Genetic analysis of response regulator activation in bacterial chemotaxis suggests an intermolecular mechanism

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Abstract

Response regulator proteins of two-component systems are usually activated by phosphorylation. The phosphorylated response regulator protein CheY~P mediates the chemotaxis response in *Escherichia coli*. We performed random mutagenesis and selected CheY mutants that are constitutively active in the absence of phosphorylation. Although a single amino acid substitution can lead to constitutive activation, no single DNA base change can effect such a transition. Numerous different sets of mutations that activate in synergy were selected in several different combinations. These mutations were all located on the side of CheY defined by $\alpha 4$, $\beta 5$, $\alpha 5$, and $\alpha 1$. Our findings argue against the two-state hypothesis for response regulator activation. We propose an alternative intermolecular mechanism that involves a dynamic interplay between response regulators and their effector targets.

Keywords: CheY; *Escherichia coli*; random mutagenesis; two-component systems; chemotaxis

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Two-component signal transduction systems mediate adaptive responses to changing environmental conditions in microorganisms and plants (for a recent review, see Stock et al. 2000). The prototype system consists of two conserved components, a histidine protein kinase and a response regulator. Extracellular stimuli generally regulate the autophosphorylation of a specific conserved histidine residue in the histidine protein kinase. The phosphoryl group is then transferred to an invariant aspartate residue in a cognate response regulator, which is thereby activated to produce a response. The best-characterized two-component signal transduction mechanism is in the network of interacting proteins that mediates chemotaxis responses in *Escherichia coli* (Falke et al. 1997; Bren and Eisenbach 2000). In this system, attractant or repellent chemicals are sensed by transmembrane chemoreceptors that control the activity of the histidine pro-

tein kinase CheA. CheA autophosphorylation is the rate-limiting step in the transfer of phosphoryl groups from ATP to the chemotaxis response regulator CheY. Phosphorylated CheY (CheY~P) binds to the flagellar motor protein FlhM to elicit reversals in the sense of flagellar rotation that cause a cell to tumble and thereby change its direction of motion (Welch et al. 1993; Alon et al. 1998; Scharf et al. 1998). Attractant stimuli inhibit CheA autophosphorylation so that tumbling is suppressed and cells tend to continue on course.

Most response regulators are two-domain proteins. They share a conserved N-terminal regulatory domain referred to as the receiver domain, but they differ in their C-terminal effector domains. A few response regulators, like CheY, consist solely of the receiver domain. This domain is a doubly wound α/β structure with a central five-stranded parallel β sheet surrounded by five α helices (Stock et al. 1989; Volz and Matsumura 1991). The site of phosphorylation in CheY, Asp57, is located on the solvent exposed loop between $\beta 3$ and $\alpha 3$. Asp57 is surrounded by four other residues, Asp12, Asp13, Thr87, and Lys109, that are highly conserved among response regulator proteins (Volz 1995). It has been proposed that the mechanism of activation in-

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volves a phosphorylation-induced shift in a preexisting equilibrium between inactive and active conformers (Volkman et al. 2001). The active conformer, thereby stabilized, goes on to generate a response. Because of the very short lifetime of phosphorylated CheY, its structure has been inaccessible. Recently, however, the structures of stably modified phosphoprotein analogs, phosphono-CheY and CheY.BeF₃⁻, and the phosphorylated receiver domains of FixJ, Spo0A, and NtrC have been solved (Birck et al. 1999; Kern et al. 1999; Lewis et al. 1999; Cho et al. 2000; Halkides et al. 2000; Lee et al. 2001a). From these structures, it is clear that phosphorylation does not cause substantial changes in the overall fold, but rather seems to act to reposition slightly the secondary structural elements identified as sites involved in protein-protein interactions with the histidine kinase and downstream effectors.

CheY has been extensively studied, and a large number of mutants have been created by site-directed or random mutagenesis. Site-directed mutagenesis at the CheY active site showed that a CheYD57A mutant protein cannot be phosphorylated in vitro, and cells expressing only CheYD57A do not tumble (Bourret et al. 1990; Alon et al. 1998). On the other hand, CheYD13K and CheYD13R mutants cause tumbles in the absence of phosphorylation (Bourret et al. 1990; Bourret et al. 1993), whereas the CheY mutants N23D, N59R, I95V, or Y106W cause excessive tumbling, but only when they are phosphorylated (Sanna et al. 1995; Zhu et al. 1996; Schuster et al. 1998; Lee et al. 2001b; Silversmith et al. 2001). What, then, are the requirements for constitutive activation of CheY? This begs the question as to whether other phosphorylation-independent activated CheY mutants can be obtained other than those possessing a positive charge at residue 13. And if so, what are the features of these activated proteins? To address these questions, we have performed a random mutagenesis screen to isolate constitutively activated CheY mutants. All the isolated active CheY mutants possess multiple missense mutations that act in synergy to generate the activated phenotype. Virtually all of the mutations that lead to activation are in surface residues that are likely involved in intermolecular interactions with other regulatory proteins in the chemotaxis system.

