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Organization of the Receptor-Kinase Signaling Array That Regulates *Escherichia coli* Chemotaxis*

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Motor behavior in prokaryotes is regulated by a phosphorelay network involving a histidine protein kinase, CheA, whose activity is controlled by a family of Type I membrane receptors. In a typical *Escherichia coli* cell, several thousand receptors are organized together with CheA and an Src homology 3-like protein, CheW, into complexes that tend to be localized at the cell poles. We found that these complexes have at least 6 receptors per CheA. CheW is not required for CheA binding to receptors, but is essential for kinase activation. The kinase activity per mole of bound CheA is proportional to the total bound CheW. Similar results were obtained with the *E. coli* serine receptor, Tsr, and the *Salmonella typhimurium* aspartate receptor, Tar. In the case of Tsr, under conditions optimal for kinase activation, the ratio of subunits in complexes is ~6 Tsr:4 CheW:1 CheA. Our results indicate that information from numerous receptors is integrated to control the activity of a relatively small number of kinase molecules.

Cellular responses to hormones, neurotransmitters, and a wide variety of environmental signals are often mediated by proteins composed of extracellular stimulus-binding domains connected by membrane spanning α-helices to intracellular signaling domains. These so called Type I receptors include the protein tyrosine kinase receptors that mediate responses to insulin, growth factors, and cytokines in vertebrate cells (1), as well as the protein histidine kinase receptors in microorganisms and plants (2). Type I receptors are generally thought to function as dimers (3). Stimulatory ligands bind to sites that bridge the dimer interface to cause conformational changes leading to altered interactions between protein kinase domains within the cytoplasm. Over the past several years the serine and aspartate receptors, Tsr and Tar, which mediate chemotaxis responses to serine and aspartate in *Escherichia coli* and *Salmonella typhimurium*, have emerged as useful models for understanding general principles of Type I receptor function (2, 4–6). Tsr and Tar are homologous 60-kDa proteins with dimeric extracytoplasmic ligand-binding domains connected by transmembrane sequences to conserved cytoplasmic coiled-coil domains that bind the dimeric histidine protein kinase, CheA (7), and an Src homolgy 3-like protein, CheW (8). CheA phosphorylates one of its own histidine residues, and the phosphoryl group is then rapidly transferred to an aspartate residue in the single domain response regulator CheY. Phosphorylated CheY binds to the flagellar motor switch, where it promotes a change in the direction a bacterium is swimming. Serine and aspartate cause changes in receptor conformation that inhibit CheA. This decreases the level of phospho-CheY so that bacteria tend to continue swimming toward these attractants.

It has generally been assumed that the chemoreceptor signaling unit is composed of a receptor dimer linked via two CheW subunits to the dimeric histidine kinase CheA. There is mounting evidence, however, that signaling entails much more extensive interactions involving numerous receptor subunits. Immunoelectron and fluorescence microscopies (9–12) and *in vivo* fluorescence studies (13) have shown that the majority of the thousands of receptor monomers in a typical *E. coli* cell are localized to one or two patches at the cell poles.

Another strong line of evidence for the involvement of higher order interactions in signaling was provided by *in vitro* studies where highly active membrane-free complexes were obtained by mixing soluble receptor cytoplasmic domain fragments from Tar or Tsr with CheA and CheW (14–16). Earlier, it had been found that appending sequences that form parallel coiled-coil dimers to the N termini of tyrosine kinase signaling domains could lead to kinase activation (17–19). Similar results were obtained when the cytoplasmic domain of Tar was linked to a leucine zipper coiled-coil dimerization motif (15, 16). The complexes formed from these constructs were purified and characterized (20, 21). The subunit composition was ~28 receptor signaling domains, 6 CheWs, and 4 CheAs.

To bridge the gap between results obtained *in vitro* with soluble receptor fragments and results derived from immunoelectron microscopy and fluorescence studies of full-length receptors in intact cells, we have investigated ternary complexes formed by adding purified CheA and CheW to Tar and Tsr receptors in *E. coli* membranes. Our data shows that, similar to the situation with the soluble complexes, in the membrane-containing complexes receptor is present in an excess over the kinase, approximately 6:1. Moreover, the molecular activity of CheA within these complexes is the same as within the soluble complexes (22). The finding that fully active signaling complexes contain numerous receptors for every kinase raises the intriguing possibility that information is integrated within the sensory receptor array.

EXPERIMENTAL PROCEDURES

Purification of Proteins and Preparation of Membranes—The *S. typhimurium* proteins CheA (23), CheW (24), CheY (25), CheR (26), and $CheB_c$, the catalytic domain of the methylesterase CheB (26), were purified from overproducing strains of *E. coli* as described previously.

