In bacterial chemotaxis, clustered transmembrane receptors and the adaptor protein CheW regulate the kinase CheA. Receptors outnumber CheA, yet it is poorly understood how interactions among receptors contribute to regulation. To address this problem, receptor clusters were simulated using liposomes decorated with the cytoplasmic domains of receptors, which supported CheA binding and stimulation. Competitive and cooperative interactions were revealed through the use of known receptor signaling mutants, which were used in mixtures with the wild type domain. Competitive effects among the receptor domains sorted cleanly into two categories defined by either stronger or weaker interactions with CheA. Cooperative effects were also evident in CheA binding and activity. In the transition from the stimulating to the inhibiting states, both the cooperativity of the transition and the persistence of stimulation by the wild type domain increased with receptor modification, as in the intact receptor. We conclude that competitive and cooperative receptor interactions both contribute to CheA regulation and that liposome-mediated assembly is effective in addressing these general membrane phenomena.

Chemotaxis enables motile bacteria to swim either up or down gradients of specific attractants and repellents, respectively, via a signal transduction pathway that modulates cell motility. The well studied pathway in *Escherichia coli* is remarkable for the capacity to detect small changes in concentration (~0.1%) via temporal comparisons (~4 s) over a large range (~10^-3 to 10^-1 M) (1, 2). The chemical stimulants are detected through specific ligand binding interactions with transmembrane receptors that are clustered in the membrane, frequently at the cell poles (3, 4).

The clusters of chemoreceptors are heterogeneous with respect to the specificity and mode of ligand binding (5). In *E. coli*, five homologous receptors participate in cluster formation. The cytoplasmic signaling domain is highly conserved throughout the Bacteria and Archaea (6), which bind to an Src homology 3-like adaptor protein, CheW, and the kinase, CheA, to form a core signaling complex consisting of receptors, CheW and CheA (7, 8). The kinase activity of CheA in the signaling complexes is several hundredfold greater than the CheA dimer alone, and the activity is inhibited by the binding of attractants to the receptors (9–12). The regulation of CheA in the assembly dictates the magnitude and duration of responses to chemotactic stimuli, which reflect inputs originating from different receptors. In reconstituted signaling complexes generated with the aspartate receptor or the serine receptor, ligand-mediated kinase inhibition can exhibit significant positive cooperativity (10, 13). These data suggest that in the cell, receptors are organized in heterogeneous clusters of dimers, which are the cooperative units that regulate CheA (13, 14). Theories that invoke coupling interactions among receptor dimers in a general way, to account for signal integration and the regulation of kinase activity, provide a satisfactory explanation for the experimental observations of attractant-mediated kinase inhibition (15–17), but the molecular basis of coupling among receptors is still poorly understood.

Receptor clusters have a hierarchical organization of subunits that is compatible with cooperative mechanisms of enzyme regulation. In this hierarchy, the receptor homodimer is the fundamental unit that defines ligand-binding specificity (reviewed in Ref. 18). Fig. 14 depicts an atomic model of a receptor dimer from *E. coli* (19). Each monomer consists of a four-helix bundle that (as a dimer) binds ligand (20), two membrane-spanning helices, and an antiparallel coiled-coil cytoplasmic signaling domain. The overall length of the dimer is ~300 Å (21); the width is about one-tenth the length. Dimers associate further through interactions among cytoplasmic domains and through interactions with CheW and CheA. Both trimer-of-dimers (19) and row-of-dimers arrangements (22) have been observed in crystals of the cytoplasmic domain fragment, which are postulated to form the basis of the receptor-CheW-CheA signaling arrays (22, 23).

Structure-guided mutagenesis and cross-linking experiments that sought to find interactions between cytoplasmic domains near the sites of CheW and CheA binding have provided evidence for such interactions. These interactions occur between receptor dimers that differ in the ligand binding specificity, which are interpreted in support of receptor signaling teams (5, 24). Point mutations in the cytoplasmic domain characterized during the course of these and other studies (25) have strong effects on signaling and are thus useful reagents for understanding CheA regulation. Some of the mutations used in

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this study are shown in Fig. 1B. In some cases the mutations can be located far from the CheA- and CheW-binding sites, but significant influences on CheA activity are observed nonetheless. The basis for these effects is not understood.

