Correlation between Phosphorylation of the Chemotaxis Protein CheY and Its Activity at the Flagellar Motor†

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ABSTRACT: Phosphorylation of the chemotaxis protein CheY by its kinase CheA appears to play a central role in the process of signal transduction in bacterial chemotaxis. It is presumed that the role is activation of CheY which results in clockwise (CW) flagellar rotation. The aim of this study was to determine whether this activity of CheY indeed depends on the protein being phosphorylated. Since the phosphorylation of CheY can be detected only in vitro, we studied the ability of CheY to cause CW rotation in an in vitro system, consisting of cytoplasm-free envelopes of *Salmonella typhimurium* or *Escherichia coli* having functional flagella. Envelopes containing just buffer rotated only counterclockwise. Inclusion of CheY caused 14% of the rotating envelopes to go CW. This fraction of CW-rotating envelopes was not altered when the phosphate potential in the envelopes was lowered by inclusion of ADP together with CheY in them, indicating that CheY has a certain degree of activity even without being phosphorylated. Attempts to increase the activity of CheY in the envelopes by phosphorylation were not successful. However, when CheY was inserted into partially-lysed cells (semienvelopes) under phosphorylating conditions, the number of CW-rotating cells increased 3-fold. This corresponds to more than a 100-fold increase in the activity of a single CheY molecule upon phosphorylation. It is concluded that nonphosphorylated CheY can interact with the flagellar switch and cause CW rotation, but that this activity is increased by at least 2 orders of magnitude by phosphorylation. This increase in activity requires additional cytoplasmic constituents, the identity of which is not yet known.

Bacteria are the simplest organisms which respond to stimuli by movement, and as such they serve as a model system for studying sensory signal transduction at the molecular level (Koshland, 1980; Adler, 1985). The most studied system of this kind is chemotaxis of bacteria such as *Escherichia coli* and *Salmonella typhimurium*, where chemical stimuli affect the direction of flagellar rotation and thereby modulate the swimming of the bacteria so as to approach attractants and to get away from repellents [see Macnab (1987), Stewart and Dahlquist (1987), Bourret et al. (1991), and Eisenbach (1991) for recent comprehensive reviews on chemotaxis]. Thus, modulation of the direction of flagellar rotation is the "heart" of the chemotaxis machinery in bacteria.

The default direction of rotation of the flagellar motor is counterclockwise (CCW) (Eisenbach & Adler, 1981; Parkinson & Houts, 1982; Ravid & Eisenbach, 1984a; Wolfe et al., 1987; Eisenbach et al., 1990). Rotation in the other direction, clockwise (CW), is achieved by interaction of the cytoplasmic chemotaxis protein, CheY, with the switch at the base of the flagellar motor (Parkinson et al., 1983; Clegg & Koshland, 1984; Ravid et al., 1986; Yamaguchi et al., 1986; Wolfe et al., 1987). The interaction of CheY with the switch is direct, without any mediators (Ravid et al., 1986). Since the number of CheY molecules per cell is orders of magnitude higher than the presumed number of switch molecules, it was suggested that CheY exists in two states, active and nonactive, and that there should be a mechanism which regulates the transition between these states in response to chemotactic stimuli (Ravid et al., 1986; Eisenbach, 1991).

A plausible regulation mechanism is phosphorylation of CheY. Recent studies demonstrated that CheY can be phosphorylated in vitro by an autophosphorylatable kinase, CheA, and undergo dephosphorylation either spontaneously or by a specific phosphatase, CheZ [Hess et al. (1987, 1988) and Wylie et al. (1988); see Bourret et al. (1991) and Stock et al. (1991) for recent reviews on phosphorylation of chemotaxis proteins]. The phosphorylation site is aspartate 57 (Sanders et al., 1989; Bourret et al., 1990). Furthermore, the rate of CheY phosphorylation can be affected in vitro by chemotactic stimuli and by modulating the level of receptor methylation in a system consisting of receptor-containing lipid or membrane vesicles, CheA, CheY, and another chemotaxis protein, CheW (Borkovich et al., 1989; Ninfa et al., 1991). These findings made the possibility of CheY regulation by phosphorylation very appealing (Parkinson, 1988; Bourret et al., 1989; Stock et al., 1990; Eisenbach, 1991). However, a few findings do not seem consistent with such a regulation mechanism: (a) CheY can cause CW rotation in the apparent absence of phosphorylating conditions (Clegg & Koshland, 1984; Ravid et al., 1986; Wolfe et al., 1987; Kuo & Koshland, 1987; Smith et al., 1988; Conley et al., 1989); (b) acetyladenylate increases the ability of CheY to cause CW rotation in the apparent absence of phosphorylating conditions both in vivo (Wolfe et al., 1988) and in vitro (Barak et al., unpublished observations); and (c) a modified CheY protein, CheY13DK, in which aspartate 13 is substituted with lysine, is active in vivo in spite of being nonphosphorylatable in vitro (Bourret et al., 1990).