Wild type Tsr from *E. coli* encoded on plasmid pHSe/Tsr (27) was overexpressed in *E. coli* PS2002, a strain deleted for CheABRWYZ, Tap, and Tar (28). Inner membranes containing the Tsr receptor were prepared as described previously (29, 30) (Preparation I). Alternatively

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(Preparation II), harvested cells were suspended in 10 mM Tris/HCl (pH 7.2), 100 mM NaCl, 10 mM EDTA and broken in a French pressure cell. Cell debris was removed by centrifugation at 4000 × *g* for 20 min. The crude extract was centrifuged at 100,000 × *g* for 1 h in an SW 28 swinging-bucket rotor (Beckman). Membranes were suspended in 7.4% sucrose (w/v), 10 mM EDTA, 10 mM Tris/HCl (pH 7.2) and mixed with Opti-Prep (Invitrogen) in a 22:12 ratio. After 25 h centrifugation at 100,000 × *g* in an SW 28 rotor, the upper membrane band was collected from the self-formed gradient, washed with 20 ml of water, suspended in Buffer A (50 mM Tris/HCl (pH 7.2), 5.0 mM $MgCl_2$, 160 mM KCl, 0.50 mM EDTA, 0.020% NaN_3) (29), divided into aliquots, snap-frozen in liquid nitrogen, and stored at −80 °C. The Tsr receptor comprised approximately 34% of total membrane protein, as estimated from the relative intensities of the receptor and total protein bands on a Coomassie-stained gel using scanning densitometry (see below).

Wild type Tar from *S. typhimurium* was overexpressed from plasmid pME98 (15) in *E. coli* PS2002. Cells were grown in tryptone broth with 100 μg/ml ampicillin and 50 μg/ml kanamycin at 30 °C and harvested at OD ~0.8. Membranes with the overexpressed Tar were isolated following the protocol described above for Preparation II. Except for the final wash solution and the storage buffer, all solutions were supplemented with a protease inhibitor mix to give the following final concentrations: 1.0 μg/ml aprotinin, 1.0 μg/ml leupeptin, 1.0 μg/ml pepstatin, 0.10 mM phenylmethylsulfonyl fluoride, 5.0 mM 1,10-phenanthroline. In parallel, membranes from the PS2002 host strain not bearing any overproducing plasmids were prepared as a control.

Protein Quantification—Protein concentrations in stock solutions were estimated by UV absorption at 276–280 nm (31). Extinction coefficients at 276 nm ($mM^{-1}\ cm^{-1}$) of 16.0 for CheA, 5.95 for CheW, 32.4 for CheR, and 6.97 (at 280 nm) for CheY were calculated from the protein sequences. Concentrations of individual proteins in mixtures were estimated from Coomassie-stained SDS-polyacrylamide gels where a range of known amounts of the corresponding pure proteins were loaded as standards. The gels were scanned with a ScanMaker4 scanner (Microtek), and the images were quantified using NIH Image 1.61 software. Unless stated otherwise, all concentrations are expressed in terms of the indicated monomeric species.

The concentration of Tsr in membrane preparations was estimated by amino acid analysis of the protein bands excised from a 10% SDS-acrylamide gel upon electrophoretic separation of proteins. The determined amino acid composition of the protein was very similar to the one predicted from the sequence of the *tsr* gene, indicating the absence of impurities in the protein band and completeness of the protein hydrolysis. A known amount of bovine serum albumin was loaded on the same gel and quantified in parallel to correct for the efficiency of the procedure. The amino acid analysis was performed in The Howard Hughes Medical Institute Biopolymer/W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. The estimated value of the concentration of the Tsr receptor was supported by the data on serine binding to the receptor. The bound serine/receptor ratio at saturation was 0.44 (32), consistent with previous results showing that the *E. coli* Tsr and Tar receptor dimers can only bind one molecule of ligand at a time (33, 34).

Concentration of Tar receptor in the membrane preparation was estimated from the optical density of the corresponding protein band on a Coomassie-stained acrylamide gel as described above using the Tsr protein as a standard.

Formation of Receptor·CheW·CheA Complexes and Determination of the Subunit Stoichiometry in the Complexes—To form the complexes, receptor-containing membranes typically were incubated in 20 μl of Buffer A with various concentrations of CheA and CheW for 4 h at 22 °C. CheA and CheW stock solutions were centrifuged for 15 min at 4 °C prior to mixing with membranes to sediment any possible precipitate (no pellet was observed). After the incubation, 2.0-μl aliquots of the mixtures were usually removed for activity measurements, and the rest of the samples, 18 μl, were centrifuged for 5.0 min at 12,000 × *g* at room temperature. The supernatants were removed, and the pellets were dissolved in SDS-sample buffer and subjected to 11% SDS-PAGE. Amounts of the proteins in pellets were estimated by densitometry of the Coomassie-stained gels as described above. During these manipulations and incubations, there were no indications of proteolytic cleavage of receptors, CheA, or CheW either from the disappearance or alteration in mobility of protein bands or from decreases in kinase activity upon further incubation. The concentration of receptor in the supernatant after centrifugation was typically less than 5% of its original concentration in the mixture. To find out what amounts of CheA and CheW were "trapped" in the pellets due to incomplete removal of the supernatant, in parallel experiments ~35 μM [*methoxy*-^{3}H]inulin, 148 mCi/g, M_r 5000 (American Radiolabeled Chemicals), was added to the mixtures. No depletion of radioactivity in the supernatant upon centrifugation was detected, indicating that inulin does not sediment with or bind to the vesicles. The amount of radioactivity in the pellet was measured in a liquid scintillation counter and the volume of the solution trapped in the pellet calculated (0.25 ± 0.05 μl). These values were used to compute the amount of the bound protein as a difference between the total amount of a protein in a pellet as determined by densitometry in a gel and the amount of the trapped protein.