Reconstituted assemblies of the intact receptor have generated important insights into the cooperative nature of CheA regulation. Yet these samples are complex (21), and the difficulties that plague the use of transmembrane proteins create significant problems for quantitative studies. To circumvent these difficulties and still retain the key features of the core signaling complex, we used template-directed self-assembly of receptor signaling complexes (Fig. 1C) (26). In this method, the histidine-tagged cytoplasmic domain fragment of the aspartate receptor (CF) has a specific binding interaction with a Ni\(^2\+\)-chelating phospholipid in the outer leaflet of unilamellar vesicles. CheW and CheA then bind to the CF to form functional signaling complexes (26). Template-directed assembly has been used here to investigate the properties of CheA regulation in receptor clusters involving mixtures of CFs. Through the use of the mutated forms of the CFs that have altered protein interaction strengths, we demonstrate that the CF alone has the necessary domain interactions to generate cooperative CheA binding and inhibition. Moreover, binary mixtures of stimulating and nonstimulating CFs reveal competition among CFs in binding and stimulating the kinase. We suggest that both processes contribute to the regulation of CheA in the cell, which taken together can help explain how chemotactic stimuli are channeled through heterogeneous receptor clusters.

**EXPERIMENTAL PROCEDURES**

**Proteins and Protein Purification**—Histidine-tagged CFs of the aspartate receptor were prepared from the expression plasmids pSM100, pHTCF, and pSM101, which produced CF in low (CF\(_{\text{ab}}\)), intermediate (CF\(_{\text{I-OE}}\)), and high (CF\(_{\text{Q}}\)) levels of glutamine modification to mimic receptor methylation (26). Primer-directed PCR mutagenesis (Stratagene, San Diego) was used to introduce the point mutations listed in Table 1. All mutations were verified by sequencing (Davis Sequencing, Davis, CA). CheY, CheA, and CheW were prepared as described previously (26).

**Lipid Vesicles**—Sonicated unilamellar vesicles were prepared from chloroform solutions of Ni\(^2\+\)-DOGS and DOPC (Avanti Polar Lipids, Alabaster, AL), which were mixed to give a 1.5:1 molar ratio, evaporated to dryness under a stream of gaseous N\(_2\), and resuspended in kinase assay buffer (75 mM Tris-HCl, 100 mM KCl, 5 mM MgCl\(_2\), 2 mM tris(2-carboxyethyl)phosphine, 5% Me\(_2\)SO) at 1 mg/ml total lipid. The suspension was sonicated for ~90 min at 25°C, which resulted in a transparent solution.

**Kinase Assay**—A coupled ATPase assay was used to measure steady-state kinase activity of CheA in template-assembled complexes (26). Typical lipid and protein concentrations were 150 μM accessible Ni\(^2\+\)-DOGS, 30 μM CF, 1.2 μM CheA, and either 4 or 15 μM CheW (with CF\(_{\text{Q}}\) and CF\(_{\text{A}}\), respectively). Complex assembly was allowed to proceed for 4 h at 25°C. After the complexes were assembled, the activity was measured immediately after 100-fold dilution into a solution that also contained 1 mM ATP and 50 μM CheY. The activity of vesicle-bound CheA (s\(^{-1}\)) was computed from the measured activity (dc/dt) divided by the concentration of vesicle-bound CheA (f\(_B\) × [CheA]\(_{\text{total}}\)). All data points are averages of at least two independent measurements, and the error bars are standard deviations.

**Measurements of Complex Assembly**—The fractions of vesicle-bound CheA and CheW (f\(_B\)) were measured under the same conditions used to assemble CF-CheW-CheA complexes. Aliquots removed prior to and after centrifugation (15 min at 60,000 rpm in a Beckman 120.2 rotor and TLX centrifuge) were resolved by SDS-PAGE and analyzed with scanning densitometry (Bio-Rad model GS-700 densitometer) to estimate the total and free concentrations of protein, respectively, from which f\(_B\) was computed.