The purpose of this study was to try to resolve the question of whether or not the phosphorylated and nonphosphorylated states of CheY correspond to the active and nonactive forms of this protein. Because CheY phosphorylation has been demonstrated in vitro only, a study of the effect of phosphorylation on the activity of CheY, as defined by its ability to

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cause CW rotation, should also be carried out in vitro. Currently, the only available system where flagellar rotation can be monitored in vitro is a system of cytoplasm-free bacterial envelopes with functional flagella (Eisenbach & Adler, 1981; Eisenbach & Matsumura, 1988). When such envelopes, prepared from *S. typhimurium* or *E. coli*, are tethered to glass by their flagella and then energized, some of them spin (Eisenbach & Adler, 1981; Ravid & Eisenbach, 1984b). Wild-type envelopes containing just buffer spin exclusively CCW (Ravid & Eisenbach, 1984a). When they contain purified CheY, some of them rotate CW (Ravid et al., 1986). We, therefore, wished to insert phosphorylated CheY into envelopes (or to phosphorylate CheY within them) and to examine whether phosphorylation increases the fraction of CW-rotating envelopes, as might be anticipated if phosphorylation indeed activates CheY.

Experimental Procedures

Bacterial Strains and Plasmids. Bacteria were grown at 35 °C in tryptone broth (*E. coli*) or nutrient broth (*S. typhimurium*) as described (Eisenbach & Adler, 1981). An *S. typhimurium* strain ST1 (Aswad & Koshland, 1975), wild type for chemotaxis, and *E. coli* RP1616, a Δ*cheZ* mutant received from J. S. Parkinson, were used for preparation of cell envelopes. *E. coli* RP1091 [Δ(*cheA-cheZ*)] (Parkinson & Houts, 1982) was used for the preparation of semienvelopes. The strains used for overproduction of CheY were *E. coli* RP1616 and RP5943 (a *tsr* mutant), received from J. S. Parkinson. CheA was overproduced in ST1. The plasmids used were pRL22(Δ*PvuII*), carrying *cheY* without *cheZ*, and pDV4, carrying *cheA* and *cheW*, both received from P. Matsumura. CheY13DK was overproduced by pRBB40.13DK in *E. coli* strain KO641*recA*, obtained from R. B. Bourret and M. I. Simon (Bourret et al., 1990).

Preparation of Envelopes and Semienvelopes. Cell envelopes were isolated from *S. typhimurium* strain ST1 or *E. coli* strain RP1616 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). The procedures for preparation of envelopes were identical for *E. coli* and *S. typhimurium*, except for the growth media of the precursor bacteria (see above) and the incubation time with penicillin (7–17 and 35 min for *E. coli* and *S. typhimurium*, respectively). Semienvelopes were isolated from *E. coli* RP1091 as described under Results essentially by the same procedure. CheY-containing envelopes or semienvelopes were prepared by inclusion of 36 μM CheY in the lysis medium. Envelopes or semienvelopes containing phosphorylated CheY were prepared by inclusion of 36 μM CheY, 2–18 μM CheA, and 4 mM ATP in the lysis medium. When included, ADP was at 4–10 mM and caged ATP at 3 mM.