Accessibility of the cytoplasmic domain of Tsr and Tar for interactions with exogenously added proteins was estimated by measuring the availability of glutamates located in this domain for methylation by the CheR methyltransferase. A suspension of the membrane vesicles in Buffer A with a final concentration of receptor of ~6 μM was incubated for 3 h at 22 °C with 5.0 μM CheR and 200 μM *S*-adenosylmethionine. Upon this treatment, 90% (Preparation I) or 73% (Preparation II) of Tsr and 80% of Tar in membranes that contain this receptor were methylated as demonstrated by shifted mobility in a 10% SDS-PAGE. These results indicate that only the corresponding fractions of the receptors have their cytoplasmic domain accessible for complex formation with CheA and CheW. The values used for calculations of receptor stoichiometry in complexes refer only to the concentrations of receptors in membranes that were subject to methylation. This estimation was validated by the observation that in both Tsr Preparations I and II; the stoichiometry of CheW:Tsr binding at saturation was 1:1. These results indicate that, depending on experimental conditions, a variable amount of receptor can be exposed for complex formation, and this value must be independently determined for each new membrane preparation.

Kinase Activity Assays—Relative levels of steady-state activities of CheA in complexes with receptor and CheW in the presence of CheY were estimated by generation of $^{32}P_i$ from [γ-^{32}P]ATP. Complexes were prepared as described above and for the activity measurements diluted 1:20 in Buffer A containing 25 μM (final) CheY and 2.0 mM (final) [γ-^{32}P]ATP, 50 Ci/mol (PerkinElmer Life Sciences, 6000 Ci/mmol). Reactions were stopped after 20-min incubation at room temperature by addition of an equal volume of 100 mM EDTA, and 1.0-μl aliquots were loaded on a polyethyleneimine-cellulose plastic sheet (Merck) and developed in 125 mM KH_2PO_4 (Fig. 1). The relative radioactivity of the $^{32}P_i$ spots (R_f ~0.55) was quantified using PhosphorImager (Molecular Dynamics). Generation of $^{32}P_i$ under these conditions was constant for up to 30 min, indicating the relatively high stability of the receptor-kinase complexes.

Specific steady-state activity of CheA in complexes with Tsr and CheW was measured in Buffer A in the presence of 50 μM CheY and 2.0 mM ATP using the spectroscopic pyruvate kinase/lactate dehydrogenase coupled assay as described previously (35). The rate of the reaction measured in the presence of 4.0 mM serine was subtracted as a background (less than 25% of total activity). The same background rate of ATP hydrolysis was determined in the absence of CheY. The concentration of CheA in complexes was determined by SDS-PAGE as described above. Specific activities of CheA (s^{-1}) were calculated as a ratio of the rates of ATP hydrolysis (μM s^{-1}) and concentration of the membrane-bound CheA dimers (μM).

RESULTS

Formation and Characterization of Complexes between Receptors, CheW, and CheA—CheA and CheW binding to the *E. coli* serine receptor, Tsr, was measured over a range of CheA and CheW concentrations (Fig. 2). Purified CheA and CheW were mixed with membranes obtained from an *E. coli* strain, PS2002, with a chromosomal deletion extending from *cheA* through *cheZ* (28), and a multicopy plasmid, pHSe/Tsr, that overproduces Tsr (27). After incubation for sufficient time to achieve maximal complex formation, samples were sedimented and the membrane pellets dissolved in SDS sample buffer and subjected to polyacrylamide gel electrophoresis. Coomassie-stained bands corresponding to Tsr, CheA, and CheW were quantified using known amounts of the corresponding proteins as standards. Measurement of the binding of CheA and CheW to Tsr and Tar using scanning densitometry of Coomassie-stained PAGE gels has allowed us to quantify all three components in the complexes simultaneously over a wide range of subunit concentrations, avoiding potential inaccuracies associated with using radiolabeled CheW and CheA proteins (29).