**Binding Experiments**—The vesicle sedimentation method was also used to estimate association constants and binding capacities of the surface-templated CFs for CheW and CheA. These experiments used vesicles with a 1.5 to 1 molar ratio of Ni\(^2\+\)-DOGS to DOPC at a total lipid concentration of 500 and 30 μM CF. Under these conditions all the CF cosedimented with vesicles (with an approximate limit of detection of 1% in the supernatant fraction). Binding isotherms were fit to a single site model using Origin 7.5 (Northampton, MA) as shown in Equation 1,

\[
[P]_{\text{bound}} = \frac{n \cdot [P]_{\text{free}}}{(K_A^{-1} + [P]_{\text{free}})} \quad (\text{Eq. 1})
\]

where [P]\(_{\text{bound}}\) and [P]\(_{\text{free}}\) are bound and free protein concentrations (CheW or CheA), respectively; K\(_A\) is the association constant, and n is the concentration of binding sites.

**Models of CheA Activity and Complex Formation**—Specific activity and ternary complex formation data were modeled to discern the nature of the competitive and cooperative interactions. The overlapping site model assumed the following: (i) that CheW (W) and CheA (A) bound competitively to the same site, or overlapping sites, on the CF (C); (ii) bound with different affinities to WT- and NS-CFs (C\(_{\text{WT}}\) and C\(_{\text{NS}}\), respectively), and (iii) the C-W and C-A affinities were influenced similarly by the mutation introduced into C, i.e., R = K\(_{C-W}\)K\(_{C-A}\) = K\(_{C-W}\)K\(_{C-W}\). The CheA activity on binary CF mixtures is then given by Equation 2,

\[
\text{Act} = \frac{\text{Act}_A[C^*A] + \text{Act}_C[C^*A]}{[C^*A] + [C^*A]} \quad (\text{Eq. 2})
\]

where Act\(_A\) and Act\(_C\) are the CheA activities bound to 100% WT-CF and 100% NS-CF arrays, respectively; and [C\(_{\text{WT}}^*\)A] and [C\(_{\text{NS}}^*\)A] are the CheA concentrations bound to WT- and NS-CFs, respectively (in the presence of CheW), estimated through the model with constraints imposed by the activity and binding data. A second competitive model based on bridging interactions was also explored (26) but proved to be unsatisfactory in

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3 The abbreviations used are: CF, cytoplasmic fragment; WT-CF, wild-type CF; NS-CF, nonstimulating CF; ST-CF, kinase-stimulating CF; f\(_B\), fraction of CheA or CheW bound to vesicles; Ni\(^2\+\)-DOGS, 1,2-dioleoyl-sn-glycero-3-phospho-N-[5-aminocarbamoyl]lummidodiacetic acid-succinyl (nickel salt); DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; NLSF, nonlinear least squares fit.

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The vesicle sedimentation method was also used to estimate association constants and binding capacities of the surface-templated CFs for CheW and CheA. These experiments used vesicles with a 1.5 to 1 molar ratio of Ni\(^2\+\)-DOGS to DOPC at a total lipid concentration of 500 and 30 μM CF. Under these conditions all the CF cosedimented with vesicles (with an approximate limit of detection of 1% in the supernatant fraction). Binding isotherms were fit to a single site model using Origin 7.5 (Northampton, MA) as shown in Equation 1,

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\]

where Act\(_A\) and Act\(_C\) are the CheA activities bound to 100% WT-CF and 100% NS-CF arrays, respectively; and [C\(_{\text{WT}}^*\)A] and [C\(_{\text{NS}}^*\)A] are the CheA concentrations bound to WT- and NS-CFs, respectively (in the presence of CheW), estimated through the model with constraints imposed by the activity and binding data. A second competitive model based on bridging interactions was also explored (26) but proved to be unsatisfactory in
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fitting both activity and binding data. The implementation of the overlapping site and bridging models are described in the Appendix and the supplemental material, respectively.

