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Bacterial chemotaxis signaling complexes: Formation of a CheA/CheW complex enhances autophosphorylation and affinity for CheY

(protein–protein interaction/protein phosphorylation/*Escherichia coli*)

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ABSTRACT **We have demonstrated that a complex of the proteins CheA ($CheA_L$ and $CheA_S$) and CheW can be isolated and constitutes a functional unit that responds to the signaling state of the chemoreceptors. The autophosphorylation rate of $CheA_L$ is much greater when $CheA_L$ and $CheA_S$ are complexed with CheW. Moreover, the presence of mutant chemoreceptors that cause cells to tumble increases this rate. At wild-type levels of expression, the isolated $CheA_L/CheA_S$/CheW complex accounts for about 10% of the total number of $CheA_L$, $CheA_S$, and CheW molecules and exists in a 1:1:1 stoichiometry. This complex is also required for $CheA_L/CheA_S$ and CheW binding to the phosphorylation substrate, CheY. A separate interaction between CheY and another chemotaxis component, CheZ, was also detected. The CheY–CheZ interaction does not require participation of the $CheA_L/CheA_S$/CheW complex.**

Signal transduction during bacterial chemotaxis is initiated upon detection of a chemical stimulus by surface receptors. Some pathways, used to monitor the concentration of amino acids, peptides, and certain sugars, are methylation dependent (1, 2) and involve methyl-accepting chemotaxis proteins (MCPs). Others, used for the detection of oxygen and other sugars, are methylation independent (3). Stimulus detection triggers modulation of a series of phosphorylation and phosphate transfer reactions (4–8), ultimately resulting in the regulation of the direction of flagellar rotation. Counterclockwise rotation promotes forward swimming, whereas a clockwise reversal allows the bacterium to change directions (9–11).

The CheA protein plays a central role in the signaling phosphorylation cascade. The *cheA* gene encodes two similar products that are translated from different, in frame starts (12). The larger CheA protein ($CheA_L$) has a molecular mass of 73 kDa. The smaller CheA protein ($CheA_S$) lacks the first 97 amino acids and has a molecular mass of 60 kDa. The larger CheA protein ($CheA_L$) has been shown to autophosphorylate and transfer the phosphate group to CheY and CheB (5). Phosphorylated CheY is thought to be the active form of CheY that binds to the flagellar motor and causes clockwise rotation. Phosphorylated CheB possesses a MCP-specific esterase activity that regulates the methylated state of the chemoreceptors to bring about sensory adaptation (13–15).

The *in vivo* and *in vitro* roles of the CheW protein have been examined. *In vivo*, CheW is required for clockwise rotation of the flagella (16, 17). *In vitro*, CheW is needed to enable chemoreceptors to control the rate of autophosphorylation of CheA (18).

Recently, physical interactions between $CheA_L$ and CheW have been demonstrated *in vitro* (19). Here, we show by immune coprecipitation and by binding to affinity columns that $CheA_L$, $CheA_S$, and CheW form complexes in the cell. *In vitro*, these complexes possess enhanced autophosphorylation activity and receptor responsiveness. When complexed with CheW, $CheA_L/CheA_S$ exhibits an increased affinity for ATP and for CheY. This and other interactions between signaling complexes are likely to play important roles in the signal transduction process.

MATERIALS AND METHODS

Strains and Plasmids. RP437 is an *Escherichia coli* K-12 strain wild-type for chemotaxis. All other strains are derivatives of this strain. RP1788, a *cheA* deletion strain (Δ*cheA*), RP1078, a *cheW* deletion strain (Δ*cheW-tap*), RP5752, a *tsr* dominant tumbly mutant, RP4793, a *tsr* dominant smooth swimming mutant, and RP5225, a strain with an amber mutation between the two *cheA* start sites, were obtained from J. S. Parkinson (University of Utah). Strain RP5225 expresses $CheA_S$ but not $CheA_L$. HCB429, a strain defective for all four chemotaxis receptors [Δ*tsr*, Δ(*tar-tap*), Δ*trg*], was obtained from H. C. Berg (Harvard University). YK4131, a strain devoid of all chemotaxis and flagellar proteins (*flhD*$^-$), was received from Y. Komeda (University of Tokyo). Plasmid pDV4 is an expression vector that places the *cheA* and *cheW* genes under the control of the *Serratia marcescens trp* operon promoter. Plasmid pDV21 expresses a fusion protein containing the amino-terminal one-third of CheY and the carboxyl-terminal two-thirds of CheA, as well as the CheW protein, from the *trp* operon promoter. Plasmid pRL22 expresses CheY and CheZ protein from the *trp* operon promoter. Plasmid pDV4ΔEcoRV is a derivative of pDV4 with the *cheW* gene deleted. All four plasmids are inducible by addition of 3-indoleacrylic acid. Plasmid pMM5 expresses only CheW protein from the *lac* promoter and is inducible by addition of lactose or isopropyl β-D-thiogalactoside.