Preparation of Inside-Out Membrane Vesicles. Inside-out membrane vesicles were prepared essentially as described by Kleene et al. (1979). ST1 cells were grown overnight in 100 mL of nutrient broth to stationary phase, centrifuged at 10000*g* for 5 min, resuspended in 4 mL of 50 mM Tris-HCl (pH 7.9), and disrupted by two passes through a French pressure cell at 18 000 pounds/in.^{2}. The lysate was centrifuged at 100000*g* for 1 h. The pellet, containing inside-out vesicles (Rosen & Tsuchiya, 1979), was resuspended in 0.5 mL of the above buffer.

Flagellar Rotation. Flagellar rotation was assayed at room temperature (25 °C) by the tethering technique (Silverman & Simon, 1974) as described (Ravid & Eisenbach, 1983), using a flow chamber (Berg & Block, 1984). For observing the rotation of envelopes, the flow medium was supplemented with D,L-lactic acid (2 mM). Each envelope was separately tested for lack of cytoplasmic remnants as described (Ravid & Eisenbach, 1984b).

Protein Purification. CheY was overproduced and purified essentially as described by Matsumura et al. (1984) with the following modifications: the overproduction was done by the plasmid pRL22(Δ*PvuII*) in RP5943 or RP1616; the bacteria were grown on Casamino medium; the overproduction was induced by addition of indoleacetic acid (Sigma, 20 mg/mL) for 3 h; and the fractions eluted from the Cibacron column were concentrated by a negative pressure protein concentrator (Bio Molecular Dynamics, Beaverton, OR). CheY13DK was purified as wild-type CheY. CheA was overproduced in strain ST1 by the plasmid pDV4 and purified as described (Hess et al., 1988).

Protein Labeling. The reaction mixture (20 μL in volume) for phosphorylation contained, as indicated, various combinations of 50 mM Tris-HCl (pH 7.9), $MgSO_4$ (5 mM), Tetren (0.1 mM), CheY (36 μM), CheA (18 μM), [γ-^{32}P]ATP (0.5 mM, 500–3000 cpm/pmol), and membrane vesicles (8.7 mg of protein/mL). It was incubated at room temperature for 3 h. The incubation was terminated by addition of 5 μL of sodium dodecyl sulfate (SDS) sample buffer. The samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE; 12% polyacrylamide gel), after which the gels were stained by Coomassie blue, destained, dried, and autoradiographed. Phosphate incorporation into CheY and CheA was quantitated by cutting the stained bands out of the dried gel and counting in a scintillation fluid.

Fluorescence Observations. CheA and CheY were labeled by fluorescein isothiocyanate (FITC) at room temperature as described (Fothergill, 1969). FITC-CheA was then separated from possible proteolytic fragments of CheA on an Ultragel AC44 column. Envelopes containing FITC-CheA or FITC-CheY were prepared by inclusion of the labeled protein in the lysis medium under the same experimental conditions used for the nonlabeled protein. The cells were tethered to a microscope cover glass in a flow chamber and observed in a Zeiss fluorescence microscope, using a 390–490-nm transmission filter for the excitation and a cutoff filter of 515 nm.

Preparation of Caged ATP. "Caged ATP", i.e., the 1-(2-nitrophenyl)ethyl phosphate ester of ATP, was synthesized in the dark from 2-nitroacetophenone, hydrazine, and ATP as described (Walker et al., 1988) and then stored at −80 °C. ATP release from this compound, either in solution or within envelopes, was determined at room temperature (25 °C) by measuring the ATP content in the solution or in the suspension of envelopes before and after 1-min illumination by a high-intensity short-arc xenon lamp (500 W; 200–400 nm; Oriel No. 6265). ATP measurements were carried out by the luciferine–luciferase method (Stanley & Williams, 1969), using a Lumac/3M Biocounter, Model 2010A. Each measurement was performed in triplicate. Envelopes containing caged ATP, CheY, and CheA were prepared in the dark by the regular procedure as described above.

Results

Comparison of Activity of Crude and Purified CheY. All the experiments reported here were carried out with purified CheY. To confirm that its activity is not significantly different than that of nontreated CheY, we included crude CheY within envelopes and compared its activity with that of purified CheY. The protein was overproduced within a *cheZ*-deleted strain of *E. coli* (RP1616) by a plasmid which makes CheY but not

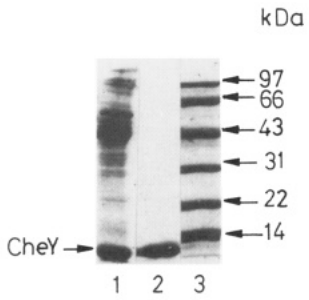


FIGURE 1: SDS–PAGE analysis of crude and purified CheY. Lane 1, crude CheY; lane 2, purified CheY; lane 3, molecular size markers. See text for details.