Results

Isolation of phosphorylation-independent activated CheY mutants

In a constant environment, motile bacteria move in a random walk of runs punctuated by brief tumbles. This pattern of motion allows cells to swarm in semisolid agar plates. On the other hand, cells that always run do not swarm in semisolid agar (Wolfe and Berg 1989). We took advantage of this property in order to select for constitutively activated

CheY mutants (i.e., CheY variants that do not need to be phosphorylated to bind to the flagellar motor and promote tumbles). Plasmids carrying randomly mutagenized *cheY* were transformed into an *E. coli* strain deleted for all *che* genes (PS2002) and were plated onto semisolid agar. Cells able to form swarming colonies on semisolid agar plates were isolated and further characterized (see Materials and Methods). To overcome the potential problem of selecting activating mutations that result from the phosphorylation of CheY by cross-talk reactions from other histidine protein kinases or small molecule phosphodonors, the random mutagenesis was performed on a nonphosphorylatable CheY mutant, CheYD57A (Bourret et al. 1990). The X-ray crystal structure of CheYD57A is essentially indistinguishable from that of the wild-type apo-CheY (Sola et al. 2000), and the CheYD57A/D13K double mutant is activated in the absence of phosphorylation just as well as the CheYD13K mutant (Bourret et al. 1993).

In our first attempt to select for constitutively active CheYD57A mutants, we used a mutagenesis procedure that gave a low frequency of random mutations that resulted in *cheY* genes with mostly single base substitutions. No activated CheY mutations were obtained through this approach. The previously known constitutive CheY mutants, CheYD13K and CheYD13R, can produce an activated form of CheYD57A (Bourret et al. 1993), but in order to convert the Asp13 codon to a Lys or Arg codon, two base changes are required. Therefore, to produce the D13K/R mutations, or apparently any kind of phosphorylation-independent activating mutation, one needs a high frequency of mutagenesis so as to generate multiple base changes. This idea was confirmed in experiments in which we randomly mutagenized *cheYD57A* by performing PCR in the presence of manganese (see Materials and Methods). This technique, which gave between two and seven base changes per *cheYD57A*, resulting in two to five amino acid substitutions, lead to the isolation of several activated CheY mutants.

Constitutive CheYD57A mutants

Ten constitutively active CheYD57A mutant proteins, herein referred to as CheY* mutants, were obtained (Fig. 1). All ten CheY* mutants promoted swarming on semisolid agar plates, although most of them were slower than CheYD13K (Table 1). To check that differences in swarming ability between the mutants were not owing to differences in levels of CheY* expression, we performed Western analysis. None of the CheY* mutants were expressed at a dramatically higher level than that of wild-type CheY (Table 1). For all the CheY* mutants, the swarming rate seemed to correlate with the tumbling frequency: The greater the swarming rate on semisolid agar plates, the higher the tumbling frequency in liquid medium (Table 1).

Fig. 1. CheYD57A constitutive mutants. Amino acid substitutions in each of the activated mutants generated from the *cheYD57A* mutagenesis are shown under the appropriate position in the protein sequence. The initial D57A mutation is shown as a bold character in each sequence, unless a mutation occurred at this position. Underlined characters indicate a mutation resulting from two base changes in the same codon. Residues that were shown to interact with FliM peptide (Lee et al. 2001b) are indicated in the top sequence by background shading. The names of the mutants are indicated on the left.