Protein Expression. Strains containing various expression vectors were grown to midexponential phase in L broth or tryptone broth at 32°C and induced by addition of 100 μg of 3-indoleacrylic acid per ml or by 2% (wt/vol) lactose or 10 mM isopropyl β-D-thiogalactoside. Cells were induced for 4–16 hr, harvested, resuspended in 50 mM Tris (pH 7.5), and stored at −70°C until sonicated. Cells were thawed, sonicated for 3 min, and then centrifuged at 30,000 × *g* for 20 min. The supernatant fraction (called S-30) was used as a source of Che proteins.

Protein Purification and Membrane Preparation. CheY was purified as described by Matsumura *et al.* (20). The CheY/CheA fusion protein and the CheA, CheW, and CheZ proteins were purified by electroelution from either nondenaturing/polyacrylamide or SDS/polyacrylamide slab gels.

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Abbreviation: MCP, methyl-accepting chemotaxis protein.

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Membranes were prepared as described by Borkovich *et al.* (18)

Construction of Affinity Columns. The protein to be coupled was concentrated to 0.5–1.0 mg/ml by lyophilization and coupled to cyanogen bromide-activated Sepharose (Sigma). The final concentration of CheY in the column was 2 mg/ml of resin bed. The final concentration of the anti-CheA antibody in the column was 1 mg/ml of resin bed.

Immunoprecipitations. Lysates isolated from various mutant and wild-type strains were mixed by rotation with affinity purified anti-CheA or anti-CheW antibody for 1 hr. The antigen–antibody complexes were precipitated by addition of protein A-agarose (Sigma), and the mixture was rotated for an additional hour. The precipitates were washed four times by resuspension in 1.5 ml of 50 mM Tris (pH 7.5) and pelleted by centrifugation at 10,000 × *g* for 30 sec. For experiments involving receptors, the insoluble immune precipitates were resuspended and centrifuged at 10,000 × *g* for 30 sec with membrane fractions from wild-type and mutant strains. The precipitated proteins were either solubilized in Laemmli SDS sample buffer for Western blot analysis or used in phosphorylation experiments.

Protein Labeling. Labeling of immunoprecipitated proteins was initiated by addition of [γ-^{32}P]ATP. Reactions were terminated at various times by addition of Laemmli SDS sample buffer, and the samples were analyzed by SDS/PAGE and autoradiography. Radioactivity was measured using a radioisotope scanning system (AMBIS Systems, San Diego).

Immunoblot Analysis and Quantitation. Protein samples were separated by 7% (for CheA) or 15% (for CheZ and CheW) SDS/PAGE and transferred to aminothiophenol paper. The blots were probed with purified anti-CheA, anti-CheZ, or anti-CheW antibody, labeled with ^{125}I-labeled protein A (^{125}I-protein A) (ICN), and subjected to autoradiography. Radioactivity was measured using a radioisotope scanning system (AMBIS Systems).

