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Biochemistry

Acetylation at Lys-92 enhances signaling by the chemotaxis response regulator protein CheY

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ABSTRACT **When *Escherichia coli* cells lacking all chemotaxis proteins except the response regulator CheY are exposed to acetate, clockwise flagellar rotation results, indicating the acetate stimulus has activated signaling by CheY. Acetate can be converted to acetyl-CoA by either of two different metabolic pathways, which proceed through acetyl phosphate or acetyl-AMP intermediates. In turn, CheY can be covalently modified by either intermediate *in vitro*, leading to phosphorylation or acetylation, respectively. Either pathway is sufficient to support the CheY-mediated response to acetate *in vivo*. Whereas phosphorylation of Asp-57 is a recognized mechanism for activation of CheY to stimulate clockwise flagellar rotation, acetylation of CheY is less well characterized. We found evidence for multiple CheY acetylation sites by mass spectrometry and directly identified Lys-92 and Lys-109 as acetylation sites by Edman degradation of peptides from [^{14}C]acetate-labeled CheY. Replacement of CheY Lys-92, the preferred acetylation site, with Arg has little effect on chemotaxis but completely prevents the response to acetate via the acetyl-AMP pathway. Thus acetylation of Lys-92 activates clockwise signaling by CheY *in vivo*. The mechanism by which acetylation activates CheY apparently is not simple charge neutralization, nor does it involve enhanced binding to the FliM flagellar switch protein. Thus acetylation probably affects signal generation by CheY at a step after switch binding.**

Motile bacteria respond to changes in their chemical environment by altering their swimming behavior. In the case of *Escherichia coli*, sensory inputs modulate the frequency of switching between two swimming modes. Counterclockwise (CCW) flagellar rotation results in smooth swimming episodes called "runs," whereas clockwise (CW) flagellar rotation results in directional changes termed "tumbles" (1). A network of signal transduction proteins gathers environmental information and adjusts the concentration of the phosphorylated form of the CheY response regulator protein (CheY-P) accordingly (reviewed in ref. 2). CheY-P binds to the flagellar switch protein FliM and promotes CW flagellar rotation (3, 4). Chemotaxis thus results from a biased random walk (5) controlled by the concentration of CheY-P.

A "gutted" strain of *E. coli*, which was genetically engineered to have fully functional flagella but none of the proteins comprising the chemotaxis signal transduction pathway, lacks CheY and thus exhibits the default behavior of exclusively CCW flagellar rotation (6). Expression of CheY in a gutted strain has little effect on flagellar rotation (7). Presumably insufficient CheY-P is formed to alter behavior, because the CheA kinase that serves as the primary source of phosphoryl groups for CheY is absent from the gutted strain. Exposure of gutted cells expressing CheY to millimolar concentrations of acetate, however, rapidly results in a dramatic CW shift in flagellar rotation (7). This behavior persists as long as acetate is present but is reversed by acetate removal. Understanding the molecular basis of this "acetate effect" can potentially provide new insights into the mechanism(s) of CheY activation.

Acetate and acetyl-CoA can be interconverted by either of two metabolic pathways (Fig. 1). One branch, catalyzed by the enzymes acetate kinase (Ack) and phosphotransacetylase (Pta), utilizes an acetyl phosphate (acetyl-P) intermediate. The other, catalyzed by acetyl-CoA synthetase (Acs), utilizes an acetyladenylate (acetyl-AMP) intermediate. Both high energy intermediates are very reactive and can potentially participate in covalent modification and activation of CheY. Acetyl-P is a substrate for CheY autophosphorylation on Asp-57, and CheY-P has been estimated to be ≈100 times more active than CheY in generating CW flagellar rotation (8–10). CheY is acetylated by Acs through an acetyl-AMP intermediate, and CheY-Ac has been estimated to be ≈10,000 times more active than CheY in generating CW flagellar rotation (11).

Some information is available concerning the relevance of these CheY modifications to the acetate effect. Dailey and Berg (12) found that an *ack* mutation abolished the CW response to acetate and concluded that CheY-P generated from acetyl-P is probably responsible for the acetate effect. Recently, Barak *et al.* (13) observed a CW response to acetate in strains deleted for *acs* (acetyl-AMP branch in Fig. 1 blocked) or *ack pta* (acetyl-P branch blocked) but not for *ack pta acs* (both branches blocked) and concluded that both Ack and Acs are involved in the acetate effect. Here, we complete the connection between participation of Acs in the acetate effect *in vivo* and the acetylation of CheY observed *in vitro*. We identified two CheY acetylation sites and found that mutation of the primary site eliminated the Acs-mediated CW response to acetate observed in an *ack pta* mutant strain. We conclude that CheY can be activated to generate CW signals by Acs-mediated acetylation of Lys-92 in addition to the previously established phosphorylation of Asp-57 and that addition of acetate to gutted cells expressing CheY may cause CW flagellar rotation by either route.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Bacteriophage. The Δ*cheY* strain KO641*recA*, the p_{trp} *cheYZ* plasmid pRBB40, and M13*cheYZ* have been described previously (14). The p_{trp} *cheY* plasmid pRL22ΔZ has been described previously (15). The *ace*$^+$ strain RBB1106, the Δ(*ackA pta hisJ hisP dhu*) zej-223::Tn*10* strain RBB1097, and the Φ(Δ*acs*::Km-1) Δ(*ackA pta hisJ hisP dhu*) zej-223::Tn*10* strain RBB1109, each of which also carries Δ(*cheA-cheZ*)::Zeo^R, have been described previ-

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Abbreviations: CCW, counterclockwise; CW, clockwise; Ack, acetate kinase; Pta, phosphotransacetylase; Acs, acetyl-CoA synthetase; CheY-Ac, acetylated CheY; CheY-P, phosphorylated CheY.