When mapped on the secondary structure of CheY, CheY* mutations were localized mainly to the loop $\beta 1$ – $\alpha 1$; to helices $\alpha 1$, $\alpha 4$, and $\alpha 5$; and to the $\beta 2$ strand (Fig. 1). Remarkably, nine out of the 10 mutants carry at least one substitution that has previously been described as a CheY activating mutation or that affects residues involved in CheA, CheZ, and/or FliM binding (Sackett et al. 1992; Sanna et al. 1995; Swanson et al. 1995; Zhu et al. 1997; Schuster et al. 1998; Shukla et al. 1998; Welch et al. 1998). The previously described mutations we selected were D13R, N23D, K26E, I95V, A103V, and A113P. CheY mutants N23D, K26E, I95V, and A103V have been shown to cause a phosphorylation-dependent tumbly bias (Sanna et al. 1995), and the CheYA113P mutant has been observed to cause reversals in a $\Delta cheA$ host (Bourret et al. 1993). In the case of the CheYI95V mutant, the phosphorylated protein exhibits a two- to sevenfold increase in binding affinity for FliM compared with that of the wild-type CheY–P (Schuster et al. 1998, 2000), and structural studies indicate that I95 is directly involved in FliM as well as CheA bind-

ing (McEvoy et al. 1998; Gouet et al. 2001; Lee et al. 2001b). Some of the described mutations, such as N23D, I95V, A103V, or A113P, were selected more than once in our screen, sometimes in combination (Fig. 1). The A113P mutation seems to have a dramatic effect on CheY*10 mutant activity (compare CheY*4 and CheY*10; Table 1). Such synergistic effects appear to be specific to certain combinations of mutations because CheY*6 also carries the A113P mutation but is only weakly activated.

Constitutive CheYD57A/I95V mutants

The fact that we obtained multiple alleles carrying previously described phosphorylation-dependent mutations (i.e., N23D and I95V) indicated that the presence of these substitutions could increase the probability of selecting other mutations that, in combination with these previously known mutations, would yield phosphorylation-independent mutants. To test this hypothesis directly, we selected for activating mutations in a randomly mutagenized *cheYD57A/I*

Table 1. Swarming rate, quantitation, and tumbling frequency of D57A CheY* mutants

Mutants	Mutations ^a	% increase in swarming rate ^b	Expression level (%) ^c	Tumbling frequency ^d	
				5 μ M	20 μ M IPTG
CheYD13K ^e	D13K	87	148 $\pm$ 32	+/++	+++
CheY*1	N23D/K26E/A57G (T16I/E35Q)	61	91 $\pm$ 16	+/++	+++
CheY*2	D13N/A113P (E93V)	39	142 $\pm$ 43	+/++	+++
CheY*3	D13H/A113P/(N94Y/N121Y)	25	116 $\pm$ 25	+	++/+++
CheY*4	N23S/I95V/A103V	8	142 $\pm$ 32	–	–/+
CheY*5	D13R (E24G)	62	121 $\pm$ 35	+	+++
CheY*6	D12E/A113P	9	98 $\pm$ 26	–	–/+/++
CheY*7	N23D/A57D (E35G)	18	117 $\pm$ 29	–	–/+
CheY*8	D13Y/I95V	7	127 $\pm$ 32	–	+
CheY*9	D13G/L28M/F124S	9	57 $\pm$ 19	–	–/+
CheY*10	N23S/I95V/A103V/A113P	122	ND ^f	++/+++	+++

^a Mutations in parenthesis do not appear to cause any substantial CheY activation.

^b Swarming rate was measured in the presence of 20 μ M IPTG (see Materials and methods). CheY proteins were expressed from the pUC-lac1 vector in the (Δ cheA-cheZ) PS2002 strain of *Escherichia coli*.

^c Protein expression was induced by 20 μ M IPTG in tryptone media at 30°C. Data are the average of three or four experiments, and are expressed as a % of wild-type CheY expression $\pm$ SD (see Materials and methods).

^d CheY proteins were expressed from the pUC-lac1 vector in the (Δ cheA-cheZ) PS2002 strain of *E. coli* at 30°C. Tumbling frequency (TF) was observed at mid-log phase. – indicates no tumblers; +, medium TF; ++, high TF; and +++, very high TF. Several levels of TF for a single mutant mean that a fair number of individual cells were tumbling at different levels.

^e Known constitutively active CheYD13K mutant expressed from the pUC-lac1 vector in the PS2002 strain.

^f ND indicates not determined.

I95V gene. Our results clearly show that the presence of the I95V mutation favors the selection of phosphorylation-independent swarming mutants. We obtained 16 CheYD57A/I95V activated mutants (hereafter referred to as CheY**; Fig. 2), despite screening only half the number of transformants compared with the first screen (see Materials and Methods). Most of the CheY** mutants caused cells to swarm significantly faster than did the mutants selected from the *cheYD57A* screen (Table 2). Apart from CheY**11, which had a twofold lower level of expression of CheY, and CheY**2, which had a 2.5-fold higher level of expression, all the other CheY** proteins were expressed at a similar level to that of wild-type CheY in a matched control strain (Table 2).