RESULTS

Isolation of Soluble CheA/CheW Complexes. To demonstrate the existence of a stable CheA/CheW complex, anti-CheA antibodies were used to isolate CheA and CheW from soluble cell extracts. Affinity-purified anti-CheA antibody raised against the carboxyl-terminal end of CheA was covalently attached to Sepharose. This affinity column was used to selectively remove $CheA_L$ and $CheA_S$ from S-30 lysates of cells of an *flhD* mutant that overproduced CheA and CheW but did not produce other chemotaxis proteins. We found that, in addition to $CheA_L$ and $CheA_S$, CheW was also retained on the anti-CheA column. Fig. 1 shows a Coomassie blue-stained SDS/polyacrylamide gel of the proteins isolated from the column. Lane 2 shows the S-30 lysate of cells that carry pDV4 and overproduced CheA and CheW. Lane 3 shows the proteins that did not bind to the column. Lane 5 shows the proteins that remained bound to the column after washing with 100 column volumes of 0.5 M NaCl but were eluted with the chaotropic agent 3 M KSCN—namely, $CheA_L$, $CheA_S$, and CheW. Minor bands below the CheA bands reacted with anti-CheA antibody and are most likely products of CheA degradation. CheW protein, when overexpressed by itself, did not bind to the anti-CheA column (lane 4), indicating that CheW must be retained through association with $CheA_L$ or $CheA_S$. Since the CheW association was stable to extensive washing with 0.5 M NaCl, the CheA/CheW interaction is apparently not a simple electrostatic interaction. The isolated complexes are free of major contaminants and do not require other flagellar or chemotaxis proteins for formation or stability. This complex was only seen when the *cheA* and *cheW* genes were expressed in the same cells. Mixing of S-30 lysates of cells from a *cheA* deletion strain and cells from a *cheW* deletion strain did not form complexes.

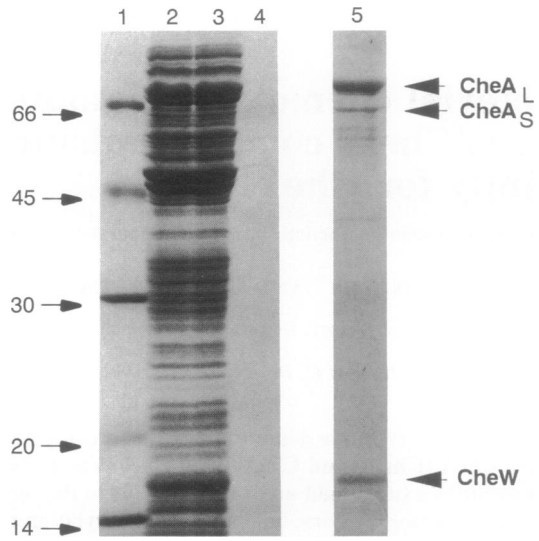

FIG. 1. Immunoadsorption of CheA and CheW proteins to CheA antibody column. Proteins were analyzed by 12.5% SDS/PAGE and stained with Coomassie blue. Lanes: 1, molecular mass standards (shown in kDa); 2, S-30 lysate with overexpressed levels of CheA and CheW proteins (pDV4 in strain YK4131); 3, proteins from this lysate that failed to bind the column; 4, proteins from an S-30 lysate with an overexpressed level of CheW protein only that bound and were eluted from the column (pMM5 in strain YK4131); 5, proteins from the lysate of lane 2 that bound and were eluted from the column.

Autophosphorylation of Coprecipitated CheA/CheW Complexes. The autophosphorylation activity of $CheA_L$ was examined in the presence and absence of complexed CheW by measuring PO_4 incorporation into immunoprecipitated $CheA_L$. In Fig. 2, the filled circles represent $CheA_L$ that was coprecipitated as a complex with CheW using anti-CheW antibody. The open circles represent $CheA_L$ that was precipitated from a *cheW* deletion strain using anti-CheA antibody. The initial rate of $CheA_L$ autophosphorylation was 16-fold greater when $CheA_L$ and $CheA_S$ were isolated as a complex with CheW. This difference was not due to an effect caused by antibody binding. The addition of either anti-CheA or anti-CheW antibody had no appreciable effect on the autophosphorylation rates of anti-CheW and anti-CheA precipitates (data not shown).

The increased autophosphorylation activity of the CheA/CheW complex was found to be due to an increased affinity for ATP rather than an increased V_{max}. In Fig. 3, the initial rates of the $CheA_L$ autophosphorylation reactions are shown at various concentrations of ATP. The binding of CheW to $CheA_L$ did not have an appreciable effect on the maximum velocity of the reaction but did decrease the K_m by 68-fold. Thus, the binding of CheW to CheA increases the phosphorylation rate by increasing $CheA_L$'s affinity for ATP.