Table I: Rotation of Tethered Envelopes[a]

contents of envelopes	fraction of CW-rotating envelopes[b] (%)	no. of determinations[b]
buffer	0	>3000
purified CheY	14	189
crude CheY	9	67
CheY,[c] CheA, ATP	16	81
CheY,[c] CheA, caged ATP	14	57
CheY,[c] ADP	12	64
CheY13DK[c]	16	44

[a] The experiments were carried out at pH 7.5 or 7.9. [b] The total number of rotating envelopes was considered as 100%. (Each rotating envelope constituted a separate determination.) [c] In a purified form.

CheZ [pRL22(Δ*PvuII*)]. The bacteria with overproduced CheY were sonicated and spun down to get rid of cells and debris. Envelopes containing crude CheY were prepared by lysing penicillin-treated bacteria within a lysis medium containing the supernatant. As shown in Figure 1, CheY was the major protein in the supernatant. By scanning the protein profile on the gel (Figure 1), the fraction of CheY out of the total protein content in the supernatant was estimated to be 13%. Because of problems with high viscosity, we could not include within the lysis medium as high a concentration of crude CheY as was used in the experiments with purified CheY (36 μM). We therefore included in the lysis medium 0.8 mg of total protein/mL, corresponding to about 10 μM CheY. As shown in Table I, CW rotation was observed with both the crude and purified preparations. This indicates that the purification of CheY does not significantly alter its activity.

Activity of Phosphorylated CheY within Envelopes. Since CheY dephosphorylates spontaneously (Hess et al., 1988; Wylie et al., 1988), we first had to find conditions under which a sufficient fraction of the inserted CheY molecules would be phosphorylated for the duration of the assay (about 2 h). For this purpose, we incubated various combinations of [γ-^{32}P]ATP and CheA with CheY and found that when their concentrations were 0.5 mM, 18 μM, and 36 μM, respectively, CheY was phosphorylated for at least 2 h at room temperature (Figure 2). To make the conditions as close as possible to those within envelopes, we repeated the phosphorylation assay in the presence of inside-out membrane vesicles. The presence of such membrane vesicles doubled the fraction of CheY molecules which were phosphorylated under these steady-state conditions, from 0.6–1.7% to 1.0–3.2%, in accordance with the observations of Borkovich et al. (1989). On the basis of these measurements and considering the average volume of one envelope to be 2.3×10^{-15} L (Lelkes et al., 1984), we calculated that under these conditions there should be, on average, about 1200 phosphorylated CheY molecules within each envelope (out of the 50 000 CheY molecules actually inserted). We anticipated that if indeed phosphorylation significantly increases the activity of CheY, the effect of 1200 phosphorylated CheY molecules per envelope on flagellar rotation should be at least detectable. This was not the case. Such envelopes, prepared by inclusion of ATP, CheA, and CheY in the lysis medium, were tethered to glass, and their rotation was measured. As shown in Table I, the fraction of CW-rotating envelopes was independent of whether or not the envelopes contained CheA and ATP, in addition to CheY.

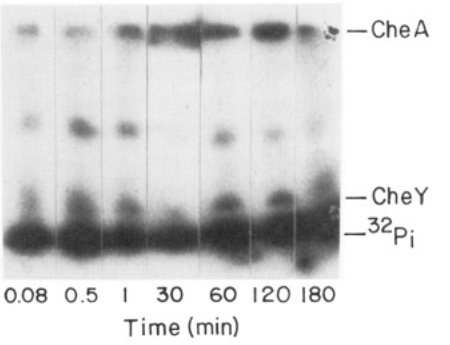


FIGURE 2: Autoradiogram of CheA and CheY labeling by [γ-^{32}P]ATP as a function of time. The experimental conditions were similar to those used for assaying flagellar rotation in envelopes. The reaction mixture contained CheA (18 μM), CheY (36 μM), $MgSO_4$ (5 mM), Tetren (0.1 mM), [γ-^{32}P]ATP (0.5 mM, 3000 cpm/pmol), and 50 mM Tris-HCl (pH 7.9). Labeling was carried out as described under Experimental Procedures.