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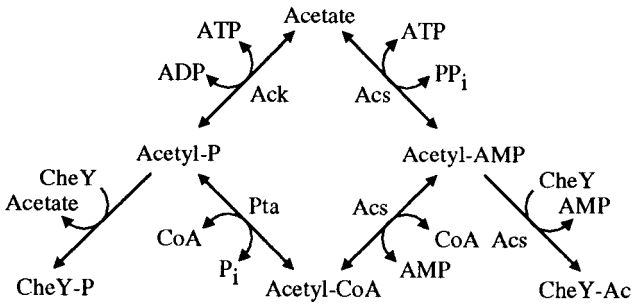

FIG. 1. Connections between CheY activation and the two branches of acetate metabolism.

ously (13). Note that RBB1097, RBB1106, and RBB1109 are not completely gutted strains, as they still contain the Tsr, Trg, and Aer chemoreceptors.

Site-specific mutagenesis was performed by the method of Kunkel *et al.* (16). Mutagenic oligonucleotide primers replacing the Lys-92 codon of *cheY* with codons for Arg, Gln, Glu, or Met were synthesized at the UNC Lineberger Comprehensive Cancer Center Nucleic Acids Core Facility. Candidate M13*cheYZ* bacteriophage carrying the desired mutations were identified by DNA sequencing. Mutant *cheY* alleles were subcloned into pRBB40 on a 0.5-kb restriction fragment extending from the *Bst*XI site in *cheB* to the *Bsm*I site in *cheZ* by using standard techniques and transformed into KO641*recA*. The mutations were reconfirmed by sequencing plasmid DNA. The *Bst*XI/*Bsm*I fragment carrying the *cheY92KR* mutation was also subcloned from pRBB40 into pRL22ΔZ.

Behavioral Assays. Swarm assays were performed as described previously (14). For measurement of flagellar rotational bias, cultures were grown and tethered as described previously (17), except that the media for some cultures contained 5 or 10 mM NaOAc (pH 7.0) where indicated. Multiple fields of view were recorded on videotape simultaneously with a vertical interval time code. To observe the acetate effect, fluid in the sample chamber was replaced with fresh tethering buffer containing 5 or 10 mM NaOAc (pH 7.0), and multiple fields of view were recorded after a 2-min interval. Videotapes were played back at slow (≈⅕) speed for accurate scoring. Behavior was analyzed with The Observer 3.1 Video Tape Analysis software (Noldus Information Technology, The Netherlands), which allows correlation of the videotape time code with CCW, CW, or pause episodes entered manually on a computer keyboard. Thirty seconds of behavior were scored for each cell. Cells that paused >5% of the time were not included in the calculation of bias (defined as the fraction of time spent in CCW rotation).

Protein Biochemistry. CheY was purified from KO641*recA*/pRBB40 as described previously (18), with two minor modifications: 25 mM Tris (pH 7.5)/5 mM $MgCl_2$/10% glycerol was used instead of TEDG buffer, and the ion exchange column was DE52 (Whatman) instead of DEAE-Sepharose CL-6B.

CheY was acetylated by using a method described previously (11) with modifications suggested by R. Barak (personal communication). The 50-μl reaction mix contained 200 μM CheY, 5 mM ATP, 5 mM $MgSO_4$, 1.75 mM [2-^{14}C]acetate (44 mCi/mmol, Sigma), 0.01 unit of inorganic pyrophosphatase (Sigma), and 0.03 unit of yeast Acs (Sigma) in 50 mM Tris·HCl (pH 7.9). Pyrophosphatase was added to drive the labeling reaction to completion by breaking down the PP_i generated. The reaction mixture was incubated at 37°C for 6 h. CheY was separated from free acetate and the other proteins on a C4 reversed phase HPLC column with a 5–60% acetonitrile gradient in 0.1% trifluoroacetic acid. Fractions containing CheY were identified by scintillation counting.

For mass spectrometry, acetylated CheY was dialyzed overnight against 25 mM ammonium bicarbonate. Electrospray ionization mass spectrometry was performed at the Duke University Medical Center Biomolecular Mass Spectrometry Laboratory. Measurements were made on a Micromass (formerly Fisons)-VG Quattro BQ triple quadrupole mass spectrometer equipped with a pneumatically assisted electrostatic ion source operating at atmospheric pressure. Samples were introduced by loop injection into a stream of 50% aqueous acetonitrile containing formic acid (1% vol/vol) flowing at 6 μl/min. Mass spectra were acquired in the multichannel analyzer mode from m/z 700-1400 with a scan time of 10 s. The mass scale was calibrated with horse heart myoglobin (M_r 16951.48) with a resolution corresponding to a peak width at a half-height of 0.8 Da for m/z 893. The raw electrospray ionization-MS spectra were transformed to a molecular mass scale by using a maximum entropy-based method (MaxEnt), which uses the MemSys5 program (MaxEnt Solutions Ltd., Cambridge, UK) and is part of the VG MassLynx software suite.