Effect of Chemoreceptors on Formation and Autophosphorylation of CheA/CheW Complexes. The CheA/CheW complex detected by immune coprecipitation was found in similar ratios in wild-type, receptor-free strains and in receptor mutants (which were either constantly tumbly or smooth swimming). In all cases, immune precipitations with anti-CheW antibody coprecipitated ≈100 mmol of $CheA_L$ and $CheA_S$ per mol of CheW. Even though the signaling state of the receptors did not alter the amount of CheA bound to CheW, the receptors did have a marked effect on the biochemical activity of the complexed CheA (Fig. 4). The autophosphorylation activities of antibody-precipitated complexed CheA from wild-type and receptor mutants were

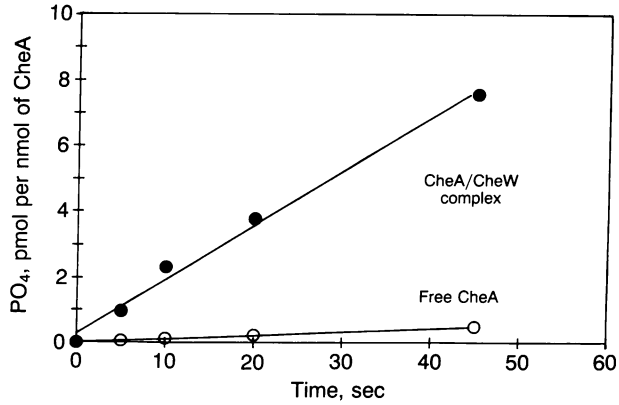

FIG. 2. Phosphorylation of $CheA_L$ in the presence and absence of CheW protein. CheA bound to CheW was immunoprecipitated from a wild-type lysate (strain RP437) using anti-CheW antibody. Uncomplexed CheA was immunoprecipitated from a lysate devoid of CheW protein (strain RP1078) using anti-CheA antibody. Phosphorylation assays were performed on immunoprecipitated proteins resuspended in 50 mM Tris/50 mM KCl/5 mM $MgCl_2$, pH 7.5. Reactions were initiated by addition of [γ-^{32}P]ATP to a final concentration of 10 μM and terminated after 5, 10, 20, and 45 sec by addition of Laemmli SDS sample buffer. The samples were analyzed by SDS/PAGE and a radioisotope scanning system. Identical samples were subjected to Western blot analysis to determine protein levels in the precipitates. ●, Phosphorylation of $CheA_L$ protein bound to CheW; ○, phosphorylation of uncomplexed $CheA_L$ protein. The plots represent an average of experiments with lysates from two sets of cultures, each performed in duplicate; the deviation from the mean was <10% of each sample.

compared in the presence of different membrane fractions. When the precipitates and the membranes were obtained from the same source, the autophosphorylation activity was highest with the receptor mutants that were constantly tumbly (RP5752) and lowest with receptor mutants that were smooth-swimming (RP4793) or with mutants devoid of receptors (HCB429) (Fig. 4).

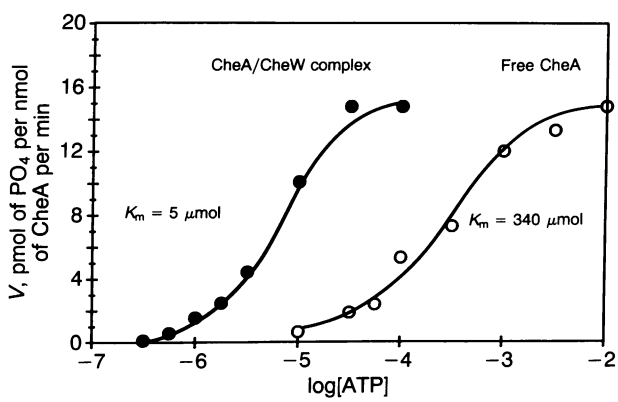

FIG. 3. $CheA_L$ autophosphorylation as a function of ATP concentration. Phosphorylation assays were performed on $CheA_L$ bound to CheW and immunoprecipitated with anti-CheW antibody and on uncomplexed $CheA_L$ immunoprecipitated with anti-CheA antibody (as in Fig. 2). Reactions were initiated by addition of various amounts of [γ-^{32}P]ATP and terminated after 5, 10, 20, and 45 sec by addition of Laemmli SDS sample buffer. The samples were analyzed by SDS/PAGE and a radioisotope scanning system. Identical samples were subjected to Western blot analysis to determine the levels of protein in the precipitates. The initial rate of $CheA_L$ autophosphorylation is plotted as a function of the log of ATP concentration. The K_m was determined as the midpoint of inflection of the curve. The plots represent an average of experiments with lysates from two sets of cultures, each performed in duplicate; the deviation from the mean was <10% of each sample.

