# Acetyladenylate or Its Derivative Acetylates the Chemotaxis Protein CheY in Vitro and Increases Its Activity at the Flagellar Switch†

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ABSTRACT: CheY, a key protein in the mechanism of bacterial chemotaxis, is known to interact with the flagellar switch and thereby cause clockwise rotation. This activity of CheY was significantly increased by producing acetyladenylate (AcAMP) within cytoplasm-free bacterial envelopes containing purified CheY. This was achieved by including in the envelopes the enzyme acetyl-CoA synthetase (ACS) and ATP, and adding acetate externally. The fraction of clockwise-rotating envelopes, tethered to glass by their flagella, increased from 14% to 58% by the presence of AcAMP (or its derivative). In parallel experiments carried out with [$^{14}$C]acetate under similar conditions, CheY became acetylated: [1-$^{14}$C]acetate was as effective as [2-$^{14}$C]acetate in labeling CheY, and ACS-dependent labeling of CheY by [$\alpha$-$^{32}$P]ATP was not detected. The switch proteins, FliG, FliM, and FliN, isolated to purity, were not acetylated. The acetylation was specific for CheY and dependent on its native conformation. The acetylated form the CheY was estimated to be more active than its nonacetylated form by 4–5 orders of magnitude. Acetylated CheY was stable in the presence of the strong nucleophiles hydroxylamine or ethanolamine, indicative of N-acetylation. There was a correlation between the activity of CheY in vivo and its ability to be acetylated in vitro. Thus, proteins with a single substitution at their active site, CheY57DE and CheY109KR, are not active in vivo and accordingly were not acetylated in vitro; in contrast, the protein CheY13DK is active in vivo and was normally acetylated in vitro. The possibility that CheY acetylation plays a role in bacterial chemotaxis is discussed.

Bacteria such as *Escherichia coli* and *Salmonella typhimurium* can respond to chemical changes in their environment by altering their flagellar rotation. Attractants or repellents increase the probability of counterclockwise (CCW)[1] or clockwise (CW) rotation, respectively, resulting in migration toward higher attractant concentrations or away from a repellent source [see Eisenbach (1991), Macnab (1987), and Stewart and Dahlquist (1987) for recent general reviews on bacterial chemotaxis]. The direction of flagellar rotation is determined by a switch at the base of the flagellar motor. Signaling between the receptors, which sense the chemotactic stimuli, and the switch is done by a sophisticated processing system, which involves chemical interactions between cytoplasmic chemotaxis proteins as well as three chemical modifications: phosphorylation of the chemotaxis proteins CheA, CheB, and CheY; methylation of the membrane receptors; and deamidation of the latter [see Bourret et al. (1991), Eisenbach (1991), and Stewart and Dahlquist (1987) for recent reviews]. The actual interaction with the switch is done by the CheY protein with a resultant CW rotation. This was shown by second-site suppression analysis (Parkinson et al., 1983; Yamaguchi et al., 1986), by expression or overexpression of CheY within bacteria missing the other cytoplasmic chemotaxis proteins (Clegg & Koshland, 1984; Wolfe et al., 1987), and by inclusion of purified CheY within an in vitro system (Ravid et al., 1986) made up of cytoplasm-free, bacterial envelopes with functional flagella (Eisenbach & Adler, 1981; Eisenbach & Matsumura, 1988). The latter approach indicated that the interaction between CheY and the switch is direct, without any mediators (Ravid et al., 1986).

Studies with CheY-containing envelopes implied that CheY may be in active and nonactive forms, the activity defined as the ability of CheY to interact with the switch and cause CW rotation [Eisenbach & Matsumura, 1988; Ravid et al., 1986; see Eisenbach (1991) for a review]. The mechanism which regulates this activity is not yet known. Two recently discovered phenomena may be part of this mechanism: (i) CheY phosphorylation by CheA (Hess et al., 1987, 1988; Wylie et al., 1988). This phosphorylation has been detected so far only in vitro (Stock et al., 1989), but its extent and rate were shown to be modulatable by chemotactic stimuli in an in vitro system consisting of receptor-containing vesicles and the chemotaxis proteins CheY, CheA, and CheW (Borkovich et al., 1989; Ninfa et al., 1991). The phosphorylated form of a CheY molecule is about 100-fold more active at the switch than the nonphosphorylated form (Barak & Eisenbach, 1992). However, this higher activity requires additional cytoplasmic constituents, the identity of which is not known (Barak & Eisenbach, 1992). (ii) Acetate increases the probability of CW rotation in vivo in bacterial cells of "gutted" strains (i.e., strains deleted for the receptors and for the cytoplasmic chemotaxis proteins) containing intact CheY. This phenomenon was attributed to acetylaldenylate (AcAMP) formed from acetate by the enzyme acetyl-CoA synthetase (ACS)

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[1] Abbreviations: AcAMP, acetyladenylate; ACS, acetyl-CoA synthetase; CCW, counterclockwise; CW, clockwise; IPTG, isopropyl $\beta$-D-thiogalactopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Table I: Bacterial Strains and Plasmids Used in This Study

| strain/plasmid | relevant characteristics | source/reference |
|---|---|---|
| *E. coli* strains | | |
| BL21(DE3)pLysS | host | Studier et al. (1990) |
| KO641*recA* | Δ*cheY* mutant | Bourret et al. (1990) |
| RP437 | wild type for chemotaxis | Parkinson (1978) |
| RP1616 | Δ*cheZ* mutant | J. S. Parkinson |
| RP5943 | *tsr* mutant | J. S. Parkinson |
| *S. typhimurium* | | |
| ST1 | wild type for chemotaxis | Aswad & Koshland (1975) |
| plasmids | | |
| pDV4 | carrying *cheA* and *cheW* | P. Matsumura |
| pKOT113 | carrying fliG | K. Oosawa, T. Ueno, & S.-I. Aizawa |
| pKOT179 | carrying *fliM* and *fliN* | K. Oosawa, T. Ueno, & S.-I. Aizawa |
| pRBB40.13DK | carrying *cheY13DK* | Bourret et al. (1990) |
| pRBB40.57DE | carrying *cheY57DE* | Bourret et al. (1990) |
| pRBB40.109KR | carrying *cheY109KR* | R. B. Bourret & M. I. Simon |
| pRL22(Δ*PvuII*) | carrying *cheY* without *cheZ* | P. Matsumura |

(Wolfe et al., 1988):

$$\mathrm{Ac} + \mathrm{ATP} \overset{\mathrm{ACS}}{\rightleftarrows} \mathrm{AcAMP} + \mathrm{PP_i} \qquad \text{(i)}$$

AcAMP is functional only as a complex with the enzyme (Berg, 1956; Londesborough & Webster, 1974; Metzler, 1977; Stadtman, 1973; Webster, 1967). When CoA is available, the complex interacts with it to form acetyl-CoA:

$$\mathrm{AcAMP} + \mathrm{CoA} \overset{\mathrm{ACS}}{\rightleftarrows} \mathrm{AcCoA} + \mathrm{AMP} \qquad \text{(ii)}$$

Here we report that AcAMP (or a closely-related substance) acetylates CheY and significantly increases its activity. This acetylation of CheY is the fourth type of chemical modification which may be involved in bacterial chemotaxis.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains and Plasmids.* The strains and plasmids used in this study are listed in Table I. Bacteria were grown at 35 °C in tryptone broth (*E. coli*) or nutrient broth (*S. typhimurium*) as described (Eisenbach & Adler, 1981).