The mutations arising from the *cheYD57A/I95V* mutagenesis were more dispersed on the secondary structure than those from the *cheYD57A* screen, but as observed for the first screen, a number of mutations were repeatedly isolated. Mutations D13G, L28Q, A103V, A113P, and F124L were found between two and five times each (Fig. 2). Some of these substitutions were combined in the same protein, and several of these mutants induced the highest swarming rates and tumbling frequency (see mutants CheY**3, 6, 8, 10, 11, and 12; Table 2). These results strongly indicate that the mutations D13G, L28Q, A103V, A113P, and F124L have an additive effect when combined with the I95V mutation.

Among the CheY** activated mutants, six had an A57D reversion (Fig. 2). In this case, we cannot exclude the possibility that the reverting A57D CheY** mutants are activated in vivo by phosphorylation from small molecule

phosphodonors or cross-talk reactions from other two-component histidine kinases in *E. coli* (Lukat et al. 1992). However, when expressed in a *cheA*⁺ background (Δ cheB,Y,Z PS2001 strain), these CheY** mutants caused a dramatic increase in the tumbling frequency of the cells, indicating that they were highly activated by CheA (data not shown). Thus, in a Δ cheA background, the CheY** mutants that have a normal phosphorylation site are not very efficiently phosphorylated by cross-talk reactions or small phosphodonor molecules.

Activating mutations are localized to four distinct regions of the CheY surface

Among the mutant *cheY* genes that were selected for their ability to confer increased swarming in a phosphorylation-deficient background, there were a total of 96 base changes, split roughly equally between transitions and transversions. Of these, 20 are silent mutations. Among the remaining 76 mutations, 55 caused missense mutations that appear to contribute to the swarming phenotype, whereas the remaining 21 do not appear to play a significant role. Whereas the 20 silent base changes and the 21 insignificant missense mutations are all unique, among the 55 base changes with physiologically significant consequences, there are only 17 different amino acid changes at 14 different positions. Among these, six are *cheY* mutations that have previously been associated with increased activation, three are novel amino acid substitutions at positions corresponding to previously identified activating mutations, and eight are novel

Fig. 2. CheYD57A/I95V constitutive mutants. Amino acid substitutions in each of the activated mutants generated from the *cheYD57A/I95V* mutagenesis are shown under the appropriate position in the protein sequence. The initial D57A and I95V mutations are shown as bold characters in each sequence, unless a mutation occurred at these positions. Underlined characters indicate a mutation resulting from two base changes in the same codon. Residues that were shown to interact with FlhM peptide (Lee et al. 2001b) are indicated in the top sequence by background shading. The names of the mutants are indicated on the left.

activating mutations at positions that have not previously been associated with CheY activation. Another seven amino acid substitutions that have previously been reported to cause some degree of CheY activation on phosphorylation (besides D13K that does not need phosphorylation) were not selected in our screens, but five of these require two base changes to effect the relevant amino acid codon change (Fig. 3A). In sum, from this work and previous studies, 24 different amino acid changes at 18 different positions have been identified that can contribute to CheY activation (Fig. 3A).

Virtually all of the 18 residues with mutation that can contribute to CheY activation are surface exposed. These residues are located within four distinct clusters on the CheY surface (Fig. 3B). The three active-site aspartate residues and N59, all of which are involved in Mg(II) coordination and participate in the phosphotransfer chemistry, are all subject to activating mutagenesis (Fig. 3A). In addition to the previously identified D13K and D13R mutations—which appear to activate CheY to the same extent as phosphorylation—D12E, D13G, D13N, and D13Y, as well as D57G, can act synergistically with other activating muta-

Table 2. Swarming rate, quantitation, and tumbling frequency of D57A/I95V/CheY** mutants