To determine the sites of acetylation, radiolabeled CheY was dried under vacuum and resuspended in 70% formic acid. Excess cyanogen bromide (CNBr) was added, and the protein was incubated for 2 h at room temperature under nitrogen in the dark. Peptides were separated on tandem C4 and C8 reversed phase HPLC columns with a 5–60% acetonitrile gradient in 0.1% trifluoroacetic acid. Radioactive fractions were identified by scintillation counting and analyzed at the UNC/PMBB Micro Protein Chemistry Facility. One aliquot was used for N-terminal amino acid sequencing to identify the peptide. A second aliquot was used to determine the radioactivity released in each cycle of Edman degradation and hence the sites of acetylation.

For FliM binding, CheY was acetylated as described above by using nonradioactive sodium acetate. CheY was purified over a Superdex-75 FPLC gel filtration column and concentrated. CheY-FliM binding was measured by using a previously described modification (19) of the method of Bren *et al.* (20).

RESULTS

Identification of the CheY Acetylation Sites. The [^{14}C]acetyl groups on CheY are stable under treatment with hydroxylamine or ethanolamine, indicating that acetylation occurs on amino groups rather than hydroxyl groups (11). Each of the 11 Lys residues in CheY (21) and the N terminus are thus potential acetylation sites. We first sought to determine how many actual acetylation sites there are in CheY. A CheY acetylation reaction was performed as described under *Experimental Procedures*, and the products were subjected to electrospray ionization mass spectrometry. Three species were observed that clearly correspond to CheY with zero, one, or two acetyl groups (Fig. 2). A minor peak that probably represents CheY with three acetyl groups was also observed. The measured molecular masses were in excellent agreement with predicted values: 13966.0 (13965.2 expected), 14008.0 (14007.3 expected), 14049.0 (14049.3 expected), and 14090.0 (14091.3 expected). A time course of [^{14}C]acetate incorporation into CheY suggests that acetylation is near maximal under the reaction conditions used (data not shown).

To determine the sites of acetylation on CheY, the protein was labeled with [^{14}C]acetate in the presence of Acs, ATP, and pyrophosphatase. Radiolabeled CheY was separated from the other components of the reaction by HPLC and digested with CNBr. The peptides thus generated were again separated by HPLC (Fig. 3*A*). Subsequently, one half of each radioactive sample was subjected to N-terminal amino acid sequencing to identify the peptide. The other half of the sample also was subjected to Edman degradation, but the cleavage products were saved in a fraction collector to determine the radioac-

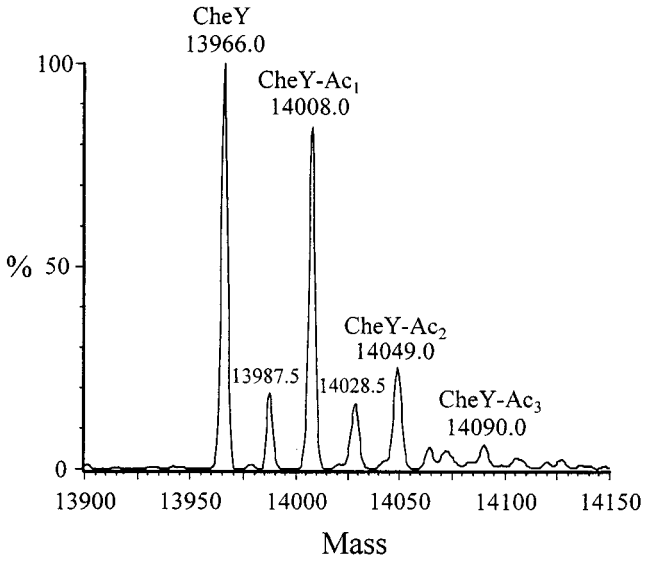

FIG. 2. Mass spectrometry reveals the presence of CheY with zero, one, two, or possibly three acetyl groups following incubation of CheY with Acs, acetate, ATP, and pyrophosphatase as described under *Experimental Procedures*. The peaks at 13987.5 and 14028.5 are the Na^+ adducts of CheY and CheY-Ac, respectively.

tivity released in each cycle rather than routed to the HPLC for amino acid identification. This mapping procedure was carried out on three different CheY acetylation reactions. The first radioactive peak (Fig. 3*A*, fraction 5) was seen in each case but did not yield any peptide sequence and presumably corresponds to free acetate. Consistent with the interpretation that this is the elution position of small molecules, a peak of ^{32}P (presumably representing P_i) is found in the corresponding fraction on similar treatment of [^{32}P]CheY57DN-P (J. Appleby, personal communication). The second radioactive peak (Fig. 3*A*, fraction 44) is also highly reproducible (seen in all three experiments) and corresponds to the C-terminal CNBr fragment of CheY (Val-86 to Met-129). This peptide contains six Lys residues. Radioactivity was detected in the fractions corresponding to Lys-92 and Lys-109 (Fig. 3*B*). The third radioactive peak (Fraction 52) was observed only in the particular experiment displayed in Fig. 3*A* and was found to contain the N terminus of CheY. No ^{14}C was released from the material in Fraction 52 during 20 cycles of Edman degradation. Either of two circumstances could yield this result. (*i*) CheY could be acetylated on the N terminus, which would block the Edman degradation chemistry, or (*ii*) some CheY acetylated on Lys-92/Lys-109 might have survived CNBr digestion intact. In any case, the mapping data demonstrate that there are multiple acetylation sites on CheY, consistent with the mass spectrometry results. Furthermore, identification of Lys-109 as one of multiple acetylation sites is consistent with a previous report that replacement of Lys-109 with Arg reduces but does not eliminate CheY acetylation (11).