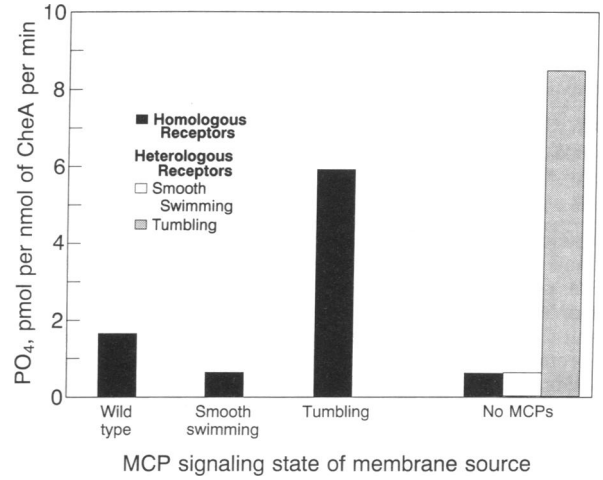

FIG. 4. Phosphorylation of $CheA_L$ in the presence of mutant or wild-type Tsr receptors. Phosphorylation assays were performed on $CheA_L$ coprecipitated with CheW using anti-CheW antibody. The precipitated CheA/CheW complexes were mixed with suspensions of membranes either from the same strain (black bar graph) or from a heterologous strain (white or stippled bar graph). Reactions were initiated by addition of [γ-^{32}P]ATP to 10 μM and terminated after 10 sec by addition of Laemmli SDS sample buffer. The samples were analyzed by SDS/PAGE and a radioisotope scanning system. Identical samples were subjected to Western blot analysis to determine the levels of protein in the precipitates.

When the precipitates were obtained from receptor-less mutants (HCB429) and the membranes from the tumbly mutant (RP5752) and the smooth-swimming mutant (RP4793), the activity was high with the tumbly mutant membranes and remained low with the smooth-swimming mutant membranes. Thus, CheA/CheW complexes formed and isolated in the absence of receptors were nevertheless able to respond *in vitro* to membrane fractions isolated from mutants with receptors that cause cells to tumble.

Binding of CheA/CheW Complexes to CheY. Complexes of CheA and CheW bound to an immobilized CheY column; however, $CheA_L$, $CheA_S$, and CheW, when tested alone, did not. Fig. 5 shows a gel stained with Coomassie blue (lanes 2–4) and an immunoblot (lanes 6–8) of proteins that bound to an immobilized CheY column. Lanes 1 and 5 are the S-30 lysates of cells overproducing CheA and CheW. Clearly, the

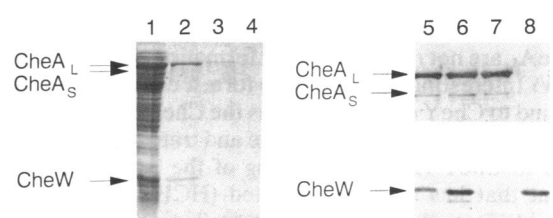

FIG. 5. Binding of CheA and CheW from S-30 lysates to a CheY affinity column. Lanes 1–4, proteins analyzed by 15% SDS/PAGE and stained with Coomassie blue. Lanes 5–8, immunoblots with anti-CheA antibody (top panel; 7% polyacrylamide gel) and with anti-CheW antibody (bottom panel; 15% polyacrylamide gel). Lanes: 1, S-30 lysate from cells overproducing CheA and CheW (pDV4 in strain YK4131); 2, proteins bound to column from this lysate; 3, protein bound to column from an S-30 lysate of cells producing CheA only (pDV4ΔEcoRV); 4, proteins bound to column from an S-30 lysate of cells overproducing CheW only (pMM5); 5, S-30 lysate of cells overproducing CheA and CheW (as above); 6, proteins bound to column from this lysate; 7, proteins bound to column from cells producing CheA only; 8, proteins bound to column from cells producing CheW only. Note that samples in lanes 7 and 8 contained 15-fold greater amount of protein than those in lane 6. This was done to obtain an antibody reaction significantly over background.

