Phosphorylating and Dephosphorylating Protein Complexes in Bacterial Chemotaxis

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During optimal motility conditions, a 1:1 stoichiometry of CheA_L (654 amino acids) to CheA_S (557 amino acids) was determined. It was also found that CheZ binding to CheA_S was inhibited by CheA_L-CheA_S-CheW interaction. This suggests that CheA_S has different functions in the phosphorylating complex (CheA_L-CheA_S-CheW) and in the dephosphorylating complex (CheA_S-CheZ).

The regulation of the chemotaxis signal is based on the level of phosphorylated CheY (1, 3, 7). The amount of phosphorylated CheY is, in turn, a function of the rate of the autophosphorylation activity of CheA and the dephosphorylation activity of CheZ (7, 12). We have previously demonstrated that a CheA_L-CheA_S-CheW complex can be isolated from cells and that the rate of the autophosphorylation of CheA_L (654 amino acids) in this complex is responsive to the signalling state of the receptors (8). We have also demonstrated that a CheA_S-CheZ complex can be isolated from cells and that this complex formation results in enhanced dephosphorylation activity of CheZ (12). These two complexes have opposite activities, yet they have a common component, CheA_S (557 amino acids). In this study, physical interactions among CheA_L, CheA_S, CheZ, and CheW were further examined. The results indicate that a CheA_L-CheA_S-CheW complex, with an apparent 1:1:1 molar ratio, exists *in situ*. Consistent with this stoichiometry, we found that the relative amounts of CheA_L and CheA_S in wild-type chemotactic cells at maximum motility (post-exponential phase) are 1:1. We also found that the CheA_S-CheZ interaction is reduced by the CheA_S-CheW interaction.

Physiological amounts of CheA_L and CheA_S in wild-type cells. Because CheA_L can form a functional homodimer *in vitro* (4, 5, 12) and because the cloned, overexpressed *cheA* gene produces predominantly CheA_L (6, 10, 11), the amounts and ratios of CheA_L and CheA_S were determined to see if their heterodimer formation is consistent with the relative concentrations. Wild-type cells (RP437) were grown in tryptone broth at 30°C, and the amounts of CheA_L and CheA_S were quantified at all growth phases by immunoblotting (12). The amounts of both proteins changed at different growth stages, with a 0.65×10^3 to 2.7×10^3 molecule/cell range for CheA_L and a 0.15×10^3 to 2.5×10^3 molecule/cell range for CheA_S. Both proteins peaked at exponential and post-exponential phases, and the molar ratio of the two proteins was constant during these growth stages. Figure 1 shows that the molar ratio of CheA_L to CheA_S at maximum motility (2) is 1:1.

CheA_L-CheA_S-CheW complex isolated from cell extracts. Although *in vitro*-formed complexes of CheA were homodimers of CheA_L (4, 5, 12), we found that an *in vivo*-formed CheA_L-CheA_S-CheW complex could be isolated from lysates on an immobilized CheY column (8). In these experiments, we

concluded that a complete complex bound to the CheY column rather than being formed on the CheY column. Here, we used coprecipitation to demonstrate that this complex exists prior to binding to CheY. CheA_L, CheA_S, and CheW were overproduced in a strain which did not express any flagellar or chemotaxis proteins (RP3098). Lysates isolated from the cells were then precipitated with an affinity-purified anti-CheW antibody or with a monoclonal anti-CheA antibody that was specific for CheA_L. The immunoprecipitates, as well as the lysates, were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analyses. As shown in Fig. 2, CheA_S and CheW were coprecipitated with CheA_L by the anti-CheA_L antibody (lane 7) from the lysate containing overproduced CheA_L, CheA_S, and CheW (lane 3), while anti-CheW antibody precipitation of the same lysate resulted in the coprecipitation of CheA_L and CheA_S with CheW (lane 8).

In control experiments, lysates were isolated from RP3098 cells harboring plasmids overproducing only CheW (lane 1) or CheA_S (lane 2) or CheA_S and CheA_L but no CheW (lane 4). The lysates were then precipitated with the anti-CheW antibody or the anti-CheA_L antibody. These experiments indicated that at equivalent protein levels, the anti-CheW antibody did not cross-react with the two CheA proteins (lane 9), nor did the anti-CheA_L antibody show any detectable affinities for CheW (lane 5) or CheA_S (lane 6). These data demonstrated that the three proteins can form a stable CheA_L-CheA_S-CheW complex *in situ*.

Stoichiometry of the CheA_L-CheA_S-CheW complex. The stoichiometry of the ternary CheA_L-CheA_S-CheW complex was analyzed by quantitating the protein amounts in the immunoprecipitates with purified CheA and CheW proteins of known concentrations as standards. In the anti-CheW antibody precipitation, the ratio of the amounts of CheA_L and CheA_S that coprecipitated with CheW reflected the CheA_L/CheA_S molar ratio in the complex, while the CheA_S/CheW molar ratio in the complex could be deduced from the amounts of CheA_S and CheW that coprecipitated with CheA_L in the anti-CheA_L antibody precipitation. The results listed in Table 1 indicate that the amounts of CheA_L and CheA_S that coprecipitated with CheW had a 2.72/2.82 molar ratio and that the amounts of CheA_S and CheW that coprecipitated with CheA_L had a 2.14/1.89 molar ratio. Thus, the CheA_L-CheA_S-CheW complex most likely has a molar composition of 1:1:1.