Lys-92 Is the Biologically Relevant Acetylation Site. Only 0.01% of the CheY molecules were estimated to have been acetylated in the experiment when the CW signaling activity of CheY-Ac was originally discovered (11). It is therefore highly unlikely that multiply acetylated CheY molecules were present, implying that acetylation at a single site is sufficient to activate CW signal generation by CheY. In the experiment of Fig. 3*B*, more radioactivity was associated with Lys-92 than with Lys-109, suggesting that Lys-92 is the preferred acetylation site. Consistent with the inference that acetylation occurs faster at Lys-92 than at Lys-109, the ratio of radioactivity observed at Lys-92 to that found at Lys-109 increased from ≈2 to ≈6 when the incubation time of the acetylation reaction was

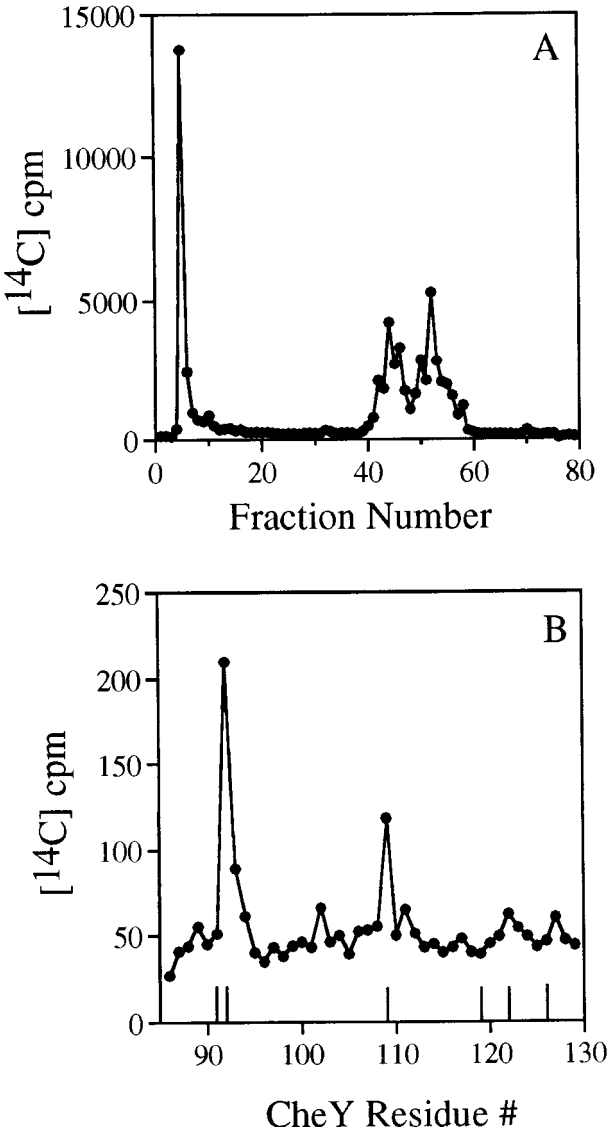

FIG. 3. Mapping the CheY acetylation sites. (*A*) HPLC fractionation of CNBr-digested [^{14}C]CheY-Ac. Radioactive peptides were identified by amino acid sequencing. Fraction 5 did not yield any sequence, whereas fractions 44 and 52 contained the C- and N-terminal CNBr fragments of CheY, respectively. Note that the fraction 52 peak was observed in only one of three independent experiments. (*B*) Radioactivity released during each cycle of Edman degradation of the peptide in fraction 44 of *A*. The vertical lines below the radioactivity profile indicate the positions of the six Lys residues in this peptide. The two peaks of radioactivity that rise above background correspond to Lys-92 and Lys-109. The cpm values are relatively modest because only 1% of the material released in each cycle was subjected to scintillation counting.

shortened from 6 to 2 h (data not shown). Taken together, these two lines of evidence suggest that the biological activity of CheY-Ac can be attributed to acetylation of Lys-92.

Removal of the Primary Acetylation Site Does Not Affect Chemotaxis. To investigate the possible role of CheY acetylation in the chemotactic responses of intact rather than gutted bacteria, a mutant was created in which Lys-92 of CheY was replaced with Arg. This substitution should prevent acetylation of the primary site in CheY. The CheY92KR mutant formed chemotactic swarms of normal appearance that expanded at 72% of the wild-type rate (data not shown). This result is consistent with the observation that cells carrying a deletion in *acs*, which therefore should be defective in CheY acetylation, exhibit wild-type chemotaxis (S. Kumari and A. J. Wolfe,

personal communication; R. Tishel and M. Eisenbach, personal communication). The secondary acetylation site, Lys-109, is a critical active site residue that is highly conserved among response regulator proteins (22). Replacement of CheY Lys-109 is known to abolish chemotaxis (23, 24), but there is no evidence that this consequence is related to the role of Lys-109 as an acetylation site.

Removal of the Primary Acetylation Site Abolishes the Acs-Mediated Acetate Effect. Acs has been shown to mediate both acetylation of CheY *in vitro* (11) and the acetate effect *in vivo* (13). A logical extension of these results would be that Acs mediates the CW response to acetate by acetylating CheY. To test this hypothesis, the ability of cells lacking most chemotaxis proteins except CheY to respond to acetate when both, one, or neither of the branches of the acetate metabolism pathway were operational and when the primary CheY acetylation site was either present or absent was examined (Table 1). Expression of *E. coli* Acs activity is inducible by acetate (25), so the effect of growing cells in the presence of acetate before tethering was also examined.