Cooperative changes in CheA activity and the fraction of bound CheA, $f_{o}$, were fit to Equation 3,

$$Act = Act^o - \Delta Act \frac{X^o}{X_{1/2}^o + X^o} \quad (\text{Eq. 3})$$

where Act is the activity (s$^{-1}$) of vesicle-bound CheA for a given value of $X$, the NS-CF mole fraction. Act$^o$ is the activity when $X = 0$, and $\Delta Act$ is the difference in activity at $X = 0$ and $X = 1$. $X$ is equal to $X_{1/2}$ when Act equals Act$^o - \Delta Act/2$, and $\alpha$ is the cooperativity coefficient. Equation 3 was also used to analyze cooperative changes in $f_{o}$, except that Act, Act$^o$, and $\Delta Act$ were replaced by the experimental values of $f_{o}$, an estimate for $f_{o}$ with vesicles of 100% WT-CF ($f_{o}^\text{WT}$), and the difference in $f_{o}$ between 100% WT-CF and 100% NS-CF ($\Delta f_{o}$).

RESULTS AND DISCUSSION

Assembly and Activity of Receptor Complexes—Fig. 1C depicts CF-CheW-CheA complexes assembled on a lipid bilayer. Previously, these complexes had been assembled on vesicles to demonstrate that wild type CF (WT-CF) stimulated CheA at all levels of adaptational modification (26). Here these template-assembled complexes were used to determine the requirements for kinase stimulation. Several CFs, each containing a point mutation known to interfere with stimulation (NS-CFs), were tested and compared with WT-CF. The positions of these mutations in the sequence of the intact receptor and their known properties are summarized in Table 1. Vesicles were also decorated with equimolar WT-CF/NS-CF mixtures to assess the impact of having stimulating and nonstimulating CFs present simultaneously. The specific activities (s$^{-1}$) of the CheA bound to vesicles decorated with WT-CF, NS-CF, or a 1:1 WT-CF/NS-CF mixture are plotted in Fig. 2, A–D, and E–H shows the fraction of CheA bound to these vesicles ($f_{o}$ (CheA)), and I–L shows the fraction of bound CheW ($f_{o}$ (CheW)). The activities reported in Fig. 2, and elsewhere under “Results,” assumed that only vesicle-bound CheA contributed significantly to activity, which was possible because the specific activity of CheA in solution was small by comparison (<0.1 s$^{-1}$), and in the majority of cases the activities in the presence of CF-decorated vesicles and CheW were at least 10-fold greater (>1.0 s$^{-1}$). The experiments depicted in Fig. 2 were conducted with CFs in the two extremes of covalent modification, where glutamine (Q) residues were substituted for glutamate (E) residues at the four major methylation sites (CF$_{4E}$ and CF$_{4Q}$, filled and open symbols, respectively) (27). Glutamine substitution mimics the effect of receptor methylation, which is involved in the process of adaptation during bacterial chemotaxis (28, 29).

On the basis of kinase activity, the NS-CFs were sorted into two groups, inhibitory (Fig. 2, A and C) and permissive (Fig. 2, B and D). CheA activity dropped progressively in the inhibitory group as the mole fraction of NS-CF increased. In contrast, CheA bound to 1:1 mixtures of WT-CF and NS-CFs from the

**TABLE 1**

| Mutation   | Phenotype | Covalent modification$^a$ | Kinase stimulation$^b$ | Inhibitory/permissive$^b$ |
|------------|-----------|---------------------------|------------------------|--------------------------|
| S25L$^+$  | Constitutively CCW$^+$ | 4E and 4Q                 | Nonstimulating         | Inhibitory               |
| I375Q$^+$ | Dominant$^+$      | 4E and 4Q                 | Nonstimulating         | Permissive               |
| L376A$^+$ | Epistatic$^+$    | 4E and 4Q                 | Nonstimulating         | Inhibitory               |
| V382P$^+$ | Resistant$^+$    | 4E and 4Q                 | Nonstimulating         | Permissive               |
| E383A$^+$ | Dominant/rescue$^+$ | 4E and 4Q                 | Nonstimulating         | Permissive               |
| S461L$^+$ | Constitutively CCW$^+$ | 4E and 4Q                 | Stimulating            | Inhibitory               |
| G338L$^+$ | Kinase-stimulating$^+$ | 4E                     | Stimulating            |                          |
| V436M$^+$ | Constitutively CW$^+$ | QEQE                    | Stimulating            |                          |
| V433F$^+$ | Constitutively CW$^+$ | QEQE                    | Stimulating            |                          |
| A436V$^+$ | Constitutively CW$^+$ | QEQE                    | Stimulating            |                          |

$^a$ Amino acids at the major methylation sites, Gln-295, Glu-302, Gln-309, and Glu-491, in full-length aspartate receptor prior to modification, which are referred to by QEQE, etc. (27).