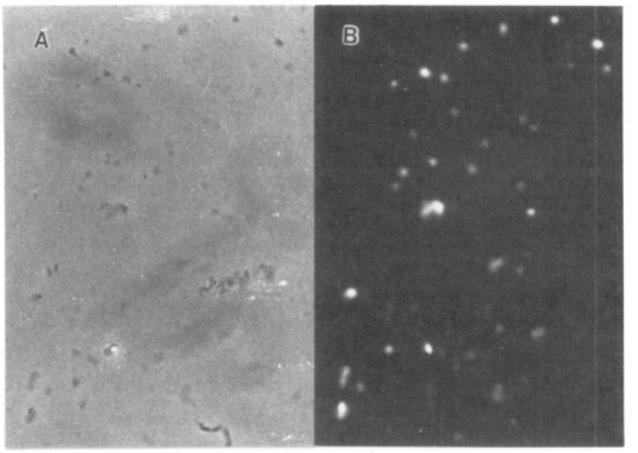
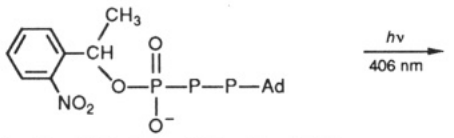


FIGURE 3: Labeling of envelopes and semienvelopes by FITC-CheA. FITC-CheA (36 μM) was included within envelopes as described under Experimental Procedures. The cells were tethered to a cover glass in a flow chamber and observed under a fluorescence microscope. Magnification: 577×. (A) Optical field of cells observed by a phase-contrast microscope. The contrast is poor because envelopes, unlike intact bacteria, are not opaque. (B) The same field observed with a fluorescence microscope.

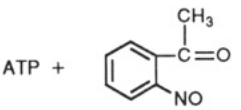
Insertion of a Fluorescent Analogue of CheA. CheA is a 146-kDa dimer (Gegner & Dahlquist, 1991), about 10-fold larger than CheY (14 kDa). In view of the lack of detectable effect of CheA and ATP, we wished to ascertain that CheA had indeed been inserted into the envelopes. For the purpose, we labeled CheA with a fluorescent probe, FITC, and prepared envelopes by using a lysis medium which contained FITC-labeled CheA. All the envelopes and partially lysed cells, unlike nonlysed bacterial cells, were fluorescent (Figure 3). A similar experiment, carried out with FITC-labeled CheY, yielded similar distribution and extent of fluorescence (not shown). This indicates that CheA was indeed inserted into envelopes (as well as into partially-lysed cells, discussed below) by its inclusion in the lysis medium and that the extent of the insertion was similar to that of CheY. This finding avoided the need for harsher insertion techniques such as liposome-

mediated insertion into envelopes (Lelkes et al., 1984).

Phosphorylation of CheY Intracellularly. Knowing now that the envelopes contained both CheA and CheY, we had to consider a possibility that the amount of ATP within the envelopes was not as high as expected, perhaps as a result of faster ATP hydrolysis within them. To address this possibility, we synthesized "caged ATP" and included it within the envelopes instead of ATP. Caged ATP is a photolabile ester analogue of ATP which, upon illumination, decomposes instantaneously to ATP and nitrosoacetophenone (Walker et al., 1988):



1-(2-nitrophenyl)ethyl phosphate ester of ATP
(caged ATP)



nitrosoacetophenone

We hoped that by fast release of large amounts of ATP within the envelopes, we could cause a burst of CheY phosphorylation and then, perhaps, observe a prominent effect on the direction of flagellar rotation.