Mutants	Mutations ^a	% increase in swarming rate ^b	Expression level (%) ^c	Tumbling frequency ^d	
				5 μ M	20 μ M IPTG
CheYD13K ^e	D13K	87	148 $\pm$ 32	+/++	+++
CheY**1	K45R/A57D/K119Q	78	94 $\pm$ 28	-/+	++/+++
CheY**2	A57G (S15Y/M85V)	100	272 $\pm$ 32	+/++	++/+++
CheY**3	D13G/A103V (K70R)	81	129 $\pm$ 18	++	+++
CheY**4	A57D/A90G (V33A)	59	127 $\pm$ 30	-	+/++
CheY**5	A57D/F124L	61	95 $\pm$ 16	-	+/++
CheY**6	D13G/L28Q	67	87 $\pm$ 27	++	+++
CheY**7	A113P	5	86 $\pm$ 16	-	+
CheY**8	D13G/L28Q/F124L	106	102 $\pm$ 30	+++	+++
CheY**9	A113P (Q100R)	9	107 $\pm$ 47	-	+
CheY**10	D13G/A103V (N62Y)	88	100 $\pm$ 44	+	+++
CheY**11	D13G/L28Q L6P)	75	54 $\pm$ 22	-	+/++
CheY**12	A113P/F124L (E5G)	85	92 $\pm$ 50	+	+++
CheY**13	A57D/K91R	63	112 $\pm$ 15	+/+	++/+++
CheY**14	D13N (I55T)	60	122 $\pm$ 36	-/+	++
CheY**15	A57D	55	129 $\pm$ 15	-	+
CheY**16	A57D (D3N/V10I)	48	144 $\pm$ 30	-	+

^a Mutations in parenthesis do not appear to cause any substantial CheY activation.

^b Swarming rate was measured in the presence of 20 μ M IPTG (see Materials and methods). CheY proteins were expressed from the pUC-lac vector in the (Δ cheA-cheZ) PS2002 strain of *Escherichia coli*.

^c Protein expression was induced by 20 μ M IPTG in tryptone media at 30°C. Data are the average of three or four experiments, and are expressed as a % wild-type CheY expression $\pm$ SD (see Materials and methods).

^d CheY proteins were expressed from the pUC-lar vector in the (Δ cheA-cheZ) PS2002 strain of *E. coli* at 30°C. Tumbling frequency (TF) was observed at mid-log phase. - no tumblers; +, medium TF; ++, high TF; +++, very high TF. Several levels of TF for a single mutant mean that a fair number of individual cells were tumbling at different levels.

^e Known constitutively active CheYD13K mutant expressed from the pUC-lac1 vector in the PS2002 strain.

tions to cause tumbling in the absence of phosphorylation (Fig. 3A). A second major cluster of activating mutations is located at the FliM binding surface formed by α 4- β 5- α 5. The activating mutations in this region include A90G, K91R, I95V or I95A, A103V, Y106W, and K119Q. A third cluster involves residues at the surface bridging α 5 and α 1. These activating mutations include N23D, K26E, L28Q, A113P, and F124L. Finally, a fourth cluster is localized to the surface formed by β 2- α 2. Mutations here include E34K, D41K, and K45R.

Discussion

Our results indicate that CheY activation by mutagenesis requires multiple base changes. All the constitutive mutants we selected resulted from a combination of several mutational events. The cumulative effect of several amino acid substitutions is needed to obtain constitutive activation of CheYD57A, except D13R or D13K. These "exceptions" prove the rule, because each still requires two base substitutions. Interestingly, some of the previously described phosphorylation-dependent activating mutations—such as N23D, K26E, I95V, or A103V—were found in combination within the same protein. It thus seems that these activating mutations have an additive effect overcoming the necessity

for phosphorylation (Tables 1, 2). A similar effect of combinations of activating mutations has also been observed for the PhoP and NtrC response regulators in which the combination of several weakly constitutive mutations into one protein resulted in a synergistic effect (Nohaile et al. 1997; Gunn et al. 2000). From this, one would expect that introduction of the phosphorylation-dependent hyperactive mutation I95V would dramatically increase the chances of getting constitutively active mutants by random mutagenesis, and this is, in fact, what we observed.

The requirement of multiple mutagenic events for constitutive CheY activation may serve a functional advantage insofar as it prevents the spontaneous generation of activated response regulator variants. The limitations imposed on mutagenic pathways by specific codon usage may be a much more important underlying principle of protein design in protein evolution than has hitherto been realized.

Because of the high mutation rate we used to obtain constitutively active CheY mutants, it is unlikely that all the amino acid substitutions in one particular mutant were responsible for activation. Residues 13 and 57 were "hot spots" for mutation. They were substituted 11 and nine times, respectively, out of 25 total mutants (Figs. 1, 2). Only two substitutions occurred at residue 57: G (twice) or D, the latter restoring a wild-type active site in seven mutants. Residue 13 was substituted by six different amino acids, the