$^b$ Classified by the activity generated in the template-assembled assay (Figs. 2, 6, 7, and 9).

$^c$ Terminology adopted for the trimers-of-dimers mutations generated by Ames et al. (5).

$^d$ In this study, G338L-CF displayed strong CheA and CheW binding and stimulated activity.
permissive group remained fully stimulated. In some cases the retention of activity depended on covalent modification (Fig. 2, B and D). Operationally, the retention of full CheA activity in the WT-CF4Q/NS-CF4Q mixture was deemed sufficient to classify a mutation as permissive, whether or not full activity was also retained with the WT-CF4E/NS-CF4E mixture.

The inhibitory and permissive mutations also had distinct effects on CF-CheW-CheA interactions, which were evident in $f_B$ (CheA) and $f_B$ (CheW), shown in Fig. 2, E–H and I–L, respectively. None of the permissive NS-CFs were as effective as WT-CF at complex formation; $f_B$ was significantly lower for both CheA and CheW (Fig. 2, F, H, J, and L). That only small levels of CheA and CheW binding were observed with the permissive CFs (V382P especially) eliminated the possibility that CheA and CheW bound to vesicles nonspecifically. By contrast, two inhibitory NS-CFs (S325L, L376A) promoted CheA and CheW binding at extents comparable with, or greater than, WT-CF (Fig. 2, E, G, I, and K). Thus, these inhibitory NS-CFs lowered CheA activity through a strong competition for CheW and CheA. The S461L NS-CF was also classified as inhibitory, despite the fact that it was less efficient in forming signaling complexes. As described below, the S461L NS-CF inhibited CheA in a qualitatively different manner.

The binding of CheW and CheA to CF-decorated vesicles was assessed by sedimentation in a separate set of experiments. Fig. 3 plots CheW binding to the WT-CF and various NS-CFs in low and high levels of covalent modification (Fig. 3, A and B, respectively). The binding affinities and capacities, which were estimated with a single site binding model (Equation 1), are summarized in Table 2. The introduction of inhibitory mutations (S325L and L376A) generated measurable increases in binding affinity, whereas the permissive mutations (I375P, V382P, and E383A) reduced the binding strength by large amounts.

In the case of WT-CF and the strong-binding inhibitory CFs, estimates for the total concentration of binding sites were generated in fits of the data to the single-site model. These values, which ranged from 15 to 21 $\mu$M, were generally smaller than the total CF concentration used in the experiment (30 $\mu$M). Although the binding ratios calculated with these results are approximately consistent with a 1:1 CheW/CF binding ratio, which has been reported previously (11, 30), they are similarly consistent with a 1:2 interaction. The origin of the substoichiometric binding, i.e. less than one CheW per CF, is not known, but it could be because of incomplete assembly of the cytoplasmic domains on the vesicle surface, such as dimerization.

On the other hand, the effects of the mutations on the CheW binding strength could not be clearer. The inhibitory mutations S325L and L376A produced significant increases in strength,
whereas the permissive mutations (I375P, V382P, and E383A) have much lowered interaction strengths. These effects clearly impact the manner and efficiency with which the cytoplasmic domain forms active signaling complexes with CheW and CheA.

A limited number of vesicle binding experiments were conducted with CheA. Fig. 4 shows that CheA exhibited saturable binding to CF-decorated vesicles in the absence of CheW. The fits of these data to the single sites model with the parameters in Table 3.