First, we measured the extent of ATP release upon illumination of the caged ATP. In the pH range 6.3–7.0, 100% of the caged ATP decomposed to ATP within 1 min of illumination under the microscope. At pH 7.9, which is the optimal pH for CheY activity (Ravid et al., 1986), 2 min were required for full ATP release. Because the high-intensity illumination arrested the rotation of the tethered envelopes after 2 min, we illuminated them for 1 min only, during which 71 ± 12% of the caged ATP turned into ATP. Addition of Mg^{2+} did not affect the efficiency of the ATP release. After confirming that the side product of the reaction (nitrosoacetophenone) does not interfere with flagellar rotation, we prepared, in the dark, envelopes containing CheY, CheA, and caged ATP. The envelopes were tethered to a glass slide and illuminated under the microscope. The results were no different than before: none of the CCW-rotating envelopes changed its direction of rotation in response to ATP release, nor was there any change in the fraction of CW-rotating envelopes within the total population (Table I).

Activity of Nonphosphorylated CheY. At this stage we had to consider a possibility that the reason for the lack of effect was that CheY in the envelopes was already phosphorylated in spite of the absence of CheA and ATP. This phosphorylated state could perhaps be achieved by "cross talk" with membrane-bound autophosphorylated kinases such as EnvZ, PhoR, and CpxA (Igo & Silhavy, 1988; Stock et al., 1988; Bourret et al., 1989) which, in the absence of their usual substrate proteins, may transfer their phosphate groups to CheY within the envelopes. We addressed this possibility by reducing the phosphate potential within the envelopes. We prepared envelopes containing 4–10 mM ADP, in addition to CheY, and thereby significantly decreased the possibility of CheY being phosphorylated within the envelopes by any kinase. (The effectiveness of ADP will be demonstrated below.) As a matter of fact, in the presence of such high ADP concentrations, it seems unlikely that even a small fraction of CheY is phosphorylated. As shown in Table I, the fraction of CW-rotating envelopes was similar in the presence and absence of ADP,

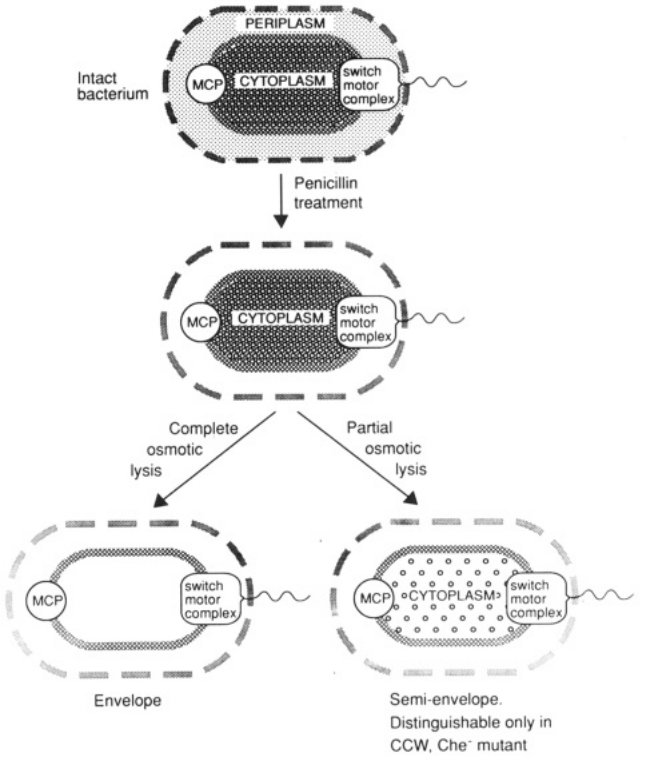


FIGURE 4: A schematic description of the preparation of envelopes and semienvelopes.

indicating that nonphosphorylated CheY is active.

Comparison of Activity of Wild-Type CheY and CheY13DK within Envelopes. In the modified CheY protein, CheY13DK, aspartate 13 is substituted by lysine (Bourret et al., 1990). The consequence of this substitution is that the modified protein is nonphosphorylatable in vitro but nevertheless appears to be active in vivo. The latter is evident from the swimming behavior of the mutant, a swimming typical of CW-biased flagellar rotation (Bourret et al., 1990). We wished to examine whether or not this higher activity of CheY13DK at the motor would be also expressed in envelopes. We therefore prepared envelopes containing CheY13DK and studied their flagellar rotation. As shown in Table I, the activity of CheY13DK within envelopes was similar to that of wild-type CheY.

