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Signal termination in bacterial chemotaxis: CheZ mediates dephosphorylation of free rather than switch-bound CheY

(signal transduction/response regulator/flagellar switch/phosphatase/cross-linking)

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ABSTRACT **Chemotaxis in bacteria is controlled by regulating the direction of flagellar rotation. The regulation is carried out by the chemotaxis protein CheY. When phosphorylated, CheY binds to FliM, which is one of the proteins that constitute the "gear box" (or "switch") of the flagellar motor. Consequently, the motor shifts from the default direction of rotation, counterclockwise, to clockwise rotation. This biased rotation is terminated when CheY is dephosphorylated either spontaneously or, faster, by a specific phosphatase, CheZ. Logically, one might expect CheZ to act directly on FliM-bound CheY. However, here we provide direct biochemical evidence that, in contrast to this expectation, phosphorylated CheY (CheY∼P), bound to FliM, is protected from dephosphorylation by CheZ. The complex between CheY∼P and FliM was trapped by cross-linking with dimethylsuberimidate, and its susceptibility to CheZ was measured. CheY∼P complexed with FliM, unlike free CheY∼P, was not dephosphorylated by CheZ. However, it did undergo spontaneous dephosphorylation. Nonspecific cross-linked CheY dimers, measured as a control, were dephosphorylated by CheZ. No significant binding between CheZ and any of the switch proteins was detected. It is concluded that, in the termination mechanism of signal transduction in bacterial chemotaxis, CheZ acts only on free CheY∼P. We suggest that CheZ affects switch-bound CheY∼P by shifting the equilibrium between bound and free CheY∼P.**

The response regulator CheY is apparently the central control site of signal transduction in bacterial chemotaxis (for a recent review, see ref. 1). It is phosphorylated by a specific kinase, CheA, and dephosphorylated by a specific phosphatase, CheZ (2, 3). Upon phosphorylation, CheY is released from the quaternary complex receptor:CheW:CheA:CheY (4, 5). Phosphorylated CheY (CheY∼P) has then two targets: FliM, which is one of the proteins that constitute the "gear box" (termed a "switch") of the flagellar motor, and CheZ (refs. 6 and 7, respectively, and references cited there). Binding to the switch results in shifting the direction of flagellar rotation from the default direction of rotation, counterclockwise, to clockwise (8–11). Binding to CheZ results in CheZ oligomerization (12) and activation (13). The consequence of CheZ activation is dephosphorylation of CheY∼P and termination of CheY binding to the switch. Termination of the binding could be performed by two ways (Fig. 1): CheZ may act on both switch-bound CheY∼P and free CheY∼P (Fig. 1*A*), or it may act only on free CheY∼P and affect switch-bound CheY by shifting the equilibrium between bound and free CheY (Fig. 1*B*). The simplest mechanism of clockwise termination seems to be direct action of CheZ on switch-bound CheY∼P. However, Sanna *et al.* (14) found that *cheY* mutations which confer resistance to the phosphatase activity of CheZ map near to the presumed FliM-binding surface of CheY. This implies that the CheY∼P surfaces that bind to FliM and CheZ might overlap, and therefore binding of CheY∼P to the switch might sequester the CheZ-binding site(s) on CheY∼P. Resolving this question is essential for understanding the mechanism of CheY∼P deactivation and clockwise signal termination.