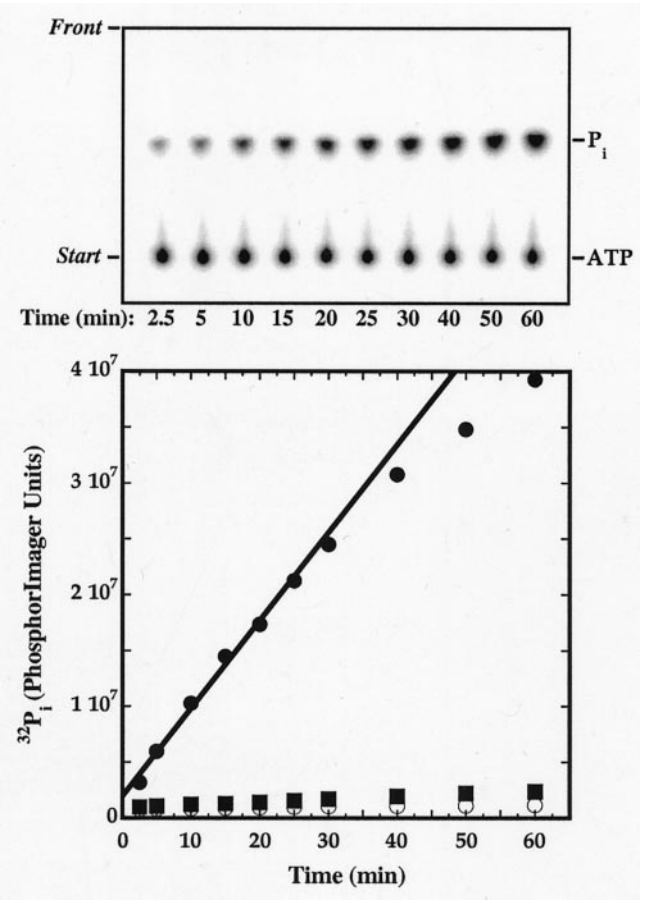

FIG. 1. **Measurement of the steady-state activity of CheA by generation of $^{32}P_i$.** Generation of $^{32}P_i$ was determined by quantitative analysis of the phosphorimaged TLC plates where aliquots removed at various times from the reaction mixtures containing Tsr·CheW·CheA (●), Tsr·CheW (○), or Tsr·CheA (■) complexes, CheY, and [γ-^{32}P]ATP were loaded (see "Experimental Procedures"). In the experiment shown here, the complexes were formed in incubation mixtures containing 6.4 μM Tsr (Preparation II, see "Experimental Procedures"), 20 μM CheW, and 10 μM CheA. Due to the rapid hydrolysis of phospho-CheY, the CheA·CheY mixture essentially constitutes an ATPase: CheA + CheY + ATP → CheA-*p* + CheY +ADP → CheA + CheY-*p* + ADP → CheA + CheY + ADP + P_I, and the rate of the reaction can be measured by generation of P_i. When CheY is provided in sufficient concentrations, the rate of CheA autophosphorylation is limiting (experimentally, the rate of the reaction varies linearly with the concentration of the ternary complex).

Kinase activities of the complexes were measured in parallel. The complexes formed by Tsr, CheW, and CheA appear to be rather stable. When diluted 20–40-fold into buffer their activity does not change for at least half an hour (Fig. 1).

CheW binds to the *E. coli* serine receptor, Tsr, with a 1:1 stoichiometry and an apparent K_d of 10 μM in the absence of CheA (Fig. 2*A*). This result is consistent with previous studies indicating that CheW can bind with 1:1 stoichiometry to Tsr in membranes (29) or to a fragment of the cytoplasmic domain of Tar (15). CheA appears to compete with CheW, *e.g.* 10 μM CheA causes about a 30% reduction in the level of CheW bound.

CheA binds to Tsr with a significantly higher affinity than CheW, K_d 1–2 μM (Fig. 2*B*). In contrast to CheW, binding of CheA to Tsr is substoichiometric with a ratio of CheA:Tsr at saturation of only about 1:10. Low concentrations of CheW increase CheA binding to maximal levels of 1 CheA subunit per six Tsr subunits without causing a substantial change in binding affinity. This fits the notion that CheW plays a role in organizing the receptor array to accommodate more CheA, in line with the observation that CheW is essential for receptor clustering *in vivo* (9).

High concentrations of CheW have previously been shown to inhibit CheA binding to Tsr (29, 36). This result has been interpreted in terms of the idea that CheW functions as an adapter to attach CheA to the receptors, so that at sufficiently high concentrations, CheW monomers bind independently to both the receptors and CheA, thereby jamming the process of complex assembly (15, 29). But CheA can bind to receptors in the absence of CheW, and the affinity of receptors for CheA is not substantially affected by the presence of up to 20 μM CheW. Furthermore, the inhibitory effects of high concentrations of CheW on CheA binding are paralleled by inhibitory effects of high concentrations of CheA on CheW binding. The reduction in CheW binding associated with high concentrations of CheA involves the displacement of two CheW subunits for each CheA subunit. The inhibitory interaction between CheW and CheA may be due, at least in part, to competition for overlapping binding sites on the receptor, consistent with the fact that CheW is homologous to the receptor interaction domain of CheA (7, 8).

Although CheW is not required for CheA binding to receptors, it is essential for kinase activation. At concentrations of CheW and CheA that give the highest total kinase activity, membrane signaling complexes are saturated at a subunit stoichiometry of ~6 Tsr:4 CheW:1 CheA (Fig. 2*A*).

The results obtained with the serine receptor, Tsr, were confirmed in experiments with the *Salmonella* aspartate receptor, Tar. Membranes enriched for Tar were prepared from *E. coli* PS2002 cells that contained a Tar overproducing plasmid. As with Tsr, substantial binding of CheA was observed in the absence of CheW, and CheW enhanced this value about 2-fold. The maximum values obtained for the ratio of bound CheA to Tar subunits, were essentially the same as with Tsr, ~1:6 (Fig. 3). As with the Tsr receptor, CheW was essential for kinase activation (data not shown). Although Tar was one of the major proteins in these membrane preparations, its level of overproduction was much lower than that obtained with the Tsr overproducing strain. This precluded direct measurements of membrane-bound CheW on Coomassie-stained polyacrylamide gels.