Comparison of Activity of Phosphorylated and Nonphosphorylated CheY within Semienvelopes. Under all the conditions employed so far, the activity of CheY appeared to be very similar (Table I). This indicated either that phosphorylation does not increase the ability of CheY to cause CW rotation or that the envelopes lack a cytoplasmic component(s) needed for expressing the effect of CheY phosphorylation in them. To distinguish between these possibilities, we wished to have a preparation where the effect of CheY phosphorylation in the presence of the original cytoplasmic constituents could be studied. We employed for the purpose partially-lysed cells (called here semienvelopes) from RP1091, which is a mutant strain lacking the cytoplasmic chemotaxis proteins owing to a deletion of the genes from *cheA* to *cheZ* (Parkinson & Houts, 1982). As shown in Figure 4, semienvelopes are always obtained along with envelopes and contain, in addition to the inserted substances, various amounts of the original cytoplasmic constituents (i.e., during the partial lysis these cells lose variable amounts of cytoplasmic contents and, at the same time, gain some of the extracellular ingredients). The presence of cytoplasm is evident from their being darker than cytoplasm-free envelopes and from their ability to rotate in the absence of an external energy source.[1] RP1091 semienvelopes,

Table II: Rotation of Tethered Semienvelopes

additions to lysis medium[a]	relative activity of CheY (%)[b]	total no. of rotating cells
CheY	100	1141
CheY + CheA	225	665
CheY + CheA + ADP	100	1238
CheY + CheA + ATP	285	265

[a] The lysis medium for the preparation of semienvelopes was 50 mM Tris-HCl (pH 7.9), 5 mM $MgSO_4$, and 0.1 mM Tetren. The concentrations of CheY, CheA, ADP, and ATP were 36 μM, 3.6–7.2 μM, 4–8 mM, and 4 mM, respectively. [b] The activity was determined according to the fraction of CW-rotating semienvelopes. They include both those which rotated CW only and those which alternated between both directions of rotation. The value of 100% was defined as the activity of CheY alone, activity which caused 2% of the total number of rotating cells (lysed, partially-lysed, and unlysed cells together) to go CW. (Note that these cells belong to RP1091, a CCW mutant.) Each semienvelope was monitored for 2 min.

like their precursor bacterial cells, rotate their flagella only in the default direction of rotation, CCW, because of the absence of chemotaxis proteins. However, when they contain inserted CheY, they should rotate CW and therefore be easily distinguishable.[2] As shown in Table II, this was indeed the case: inclusion of nonphosphorylated CheY within semienvelopes caused some of them to rotate CW. These included semienvelopes which occasionally reversed and altered their direction of rotation (unlike envelopes, which always rotated in one direction, either CCW or CW). In the absence of CheY all the envelopes and semienvelopes rotated CCW. Inclusion of both CheY and CheA within the semienvelopes increased the fraction of CW-rotating cells 2-fold. This increase was due to phosphorylation of CheY, because in the presence of ADP, which was added to reduce the phosphate potential and thereby avoid CheY phosphorylation, the activity of CheY returned to that observed in the absence of CheA (Table II). This demonstrates the efficiency of ADP in prevention of phosphorylation. Inclusion of ATP in the semienvelopes, in addition to CheY and CheA, increased the activity of CheY further, reaching a value almost 3-fold higher than that of nonphosphorylated CheY. This indicates that the concentration of ATP within the semienvelopes is limiting. This further indicates that phosphorylation of CheY indeed increases the activity of the protein at the motor, but that cytoplasmic constituents are required for the expression of this increase in activity.

Discussion

In this study we have shown that nonphosphorylated CheY is active in causing CW rotation and that this activity is further increased by phosphorylation. We have also demonstrated that there are additional requirements for the expression of this increase in activity. Each of these findings is discussed below.