*Preparation of Cell Envelopes.* Cell envelopes were isolated from strain ST1 by penicillin treatment and subsequent osmotic lysis as described (Ravid & Eisenbach, 1984a), except that the lysis medium and the flow medium contained 50 mM Tris-HCl (pH 7.9), 5 mM $MgSO_4$, and 0.1 mM tetraethylenepentamine (Tetren). Where indicated, CheY (16 μM), ATP (4 mM), and acetyl-CoA synthetase (ACS, Sigma) (0.04 unit/mL) were inserted into the envelopes by their inclusion in the lysis medium. Sodium acetate (0.5 mM) was added externally by inclusion in the flow medium where indicated. Flagellar rotation was assayed at room temperature by the tethering technique (Silverman & Simon, 1974) as described (Ravid & Eisenbach, 1983), using a flow chamber (Berg & Block, 1984). For observing the rotation, the flow medium was supplemented with DL-lactic acid (2 mM). Rotating tethered envelopes were examined for lack of cytoplasmic remnants as described (Ravid & Eisenbach, 1984b).

*Preparation of Vesicles, Liposomes, and Cell-Free Extract.* Inside-out membrane vesicles were prepared by French pressure cell as described (Barak & Eisenbach, 1992). Liposomes were prepared from lipids extracted from wild-type cells: RP437 cells were disrupted to completion by sonication (Branson Sonifier; $^3/_8$th-in. tip); the membrane fraction of the sonicate was obtained by centrifugation (90000*g* for 1 h at 4 °C), and lipids were extracted from it by the chloroform/methanol/water technique according to Bligh and Dyer (1959), except that we used a ratio of 8:1:1; liposomes were obtained from these lipids by sonication in a Laboratory Supplies bath-type sonicator (Blumenthal et al., 1977) and kept in liquid nitrogen. The phospholipid content of the liposomes was estimated by phosphorus determination (Bottcher et al., 1961). For the preparation of cell-free extract, RP437 cells were grown overnight in Luria broth (L broth), washed in Tris-HCl (50 mM, pH 7.9), and sonicated (Branson sonifier). The resulting extract was centrifuged at 110000*g* for 1 h at 4 °C to spin down unlysed cells and membrane fragments, yielding a supernatant of cell-free extract.

*Protein Purification.* CheA and CheY were isolated and purified as described (Barak & Eisenbach, 1992). CheY13DK, CheY57DE, and CheY109KR were overproduced in KO-641*recA* from the plasmids indicated in Table I and purified as wild-type CheY. The switch proteins were overproduced in BL21(DE3)pLysS from the plasmids indicated in Table I. BL21(DE3)pLysS cells were grown in L broth supplemented with chloramphenicol (30 μg/mL) and ampicillin (50 μg/mL) with good aeration at 37 °C. When the cells reached $OD_{590}$ = 0.5, overproduction was induced by isopropyl β-D-thiogalactopyranoside (IPTG, 1 mM final concentration). After 2 additional h of growth, the cells were harvested and washed in TE buffer (50 mM Tris-HCl, pH 7.9, and 1 mM EDTA) and then disrupted by sonication (Branson sonifier) to completion. For both plasmid-containing strains, inclusion bodies and membrane vesicles were pelleted by centrifugation (114000*g* for 1 h). From this stage, two purification procedures were used.

(*i*) *Purification to Homogeneity.* The pellet was washed once in TE buffer, recentrifuged, resuspended in 6 M urea to solubilize the proteins, and recentrifuged as before. The supernatant was dialyzed 3 times (each time for 2–4 h, the last dialysis for overnight) against 2 L of TE buffer and concentrated to a final volume of ca. 30 mL by reverse dialysis against dry poly(ethylene glycol) ($M_r$ = 20 000). Proteins were isolated by ammonium sulfate (40% saturation) precipitation and applied to a Sephadex G-50 column. A broad single peak was eluted from the column in both FliG and FliM-N cases, collected, and concentrated prior to resolution by HPLC. The concentrated samples were applied to a reversed-phase C-18 Vydac column in 10% acetonitrile and eluted in a linear 20–80% gradient of this solvent. The peaks containing the switch proteins were collected, lyophilized, and resuspended in TE buffer. The FliG, FliM, and FliN proteins were eluted out as single peaks, judged pure by one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

(*ii*) *Enrichment under Mild Conditions.* Partial purification, without a denaturation step, was achieved by titrating

ammonium sulfate (usually 10–15% saturation) to the soluble fraction obtained after the first centrifugation step (prior to the addition of urea). The switch proteins comprised 60% or 30% of the total protein content in this preparation of FliM + FliN or FliG, respectively, as determined by the 215-nm absorbance of the HPLC fractions and by densitometry of the gel.

*Protein Labeling by [$^{14}C$]Acetate.* The reaction mixture contained, as indicated for each experiment, various combinations of 50 mM Tris-HCl (pH 7.9), $MgSO_4$ (5 mM), Tetren (0.1 mM), CheY (140–200 $\mu$M, assuming a size of 14 kDa), CheA (40 $\mu$M, assuming a size of 156 kDa), ATP (0.5–5 mM), ACS (0.35–2.5 units/mL, equivalent to 0.2–0.5 mg of protein/mL, depending on the batch of enzyme used), sodium [1-$^{14}$C]- or [2-$^{14}$C]acetate (unless otherwise specified, 1.6–2 mM, 52–59 mCi/mmol), and membrane vesicles (4 $\mu$L, 8.7 mg of protein/mL) in a total volume of 40 $\mu$L. The reaction mixture was incubated for 2 h at room temperature or at 37 °C as indicated. The reaction was terminated by 10 $\mu$L of SDS sample buffer. The samples were subjected to SDS–PAGE (12% polyacrylamide gel), after which the gels were stained by Coomassie blue, destained, dried, and autoradiographed. Alternatively, the gels were counted for 1–4 h by a BIODET system, as described below. Protein concentration was determined by biuret, using bovine serum albumin and $\gamma$-globulin as standards.