MATERIALS AND METHODS

Protein Purification. All proteins used in this study were originated from *Salmonella typhimurium*. CheY was purified as described (12). CheZ was purified from RP3098 (15) cells containing pEWW7 (13) as described (12). FliM was purified from *Escherichia coli* strain BL21(DE3)/pLysS (16), carrying the plasmid pKOT179 which overexpresses FliM and FliN (17). Bacteria were grown at 35°C in 1.5 liters of Luria broth. When the cells reached OD_{590} = 1.0, they were incubated with 1 mM isopropyl β-D-thiogalactopyranoside for 3 h at 35°C. All further steps were carried out at 4°C. The cells were harvested, resuspended in 80 ml of buffer A (50 mM Tris·HCl, pH 7.9/0.1 mM EDTA) and sonicated. Inclusion bodies were collected by centrifugation at 72,000 × *g* for 30 min. Membranes were removed from the white pellet of inclusion bodies by gently wiping off the upper brown layer. The inclusion bodies were solubilized in buffer B (buffer A containing 7 M urea), and nonsoluble materiel was removed by centrifugation at 186,000 × *g* for 30 min. The clear solution was loaded onto a 20 ml Sepharose CL-6B column, pre-equilibrated with buffer B. The column was washed with 50 ml buffer B, and FliM was eluted with a 200 ml linear gradient of buffer B containing 0–0.5 M NaCl. For refolding the protein, the FliM-containing fractions were combined, diluted in buffer B to a final protein concentration of 30 μg/ml (as determined by Bradford with immunoglobulin G as standard), and dialyzed against buffer A containing 10% (vol/vol) glycerol. Refolded FliM was concentrated to a final concentration of 600–750 μg/ml by ultrafiltration through a 10-kDa cut-off membrane in an Amicon chamber (model 52), and stored at −80°C.

Trapping CheY-FliM Complexes by Chemical Cross-Linking. The reaction mixture (500 μl) contained 50 mM KP_i (pH 8.0), 22 mM acetyl phosphate (AcP), 5 mM $MgCl_2$, 50 μM CheY, and 7.9 μM FliM. The reaction was initiated by addition of the cross-linker dimethylsuberimidate (11 mM final concentration). After 3 h incubation at room temperature (22–25°C), the nonreacted cross-linker and AcP were removed by filtration through a 1 ml G-50 mini-column, followed by dialysis against 50 mM Tris·HCl (pH 7.5). The resulting cross-linked complexes were stored at −80°C.

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Abbreviations: AcP, acetyl phosphate; CheY∼P, phosphorylated CheY.

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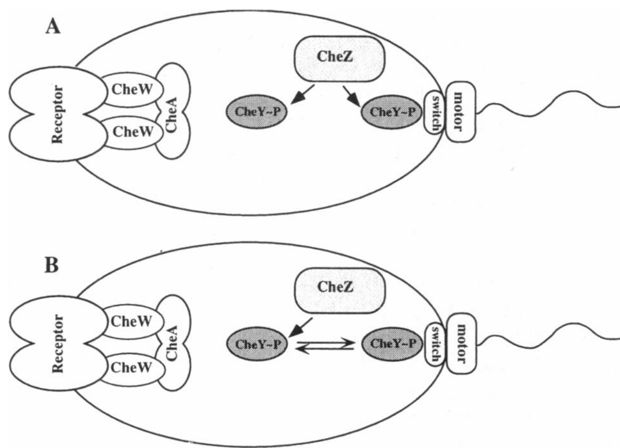

FIG. 1. Two alternative mechanisms for terminating the interaction of CheY~P with the switch. See text for details.

CheZ-Mediated Dephosphorylation of CheY~P Complexed with FliM. Cross-linked complexes (1.13 mg total protein/ml as determined by Bradford with immunoglobulin G as standard; this value is equivalent to ≈0.62 mg/ml after correction for the concentration of CheY as measured by Biuret or absorbance at 280 nm) were incubated at room temperature (22–25°C) with 5 mM $MgCl_2$ and 6.3 mM ^{32}P-labeled AcP [310 cpm/pmol, synthesized as described (18)] for 3 min. During this period of time, three 20 μl aliquots were removed for analysis. After 3 min, 0.14 mg/ml CheZ (equivalent to 6 μM monomeric CheZ) was added, and three additional aliquots (22 μl each) were removed. All the aliquots were quenched, immediately after removal, by the addition of 100 μl of 10% ice-cold trichloroacetic acid (TCA). The proteins were precipitated by centrifugation, washed once again with 100 μl of 10% TCA at 0°C (we verified that TCA does not hydrolyze phosphorylated CheY under our experimental conditions), and analyzed by SDS/PAGE as described (7).