Chemotaxis receptors are encoded with several specific glutamate or glutamine residues in their cytoplasmic domains that are subject to methyl esterification by a specific *S*-adenosylmethionine-dependent methyltransferase, CheR (37), or demethylation/deamidation by a specific esterase/amidase, CheB (38). The Tsr and Tar receptors used in these studies were not modified, because they were expressed in a strain that lacks CheR and CheB. We have examined the effect of methylation and deamidation on formation of receptor signaling complexes by generating the complexes in the presence of CheR plus *S*-adenosylmethionine or CheB plus ATP. Neither methylation nor deamidation had any significant effect (less than 26%) on the stoichiometry of the signaling complexes (Table I).

Kinase Activity in Receptor Signaling Complexes—It has previously been shown that the histidine kinase activity of CheA can be increased more than 100-fold in the presence of CheW plus receptors (39). CheW is essential for the membrane receptor-mediated CheA activation. Similar results were obtained earlier with soluble fragments of the Tsr cytoplasmic domain (14). Histidine kinase activity per mole of receptor-bound CheA increases linearly with increasing amounts of bound CheW, independent of the total amount of CheA bound (Fig. 4*A*). Linearity extends from levels of CheA that are high enough to displace CheW to levels of CheW that are sufficiently high to begin to displace bound CheA. In the absence of receptors, micromolar concentrations of CheW have no apparent affect on CheA activity; but although the total intracellular concentra-