*B-Counting by BIODET.* The BIODET system, a bi-dimensional imaging device for mapping biological samples labeled with $\beta$-emitters, was developed at the Weizmann Institute of Science by A. Breskin, E. Dafni, and R. Chechik of the Department of Nuclear Physics. The system is comprised of a multistage gaseous proportional detector and a bi-dimensional localization readout with signal processing electronics and PC-based data acquisition software. It offers an ultimate localization resolution of 0.4 mm (FWHM) for $^{14}$C $\beta$-electrons emitted from a flat sample coupled to the detector's window. The efficiency of the system is typically 60% (over $2\pi$) for $^{14}$C-labeled samples. It is fully linear and digital, and thus provides reliable absolute and relative counting rates from the imaged samples, over a broad dynamic range of sample activity levels. The data are stored in the computer's memory for further analysis and reference, while the gel remains intact and can be reanalyzed by the same technique or by films. The BIODET system is 50–100 times faster than films.

## RESULTS

*Effect of AcAMP on Flagellar Rotation in Envelopes.* In order to examine the effect of AcAMP (or its derivative) on the rotation of tethered envelopes, we had to insert this compound into them. However, because AcAMP (including chemically-synthesized AcAMP) is functional only as a complex with the enzyme (Berg, 1956; Londesborough & Webster, 1974; Metzler, 1977; Stadtman, 1973; Webster, 1967), it was pointless to include pure AcAMP in the envelopes. Instead, we generated it continuously within the envelopes by including in them an AcAMP-generating system. The generating system consisted of the enzyme ACS from yeast (because of the unavailability of the enzyme from *E. coli*), ATP, and $Mg^{2+}$ (see reaction i). Acetate was added externally. (ATP was included in the lysis medium rather than added externally because it is membrane-impermeant.) The envelopes presumably do not contain CoA (each rotating envelope is assayed for lack of cytoplasmic remnants), as a result of which reaction ii cannot occur and the major product of the reaction should be AcAMP. Since the magnitude of the fraction of CW-rotating envelopes depends on the intracellular concentration of CheY and pH (Ravid et al., 1986), we kept in this study the concentration of CheY and the pH constant (16 $\mu$M and pH 7.9), conditions under which the rotation of 14 ± 4% of the rotating envelopes was in the CW direction (the rest 86% rotated CCW). As shown in Figure 1, the fraction of CW-rotating envelopes was significantly larger when the envelopes contained, in addition to CheY, the AcAMP-generating system. As a matter of fact, under these conditions, more than half of the envelopes rotated CW. This effect of AcAMP (or its derivative) was totally dependent on the presence of CheY in the envelopes (Figure 1). This in vitro observation endorses and extends the in vivo observation of Wolfe et al. (1988) described in the introduction. It should be pointed out that, as in the case of envelopes containing just CheY (Ravid et al., 1986), all the envelopes in this study rotated exclusively in one direction, either CCW or CW, and they never reversed.

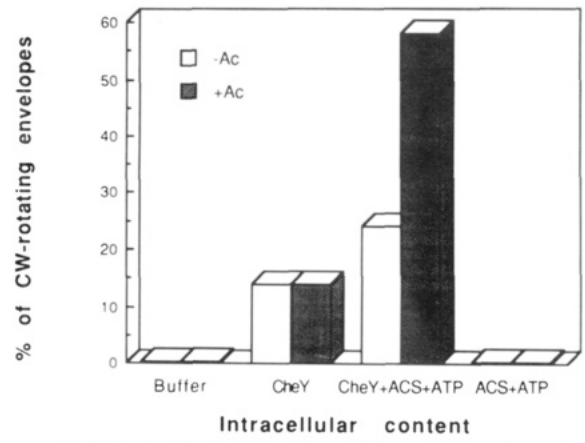


FIGURE 1: Effect of AcAMP on the rotation of tethered envelopes. The experimental conditions were as detailed under Experimental Procedures. The total number of rotating envelopes (each rotating envelope constituted a separated determination) under each set of conditions was considered as 100%. These were, from left to right, >3000, 189, 81, and 26. In 4 out of 10 experiments, the addition of lactate (as an energy source) to envelopes containing an AcAMP-generating system was sufficient for increasing the fraction of CW-rotating envelopes, possibly as a result of the action of the membrane-bound lactate oxidase which converted lactate to acetate. This is the reason for the somewhat higher fraction of CW-rotating envelopes in the absence of acetate in envelopes containing CheY + ACS + ATP (relative to the control of CheY-containing envelopes). A similar effect of lactate was observed in vivo (Wolfe et al., 1988). It should be noted that the concentration of acetate (0.5 mM) used in this study was below the concentration range at which acetate causes rotating envelopes to stop (Eisenbach et al., 1990).

Since AcAMP in the presence of ACS acetylates CoA (reaction ii), we examined the possibility of acetylation of a chemotaxis protein by AcAMP. Because the effect of AcAMP in envelopes (Figure 1) and in intact bacteria (Wolfe et al., 1988) was dependent on the presence of CheY, and because CheY interacts with the flagellar switch, we considered CheY and the switch proteins as the best candidates for acetylation.

*CheY Is Acetylated by AcAMP.* We incubated [2-$^{14}$C]-acetate with various combinations of CheY, ATP (in the presence of $Mg^{2+}$), and ACS, and looked for labeling of the proteins. The incubation time was 2 h, like the average period of time between the formation of envelopes (i.e., the lysis time of the penicillin-treated cells) and the observation of flagellar rotation. As shown in Figure 2, which contains the coomassie blue-stained gel and its autoradiogram, ACS was labeled by acetate (lane 3) and in the presence of CheY it transferred the label to the latter (lane 4). The labeling of CheY by