Spontaneous Dephosphorylation of CheY~P Complexed with FliM. CheY–FliM complex was phosphorylated by ^{32}P-labeled AcP as described above, except that only one sample was removed after the 3 min of phosphorylation. After phosphorylation by ^{32}P-labeled AcP, 50 mM nonlabeled AcP was added, and 22 μl aliquots were removed, quenched, and analyzed by SDS/PAGE. Quantitation was made by a PhosphoImager Fujix BAS 1000.

RESULTS

CheZ readily enhances the dephosphorylation of free CheY~P *in vitro* (2, 3). To determine whether CheZ can also act on FliM-bound CheY~P, we had first to form a stable complex between CheY~P and FliM. Since a complex between CheY and FliM can be formed only under phosphorylating conditions (6, 19) and the process appears to be reversible (A.B. and M.E., unpublished data), we trapped the complex by chemical cross-linking with the amine-reactive cross-linker dimethylsuberimidate (20). As shown in Fig. 2, a 1:1 CheY–FliM cross-linked complex was obtained only under phosphorylating conditions [presence of both the phosphodonor AcP (21) and Mg^{2+}], indicating that this cross-linked complex represents molecules trapped during specific interactions rather than molecules which collided randomly.

To determine the susceptibility of FliM-bound CheY~P to the phosphatase activity of CheZ, we phosphorylated the CheY–FliM cross-linked complex with ^{32}P-labeled AcP and then exposed it to CheZ. As shown in Fig. 3, CheY~P complexed with FliM was not dephosphorylated by CheZ. In contrast, free CheY~P and nonspecific cross-linked CheY

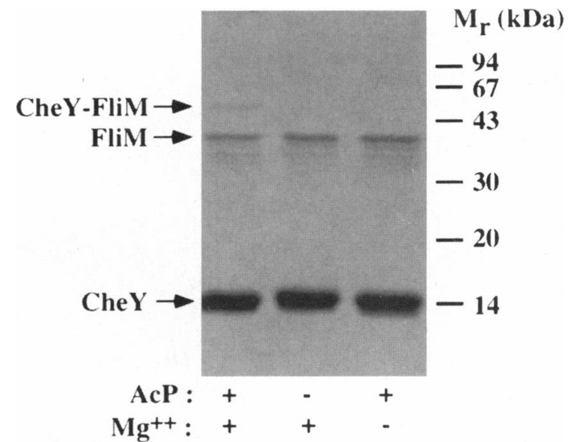

FIG. 2. Phosphorylation-dependent generation of CheY–FliM cross-linked complex. The figure shows a Coomassie-blue stained gel, with samples of 8 μg total protein run on 15% SDS/PAGE. Where indicated, 5 mM Mg^{2+} and 22 mM AcP were included in the cross-linking reaction mixture.

dimers (always formed to a very small extent in such experiments; they cannot be observed in Coomassie-blue stained gels, only in autoradiograms) were susceptible to the action of CheZ and were dephosphorylated. This indicated that the resistance of FliM-bound CheY~P to the action of CheZ is an intrinsic property of the complex rather than the result of chemical modifications caused by the cross-linker (see below). Qualitatively similar results were obtained also with another cross-linker, *o*-phthaldialdehyde.

