellar switch, a Mg(II) ion binds to the phosphoryl group and breaks any remaining electrostatic interaction between Lys[109] and the acylphosphate. The Mg(II) and repositioned Lys[109] interact with the switch to cause CW flagellar rotation. B, activation by mutation. A positively charged Lys or Arg side chain at position 13 mimics the position of the surface Mg(II) in phosphorylated wild-type CheY. This positive charge also disrupts the Asp[57]-Lys[109] electrostatic interaction, together yielding partial activation.

teraction is provided by the *cheYD13K D57A* and *cheYD13R D57A* mutations, which prevent salt bridge formation yet retain signaling (Table II). Finally, although CheYK109R is stably phosphorylated (12), it is proposed to be incapable of generating CW flagellar rotation because the Arg[109] side chain cannot interact properly with the switch (Fig. 7B). All mutants lacking Lys[109] would similarly be defective (Table I).

Interaction of CheY and the Flagellar Switch

A face of the CheY molecule that interacts with the flagellar switch has been tentatively identified, based on the location of *cheY* mutations that suppress mutations in genes encoding switch proteins (23, 32), as illustrated in Fig. 1B. We have made three further observations consistent with the proposal that this region is responsible for interaction of CheY with the flagellar switch. First, the region includes Lys[109], which is essential for generation of CW flagellar rotation by either phospho-CheY or the activated mutant CheY proteins (Tables I and II). Second, the putative switch interaction region is adjacent to the phosphorylation site, and activation of CheY

can occur in the absence of a long range conformational change (Fig. 5). Third, evidence has been presented that Ala[88], which is located on the border of the proposed docking region, could directly interact with the switch (Fig. 1B).

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