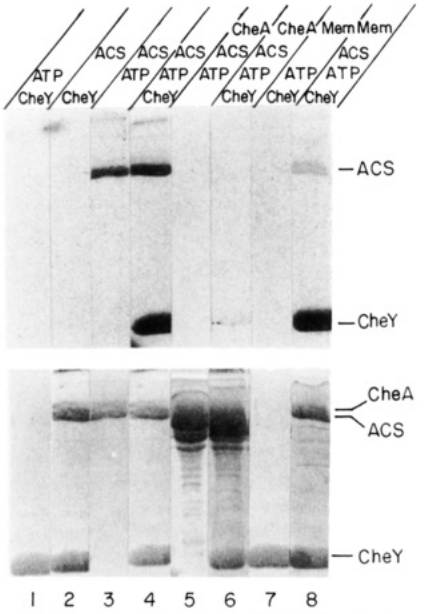


FIGURE 2: Electrophoretic assay of CheY labeling by [$^{14}$C]acetate in the presence of ACS and 5 mM ATP. The reaction mixture contained combinations of CheY, CheA, ATP, sodium [2-$^{14}$C]acetate, and membrane vesicles, as indicated at the top of the figure. The procedure (carried out at room temperature) and the concentrations were as given under Experimental Procedures. Lower panel, the Coomassie blue-stained gel; upper panel, autoradiogram of the same gel.

labeled acetate was fully dependent on the presence of ACS and ATP (lane 4 vs lanes 1 and 2, respectively). CheA, the kinase of CheY, inhibited the labeling of ACS by acetate (lane 5) and consequently hampered the labeling of CheY (lane 6). CheA itself was not labeled. Therefore, we tried first to label ACS for 1 h and only then to add CheA and CheY to the reaction mixture. The results were similar: CheA did not become labeled, ACS remained labeled and the label on CheY was only 27% of that in the absence of CheA (determined by BIODET). In a similar type of experiment, CheY was only 10% labeled when added 30 min after CheA. To make the conditions as close as possible to those within the envelopes, the experiments were repeated in the presence of inside-out membrane vesicles. The labeling of CheY in the presence of the vesicles (lane 8) was similar to (sometimes more than) that observed in their absence (lane 4). To confirm that the label was indeed transferred from ACS to CheY, we reduced the concentration of ATP 10-fold (Figure 3). Under these conditions, a reduction in the intensity of the label of ACS could be observed concomitantly with the labeling of CheY (lane 3 vs. lane 2).

To distinguish between acetylation and methylation of CheY, we repeated the experiment shown in Figure 2 but we used [1-$^{14}$C]acetate instead of [2-$^{14}$C]acetate. The results (not shown) were the same as in Figure 2, indicating that both carbon atoms of acetate become associated with ACS and then with CheY. To examine possibilities of adenylation of CheY or of adsorption of AcAMP to CheY, we incubated, under the conditions of Figure 2, [$\alpha$-$^{32}$P]ATP and cold acetate with CheY and ACS. As shown in Figure 4, some ATP was bound or adsorbed to CheY in the absence of ACS [cf. Kar et al. (1988), Smith et al. (1988), and Wong et al. (1988)], and addition of ACS not only did not increase the labeling of CheY but rather decreased it. These results indicate that ACS catalyzed the acetylation of CheY rather than any other process or chemical modification.

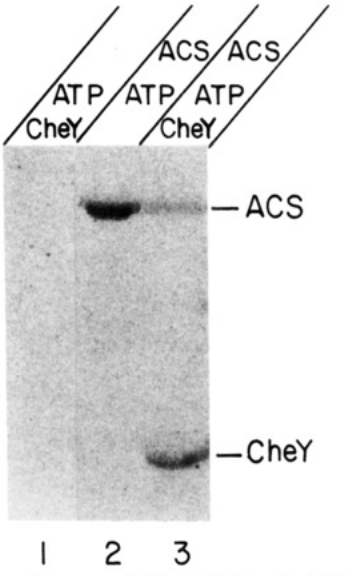


FIGURE 3: Autoradiogram of CheY labeling by [$^{14}$C]acetate in the presence of ACS and 0.5 mM ATP. The experiment was carried out at room temperature as described under Experimental Procedures.

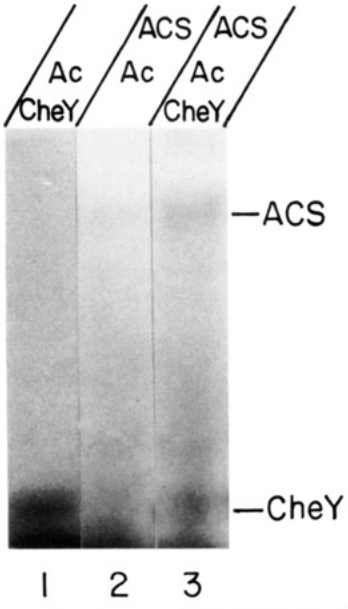


FIGURE 4: Electrophoretic assay of CheY labeling by [$\alpha$-$^{32}$P]ATP. The labeling procedure with [$\alpha$-$^{32}$P]ATP (carried out for 2 h at room temperature) was as for labeling with [$^{14}$C]acetate (as described under Experimental Procedures), except that the reaction mixture contained combinations of CheY (200 $\mu$M), ACS (1.4 units/mL), sodium acetate (2 mM), and [$\alpha$-$^{32}$P]ATP (1 mM, specific activity 40 mCi/$\mu$mol) as indicated in the figure.

The intensity of the label on ACS was dependent on the concentrations of the substrates, and the acetylation of CheY was dependent on the enzyme concentration (Figure 5), indicating that the sequence of events is

$$\text{Ac} + \text{ATP} + \text{ACS} \rightleftarrows \text{AcAMP·ACS} + \text{PP}_i \quad \text{(iii)}$$

$$\text{AcAMP·ACS} + \text{CheY} \rightleftarrows \text{AcCheY} + \text{AMP} + \text{ACS} \quad \text{(iv)}$$

With 150 $\mu$M CheY, 5 mM ATP, 10 mM acetate, and 2.5 units/mL ACS, the level of acetylation (i.e., the stoichiometry between acetylated CheY and total CheY) was 5.5% or 31% at 24 or 37 °C, respectively. However, under the exact conditions of the assays with envelopes, the concentration of ACS was 62-fold lower, and those of CheY and acetate were 10-fold and 20-fold lower, respectively. Therefore, the extent of CheY labeling under these conditions was undetectable.