The resistance of FliM-bound CheY~P to dephosphorylation by CheZ could result either from general stabilization of the phosphorylated conformation of CheY or from a specific perturbation of the interaction with CheZ. To distinguish between these possibilities, we examined whether autodephosphorylation of CheY~P is affected by complex formation with FliM. This was done by adding excess of nonradiolabeled AcP to FliM-bound CheY~P which had been prephosphorylated by ^{32}P-labeled AcP, and monitoring the loss of the ^{32}P label from free and bound CheY. As shown in Fig. 4, the autodephosphorylation rate of CheY~P was not reduced, but even slightly increased by the binding to FliM. The specific resistance of FliM-bound CheY~P to dephosphorylation by CheZ, but not to spontaneous dephosphorylation, indicates that the resistance to the action of CheZ is caused by a specific perturbation of the interaction of CheY~P with CheZ.

Deactivation of CheY~P at the switch can also be mediated by direct interactions between CheZ and the switch as suggested by genetic suppression analysis (22–25). To examine this possibility,

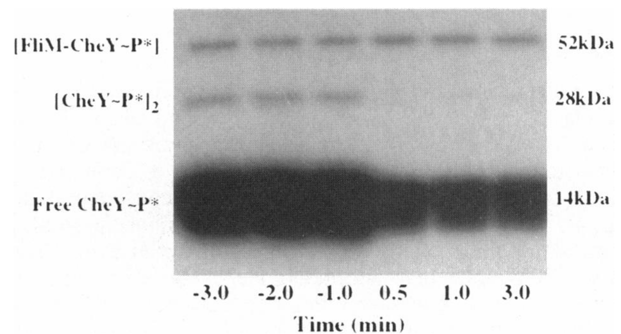

FIG. 3. CheZ-mediated dephosphorylation of CheY~P complexed with FliM. The results are shown in the form of an autoradiogram of an experiment carried out as described. ^{32}P-labeled AcP was added at t = −3 min, CheZ was added at t = 0. The asterisk symbolizes radioactive phosphate.

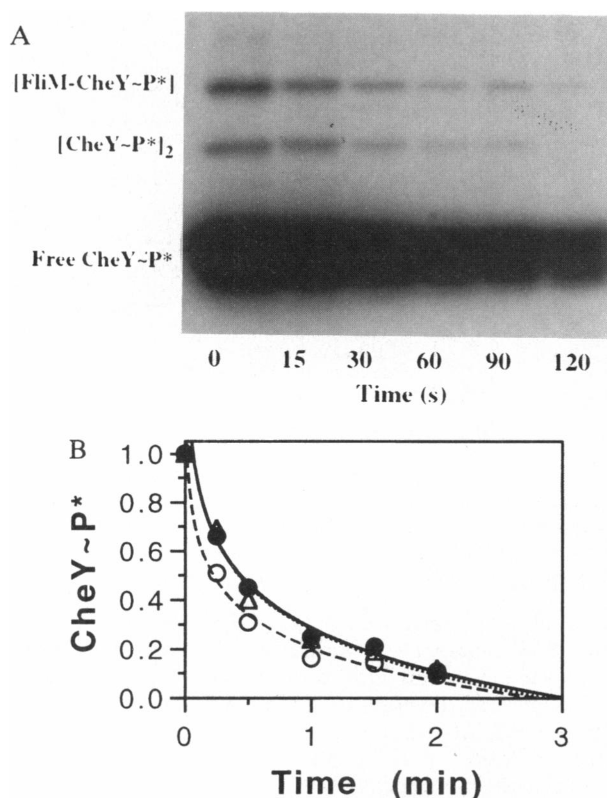

FIG. 4. Autodephosphorylation of CheY~P complexed with FliM. (*A*) An autoradiogram of 15% SDS gel, carried out as described. Nonlabeled AcP was added at $t = 0$. (*B*) Quantification of the results shown in *A*. The level of phosphorylation is given in relative units; the level before the addition of nonlabeled AcP was considered as 1. ●, Monomeric CheY~P* (solid line); △, dimeric CheY~P* (dotted line); ○, FliM-bound CheY~P* (dashed line). The asterisk symbolizes radioactive phosphate.