Table 2: Parameters of CheW binding to template-assembled CFs

| CF           | $K_{CW}$ | Concentration of sites |
|--------------|----------|------------------------|
|              | CF$_{4E}$ | CF$_{4Q}$ |
| WT           | 0.08 ± 0.01 | 0.13 ± 0.02 | 21.0 ± 2.1 | 18.8 ± 2.4 |
| S325L        | 0.20 ± 0.02 | 0.37 ± 0.06 | 18.7 ± 1.5 | 15.1 ± 0.9 |
| L376A        | 0.26 ± 0.03 | 1.42 ± 0.25 | 19.3 ± 3.4 | 19.1 ± 1.4 |
| S461L        | 0.01 ± 0.01 | 0.01 ± 0.01 | 20*        | 20*        |
| I375P        | <0.01      | <0.01      | 20*        | 20*        |
| V382P        | <0.01      | <0.01      | 20*        | 20*        |
| E383A        | <0.01      | <0.01      | 20*        | 20*        |

* The binding site concentrations were fixed to this value.

Table 3: Parameters of CheA binding to template-assembled CFs

| CF           | $K_A$ | Concentration of sites |
|--------------|-------|------------------------|
| S325L-CF$_{4Q}$ | 3.9 ± 0.5 | 1.37 ± 0.13 |
| WT-CF$_{4Q}$  | 0.74 ± 0.08 | 1.4* |
| I375P-CF$_{4Q}$ | 0.17 ± 0.03 | 1.4* |
| I375P-CF$_{4E}$ | 0.02 ± 0.01 | 1.4* |

* The binding site concentrations were fixed to this value.

FIGURE 4. CheA binding to WT-CF and NS-CFs templated at 30 μM (CF)$_{TOT}$. WT-CF$_{4Q}$ (●); S325L-CF$_{4Q}$ (△); I375P-CF$_{4Q}$ (♦); and I375P-CF$_{4E}$ (○). Fitted curves were generated with a single sites model with the parameters in Table 3.
This mutation is known not to disrupt the formation of receptor clusters (5), and we observed that it did not interfere with the stimulation of CheA in CF mixtures at either level of modification (Fig. 2, B and D).

From these results we concluded that the loss of CheA stimulation by NS-CFs was produced by two general mechanisms as follows: through the disruption of the binding interactions between CF, CheW, and CheA and through stronger interactions with NS-CF relative to WT-CF. These properties are relevant for receptor function, where covalent modification can balance natural influences on receptor clustering, such as ligand binding.

**Competition among CFs for CheW and CheA**—The results demonstrated that single amino acid substitutions in the CF can significantly alter the strength of the interaction with CheA and CheW. This property was exploited to determine the underlying mechanism of CheA regulation through the competition that was evident between WT- and NS-CFs. Binary mixtures of two different WT-CF/NS-CF pairs were investigated in detail for this purpose. One pair consisted of WT-CF and the S325L inhibitory NS-CF, which displayed strong CF-CheW interactions. The other set of mixtures consisted of WT-CF and the E383A permissive NS-CF. Fig. 6 plots the CheA activity and $f_B$ (CheA) as a function of the NS-CF mole fraction. The curvature in the activity data reflected the extent to which CheW (and CheA) bound to the NS-CFs, either more or less, than the WT-CF. In Fig. 6A, the dotted diagonal line is shown for emphasis, to depict the activity dependence that would arise if CheA (and CheW) were distributed without preference between the NS- and WT-CFs. The observed dependence fell below this diagonal line for the S325L NS-CF and above it for the E383A NS-CF, which are manifestations of relatively stronger and weaker binding, respectively, to CheA and CheW. Increasing the CheW concentration from 4 to 15 μM (Fig. 6A, closed and open symbols, respectively) decreased the deviation from the diagonal line for both the inhibitory and permissive mixtures, a trend that was consistent with a decrease in competition. At 15 μM, complex formation was influenced more by the relative amounts of WT-CF and NS-CF present rather than the relative affinities for CheW.

Such competitive effects can influence signaling in the cell. The strong binding of CheA and CheW to the S325L and L376A inhibitory NS-CFs can explain the chemotaxis-null phenotypes observed with *E. coli* cells expressing both a wild type receptor and a receptor containing an inhibitory mutation; tight binding by the inhibitory receptor out-competes wild type receptors for CheW and CheA, generating an imbalance in signaling. The rates of chemotaxis of *E. coli* cells in the soft-agar swarm assay were used to obtain evidence of functional interference. Normal chemotaxis was disrupted when an inhibitory mutation (S325L or L376A) was introduced into the aspartate receptor. By contrast, the introduction of a permissive mutation (I375P) in the aspartate receptor did not interfere with chemotaxis-mediated signaling through wild type (serine and ribose/galac-
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TABLE 4
Parameters determined in a combined fit of CheA activity and binding data to the overlapping site model