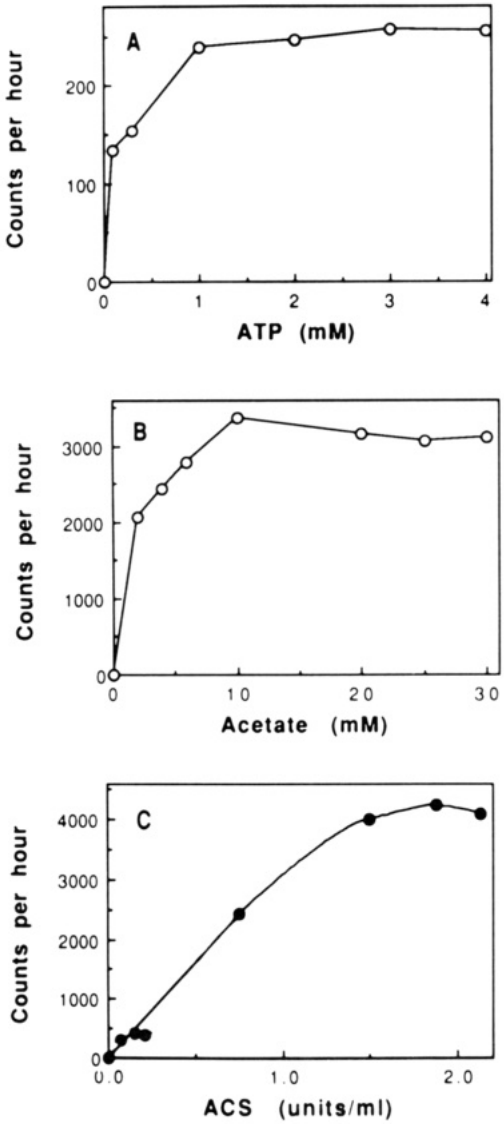


FIGURE 5: Dependence of labeling on the concentrations of the substrates and the enzyme. The experiments were carried out at room temperature as described under Experimental Procedures. The counts per hour are those made by the BIODET system; 100 counts per hour are equivalent to 170 dpm. The specific activity of the [2-$^{14}$C]acetate was 52 Ci/mol. (○) ACS labeling; (●) CheY labeling. (A) Dependence of ACS labeling by [$^{14}$C]acetate on the ATP level. The acetate and ACS levels were 1.9 mM and 1.3 units/mL, respectively. The $MgSO_4$ concentration was 3-fold higher than the given ATP concentration. (B) Dependence of ACS labeling on the [$^{14}$C]acetate level. The ACS, ATP, and $MgSO_4$ concentrations were 1.5 units/mL, 2 mM, and 10 mM, respectively. (C) Dependence of CheY labeling on the ACS level. The concentrations used were 10 mM [$^{14}$C]acetate, 10 mM $MgSO_4$, 2 mM ATP, and 140 $\mu$M CheY.

On the basis of titration curves like those shown in Figure 5, the acetylation level of CheY in the envelopes was estimated to be 0.01%. The significance of this calculated value is discussed below.

*The Switch Proteins Are Not Acetylated by AcAMP.* The switch proteins FliM, FliN, and FliG were isolated and purified to homogeneity as described under Experimental Procedures, and then examined for their ability to be acetylated. As shown in Figure 6, none of the switch proteins became labeled nor did they affect the ability of added CheY to be acetylated, independently of whether they were examined individually or mixed together. On the basis of the data of the HPLC column used for the purification of the switch proteins, it is possible that the switch proteins are hydrophobic. Therefore, the assay was carried out in the presence of liposomes derived from *E.*

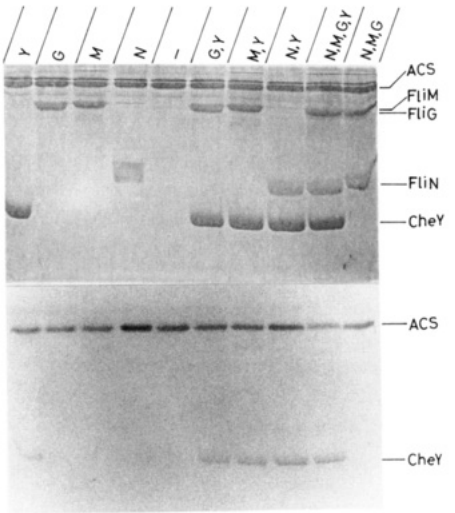


FIGURE 6: Ability of the switch proteins to be acetylated. The switch proteins (50 $\mu$g of each), purified to homogeneity as described under Experimental Procedures, were incubated under the acetylating conditions either alone or in the combinations indicated. Liposomes (corresponding to 50–300 ng of phosphate as phospholipids) were added to each reaction mixture. The other experimental conditions were as described under Experimental Procedures for CheY acetylation, except that reagents were added in amounts to accommodate a reaction volume of 60 $\mu$L rather than 40 $\mu$L. Incubation was for 2.5 h at room temperature. Note that ACS from Sigma usually has two bands. In Figures 2 and 7, the two bands are close together and may seem to be one broad band. Here the bands are more separated. This difference may make the impression of appearance of new bands, but this is not the case. Upper panel, gel; Lower panel, autoradiogram.

*coli* membranes. However, the presence or absence of liposomes made no difference with regard to the acetylation. To eliminate the possibility that the switch proteins were not labeled due to loss of their native conformation, we (a) refolded them by addition of urea to 6 M concentration followed by gradual dilution to 0.5 M and (b) enriched them under mild conditions (instead of purification to homogeneity) as described under Experimental Procedures.[2] Here, too, very little labeling was observed, independently of whether CheY was present or not. To examine whether our renaturation conditions were effective, CheY was denatured by urea and put through the same refolding process as were the switch proteins. The ability of CheY to be acetylated remained unimpaired. In order to establish the ability of the switch proteins to be acetylated in a more physiological environment, we examined the membrane fractions for labeling by [$^{14}$C]acetate. To this end, we removed the inclusion bodies from the sonicate of the overproducer by low-speed centrifugation and only then sedimented the membrane fraction as detailed under Experimental Procedures. In no case was labeling, other than that of added CheY and ACS, observed.

*Specificity of the Acetylation.* To determine the specificity of the acetylation, we examined several other proteins for their ability to be acetylated under the conditions of CheY acetylation. We have already found that under these conditions CheA, FliG, FliM, and FliN are not acetylated (Figures 2 and 6). We tested, on the one hand, a cell-free extract of RP437 and, on the other hand, arbitrarily-chosen proteins at concentrations (in milligrams per milliliter) higher than CheY. In four out of six experiments, neither ACS nor any of the proteins in the extract were labeled. Apparently, a constituent of the extract perturbed the labeling of ACS but not its activity,

[2] When these procedures were not used, purified FliM was insoluble in buffer and had to be used in suspension.