Δ(*cheA-cheZ*) cells expressing wild-type CheY exhibited a strong CW rotational shift on acetate addition (Table 1, lines 1 and 4). This is the previously described acetate effect. The acetate effect was observed even in the absence of Ack and Pta (Table 1, line 2), although the magnitude of the response was reduced somewhat. Both aspects of the response (occurrence and magnitude) in Δ(*ackA pta*) cells are consistent with the recent report of Barak *et al.* (13). The previous observation (13) that removal of Acs, Ack, and Pta abolishes the CW response to acetate was also confirmed (Table 1, line 3). Thus, Acs can mediate the acetate effect (Table 1, cf. lines 2 and 3). However, the Acs-mediated CW response to acetate depended on prior growth in acetate (presumably to induce Acs expression) (Table 1, cf. lines 2 and 5). CheY92KR also supported a strong CW response to acetate (Table 1, lines 1 and 4) but only when Ack and Pta were present (Table 1, cf. lines 1 and 2). In contrast to wild-type CheY, CheY92KR did not support an observable acetate effect when only the Acs branch of the acetate metabolic pathway was present. Therefore, the ability of Acs to support a CW response to acetate depends on the presence of the primary CheY acetylation site.

The prestimulus biases of the strains in Table 1 diverge substantially. The differences do not appear to reflect the consequences of *ace* genotype, *cheY* allele, or growth with or without acetate. Rather, our experience has been that in marked contrast to *che*$^+$ bacteria, many CheY overexpression strains exhibit culture to culture variability in resting bias (data not shown). Such deviation may arise from the highly nonlinear relationship between CheY and flagellar rotational bias (26, 27). The critical feature is that the presence or absence of a rapid CW response to acetate addition is reproducible, irrespective of the initial bias of a particular culture (data not shown).

Tests of Potential Mechanisms by Which Acetylation Might Activate CheY. The results described above provide independent verification of the conclusion by Barak *et al.* (11) that acetylation activates CheY for CW signal generation. Two possible mechanisms for this activation were explored.

Electrostatic charge appears to be a critical aspect of the mechanism for response regulator activation by phosphorylation. Several highly conserved features of the response regulator active site are charged, including one Lys and three Asp residues (22). In addition, a divalent cation (typically Mg^{2+}) in the active site is essential for catalysis of phosphorylation and dephosphorylation reactions (28). Introduction of a negatively charged phosphoryl group into the active site presumably results in repositioning of charged moieties. For example, there is evidence that both the binding affinity and the amino acid side chains involved in Mg^{2+} coordination are different in CheY-P than in CheY (4, 23). Furthermore, introduction of charged amino acids in the vicinity of the active site results in the phosphorylation-independent activation of several response regulators, including CheY (14, 24, 29–31). Thus it is plausible that neutralization of the positive charge on Lys-92 by acetylation results in a conformational change in CheY, which in some important way mimicks that caused by phosphorylation and thus activates CheY. To test this hypothesis, we made mutants in which Lys-92 was replaced by Gln, Met, or Glu and asked whether elimination of the Lys-92 positive charge by mutation results in constitutive CW activity similar to that observed following acetylation of CheY. The CheY92KM and CheY92KQ mutant proteins bearing the neutral charge substitutions Met and Gln supported chemotaxis at 66 and 42% of the wild-type swarm rates, respectively. Tethered *cheY92KM* and *cheY92KQ* mutant bacteria exhibited intermediate flagellar rotational biases and frequent reversals of direction (data not shown), consistent with their ability to form swarms. CheY with the negative charge substitution Glu at position 92 did not support chemotaxis and resulted in CCW flagellar rotation (data not shown). Thus there is no evidence that neutralization of the positive charge at residue 92 results in increased CW flagellar rotation.

Phosphorylation of CheY results in enhanced binding to the FliM flagellar switch protein (3, 4), which in some as yet undefined manner then results in CW flagellar rotation. Thus another possible mechanism for activation by acetylation is that acetylation increases the binding affinity of CheY for FliM. The ability of CheY-Ac to bind to FliM *in vitro* was examined in the presence or absence of acetyl-P by using a previously described assay (19, 20). Binding of CheY-Ac to FliM was indistinguishable from that observed with nonacetylated CheY under nonphosphorylating conditions (data not shown). Furthermore, phosphorylating conditions enhanced

Table 1. CheY92KR does not support the Acs-mediated CW response to acetate

	Mean flagellar rotational bias ± SEM (n)†			
	Wild-type CheY‡		CheY92KR	
Relevant genotype*	Prestimulus	+Acetate	Prestimulus	+Acetate
Cells grown with acetate				
1. Δ(*cheA-cheZ*) *ace*$^+$	0.93 ± 0.04 (30)	0.04 ± 0.03 (31)	0.93 ± 0.04 (28)	0.14 ± 0.05 (30)
2. Δ(*cheA-cheZ*) Δ(*ackA pta*)	0.83 ± 0.06 (29)	0.27 ± 0.07 (29)	0.82 ± 0.05 (30)	0.75 ± 0.07 (30)
3. Δ(*cheA-cheZ*) Δ(*ackA pta*) Δ*acs*	0.69 ± 0.11 (15)	0.68 ± 0.16 (8)	0.89 ± 0.08 (12)	0.78 ± 0.20 (5)
Cells grown without acetate				
4. Δ(*cheA-cheZ*) *ace*$^+$	0.44 ± 0.06 (39)	0.00 ± 0.00 (32)	0.99 ± 0.00 (30)	0.03 ± 0.03 (30)
5. Δ(*cheA-cheZ*) Δ(*ackA pta*)	0.96 ± 0.03 (21)	0.99 ± 0.01 (31)	1.00 ± 0.00 (20)	1.00 ± 0.00 (14)
6. Δ(*cheA-cheZ*) Δ(*ackA pta*) Δ*acs*	0.77 ± 0.08 (25)	0.72 ± 0.14 (11)	0.92 ± 0.07 (14)	0.90 ± 0.06 (16)