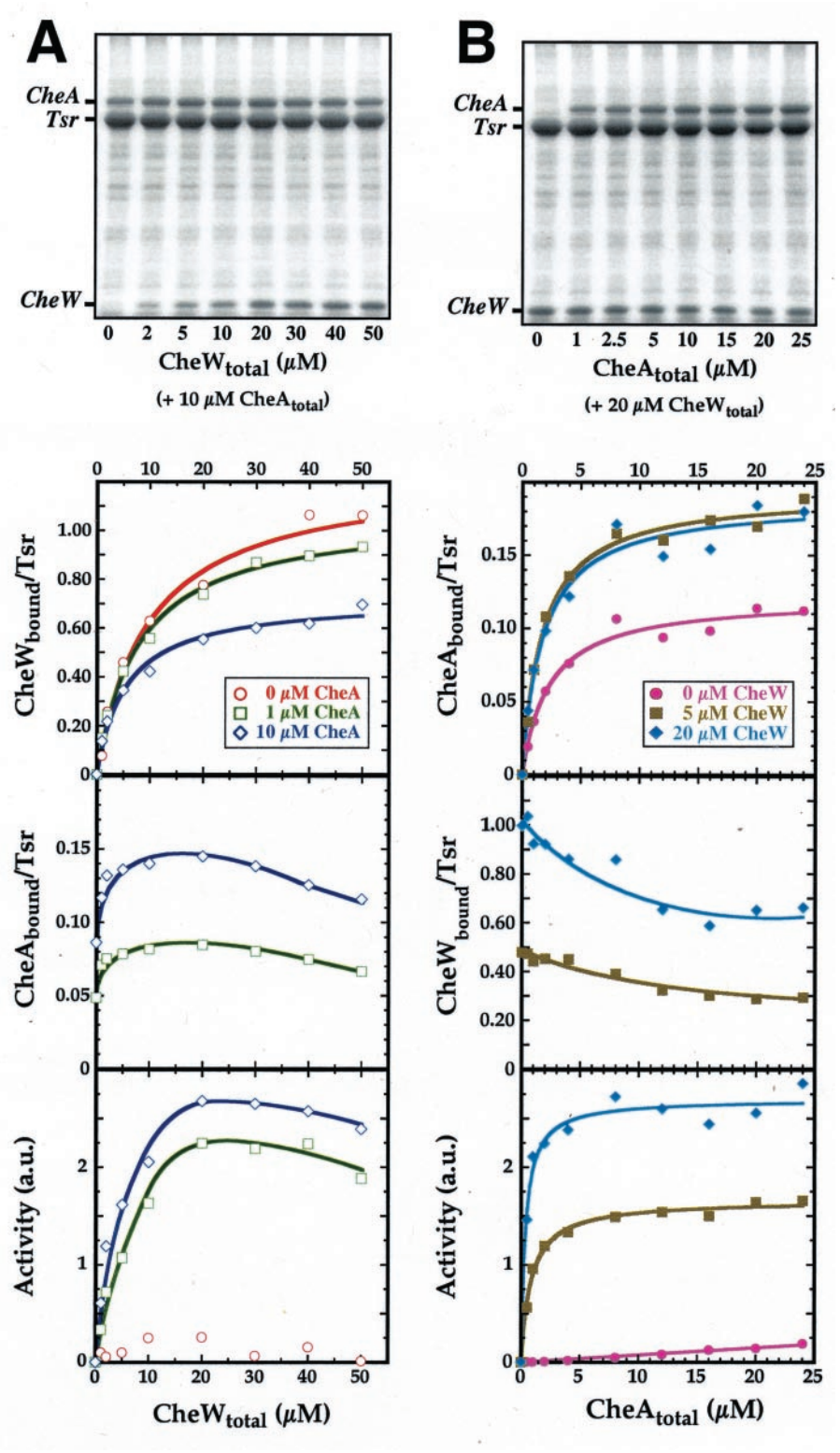

FIG. 2. **Composition and activity of Tsr·CheW·CheA complexes.** Complexes were formed, isolated, and analyzed by scanning densitometry of polyacrylamide gels (examples in the *top row*) to determine the amounts of bound CheW and CheA. Kinase activity (expressed in arbitrary units, *a.u.*) was measured by generation of $^{32}P_i$ (see "Experimental Procedures"). The $CheW_{bound}$/Tsr and $CheA_{bound}$/Tsr ratios were calculated as described under "Experimental Procedures." Complexes were formed by incubating mixtures containing 6.4 μM Tsr plus the indicated concentrations of CheW and 0, 1.0, or 10 μM CheA (*A*) and the indicated concentrations of CheA and 0, 5.0, or 20 μM CheW (*B*). Essentially the same results were obtained with two different Tsr membrane preparations (Preparations I and II, see "Experimental Procedures"). The SDS-PAGE gels pictured in this figure were obtained using Preparation I, and the quantitative data shown in the figure were obtained by scanning gels from experiments where Preparation II was used.

tion of CheW in wild type *E. coli* cells is in the micromolar range (29), within receptor signaling complexes the effective local concentration would be orders of magnitude higher. The catalytic ATP-binding domain of CheA is homologous to the ATP-binding domains of the Hsp90/topisomerase II/MutL family of ATPases (7). These proteins all undergo complex cycles of reversible domain interactions that are driven by the energy released by ATP hydrolysis (40). ATP binding results in the closure of a relatively disorganized loop termed the ATP-binding lid, which produces a surface for the subsequent binding of other protein domains (41). This leads to phosphotransfer to water or, in the case of CheA, to a histidine side chain in the histidine phosphotransfer (HPt)[1] domain, followed by dissociation of this domain and release of ADP (Fig. 4*B*). Kinetic analysis of CheA activity indicates that activation in receptor signaling complexes results from enhanced formation of the phosphotransfer complex between the ATP binding catalytic

[1] The abbreviation used is: HPt, histidine phosphotransfer.

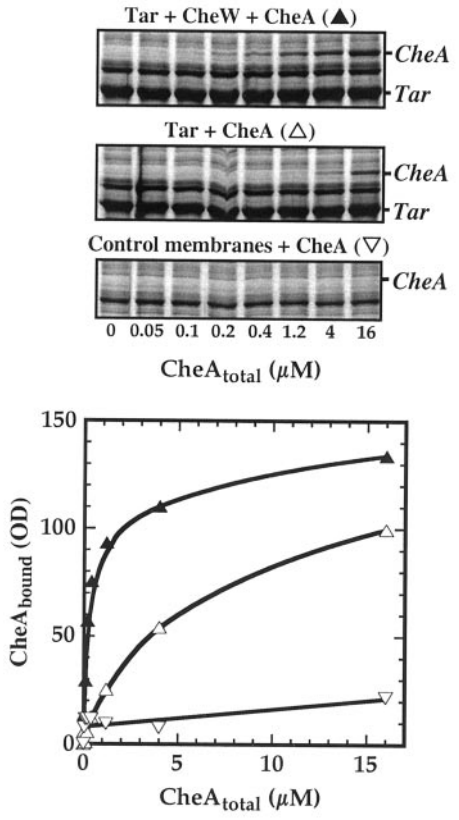

FIG. 3. **Binding of CheA to Tar.** The indicated concentrations of CheA were incubated in the presence of 5.0 μM CheW with membranes enriched for Tar, in the absence of CheW with membranes enriched for Tar, or in the absence of CheW with control membranes lacking Tar. Amounts of membrane-associated CheA are given in terms of relative optical density of bands on polyacrylamide gels as determined by scanning densitometry.

TABLE I
Effects of Tsr glutamyl modification status on CheA and CheW binding to Tsr

Tsr·CheW·CheA complexes were formed by incubation of the mixture containing 6.4 μM Tsr, 5.0 μM CheW, 1.0 μM CheA and, where indicated, 5.0 μM CheR (with or without 176 μM *S*-adenosylmethionine), or 5.0 μM $CheB_c$, or 5.0 μM CheB + 1.8 mM ATP. Complexes were formed and their relative composition estimated by comparing Coomassie-stained SDS-PAGE gels as described under "Experimental Procedures." Data represent averages of two experiments, deviations did not exceed 10%.

Components in the incubation mixture	CheA bound	CheW bound
	%	
Tsr + CheW + CheA	100	100
Tsr + CheW + CheA + CheR	113	118
Tsr + CheW + CheA + CheR + SAM	104	106
Tsr + CheW + CheA + $CheB_c$	123	126
Tsr + CheW + CheA + CheB + ATP	121	125

domain and the phosphoaccepting HPt domain of CheA (21). CheW could accomplish this by simply binding to CheA so as to shift the equilibrium toward the HPt-bound form.