Table 1. Binding of overexpressed Che proteins to CheY column

Proteins overexpressed	Plasmid	$CheA_L$ nmol*	$CheA_L$ %†	$CheA_S$ nmol*	$CheA_S$ %†	CheW nmol*	CheW %†
$CheA_L$, $CheA_S$, CheW	pDV4	1.05	24.7	0.2	10.9	1.48	21.5
$CheA_L$, $CheA_S$	pDV4ΔEcoRV	1.05	1.9	0.16	2.1	‡	ND
CheW	pMM5	‡		‡		1.48	1.2

Protein levels were measured by Western blot analysis. Supernatant and eluted samples were run on 7% (CheA) or 15% (CheW and CheZ) polyacrylamide gels. Proteins were transferred to aminothiophenol paper and immunoblotted with anti-CheA, anti-CheZ, or anti-CheW antibodies. The primary antibody was labeled with ^{125}I-protein A. The radioactivity was quantitated using a radioisotope scanning system. Standard curves were developed using known concentrations of purified CheA, CheZ, and CheW proteins. Each determination includes values from six independent measurements. ND, not determined.
*Nanomol of protein loaded.
†Percent bound.
‡Wild-type levels.

S-30 lysate that contained CheA and CheW (lane 2) showed more binding than either S-30 lysate that contained $CheA_L$/$CheA_S$ or CheW alone (lanes 3 and 4, respectively). The quantitation of this binding is shown in Table 1. The different Che proteins were expressed on multicopy plasmids in a strain that otherwise produced them at wild-type levels. When $CheA_L$, $CheA_S$, and CheW were all overexpressed in the same cell, ≈20–25% of the $CheA_L$ and CheW bound to the CheY column and about 10% of the $CheA_S$ bound. When $CheA_L$/$CheA_S$ and CheW were overexpressed separately, only 1–2% of each bound to the CheY column. An equal amount of CheA or CheW protein was added to the CheY columns in each experiment.

Complexes Isolated from Wild-Type and Mutant Cells. The binding of the CheA/CheW complex to an immobilized CheY column was repeated with lysates from cells producing proteins at wild-type levels. As shown in Fig. 6 and Table 2, we obtained the same qualitative results—that is, CheA and CheW must be present for binding to the immobilized CheY column (Fig. 6, lanes 1 and 2). With protein obtained from a *cheA* deletion mutant, no CheW bound to the CheY column (Fig. 6, lane 3), and with protein obtained from a *cheW* deletion mutant, no CheA bound (Fig. 6, lane 4). By using a mutant that does not make $CheA_L$ (RP5225), we found that a $CheA_S$/CheW complex was able to bind to the immobilized CheY column (Fig. 6, lane 5). Thus, the first 97 amino acids of $CheA_L$ are not required for the formation of a complex with CheW. Interestingly, $CheA_S$ can form a complex with CheW and bind to CheY even though it is the $CheA_L$ species that has been shown to autophosphorylate and transfer the phosphate group to CheY (17). The binding of the S-30 lysate from a mutant that has all MCPs deleted (HCB429) was indistinguishable from that of the wild type (lane 1). Therefore, the MCPs do not appear to be required for formation of the

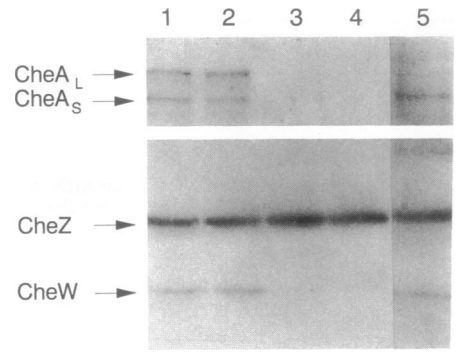

FIG. 6. Binding of wild-type levels of CheA, CheZ, and CheW proteins to the CheY column. Bound proteins were eluted with 3 M KSCN and analyzed by 7% (top panel) or 15% SDS/PAGE (bottom panel). Proteins were transferred to aminothiophenol paper for immunoblotting with anti-CheA antibody (top panel) or anti-CheZ and anti-CheW antibodies (bottom panel). The antibody was labeled with ^{125}I-protein A. The protein for the different lanes was obtained from S-30 lysates of the following strains (see Table 2): HCB429 (lane 1), RP437 (lane 2), RP1788 (lane 3), RP1078 (lane 4), and RP5225 (lane 5).