Fig. 3. CheY activating mutations. Novel mutations arising from this work (magenta), mutations arising from this work that have been already described (cyan; Bourret et al. 1993; Sanna et al. 1995), and previously known CheY activating mutations that we did not obtain (dark green; Bourret et al. 1990; Zhu et al. 1996; Schuster et al. 2000; Silversmith et al. 2001) are shown: (A) Under the appropriate position in the CheY protein sequence. Underlined characters indicate a mutation resulting from two base changes in the same codon. Residues that were shown to interact with FlIM peptide (Lee et al. 2001b) are indicated in the top sequence by background shading. (B) In the ribbon diagram of CheY. The two views are related by a 180° rotation around the indicated axis. The four clusters are shown circled in red. The structure is made from the 3CHY entry from the Protein Data Bank (PDB) (Volz and Matsumura 1991). (C) In the space-filling representation of CheY with the same orientation as in B. The two views are related by a 180° rotation around the indicated axis. The figure was created with the program MOLMOL (Koradi et al. 1996).

mutation D13G being found in six mutants. The high variability of residues allowed at position 13 in activated CheYD57A mutants indicates that the Asp at this position might be important for the stabilization of the inactivated form of CheYD57A. It is probable, however, that all the mutations in the neighborhood of the phospho-accepting D57 account, at least in part, for the acquisition of the constitutively activated phenotype. The other mutations most likely to be involved in the constitutive activation of CheY are the substitutions that are recurrent among different mutants, as well as unique mutations within a specific

combination of mutations. Mutations at residues N23, K26, E34, K45, A90, K91, I95, A103, A113, K119, and F124 (Fig. 3) seem to be important within specific combinations for constitutively activating CheY. Several of these residues were found to exhibit chemical shifts on CheY phosphorylation (Lowry et al. 1994) or in phosphono-CheY (Halkides et al. 2000), and most of them are localized to loops and helices that are rearranged on activation. Indeed, small changes associated with the repacking near the active site result in a repositioning of loops $\alpha 4$ – $\beta 4$ and $\alpha 5$ – $\beta 5$ plus helices $\alpha 4$ and $\alpha 5$ in CheY–P, in the CheY–P analogs

phosphono-CheY and CheY.BeF₃⁻, and in the structure of the phosphorylated receiver domains of FixJ and NtrC (Fig. 4; Drake et al. 1993; Birck et al. 1999; Kern et al. 1999; Cho et al. 2000; Halkides et al. 2000). Small shifts were also observed in the loop β1-α1 in phosphorylated FixJ (FixJ~P; Birck et al. 1999). These modest but significant changes alter both topological and electrostatic features of the molecular surface; the same effects could result from a combination of mutations at the surface of the protein. It should be noted, however, that the CheY crystals were formed with different crystal packings, which may affect the side-chain orientations. In general, none of the changes seen in these structures of isolated CheY seem sufficient to constitute a true conformational change to an active form.

The residues that seem to be important for the constitutive activity of our mutants, as well as those previously ascribed to phosphorylation-dependent hyperactivation, delineate four distinct “patches” on the CheY surface, which mostly cluster on the face that is defined by α1, α4, β5, and α5 (Figs. 3B, 3C). Comparison of the conformations of these residues in different X-ray structures shows that on phosphorylation, most of them undergo either a displacement of their backbone positions or a reorientation of their side-chain (Fig. 5). Interestingly, although the overall backbone conformation of the activated mutant CheYD13K is almost indistinguishable from that of apo-CheY (Fig. 4), the side-chain of several residues from the patches adopts an intermediate conformation between the inactive and active conformations (Fig. 5). One particularly striking intermediate conformation is that of the side-chain of residue Y106, which is halfway between the “inside” position found in the activated form of CheY, CheY.BeF₃⁻, and the “outside” position found in inactive CheY (Fig. 5B). High-resolution structures of CheY.BeF₃⁻, and those of two other phosphorylated response regulators, showed the concerted movement of the conserved Thr (T87 in *E. coli*) and Phe/

Fig. 4. Conformational changes on activation. X-ray structures of apo-CheY (PDB entry 2CHF; yellow), CheY complexed with Mg²⁺ (PDB entry 2CHE; orange), CheYD13K (PDB entry 1EHC; green), CheYI95V (PDB entry 1D4Z; magenta), CheY.BeF₃⁻ (PDB entry 1FQW; dark blue), and CheY.BeF₃⁻ in complex with FliM (PDB entry 1F4V; cyan) were superposed through their α carbon using the SwissPdb Viewer program (Guex and Peitsch, 1997).

Fig. 5. Clusters of activating mutations. Backbone and side-chains of residues in the four clusters defined in Fig. 3 were superposed as described in Fig. 4. Each cluster is represented in a panel, from A to D. Color code is the same as in Fig. 4. The figure was created with the SwissPdb Viewer program (Guex and Peitsch 1997).