| NS-CF | [W]_{TOT} | K_{Cw} | K_{CA} | R (K_{Cw}/K_{CA}) | OS model | CheA binding | CheW binding | Act* | Act* | f_{B*} | f_{A*} |
|-------|-----------|--------|--------|-------------------|----------|--------------|--------------|------|------|--------|--------|
|       | μM       | μM⁻¹   | μM⁻¹   | μM⁻¹             |          |              |              |      |      |        |        |
|       | (0.9–1.8) | (0.12–0.14) | (0.85–1.1) | <0.13           |          |              |              |      |      |        |        |
| E383A | 4         | 1.3    | 0.13   | 0.039 ± 0.006    | <0.13    | ND           |              |      |      | 0.77   | 0.13   |
|       |           | (0.9–1.8) | (0.12–0.14) | (0.85–1.1) |          |              |              |      |      | (0.75 ± 0.04) | (0.16 ± 0.02) |
|       | 15        | 1.3    | 0.99   | 4.6 ± 0.7        | 2.5 ± 0.4 | 5.3 ± 3.6    |              | 9.6 ± 0.3 | 0.12 | 0.20 | 0.77   | 0.94   |
| S325L | 4         | 1.3    | 0.13   | 4.6 ± 0.7        | 2.5 ± 0.4 | 5.3 ± 3.6    |              | 9.6 ± 0.3 | 0.12 | 0.20 | 0.77   | 0.94   |
|       |           | (0.9–1.8) | (0.12–0.14) | (2.8 ± 0.6) |          |              |              |      |      | (0.83 ± 0.06) | (0.92 ± 0.02) |
|       | 15        | 1.3    | 0.99   | 4.6 ± 0.7        | 2.5 ± 0.4 | 5.3 ± 3.6    |              | 9.6 ± 0.3 | 0.12 | 0.20 | 0.77   | 0.94   |

*The values of R below OS model, CheW binding, and CheA binding were estimated by the overlapping site model or by binding data to W-CF₄E, W-CF₄Q (in parentheses), and A-CF₄E (in parentheses), from Tables 2 and 3. These uncertainties were estimated by propagation of errors.

ND indicates not determined.

tose) receptors that were also present (data not shown). Altogether, these observations imply that regulated interactions between receptors, CheW, and CheA are critical for chemotactic function. Although mutations may generate extreme imbalances in the distribution of CheW and CheA among receptors, they are generally illustrative of a competitive mechanism. Under normal conditions, changes in the strengths of interactions in the distribution of CheW and CheA among receptors, CheW, and CheA with the receptor are expected to exhibit simultaneously the properties of synergistic and competitive binding. Competition is a result of CheA and CheW binding to the same or similar sites on the receptor and is an expected consequence of the structural homology between CheW and CheA (34, 35). Synergy in binding is a plausible consequence of the direct binding interaction that occurs between CheW and CheA (11, 22, 33). To determine whether the CheA activity and binding data in Fig. 6 were consistent with these two interaction modes, the data were analyzed by two models of receptor complex assembly based on the following: (i) a receptor–CheW–CheA bridging interaction, and (ii) overlapping interactions between CheW and CheA with the receptor. Fits generated with the overlapping site model agreed well with the activity and the complex formation data (Fig. 6, A and B, solid lines). The bridging model failed to produce good agreement. The curves generated by the bridging model and the associated model-generated parameters are published online as supplemental Fig. S1 and Table S1.