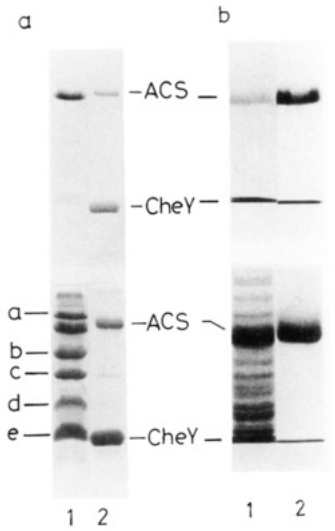


FIGURE 7: Ability of irrelevant proteins to be acetylated. Lower panels, the Coomassie blue-stained gels; upper panels, autoradiograms of the gels. (A) Ability of cell-free extract of RP437 to be acetylated. Lane 1 contained (in addition to acetate, ACS, and ATP as in Figure 2) 100 $\mu$g of cell-free extract and 0.9 $\mu$g of added CheY. Lane 2 contained 0.9 $\mu$g of CheY and no extract for comparison. (B) Ability of molecular weight markers, used here as irrelevant proteins, to be acetylated. Lane 1 contained 20 $\mu$g of each of the proteins phosphorylase *b*, ovalbumin, carbonic anhydrase, trypsin inhibitor, and $\alpha$-lactalbumin (designated as a–e, respectively). Lane 2 contained 9 $\mu$g of CheY for comparison. The experiment was carried out at 37 °C.

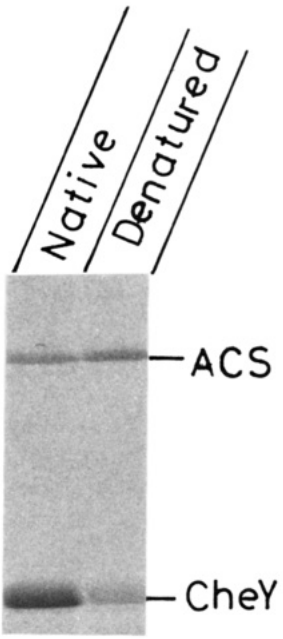


FIGURE 8: Comparison between the abilities of native and denatured CheY to be acetylated. The experiment was carried out as in Figure 2. The intensity of the label on denatured CheY was 27% of the label intensity on native CheY (as determined by BIODET).

because externally-added CheY was labeled by ACS even in the presence of the extract. In the other two experiments, ACS was labeled, but as shown in Figure 7a, labeling could not be detected in any of the proteins of the extract. Similarly, phosphorylase *b* ($M_r$ 94 000), ovalbumin ($M_r$ 43 000), carbonic anhydrase ($M_r$ 30 000), and trypsin inhibitor ($M_r$ 20 100), all at a concentration higher than that of CheY, did not become acetylated under the conditions of this study; $\alpha$-lactalbumin ($M_r$ 14 400) was very slightly labeled (Figure 7b). Lysozyme ($M_r$ 14 400), on the other hand, was labeled but to a lesser extent than CheY (22 ± 12% of the label on CheY, ±SD, 10 experiments; not shown in the figure). Thus, out of the 10 proteins (other than CheY) examined for being acetylated, and many more proteins in the cell-free extract [some of which were at concentrations similar to that of CheY (Figure 7a)], only 1 protein became acetylated but to a lesser extent. Therefore, although the reason for the low-level acetylation of lysozyme is not yet known, it seems that the acetylation of CheY by AcAMP is specific.

*The Ability of CheY To Be Acetylated Depends on Its Conformation.* To determine the dependence of the acetylation on the conformation of CheY, we unfolded the protein by boiling. As shown in Figure 8, 5 min of boiling was sufficient to reduce the ability of the protein to be acetylated to one-fourth of its original level. Boiling for 30 min was as efficient as 5 min. This indicates that CheY acetylation is dependent on the conformation of the protein.

*Distinction between N-Acetylation and O-Acetylation.* Acetylation of proteins commonly takes place at the amino group of the N-terminus or at the $\epsilon$-amino group of specific lysine residues (Allfrey et al., 1984). However, in principle, O-acetylation can also occur on serine, threonine, or tyrosine residues (Riordan & Vallee, 1967a). One way to distinguish between N-acetylation and O-acetylation is to examine the resistance of the acetylated protein to strong nucleophiles such as hydroxylamine or ethanolamine [see, e.g., Riordan and Vallee (1967a)]. For this purpose, we labeled CheY for 1 h by [2-$^{14}$C]acetate as described under Experimental Procedures. Then we separated the proteins (CheY and ACS) from the low molecular weight substances by centrifugation of the reaction mixture through a Sephadex G-50 column at 91*g* for 30 s, and treated them with hydroxylamine (1 M, pH 7.9) or ethanolamine (0.3 M) for 90 min. Even though 10 min should be sufficient for label removal, e.g., from *O*-acetyltyrosine (Riordan & Vallee, 1967b), neither hydroxylamine nor ethanolamine had a significant effect on the acetylation level of CheY: the level was 106 ± 22% or 96 ± 12% in the presence of 1 M hydroxylamine or 0.3 M ethanolamine, respectively (three experiments each). However, when hydroxylamine was added as a control at the beginning of the acetylation, i.e., when CheY was incubated with ACS in the presence of hydroxylamine, the acetylation was prevented. This indicates that CheY is acetylated on an N residue. Since the acetylation was found to be dependent on the conformation of CheY, it is perhaps reasonable to presume that the acetylation site is on a lysine residue rather than on the N-terminus.

*CheY109KR Is Not Acetylated.* A likely candidate for the acetylated residue in CheY may be Lys109, which has been shown to be conserved in all the regulators which belong to the CheY family (Stock et al., 1989). Moreover, a *cheY* mutant that has a CheY protein in which arginine replaces Lys109 (CheY109KR) is nonchemotactic in spite of being phosphorylatable to a high degree (Lukat et al., 1991). In order to address the possibility that Lys109 is the acetylation site, we assayed the ability of CheY109KR to be acetylated in vitro. This CheY had a dual effect: it inhibited the labeling of ACS, and it did not become acetylated. To circumvent the apparently inhibitory effect of CheY109KR on ACS labeling, we first labeled ACS and only then added CheY109KR. As shown in Figure 9, this CheY, unlike wild-type CheY, was hardly labeled. Although this does not prove that the acetylation site is Lys109, it is well in line with this possibility. This question of the acetylation site is currently being addressed directly.