We have estimated the specific activity (turnover number) of CheA in Tsr·CheW·CheA complexes using the coupled spectrophotometric assay for reaction rate measurements and densitometry of the Coomassie-stained gels for determination of CheA concentrations in the complexes (see "Experimental Procedures"). The complexes were produced and the specific activity measured at four sets of concentrations of CheA and CheW that cover the range estimated for the *E. coli* cytoplasm (29, 42). The average specific activity of CheA dimers in membrane receptor complexes was 60 ± 14 s^{-1} (Table II). This is 570 ± 150 times higher than the specific activity of pure CheA dimers measured under the same conditions (0.106 ± 0.008 s^{-1}).

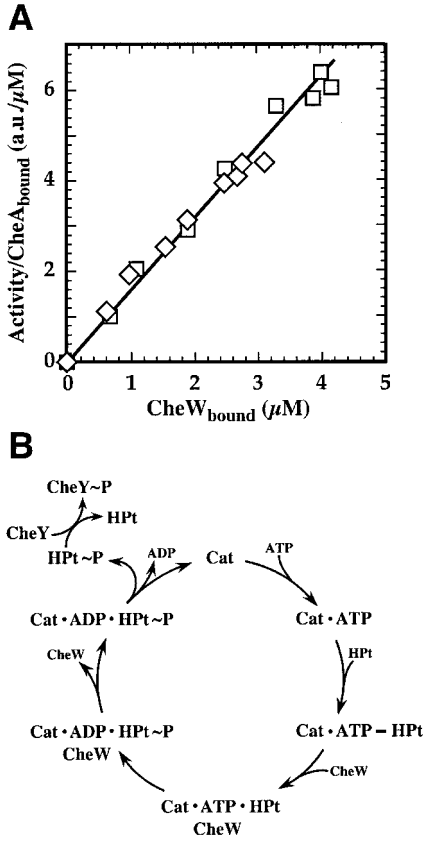

FIG. 4. **Specific activity of CheA in Tsr·CheW·CheA complexes is proportional to the total level of bound CheW.** *A*, specific activity of CheA and concentration of CheW associated with receptor signaling complexes were determined as described in the legend to Fig. 2*A* using Preparation II of Tsr membranes. Complexes were formed in the presence of 1.0 μM (*squares*) or 10 μM (*diamonds*) CheA and the indicated concentrations of CheW. *B*, model for CheA catalytic cycle. The catalytic domain of CheA, Cat, binds ATP. This causes the ATP-binding lid to close, creating a binding surface for the HPt domain from a second subunit of CheA. CheW binding facilitates tight binding of HPt to accelerate the phosphotransfer reaction. The resulting phosphorylated HPt domain then dissociates from the catalytic domain and readily transfers its phosphoryl group to the response regulator, CheY. Finally, the ATP-binding lid is released, and ADP dissociates to complete the cycle.

TABLE II
Specific activity and activation of CheA

Protein concentration in incubation mixture (μM)			Specific activity[a]	Activation factor[b]
Tsr	CheW	CheA		
			s^{-1}	
6.4	5	1	46 ± 2	427
6.4	20	1	68 ± 2	640
6.4	5	10	56 ± 4	527
6.4	20	10	74 ± 8	697

[a] Specific steady-state activity (turnover number) of the receptor-bound CheA dimers (mean ± S.D. of four experiments) was measured using the spectroscopic assay (see "Experimental Procedures").

[b] Compared with the activity of CheA dimers alone under these conditions, 0.106 ± 0.008 s^{-1}.

DISCUSSION

It has generally been assumed that the basic signaling unit in bacterial chemotaxis is a complex composed of a receptor dimer and a dimer of CheA held together by two CheW subunits (42, 43). Most of the evidence supporting this hypothesis

has been circumstantial, however. The only direct experimental basis for the existence of the 2:2:2 receptor·CheW·CheA complex is a set of experiments where binding of radiolabeled CheA and CheW to Tsr-enriched membranes was monitored by depletion of radioactivity in the supernatant after pelleting the membrane vesicles (29). Estimates of the total number of binding sites for CheA and CheW were computed from an extrapolation of a Scatchard plot of the binding data. We have directly measured the binding of CheA and CheW to Tsr and Tar using scanning densitometry of Coomassie-stained PAGE gels. This technique allowed us to quantify all three components simultaneously over a wide range of subunit concentrations without using radiolabeled proteins. Our results argue strongly against the 2:2:2 dimer signaling model. There are three fundamental inconsistencies between our results and the previous model: (i) the stoichiometry of CheA binding to receptors is far below the 1:1 value required for the 2:2:2 model; (ii) CheA can bind to receptors independently of CheW; and (iii) CheA and CheW compete for binding.