*Host strains used were RBB1106, RBB1097, and RBB1109.
†Bias, fraction of time spent in CCW rotation; n, number of cells observed.
‡CheY proteins expressed from pRL22ΔZ.

binding of CheY-Ac and nonacetylated CheY to FliM to a similar degree (data not shown). These observations apparently rule out the hypothesis that acetylation enhances CheY binding to FliM. Interpretation of the binding assay is somewhat complicated by the mixture of CheY acetylation states present, including a prominent nonacetylated subpopulation (Fig. 2). However, the assay should have been sufficiently sensitive to detect enhanced FliM binding if that were responsible for the dramatic CW activity of CheY-Ac.

DISCUSSION

A Comprehensive Explanation for the Acetate Effect. The data reported here provide the final link in a line of inquiry begun a decade ago. In 1988, Wolfe *et al.* (7) reported that cells lacking chemotaxis proteins other than CheY rotated their flagella CW following exposure to acetate and suggested Acs and acetyl-AMP might be involved. In 1992, Barak *et al.* (11) significantly strengthened the case for the possible involvement of Acs with the twin revelations that Acs acetylated CheY and CheY-Ac had CW signaling ability in cell envelopes. However, in the same year, Lukat *et al.* (8) found that acetyl-P was a substrate for CheY autophosphorylation, thus supplying an apparently satisfying explanation for the acetate effect in familiar terms (namely activation of CheY by phosphorylation). Furthermore, Dailey and Berg (12) provided evidence in 1993 that Ack (and hence acetyl-P) was involved in the acetate effect. Resolution of the conflicting accounts regarding which branch of the acetate metabolism pathway is responsible for the CW response to acetate had to await construction of strains with the appropriate mutations. Deletions of *ackA* and *pta* had long been available (32), but it was not until 1995 that Kumari *et al.* (33) created a deletion of the *E. coli acs* gene. Recently, Barak *et al.* (13) used these deletion mutations to demonstrate that acetate provoked a CW response in strains lacking either branch of the acetate metabolic pathway but not in a strain lacking both branches. Thus the acetate effect may be mediated by either Ack/Pta or Acs. Our work completes the story by demonstrating that the Acs-mediated CW response to acetate is achieved by acetylation of CheY Lys-92. Depending on which acetate metabolism pathways are available, addition of acetate to cells results in synthesis of acetyl-P and/or acetyl-AMP, which in turn activate CheY for CW signal generation by phosphorylation or acetylation, respectively.

An essential feature of signal transduction strategies is that regardless of the biochemical mechanism utilized, a transient modification is required. Old information must be erased and replaced with new information on a time scale that is faster than changes in the environmental conditions being monitored. Activation of CheY by phosphorylation is such a mechanism. CheY-P rapidly autodephosphorylates, and dephosphorylation is further accelerated by another protein in the chemotaxis signaling pathway, CheZ (2). In contrast, activation of CheY by acetylation presents a logical difficulty as a signaling mechanism, because the experiments reported in Figs. 2 and 3 show that CheY-Ac is stable, yet the acetate effect is reversible (7). This apparent paradox has been resolved by the demonstration that CheY acetylation is reversible in the presence of Acs, AMP, and PP_i (R. Barak and M. Eisenbach, personal communication).

How Does Acetylation of Lys-92 Activate CW Signal Generation by CheY? Phosphorylation of Asp-57 is a well established means of activating CheY; acetylation of Lys-92 now must be considered as a potential second route. It is reasonable to assume that the structural consequences of these two CheY modifications share some features in common, given that both eventually result in the same behavior. Three aspects of the position of Lys-92 on the three-dimensional structure of CheY support the notion that modification of this residue could plausibly affect CW signal generation. First, Lys-92 is near the site of phosphorylation, Asp-57, and is located in a region of the molecule that NMR data indicate is affected by phosphorylation (34). Second, Lys-92 is located on the putative FliM binding surface (15, 35). Third, Lys-92 is located between secondary structural elements on a loop of CheY that exhibits some flexibility in both x-ray crystallographic and multidimensional NMR studies (36–39), perhaps indicative of an ability to assume alternate conformations.