$CheA_L$/$CheA_S$/CheW complex and binding of this complex to CheY. Binding of CheZ to the immobilized CheY column was also demonstrated (Fig. 6). This interaction was observed in lysates from wild-type and *cheA*$^-$, *cheW*$^-$, and *MCP*$^-$ strains, so it does not involve the participation of these other chemotaxis proteins. It appears to be a separate interaction. Quantitation of the amount of each protein bound to the CheY column indicates that ≈7–10% of the $CheA_L$, $CheA_S$, and CheW proteins bind to the CheY column (Table 2). These values also indicate that the molar ratio of the $CheA_L$/$CheA_S$/CheW complex that binds to the CheY column is 1:1.1:0.96. Thus, the molar ratio of the complex is approximately the same as the molar ratio present in the cell. When the mutant lacking $CheA_L$ was used, the molar ratio of the $CheA_S$/CheW complex remained about 1:1.

DISCUSSION

We have isolated $CheA_L$/$CheA_S$/CheW complexes from strains that overproduce these proteins and from strains that produce them at normal physiological levels. The presence of a $CheA_L$/$CheA_S$/CheW complex under both sets of conditions suggests that it is always present and that it represents a minimal functional unit *in vivo*. Our $CheA_L$/$CheA_S$/CheW complexes, formed *in vivo*, are apparently more stable than those reported by Gegner and Dahlquist (19) and do not dissociate with extensive washing. A $CheA_L$/$CheA_S$/CheW complex did not form *in vitro* when S-30 lysates containing $CheA_L$/$CheA_S$ were mixed with those containing CheW (data not shown). Either coexpression of *cheA* and *cheW* may be required for proper complex formation or an additional labile factor may be lost during lysate preparation.

Table 2. Binding of Che proteins, expressed at wild-type levels, to CheY column

Proteins not expressed	Strain	$CheA_L$ pmol*	$CheA_L$ %†	$CheA_S$ pmol*	$CheA_S$ %†	CheW pmol*	CheW %†	CheZ pmol*	CheZ %†
None	RP437	75.0	7.8	66.9	11.3	75.0	7.6	361	4.8
MCPs	HCB429	75.0	8.1	61.1	10.5	90.0	6.8	327	5.1
CheW	RP1078	75.0	‡	65.6	‡	ND		387	4.6
$CheA_L$, $CheA_S$	RP1788	ND		ND		75.0	‡	328	5.2
$CheA_L$	RP5225	ND		66.9	9.7	75.6	7.8	335	5.1

Experiments were done as described in the legend to Table 1. ND, not determined.
*Picomol of protein loaded.
†Percent bound.
‡Not detectable.

The $CheA_L/CheA_S$/CheW complex exhibits an enhanced rate of $CheA_L$ autophosphorylation when tested *in vitro*. It also appears to be necessary for binding to CheY. In addition, autophosphorylation of $CheA_L$ in the CheW complex is sensitive to the signaling state of the receptor.

The existence of a CheA/CheW complex is consistent with the recent work of Borkovich *et al.* (18) and Borkovich and Simon (21), who demonstrated *in vitro* that receptors that cause cells to tumble and CheW are both required for the enhanced transfer of phosphate from phospho-CheA to CheY and for enhanced $CheA_L$ autophosphorylation. They did not see a lowering of the K_m for ATP or a higher than wild-type rate of $CheA_L$ autophosphorylation with membrane fractions isolated from mutants that cause cells to tumble. One significant difference between our results and theirs is that our $CheA_L$ was completely complexed to CheW.

Our results suggest that a stable $CheA_L/CheA_S$/CheW complex interacts with the receptor and enhances the autophosphorylation of $CheA_L$. Receptors are not required for formation of the complex, and their signaling state does not alter the amount of the complex that can be isolated. However, the signaling state of the receptors has a dramatic effect on the autophosphorylation rate of the complex. The correlation between the swimming behavior of the cells from which the receptors were obtained and autophosphorylation activity of the $CheA_L/CheA_S$/CheW complex *in vitro* suggests that it is the intact complex that responds *in vivo*.