*Correlation between CheY Activity in Vivo and Its Ability To Be Acetylated in Vitro.* X-ray data indicate that Lys109

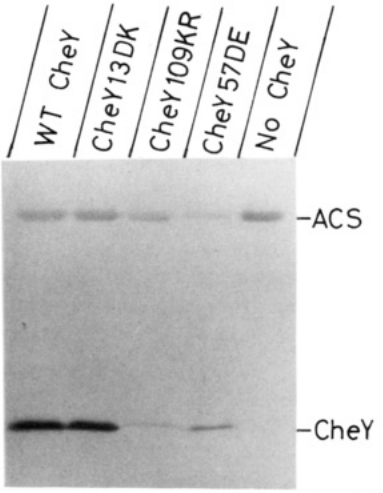


FIGURE 9: Acetylation of mutant CheY proteins. The experiment was carried out as in Figure 2, only that ACS was first labeled alone for 1 h and then the CheY proteins were added for 1-h incubation at 37 °C. Note that, in this particular experiment, the label intensity on ACS was reduced in the presence of CheY109KR to 41% of the ACS label in the presence of wild-type CheY, and to 19% in the presence of CheY57DE (determined by a densitometer).

is in close bonding contact with the carboxyl group of Asp57 (Volz & Matsumura, 1991); i.e., it is associated with the active site of CheY. To determine whether or not there exists a correlation between the acetylation process, measured in vitro, and the activity of CheY, measured in vivo, we compared the ability of CheY57DE and CheY13DK to be acetylated. Previous data indicated that Asp57 and Asp13 are associated with the active site (Bourret et al., 1990; Lukat et al., 1991; Sanders et al., 1989). CheY57DE was shown to be nonactive in vivo (Bourret et al., 1990), and Dailey and Berg (1990) found that the in vivo acetate phenomenon (i.e., the acquirement of CW bias by acetate in gutted cells containing CheY) does not occur in gutted cells containing CheY57DE instead of wild-type CheY. CheY13DK was shown to be nonphosphorylatable in vitro, but nevertheless active in vivo (i.e., cause tumbling) (Bourret et al., 1990). Thus, if a correlation between acetylation and activity of CheY exists, the prediction is that the ability of CheY57DE to be acetylated should be like that of CheY109KR whereas the ability of CheY13DK should be like that of wild-type CheY. This was indeed the case. CheY57DE behaved like CheY109KR in that it did not become acetylated and inhibited the labeling of ACS. When we first labeled the ACS for 1 h and only then added CheY57DE for another 1 h, two things happened: CheY57DE was hardly labeled (10 ± 8% labeling only; ± being the SD of six experiments) but it did withdraw most of the label from ACS; only 28 ± 7% (six experiments) of the original label on ACS remained (Figure 9). The same happened with CheY109KR, which was almost unlabeled (3 ± 2% labeling only; seven experiments) and in its presence only 28 ± 8% (seven experiments) of the ACS label remained. On the other hand, CheY13DK was labeled to the same extent as wild-type CheY, and it did not affect the labeling of ACS.

## DISCUSSION

In this work, we have shown that generating AcAMP within cytoplasm-free CheY-containing envelopes increases by more than 4-fold the fraction of CW-rotating envelopes, that under similar experimental conditions CheY becomes acetylated, that the acetylation is specific to CheY and depends on its conformation, and that there is a correlation between the activity of CheY in vivo and its ability to be acetylated in vitro. These observations taken together suggest that the activity of CheY, as defined by the ability of this protein to interact with the flagellar switch and cause CW rotation, is significantly increased by acetylation. CheY acetylation is thus the second chemical modification known to increase the activity of this protein at the flagellar switch. However, unlike phosphorylation, which was the first-discovered modification of CheY (Hess et al., 1987, 1988; Wylie et al., 1988), the acetylation process does not appear to require additional cytoplasmic constituents for being effective: phosphorylation is unable to increase CheY activity in envelopes (Barak & Eisenbach, 1992), but acetylation does (Figure 1). Below we discuss these observations and conclusions in more detail.

*Acetylation of CheY and Its Activity at the Switch.* An increase in CW rotation in envelopes may result from an effect on CheY or on the switch. Although we did not provide in this study direct evidence that CheY acetylation per se is the reason for the observed increase in activity (studies to this end are in progress), the fact that only CheY and not the switch proteins became acetylated by ACS and the correlation between the activity of CheY at the flagellar switch in vivo and its ability to be acetylated in vitro are more than suggestive that the cause of the increase in activity is the acetylation of CheY. A weak point in the comparison between the ability of the proteins to be acetylated is that although CheY, in its purified form, is functional (tested in envelopes), we do not have a similar assay for the switch proteins and we therefore cannot know whether they are functional in their purified forms and whether the purified forms are in the native conformation. We therefore tried two different purification procedures and exposed CheY to them. The observation that the acetylation of CheY, which had been shown to be sensitive to the conformation of the protein (Figure 8), was unaffected by these procedures seems to support the possibility that the increased CW activity observed in envelopes is indeed related to CheY acetylation. If this is the case, the acetylation-related increase in activity of a CheY molecule is remarkable. Since, under the steady-state conditions used with envelopes, only about 0.01% of the CheY molecules are estimated to be acetylated, and since the measured average increase in activity is 4-fold (Figure 1), the increase in activity of a single CheY molecule upon acetylation should be about 40 000-fold. (The small fraction of acetylated CheY molecules is probably the consequence of the conditions used for the preparation of envelopes, conditions which are not optimal for acetylation. A study of the optimal conditions for acetylation is in progress.)

*The Physiological Significance of the Acetylation.* An important question is whether the acetylation occurs also in vivo under physiological conditions or whether it is a phenomenon restricted to the in vitro conditions used in this study. For example, AcAMP may happen to acetylate CheY in vitro [possibly because adenylate is a good leaving group (Stadtman, 1973)], and this acetylation either does not occur in vivo or is not physiologically significant. According to this possibility, the conformation of CheY may be transformed by the acetylation to a conformation which is coincidentally similar to that achieved under physiological conditions by CheY phosphorylation. This study does not provide a direct answer to this question. However, when taken with other published data, it does provide circumstantial evidence which indicates that the in vitro phenomenon described herein occurs also in vivo and that it is physiologically significant: (i) Wolfe et al. (1988) provided evidence that the in vivo effect of acetate (i.e., causing CW rotation in gutted strains containing CheY) requires ACS, as does the acetylation of CheY (Figure 2) and