Wild type *E. coli* has five different chemotaxis receptors: Tsr, Tar, Trg, Tap, and Aer, each characterized by a variable N-terminal-sensing domain connected to the conserved coiled-coil signaling domain. Most of the receptors are clustered within one or two patches at the cell poles (9–13). It has been estimated that there are at least 2900 serine and 600 aspartate binding sites in membranes isolated from wild type *E. coli* cells (44). Because of negative cooperativity, each receptor dimer has only one binding site, so there should be roughly 3500 Tar plus Tsr receptor dimers per cell. Assuming that each of the three other *E. coli* chemotaxis receptors, Trg, Tap, and Aer, is present at about a tenth the level of the serine and aspartate receptors (45), the total number of receptor dimers per cell is roughly 5,000. Trg, which is homologous to Tsr and Tar, has been shown to form a two-dimensional crystalline array in phospholipid membranes, with a square unit cell 8.8 × 8.8 nm that contains four regular peaks of electron density, most likely corresponding to four receptor dimers (46). Assuming a similar packing in receptor clusters and considering dimensions of the ligand-binding domain dimer known from its crystal structure (47), 5000 receptor dimers would occupy a 380-nm diameter area. This is roughly the size of the polar receptor patches that have been detected by immunoelectron microscopy (9–11). Our results indicate that the maximum level of CheA binding is one CheA for every six receptors. This makes sense considering the apparent tight packing of receptors in the membrane and the relatively large size of CheA (Fig. 5). The crystal structure of a part of the cytoplasmic domain of Tsr indicates that the cytoplasmic regions of receptors form antiparallel coiled-coil structures. In the crystal, the antiparallel helices interact to form four-helix bundles that are organized in trimeric clusters (48). If this is true for the receptors in membranes and such a trimer constitutes a binding site for one of the receptor-binding domains in a CheA dimer, this would fit the observed 6 receptor:1 CheA stoichiometry of the signaling complexes. Such an arrangement would place two unbound receptor subunits within the 8-nm gap between receptor-binding domains of CheA, which is consistent with our finding that, at saturation, every CheA subunit that binds to receptors precludes the binding of two CheWs.

This organization of the signaling complexes with several interlinked chemotaxis receptors sharing the same kinase would be advantageous for processing the diverse and sometimes contradictory sensory information feeding into the system. For example, when receptors simultaneously bind an attractant and a repellent, the inhibiting and activating signals can be integrated during the signal transduction process that

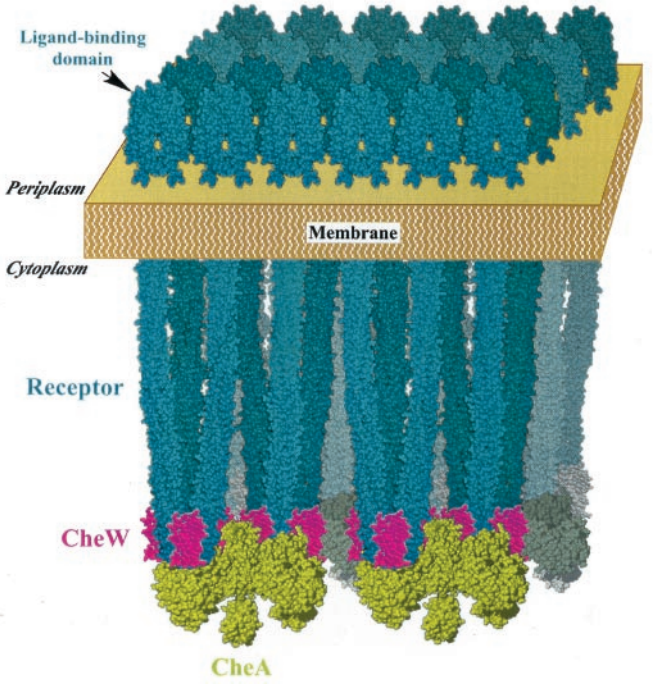

FIG. 5. **Model of the receptor/CheW/CheA array.** Atomic coordinates for the sensing domain of Tar (47), part of the cytoplasmic domain of Tsr (48), CheW (8), and the domains of CheA (7, 58, 59) were used to generate proportional space-filling models of the proteins.

leads to kinase regulation rather than at a step beyond the kinase (49). Unlike other histidine kinase signaling systems, in the chemotaxis system the kinase part and the transmembrane sensing part are separate polypeptides. The lateral interactions within the chemotaxis receptor array might also explain the phenomena that the so-called minor receptors, Trg (receptor for ribose and glucose) and Tap (receptor for dipeptides), which are present at less than 10% of the abundant Tar and Tsr can produce full scale chemotactic responses, but only when the major receptors are present (50, 51). Experimental data indicating the possibility of inter-receptor communications has been published recently (52, 53). In the future it would be interesting to investigate whether heterodimers of the ligand-binding domains are formed in membranes, which could allow bacteria to respond to a broader range of chemoeffectors, similar to the situation with the eukaryotic ErbB receptors binding epidermal growth factors where heterodimerization and clustering diversifies their ligand specificity (54).

Results from studies of receptor signaling assemblies indicate that they are very large structures composed of thousands of signal transduction components. In addition to CheW and CheA, in *E. coli* these complexes contain five different receptors, each subject to several glutamyl modifications. The complexity of this structure is staggering, it is no wonder that every individual bacterium has its own characteristic pattern of behavior (55). The primary function of the receptor signaling complexes is almost certainly much more concerned with processing information to make appropriate decisions, rather than merely sensing attractant and repellent stimuli (56). The bacterial receptor signaling apparatus with receptors, CheW, CheA, and the methylation adaptation system is highly conserved in virtually all motile prokaryotes including both Archaea and Eubacteria (6, 57). This suggests that over the past few billion years this set of interacting proteins has evolved an almost ideal mechanism to gather sensory information and use it to formulate the second to second decisions that are required to control motility.

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