Recent work has led to the proposal that the phosphorylation-induced CW signal generation pathway proceeds through a series of four separable steps: (*i*) phosphorylation of CheY, (*ii*) a change in conformation from CheY to CheY-P, (*iii*) binding of CheY-P to FliM, and (*iv*) switching the flagellar motor from CCW to CW rotation (3, 4, 40, 41). How might activation of CheY by acetylation fit into this scheme? One possibility is that the effect of acetylation depends on phosphorylation. For example, acetylation conceivably might promote Step (*i*) by inhibiting dephosphorylation of CheY-P or promote Step (*ii*) by stabilizing the phosphorylation-mediated conformational change. This class of models seem unlikely, as the CW activity of CheY-Ac has been observed both in cell envelopes and in intact cells under conditions where CheY-P is probably not formed to any significant degree because the primary sources of phosphoryl groups for CheY (the CheA kinase or acetyl-P) are absent. The FliM binding experiment apparently rules out the hypothesis that acetylation activates CheY at Step (*iii*) by enhancing the affinity of CheY-Ac for the flagellar switch (see *Results*). Thus we favor the possibility that acetylation of Lys-92 exerts its influence at Step (*iv*), a post-FliM binding step in signal transduction by CheY. The apparent action of acetylation at a step late in the CheY signaling process that is not shared with other response regulators would be consistent with the unique properties of CheY. Almost all known response regulator proteins other than CheY regulate transcription via covalently attached domains that have no homolog in CheY. Lys-92 is not conserved among response regulators (22), so acetylation is not a general mechanism for activating this class of signal transduction proteins.

How might acetylation of CheY act to change the direction of flagellar rotation [Step (*iv*)]? Replacement of Lys-92 with neutral or negatively charged amino acids did not result in constitutive CW signaling activity by CheY. This finding apparently argues against the hypothesis that neutralization of the positive charge on Lys-92 is sufficient to induce CheY to take on at least a critical subset of the structural features found in CheY-P. However, this is essentially a negative result in which the mutant side chains at residue 92 might have interfered with CheY activity. Alteration of electrostatic interactions has been suggested as a means for switching the direction of flagellar rotation (35), and it remains possible that charge change is an important feature of the mechanism by which acetylation activates CheY. Further experiments will be necessary to unravel the precise mechanism by which acetylation activates CheY.

Potential Physiological Role of CheY Acetylation. For reasons of technical convenience, both the original discovery of CW signaling by CheY-Ac (11) and our identification of Lys-92 as the critical acetylation site utilized *yeast* Acs to acetylate *E. coli* CheY *in vitro*. Our biochemical data (Fig. 3 and accompanying text) strongly argue that acetylation of Lys-92 by yeast Acs results in the CW activity of CheY-Ac observed in tethered *E. coli* envelopes (11). This discovery advances knowledge of CheY signaling mechanisms independent of physiological relevance. However, there is good evidence that the CheY modification mediated by the two *Saccharomyces cerevisiae* Acs enzymes, which share 47–49% amino acid sequence identity with *E. coli* Acs (42–44), is relevant. Our genetic data (Table 1) strongly argue that acetylation of Lys-92 by *E. coli*

Acs is responsible for the CW response to acetate exhibited by an *ack pta* strain expressing CheY (13).

The demonstrated roles of acetyl-P in regulating flagellar gene expression (45, 46) and of fumarate in switching the direction of flagellar rotation (47–49) provide precedence for linkage of physiological status to behavior. The physiological role of CheY acetylation is uncertain, but the possibilities are intriguing (A. J. Wolfe, personal communication). Motility and flagellar gene expression are maximal as *E. coli* makes the transition from exponential to stationary phase and attempts to find new nutrient sources (50). At the same time, the intracellular acetyl-P concentration is low, the extracellular acetate concentration because of excretion is high, and *acs* expression is induced (45, 51). Under such circumstances, acetyl-AMP might be a more effective means than acetyl-P by which to modulate CheY activity in relation to acetate metabolism. Irrespective of any physiological role, however, further investigation of CheY-Ac should lead to new perspectives and increased understanding of the mechanism of CheY activation.