the in vitro effect of acetate on flagellar rotation (Figure 1). (ii) An elevated level of intracellular CheA inhibited this in vivo effect of acetate (A. Wolfe, personal communication) as well as the CheY acetylation in vitro (Figure 1 and related text). (iii) The in vivo effect of acetate does not occur in a gutted strain containing CheY57DE instead of wild-type CheY (Dailey & Berg, 1990); accordingly, we found that CheY57DE is not acetylatable by ACS in vitro (Figure 9). (iv) There is a correlation between the in vivo and in vitro phenomena in all the *cheY* mutants tested by us: the mutants *cheY57DE* and *cheY109KR* do not tumble (tumbling is a chaotic angular motion which results from flagellar rotation in the CW direction), whereas *cheY13DK* tumbles frequently (Bourret et al., 1990; Lukat et al., 1991); accordingly, CheY57DE and CheY109KR are nonacetylatable by ACS in vitro whereas CheY13DK is acetylatable (Figure 9). (v) Unfolded CheY can be acetylated to a much lesser extent than native CheY (Figure 8); were the acetylation of CheY coincidental or irrelevant, such a dependence on the conformation would not be anticipated. (vi) The acetylation is specific to CheY (Figure 7); were the acetylation of CheY coincidental or irrelevant, other proteins should have been acetylated as well. (vii) The second possibility, according to which the conformation of CheY (and therefore its activity at the flagellar switch) achieved by acetylation might be similar to that achieved by phosphorylation, is not acceptable: unlike AcAMP (Figure 1), the effect of CheY phosphorylation on flagellar rotation could be observed in partially-lysed cells (semienvelopes) but not in envelopes (in the absence of other cytoplasmic ingredients) (Barak & Eisenbach, 1992).

*The Mechanism of CheY Acetylation.* Acetylation of proteins normally occurs at a lysine residue or at the N-terminus. Perhaps the best known examples for acetylation on a lysine residue are histones and highly-mobile proteins (Allfrey et al., 1984). Acetylation at the N-terminus is characteristic of many intracellular proteins in eukaryotes (Tsunasawa & Sakiyama, 1984). The donor of the acetyl group in these cases is acetyl-CoA, and the catalysis is made by a specific acetyltransferase (Sures & Gallwitz, 1980; Tsunasawa & Sakiyama, 1984). Here, as well, it is a case of N-acetylation, possibly at Lys109, but unlike the other known cases, the donor is AcAMP and the acetylating enzyme is ACS. Although we have used ACS in our in vitro study, this may not necessarily be the physiological enzyme; any enzyme which is capable of acetylating CheY in vivo may do. The data of Wolfe et al. (1988) do suggest, however, that ACS is involved. In this respect, it is of interest to note that the enzyme acetate kinase may be involved in signal transduction in *E. coli* (Fox et al., 1986; Lee et al., 1990; A. Wolfe, personal communication). This enzyme, like ACS, participates in conversion of acetate to acetyl-CoA, but through an acetyl phosphate intermediate. Acetyl phosphate itself was recently found to have the potential of phosphorylating CheY in vitro in the absence of its kinase, CheA (Lukat et al., 1992). It is not yet known whether or not these phenomena are related in any way to the observations made in this study, and if yes—how. It is clear, however, that the increased activity of CheY in envelopes by AcAMP (Figure 1) is not the consequence of CheY phosphorylation by acetyl phosphate, because phosphorylation alone cannot increase the activity of CheY in envelopes (Barak & Eisenbach, 1992).

*The Inhibitory Effect of CheA.* An interesting phenomenon is the inhibition of both the acetate effect in vivo (A. Wolfe, personal communication) and the acetylation of CheY in vitro (Figure 2) by elevated levels of CheA. Although the mechanism is not yet known, the inhibitory effect of CheA in vitro is almost certainly not the consequence of ATPase activity, which depletes the ATP in the reaction mixture. (i) The ATPase activity of CheA alone is very limited because, due to the absence of CheY, phosphorylated CheA cannot be dephosphorylated (Hess et al., 1988); nevertheless, CheA inhibited ACS labeling even when CheY was absent (Figure 2, lane 5). (ii) The stoichiometry of the interaction between CheA and ATP is 1:1 (Hess et al., 1987; Wylie et al., 1988), whereas we used in our experiments 5 mM ATP with only 40 $\mu$M CheA; it is hard to envisage how such a small amount of CheA (in the absence of CheY) would deplete all the ATP. (iii) An ATP concentration of 0.5 mM was as effective as 5 mM in inhibiting ACS labeling. (iv) CheA was added to the reaction mixture together with ATP; if the mechanism of inhibition of ACS labeling was by ATP depletion, at least some of the ACS should have been labeled by the time that all the ATP was exhausted; this was not the case (Figure 2). The inhibitory effect of CheA is probably not the consequence of using ACS from yeast, because a similar inhibitory effect occurred in vivo in *E. coli* (A. Wolfe, personal communication). Whatever the mechanism, and independently of whether or not the inhibition by CheA has any physiological significance, it seems that under physiological conditions the inhibition of acetylation by CheA may be smaller, if present at all: the inhibitory effect of CheA in Figure 2 was at a concentration of 40 $\mu$M, and the concentration of CheA in vivo is only about 1 $\mu$M [calculated on the basis of 1250 dimers per cell (Stock et al., 1991)].

*The Effects of CheY57DE and CheY109KR on ACS.* Like CheA, CheY57DE and CheY109KR inhibited the labeling of ACS. However, unlike CheA, these CheY proteins caused delabeling of already-labeled ACS. Again, the mechanism underlying this observation is not known. If the acetylation site in CheY turns out to be Lys109, one speculative possibility may be that in the ACS–CheY complex the acetyl group, still on ACS, forms a hydrogen bond with Asp57 of CheY. This hydrogen bond might enable the transfer of the acetyl group to Lys109. In the case of CheY57DE, the glutamate residue which substitutes for the aspartate residue at position 57 may form a hydrogen bond with the acetyl on ACS and even remove the group from the ACS, but it may be unable to transfer it to Lys109. In the case of CheY109KR, there is no lysine to receive the acetyl; instead, the substituting arginine residue may receive the acetyl in a futile manner. Whether this speculative interpretation is right or wrong, these findings may provide one end of a thread toward revealing the mechanism of CheY acetylation.

*Concluding Remarks.* In vivo (Bourret et al., 1991; Stock et al., 1989) and in vitro (Barak & Eisenbach, 1992) studies have indicated that phosphorylation of CheY regulates its activity. The current study indicates that acetylation of CheY may do so as well, but further studies are required to establish this point. Finding mutants in acetate metabolism, which are consequently defective in chemotaxis, may be helpful. If this is established, it will remain to be seen whether these two possible regulation modes are functional in parallel and complementary to each other or whether each of them is functional under different conditions.

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