When the in vitro phosphorylation of CheY by CheA was discovered, and the suggestion that phosphorylation activates CheY was raised, a phenomenon which appeared to be in conflict with this suggestion was the ability of apparently nonphosphorylated CheY to cause CW rotation. This phenomenon was observed both in vitro (Ravid et al., 1986) and in vivo (Clegg & Koshland, 1984; Kuo & Koshland, 1987; Wolfe et al., 1987; Smith et al., 1988; Conley et al., 1989), when CheY caused CW rotation in the apparent absence of its kinase, CheA, and other cytoplasmic Che proteins. Several explanations [reviewed in detail by Eisenbach (1991)] were raised by several groups to accommodate the suggestion with the apparently conflicting phenomenon. These were (i) that both the nonphosphorylated and phosphorylated forms of CheY are active but that the phosphorylated form has higher affinity for the switch, (ii) that there is cross talk between CheY and kinases, homologous to CheA, or (iii) that there is residual CheA activity within the cells. For example, even in RP1091, which lacks the cytoplasmic chemotaxis proteins, a residual kinase activity may be retained due to the presence of a CheA/CheZ fusion protein in it (Kuo & Koshland, 1989). The observations that CheY can cause CW rotation in envelopes and semienvelopes, even in the presence of high ADP concentrations (Tables I and II), support the first alternative and indicate that the other alternatives do not hold or have only a minor contribution.

It should be noted that the activity of nonphosphorylated CheY may be negligible in vivo, where the physiological concentration of CheY is about 8 μM (Kuo & Koshland, 1987). The activity may be significant only at the high CheY concentrations used in this study (36 μM) and in the in vivo studies with overproduced CheY [30 μM (Kuo & Koshland, 1987); cf. Kuo and Koshland (1987), Wolfe et al. (1987), and Smith et al. (1988) for the dependence of CW rotation on the concentration of CheY in vivo].

Another important finding is that the expression of increased activity of CheY upon phosphorylation requires additional cytoplasmic constituents. None of the conditions employed with envelopes appeared to be effective: inclusion of phosphorylated CheY; phosphorylation of CheY, already present in the envelopes, by caged ATP; and inclusion of CheY13DK which appears to be active at the motor without being phosphorylated. This lack of effect was not the consequence of some defect in the envelopes, because the fraction of CW-rotating envelopes can be increased much beyond the maximal percentage of Table I either by acetyladenylate (AcAMP), probably as a result of CheY acetylation (Barak et al., unpublished observations), or by fumarate (Barak & Eisenbach, 1992). This was also not the consequence of reduced proton-motive force in the envelopes, as judged by their rotation rate [cf. Ravid and Eisenbach (1984a)]. The increase in the number of CW-rotating cells under phosphorylating conditions in semienvelopes (Table II) but not in envelopes (Table I) indicates, therefore, that additional cytoplasmic constituents are involved in the increase in CheY activity by phosphorylation. (The fact that the amount of cytoplasm retained in each semienvelope presumably varied from one cell to another did not appear to be a problem because of our detection system which considered only CW-rotating cells.) This conclusion may be very significant to the molecular mechanisms of CheY function and signal transduction during bacterial chemotaxis. The identity of the cytoplasmic constituent(s) required for the expression of the increased activity is not known. One possibility is that the missing constituents are those required for acetylation of CheY (Barak et al., unpublished observations). Another possibility may be fumarate, which was found to increase CW rotation and restore switching ability to envelopes (Barak & Eisenbach, 1992) and to restore to *Halobacterium halobium* mutants the ability to swim back and forth (Marwan et al., 1990). Further experiments are required for identifying

[1] Cytoplasm-free envelopes stop rotating as soon as D,L-lactate, a common energy source for envelopes, is withdrawn from the suspending medium (Ravid & Eisenbach, 1984b). All envelopes are routinely exposed to this test.

[2] It should be emphasized that semienvelopes are always obtained as side products during the preparation of envelopes from any strain and under any conditions. However, they are easily distinguishable only when they are derived from a CCW mutant under conditions which make some of them rotate CW (e.g., inclusion of CheY within them).

the cytoplasmic constituent(s) and for determining the mutual effects and roles of CheY phosphorylation and acetylation.

By how much does the activity of CheY in semienvelopes increase upon phosphorylation? Since the observed 3-fold increase in activity (Table II) was presumably due to the added activity of about 2.3% of the CheY molecules which became phosphorylated under the steady-state conditions of the experiment, the actual increase in activity of a single CheY molecule should be about 2 orders of magnitude. Furthermore, the amount of CheY inserted into semienvelopes is uncontrolled because of the partial lysis; it is possible, therefore, that the actual number of phosphorylated CheY molecules within the semienvelopes is smaller and that the calculated increase in CheY activity is even larger.

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