Tyr (Y106) residues that are thought to be important for response regulator activation (Birck et al. 1999; Lewis et al. 1999; Cho et al. 2000; Halkides et al. 2000; Lee et al. 2001a). The side-chain of the Y106 residue of the hyperactive mutant CheYI95V adopts virtually the same position as in CheYD13K (Fig. 5B). That orientation of the side-chain of Y106 may allow its movement further inside on binding to FliM in order to avoid steric interference and, thus, might in part explain the constitutive activity of CheYD13K. As for CheYD13K, the overall backbone conformation of the CheYI95V mutant is very similar to that of apo-CheY (Fig. 4), but contrary to CheYD13K, the CheYI95V active site looks more similar to the active site of the inactive apo-CheY than the activated form of CheY (Fig. 5A). This observation might explain why the hyperactivity of CheYI95V depends on phosphorylation, and why almost all of our activated mutants that have the I95V mutation also carry a mutation at the active site. Those that do not have a muta-

tion at the active site all carry the A113P mutation. This substitution seems to play an important role in mutant activation (compare mutant CheY*4 to CheY*10; Table 1), but it is not sufficient by itself, or in combination with only the I95V mutation, to promote very high activation (compare mutants CheY*10, and CheY*6, CheY**7; Tables 1, 2). Other mutations are thus needed to trigger the conformational modifications that are required. Perhaps the replacement of an Ala by a Pro at the top of the $\alpha 5$ helix promotes the displacement of the $\alpha 5$ helix and the surrounding loops observed in activated CheY.

Our results indicate that combining mutations in regions that undergo phosphorylation-induced structural modifications can lead to CheY mutants that in some way mimic the activated form of CheY. The recent high-resolution X-ray structure of apo-CheY shows that the $\beta 4$ – $\alpha 4$ loop can take two distinct conformations, thought to represent an inactive form and a so-called meta-active form that is more similar to the activated conformation (Simonovic and Volz 2001). It was suggested that this meta-active form of apo-CheY would be active but unstable. Another recent report showed that binding of CheA, CheZ, and FliM peptides to CheY had a dramatic impact on CheY phosphorylation by small phosphodonor molecules (Schuster et al. 2001). The right combination of synergistic mutations could stabilize the meta-active form or produce a form of CheY having an increased affinity for FliM in the CW state and, as a result, bypass the need for phosphorylation and increase the tumbling probability.

A large body of structural information has accumulated on the general problem of response regulator activation. Attention has focused on changes at the surface defined by $\alpha 4$ – $\beta 5$, especially the movements of the conserved aromatic residue corresponding to Y106 in CheY. This surface has, after all, been shown in CheY to directly participate in binding the flagellar switch protein FliM (Lee et al. 2001b). Moreover, the analysis of the X-ray structure of the FixJ–P response regulator has indicated that the $\alpha 4$ – $\beta 5$ constitutes the critical dimer interface (Birck et al. 1999).

Although activating mutations of CheY tend to confirm the importance of the $\alpha 4$ – $\beta 5$ surface, they also highlight the potential involvement of the surface defined by $\alpha 1$ – $\beta 2$ at the opposite side of the protein. This part of the response regulator surface has been largely ignored, despite the fact that in the FixJ–P X-ray structure, the $\alpha 1$ – $\beta 2$ surface participates in more extensive dimer interactions than does the $\alpha 4$ – $\beta 5$ surface at the opposite side of the protein. In fact, the majority of activating mutations in CheY outside the active site correspond to residues in FixJ–P that are located in areas of intermolecular protein contact.

It has generally been assumed that response regulator phosphorylation stabilizes an active conformational state with a relatively high affinity for its regulatory target. This assumes that the relevant protein-protein interactions do not

entail extensive conformational changes. However, the structure of CheY–P bound to FliM at the motor apparatus might differ significantly from structures obtained in solution with pure proteins. For example, when CheY binds to the ring of FliM proteins at the motor, it may form multimers like those formed by the transcriptional activators FixJ or OmpR bound to their respective DNA binding sites (Pratt and Silhavy 1995; Ferrieres and Kahn 2002). It seems likely that the intermolecular interactions that occur during the formation of the CheY–FliM complex at the motor require conformational changes in both proteins, as well as in other proteins of the motor to which FliM and/or CheY binds. Postphosphorylation binding events at the motor have previously been suggested to be crucial for activation (Stock and Da Re 2000; Bren and Eisenbach 2001), and it may be these aspects of CheY structure that are facilitated by activating mutations rather than the type of subtle conformational variations that have been discerned in previous structural studies.