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1. Larsen, S. H., Reader, R. W., Kort, E. N., Tso, W.-W. & Adler, J. (1974) *Nature (London)* **249,** 74–77.
2. Stock, J. B. & Surette, M. G. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology*, eds. Neidhardt, F. C., Curtiss, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. & Umbarger, H. E. (Am. Soc. Microbiol., Washington, DC), pp. 1103–1129.
3. Welch, M., Oosawa, K., Aizawa, S.-I. & Eisenbach, M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8787–8791.
4. Welch, M., Oosawa, K., Aizawa, S.-I. & Eisenbach, M. (1994) *Biochemistry* **33,** 10470–10476.
5. Berg, H. C. & Brown, D. A. (1972) *Nature (London)* **239,** 500–504.
6. Wolfe, A. J., Conley, M. P., Kramer, T. J. & Berg, H. C. (1987) *J. Bacteriol.* **169,** 1878–1885.
7. Wolfe, A. J., Conley, M. P. & Berg, H. C. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6711–6715.
8. Lukat, G. S., McCleary, W. R., Stock, A. M. & Stock, J. B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 718–722.
9. Sanders, D. A., Gillece-Castro, B. L., Stock, A. M., Burlingame, A. L. & Koshland, D. E., Jr. (1989) *J. Biol. Chem.* **264,** 21770–21778.
10. Barak, R. & Eisenbach, M. (1992) *Biochemistry* **31,** 1821–1826.
11. Barak, R., Welch, M., Yanovsky, A., Oosawa, K. & Eisenbach, M. (1992) *Biochemistry* **31,** 10099–10107.
12. Dailey, F. E. & Berg, H. C. (1993) *J. Bacteriol.* **175,** 3236–3239.
13. Barak, R., Abouhamad, W. N. & Eisenbach, M. (1998) *J. Bacteriol.* **180,** 985–988.
14. Bourret, R. B., Hess, J. F. & Simon, M. I. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 41–45.
15. Roman, S. J., Meyers, M., Volz, K. & Matsumura, P. (1992) *J. Bacteriol.* **174,** 6247–6255.
16. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367–382.
17. Bray, D., Bourret, R. B. & Simon, M. I. (1993) *Mol. Biol. Cell* **4,** 469–482.
18. Hess, J. F., Bourret, R. B. & Simon, M. I. (1991) *Methods Enzymol.* **200,** 188–204.
19. Schuster, M., Abouhamad, W. N., Silversmith, R. E. & Bourret, R. B. (1998) *Mol. Microbiol.* **27,** 1065–1077.
20. Bren, A., Welch, M. & Eisenbach, M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10090–10093.
21. Matsumura, P., Rydel, J. J., Linzmeier, R. & Vacante, D. (1984) *J. Bacteriol.* **160,** 36–41.
22. Volz, K. (1993) *Biochemistry* **32,** 11741–11753.
23. Lukat, G. S., Lee, B. H., Mottonen, J. M., Stock, A. M. & Stock, J. B. (1991) *J. Biol. Chem.* **266,** 8348–8354.
24. Bourret, R. B., Drake, S. K., Chervitz, S. A., Simon, M. I. & Falke, J. J. (1993) *J. Biol. Chem.* **268,** 13089–13096.
25. Brown, T. D. K., Jones-Mortimer, M. C. & Kornberg, H. L. (1977) *J. Gen. Microbiol.* **102,** 327–336.
26. Kuo, S. C. & Koshland, D. E., Jr. (1989) *J. Bacteriol.* **171,** 6279–6287.
27. Scharf, B. E., Fahrner, K. A., Turner, L. & Berg, H. C. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 201–206.
28. Stock, J. B., Surette, M. G., Levit, M. & Park, P. (1995) in *Two-Component Signal Transduction*, eds. Hoch, J. A. & Silhavy, T. J. (Am. Soc. Microbiol., Washington, DC), pp. 25–51.
29. Moore, J. B., Shiau, S.-P. & Reitzer, L. J. (1993) *J. Bacteriol.* **175,** 2692–2701.
30. Pazour, G. J., Ta, C. N. & Das, A. (1992) *J. Bacteriol.* **174,** 4169–4174.
31. Stewart, R. C. (1993) *J. Biol. Chem.* **268,** 1921–1930.
32. LeVine, S. M., Ardeshir, F. & Ames, G. F. L. (1980) *J. Bacteriol.* **143,** 1081–1085.
33. Kumari, S., Tishel, R., Eisenbach, M. & Wolfe, A. J. (1995) *J. Bacteriol.* **177,** 2878–2886.
34. Lowry, D. F., Roth, A. F., Rupert, P. B., Dahlquist, F. W., Moy, F. J., Domaille, P. J. & Matsumura, P. (1994) *J. Biol. Chem.* **269,** 26358–26362.
35. Sockett, H., Yamaguchi, S., Kihara, M., Irikura, V. M. & Macnab, R. M. (1992) *J. Bacteriol.* **174,** 793–806.
36. Bellsolell, L., Prieto, J., Serrano, L. & Coll, M. (1994) *J. Mol. Biol.* **238,** 489–495.
37. Volz, K. & Matsumura, P. (1991) *J. Biol. Chem.* **266,** 15511–15519.
38. Moy, F. J., Lowry, D. F., Matsumura, P., Dahlquist, F. W., Krywko, J. E. & Domaille, P. J. (1994) *Biochemistry* **33,** 10731–10742.
39. Santoro, J., Bruix, M., Pascual, J., Lopez, E., Serrano, L. & Rico, M. (1995) *J. Mol. Biol.* **247,** 717–725.
40. Zhu, X., Amsler, C. D., Volz, K. & Matsumura, P. (1996) *J. Bacteriol.* **178,** 4208–4215.
41. Zhu, X., Rebello, J., Matsumura, P. & Volz, K. (1997) *J. Biol. Chem.* **272,** 5000–5006.
42. Blattner, F. R., Burland, V., Plunkett, G., III, Sofia, H. J. & Daniels, D. L. (1993) *Nucleic Acids Res.* **21,** 5408–5417.
43. De Virgilio, C., Burckert, N., Barth, G., Neuhaus, J. M., Boller, T. & Wiemken, A. (1992) *Yeast* **8,** 1043–1051.
44. Van den Berg, M. A. & Steensma, H. Y. (1995) *Eur. J. Biochem.* **231,** 704–713.
45. Prüss, B. M. & Wolfe, A. J. (1994) *Mol. Microbiol.* **12,** 973–984.
46. Shin, S. & Park, C. (1995) *J. Bacteriol.* **177,** 4696–4702.
47. Montrone, M., Oesterhelt, D. & Marwan, W. (1996) *J. Bacteriol.* **178,** 6882–6887.
48. Barak, R. & Eisenbach, M. (1992) *J. Bacteriol.* **174,** 643–645.
49. Barak, R., Giebel, I. & Eisenbach, M. (1995) *Mol. Microbiol.* **19,** 139–144.
50. Amsler, C. D., Cho, M. & Matsumura, P. (1993) *J. Bacteriol.* **175,** 6238–6244.
51. Shin, S., Song, S. G., Lee, D. S., Pan, J. G. & Park, C. (1997) *FEMS Microbiol. Lett.* **146,** 103–108.