CheA_S-CheW binding inhibits CheA_S-CheZ interaction. We have previously shown that CheA_S could enhance the phosphatase activity of CheZ by forming a CheA_S-CheZ complex (12). Since CheA_S is also found in the CheA_L-CheA_S-CheW

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FIG. 1. Growth curve and molar ratios of CheA_L and CheA_S in chemotactic wild-type cells. Amounts of the two CheA proteins in the RP437 cells were quantified by immunoblot analysis with purified CheA of known concentrations as standards. □, bacterial growth; *, CheA_L/CheA_S molar ratio.

complex, we examined whether CheZ and CheW could simultaneously bind to CheA_S.

Lysates were prepared from cells overproducing CheA_L, CheA_S, and CheZ as well as CheW (RP3098/pDV41/pRL22^{Kan}) and cells overproducing CheA_L, CheA_S, and CheZ but no CheW (RP3098/pDV41Δ*cheW*/pRL22^{Kan}). The lysates were precipitated with the anti-CheA_L antibody, and the immunoprecipitates were analyzed by immunoblot analyses as shown in Fig. 3.

The two lysates used in the experiments are shown in Fig. 3, lanes 1 and 2, respectively. Precipitation of the RP3098/pDV41Δ*cheW*/pRL22^{Kan} lysates by the anti-CheA_L antibody resulted in the coprecipitation of CheZ and CheA_S (lane 4).

FIG. 2. Anti-CheA_L and anti-CheW antibody precipitations showing the CheA_L-CheA_S-CheW complex. Immunoprecipitations of cell lysates were performed as described in the text. After the precipitations, the precipitates as well as the lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7% polyacrylamide for CheA and 15% polyacrylamide for the other Che proteins) and the proteins were transferred to APT cellulose paper and probed with the cognate polyclonal antibodies. The primary antibodies were then labelled with ¹²⁵I-protein and subjected to autoradiography. Lanes 1 to 4, lysates of RP3098/pMM5 (lane 1), RP3098/pAF1 (lane 2), RP3098/pDV41 (lane 3), and RP3098/pDV41Δ*cheW* (lane 4); lanes 5 to 7, anti-CheA_L antibody precipitates from RP3098/pMM5 lysate (lane 5), RP3098/pAF1 lysate (lane 6), and RP3098/pDV41 lysate (lane 7); lanes 8 to 9, anti-CheW antibody precipitates from RP3098/pDV41 lysate (lane 8) and RP3098/pDV41Δ*cheW* lysate (lane 9). Note that since RP3098 does not express any chemotactic proteins, the CheA_L-like protein in lanes 1 and 2 is likely due to cross-reactivity of the polyclonal anti-CheA antibody with other proteins.

TABLE 1. Quantitation of CheA_L, CheA_S, and CheW proteins precipitated by the anti-CheA_L and anti-CheW antibodies^a

Protein	Anti-CheA _L antibody precipitated (pmol)	Anti-CheW antibody precipitated (pmol)
CheA _L	22.8	2.72
CheA _S	2.14	2.82
CheW	1.89	12.5

^a Relative amounts of CheA_L, CheA_S, and CheW in the immunoprecipitates, as shown in Fig. 1, were quantitated with purified CheA and CheW proteins as standards in the immunoblot analyses. Concentrations of the purified CheA and CheW proteins were determined by a dye-binding assay (Pierce) and amino acid composition analysis.

Since CheA_S bound to CheA_L and since CheZ did not bind to CheA_L (12), the presence of CheZ in the precipitate was apparently due to its binding to CheA_S. When CheW was also overproduced in the lysates, along with CheA_L, CheA_S, and CheZ, CheW and CheA_S were coprecipitated with CheA_L by the antibody (lane 3) but the amount of CheZ being coprecipitated was significantly reduced. The amounts of CheA_L precipitated by the antibody and the amounts of CheA_S coprecipitated with CheA_L from the two lysates were equivalent; however, the amount of CheZ coprecipitated was reduced by more than 70% when CheW was present in the lysate compared with the amount coprecipitated when CheW was absent. Thus, it appears that either CheW or CheZ is able to bind to the CheA_L-CheA_S complex and that under these conditions CheW has a higher affinity than CheZ.

Although no behavioral phenotype has been assigned to CheA_S (9), the observation that CheA_S can participate in both phosphorylation and dephosphorylation complexes suggests a possible regulatory role for this protein. Perhaps the current technology of behavioral measurements is not adequate to measure the contribution of CheA_S. One intriguing possibility is that CheA_S first participates in the phosphorylation complex and that after phosphotransfer it participates in the dephosphorylation complex.

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FIG. 3. CheA_S-CheW binding blocks CheA_S-CheZ interaction. Anti-CheA_L antibody precipitations were performed as described in the legend to Fig. 2. Lanes 1 and 2, lysates of RP3098/pDV41/pRL22^{Kan} (lane 1) and RP3098/pDV41Δ*cheW*/pRL22^{Kan} (lane 2); lanes 3 and 4, anti-CheA_L antibody precipitates from RP3098/pDV41/pRL22^{Kan} lysate (lane 3) and RP3098/pDV41Δ*cheW*/pRL22^{Kan} lysate (lane 4). Note that although CheY was also overproduced by pRL22, CheY had no apparent effects on the coprecipitations of CheZ or CheW in anti-CheA_L antibody precipitation (data not shown).

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