Materials and methods

Bacterial strains and plasmids

The bacterial strains used in this study are the *che* deletion strains PS2002 ($\Delta cheA$ –*cheZ*) and PS2001 ($\Delta cheB$ –*cheY*–*cheZ*) described in Alon et al. (1998). The high copy number pUC-lac1 vector, which carries the *lac-I'* gene, was used to clone and express mutated *cheY* under the control of the inducible *lacOP* (Surette and Stock 1996).

Site-directed mutagenesis

The *cheYD57A/I95V* mutation was generated by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene) and following manual instructions. A pUC12 plasmid carrying the *cheYD57A* gene was used as the template for introduction of the I95V mutation. Subsequently, the *cheYD57A/I95V* gene was cloned into the pUC-lac1 vector.

Random mutagenesis

To select for activating mutations that are independent of phosphorylation, the *cheYD57A* gene was randomly mutagenized by performing a PCR reaction in the presence of Mn^{2+} (0.5 to 1 mM). A PCR product of the expected size was purified and cloned into the polylinker region of pUC-lac1. Ligation products were transformed into the PS2002 strain by electroporation. Approximately 200,000 transformed cells were plated as linear “rows” on motility (semisolid agar) plates (1% tryptone, 1% NaCl, 0.3% agar) containing 20 μ M IPTG and the appropriate selective antibiotics. After incubation for ~24 h at 30°C, we visually checked for swarming bubbles emerging from rows of growing transformants. Swarming bubbles were streaked on Luria-Bertani Medium plates, and individual clones were checked for their ability to swarm on IPTG induction. Plasmids from isolated swarming cells were purified and retransformed into the PS2002 strain to verify that the selected phenotype was actually linked to a mutation in the *cheYD57A* gene. The mutations responsible for the swarming phe-

notype of the mutated *cheYD57A* DNA were identified by DNA sequencing. The same procedure was used for the mutagenesis of *cheYD57A/I95V*, and roughly 100,000 transformants were screened.

Swarming assays

Strains containing mutated *cheYD57A* or *cheYD57A/I95V* genes on the pUC-lacI plasmid were grown overnight in tryptone media (1.3% bacto tryptone and 0.7% NaCl) at 30°C. The center of a motility plate containing 20 μ M IPTG, 100 μ g/mL ampicillin, and 25 μ g/mL kanamycin was inoculated with 0.5 μ L of the culture, and plates were incubated at 30°C. The swarm diameter was measured periodically. Plotting the swarm diameter against time gave a straight line, the slope of which is the swarming rate. The results were expressed as a percentage of increase of the swarming rate, that is, [(mutant swarming rate/growth rate of the nonswarming *cheYD57A/I95V* mutant) – 1] $\times$ 100.

Swimming behavior

Overnight cultures of cells expressing the CheY mutants from the pUC-lacI vector were diluted 1:50 in fresh tryptone media supplemented with selective antibiotics as appropriate. After 1.5 h of growth at 30°C, the cultures were induced with IPTG. The cells were harvested at mid-log phase ($OD_{600} = 0.45$) by centrifugation at 800g for 5 min at room temperature, washed once, and gently resuspended in motility media (7.6 mM $[NH_4]_2 SO_4$, 2 mM $MgSO_4$, 20 μ M $FeSO_4$, 0.1 mM EDTA, 60 mM potassium phosphate at pH6.8); 0.5 μ L of this suspension was observed with a Zeiss Dialux dark field microscope for swimming behavior.

CheY protein quantitation

Levels of CheY protein in cells were estimated by Western blot. PS2002 cells expressing wild type or mutated CheY from the pUC-lacI vector were grown overnight in tryptone broth supplemented with ampicillin and kanamycin at 37°C; diluted 1:100 into fresh tryptone broth supplemented with ampicillin and kanamycin, and containing 20 μ M IPTG; and grown at 30°C till OD_{600} reached ~ 1.5 . Cells were pelleted, resuspended in gel loading buffer, boiled, and subjected to 15% SDS-PAGE. Proteins were transferred onto PVDF membranes and membranes probed with polyclonal anti-CheY antibodies. The immunoblots were assayed using the ECL Plus detection system (Amersham Pharmacia) and were signal quantitated using fluorimager (Storm, Molecular Dynamics). Results were expressed as a percentage of wild-type CheY.

Electronic supplemental material

Supplemental material includes one table, which gives the codon changes responsible for the missense mutations in Tables 1 and 2.

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