we looked for binding of CheZ with either one of the switch proteins—FliG, FliM, and FliN. We used a number of techniques: (*i*) binding assays with CheZ, immobilized onto Sepharose beads, and radiolabeled FliG, FliM, and FliN; (*ii*) chemical cross-linking of CheZ and FliM; and (*iii*) fluorescence depolarization of fluorescein-labeled CheZ in the presence of FliM. In all cases, to look for potential CheZ–switch binding via CheY, the assays were also carried out in the presence of CheY (with and without AcP). Even though we had successfully employed all these techniques for the detection of binding interactions between CheZ and CheY (7, 12) or FliM and CheY (6), we could not detect significant binding between CheZ and any of the switch proteins. Thus, it appears that CheZ deactivates free CheY~P only.

DISCUSSION

The results of this study suggest that the phosphatase CheZ can only act on free CheY~P, not on switch-bound CheY~P. This conclusion is based on two main observations: (*i*) CheY~P complexed with FliM was completely resistant to dephosphorylation by CheZ (Fig. 3) but not to spontaneous dephosphorylation (Fig. 4); and (*ii*) this resistance apparently was not the result of chemical modification by the cross-linker, because the cross-linked CheY dimer that had undergone similar chemical modifications did not lose its susceptibility to the action of CheZ (Fig. 3). This conclusion, that CheZ does not act on switch-bound CheY~P, is in line with the failure of this study to detect biochemically an interaction between CheZ and any of the switch proteins in the presence and absence of CheY/CheY~P.

Sanna *et al.* (14) have characterized two mutant CheY proteins resistant to dephosphorylation by CheZ. Interestingly, the mutations were found to be located near to the presumed FliM-binding surface of CheY, suggesting that the FliM- and CheZ-binding surfaces of CheY might overlap. This possibility is in line with the resistance of FliM-bound CheY~P to dephosphorylation by CheZ, found in this study. Taken together, it seems that the inability of CheZ to dephosphorylate FliM-bound CheY~P is the result of sequestration of the CheZ-binding domain in CheY~P by FliM.

It should be noted that the results of this study cannot completely rule out two additional interpretations of the data. (*i*) A specific cross-link may occur between CheY and FliM with a resultant blockage of CheZ action. This possibility seems remote, particularly because of two reasons: (*a*) the CheY-FliM complex was formed only under phosphorylating conditions (Fig. 2) and (*b*) two cross-linkers, dimethylsuberimidate and *o*-phthaldialdehyde, that largely differ in the length of their space arms and in their reactive groups, were nevertheless similar in their ability to cross-link between CheY~P and FliM and to block CheZ action. (*ii*) Additional components of the switch (i.e., FliG and FliN) may be needed for CheZ to interact with FliM-bound CheY~P. The observation that the switch proteins FliG and FliN neither bind CheY/CheY~P nor affect its binding to FliM (6) suggests that the likelihood of this possibility is very low.

Chemoattractants bias flagellar rotation to counterclockwise by rapidly deactivating the clockwise signal CheY~P. How can the resistance of switch-bound CheY~P to dephosphorylation by CheZ be reconciled with the rapid deactivation of CheY~P in response to attractants? We can envision two potential mechanisms for the rapid attractant response. (*i*) There might be an additional CheY~P-deactivating factor, yet to be discovered, which deactivates switch-bound CheY~P. (*ii*) Dephosphorylation of free CheY~P shifts the equilibrium between switch-bound and free CheY~P toward dissociation and dephosphorylation (a shift to the left in Fig. 1*B*). In such a case, the rate of CheY~P–FliM dissociation should be at the order of at least $10\ s^{-1}$ for being compatible with the time scale of the attractant response (26). We prefer mechanism *ii*, as it does not require invoking an unknown CheY~P-deactivating factor and it is in line with all the observations.

To conclude, the mechanism of clockwise signal termination appears to involve deactivation of CheY by the action of CheZ on free CheY~P, dephosphorylation, and equilibrium shift from switch-bound to free CheY.

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