CheW appears to play a central role in linking the signaling state of the receptor to the phosphorylation cascade. We have shown that CheW greatly enhances CheA binding to CheY and that it increases the autophosphorylation rate of $CheA_L$ by increasing the affinity of $CheA_L$ for ATP. Also, CheW is a crucial member of a stable functional complex and is required for the receptor-mediated regulation of autophosphorylation of the complex. Sequence analysis of the *cheW* gene has identified a highly conserved nucleotide binding site between amino acids 128 and 160 (22). Receptor-mediated binding of ATP to this site could be a mechanism for controlling activation of the $CheA_L/CheA_S$/CheW complex and the phosphorylation cascade.

At wild-type levels, ≈10% of the $CheA_L$, $CheA_S$, and CheW proteins were isolated as a stable complex bound to immobilized CheY (Table 2), and, when overproduced, up to 25% of these proteins were isolated from a CheY column that had been washed extensively (Table 1). These data are consistent with the *in vitro* binding studies that have estimated that 20% of the $CheA_L$ should be complexed to CheW (19). Therefore, a large fraction of the $CheA_L$, $CheA_S$, and CheW is capable of forming a complex *in vivo* and interacting with the CheY column *in vitro*. However, immune precipitation of the $CheA_L/CheA_S$/CheW complex did not show appreciable CheY coprecipitation (data not shown). Either the immobilization of CheY to the solid matrix enhanced its ability to interact with the $CheA_L/CheA_S$/CheW complex or antibody interaction with CheA and/or CheW inhibits binding to CheY.

The stoichiometry of approximately 1:1:1 for the $CheA_L/CheA_S$/CheW complex bound to the CheY column is similar to the overall levels of these proteins present in wild-type cells (Table 2). CheZ binding to the CheY column was about 3-fold greater on a molar basis than that of $CheA_L$, $CheA_S$, and CheW. This result, together with the observation that CheZ binding did not require $CheA_L$, $CheA_S$, or CheW, suggests a separate and distinct interaction of CheZ with CheY. This interaction is consistent with data showing that CheZ can act to enhance dephosphorylation of CheY-phosphate (17). Since we do not know the phosphorylation state of the CheY bound to the column, it is not possible to determine if the $CheA_L/CheA_S$/CheW complex or CheZ has different binding affinities for the phosphorylated and unphosphorylated forms of CheY. Although most of the CheY can be assumed to be unphosphorylated due to the highly unstable nature of the phosphorylated form, it is possible that a small amount of phosphorylated CheY is trapped in an active form when it is bound to the affinity column and that this fraction accounts for the binding activity.

Whereas it has been shown that $CheA_L$, $CheA_S$, and CheW are present in approximately 1:1:1 molar ratio in the wild-type cell and when bound to the CheY column, the composition of an individual complex was not determined. It is clear that any complex interacting with CheY requires CheW and at least one species of CheA. A $CheA_L/CheA_S$/CheW complex would be the simplest explanation for a 1:1:1 ratio, but a mixture of $(CheA_L)_2$/CheW and $(CheA_S)_2$/CheW could also have a 1:1:1 molar ratio. This composition is unlikely, since it would predict that in the absence of $CheA_L$, the molar ratio of $CheA_S$ to CheW would be 2:1. The ratio observed was 1:0.8 (Table 2). In addition, this mixed trimer model would suggest a molar ratio of $CheA_L$ to CheW of nearly 2:1 in overproducing cells, since expression of $CheA_L$ and CheW is 5-fold greater than $CheA_S$ in these cells. The $CheA_L$/CheW ratio observed in this case was 1:1.2 (Table 1). Thus, it appears that $CheA_L$/CheW can interact with CheY in the absence of equimolar expression of $CheA_S$, and $CheA_S$/CheW can interact with CheY in the absence of $CheA_L$. Under normal physiological conditions, when $CheA_L$, $CheA_S$, and CheW are expressed at equimolar levels, a trimeric complex of $CheA_L/CheA_S$/CheW is the most likely form.

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