The parameters generated by a fit of the data in Fig. 6 to the overlapping site model, which are listed in Table 4, permitted some comparisons to be made with the measured values of CheW and CheA binding. To facilitate this comparison, a relative binding strength, R, was defined, e.g. as the ratio of the CheW binding constants to NS-CFs and to WT-CFs: R = K_{Cw}/K_{CA}. Model-generated R values indicated that WT-CF bound CheW 25-fold more tightly than the E383A-CF and about 5-fold less tightly than the S325L-CF. The estimates compared favorably to the values of R calculated from binding experiments, which are also summarized in Table 4.
CheW Promotes CheA-CF Interactions—The values of $f_B$ (CheA) in Fig. 6B provided evidence that CheW and CheA each promoted the binding of the other to the receptor cytoplasmic domain. Experimentally, this synergy was manifested as larger values of $f_B$ at the larger CheW concentration (15 μM). In the overlapping sites model, the increase in $f_B$ was manifested phenomenologically as larger values of the apparent CheA-CF association constant, $K_{CA}$ (Table 4). Synergy was also evident in the difference between model-derived estimates for $K_{CW}$ and those determined from binding experiments involving CheW and vesicle-templated CFs (Tables 2 and 4). The model-derived estimates were larger, which indicated a stronger interaction between receptors, CheW and CheA, which explains the smaller values of $K_{CA}$. $K_{CA}$ (Table 4). Synergy was also evident in the difference between model-derived estimates for $K_{CW}$ and those determined from binding experiments involving CheW and vesicle-templated CFs (Tables 2 and 4). The model-derived estimates were larger, which indicated a stronger interaction between receptors, CheW and CheA, which explains the smaller values of $K_{CA}$ obtained in these experiments.

Taken together, these results suggest that several modes of competition and cooperation are present in the interactions between receptors, CheW and CheA, including the following: (i) competition among CFs to bind CheW and CheA; (ii) competition between CheW and CheA in binding to CF; and (iii) synergy in the binding of CheW and CheA to CF. The full-length receptor exhibits similar behavior (11); the observations made in both systems are compatible with a direct interaction between CheW and CheA (22, 30, 33). Although these features of CheA recruited to stimulating complexes (an $f_B$ effect). As Fig. 7 illustrates, measurements of $f_B$ and kinase activity in two kinase-stimulating mutants of CFQEQE (V433I and A436V) supported an $f_B$ effect. The kinase activity, per mole of CheA in the sample, was greater than the wild type level when the CFs in vesicle-assembled complexes contained either the V433I or A436V mutation, but $f_B$ was also greater. The activity, per mole of bound CheA, was the same for complexes formed with wild type, V433I, or A436V CFs. For an unknown reason, a third kinase-stimulating mutation, V346M, did not retain the stimulating phenotype in template-assembled complexes; both $f_B$ and activity were lower than wild type. This result notwithstanding, the more effective recruitment of CheA produced as a result of the V433I and A436V mutations provided an explanation for the stimulating phenotype in these two cases, and demonstrated that competitive recruitment can serve to either increase or decrease kinase activity.

Cooperative Regulation of CheA Activity and Binding—Certain binary mixtures of CFs displayed cooperative regulation of CheA activity and binding. The normalized kinase activities and values of $f_B$ plotted in Fig. 8, A and B, were obtained with four different combinations of S461L-CF and WT-CF, where each CF was either in the lowest (4E) or the highest (4Q) modification level. These data were plotted as a function of the S461L-CF mole fraction ($X$). Activity and binding both decreased sigmoidally as $X$ increased, which was taken as evidence for a cooperative change from a kinase-stimulating to a kinase-inhibiting state. The data were thus fit to a Hill-like function.

**FIGURE 8.** Kinase activity and CheA binding to WT-CF/S461L-CF binary mixtures. Normalized kinase activities (A) and the percent CheA bound to template CF mixtures (B) were measured with WT-CF$_{4Q}$/S461L-CF$_{4Q}$ (○), WT-CF$_{4Q}$/S461L-CF$_{4E}$ (●), WT-CF$_{4E}$/S461L-CF$_{4E}$ (●), and WT-CF$_{4E}$/S461L-CF$_{4Q}$ (●) mixtures. CheW was present at 4 and 5 μM in mixtures of WT-CF$_{4Q}$ and WT-CF$_{4E}$, respectively. C, normalized kinase activity (●) and $f_B$ (○) as a function of the G338L-CF$_{4Q}$/L376A-CF$_{4E}$ mixture composition. CheW was present at 4 μM. The parameters for the fitted curves (Tables 1 and 2) are listed in Table 5. D, schematic representation of the cooperative changes in template-assembled CheA activity and binding. CheA (dimeric rounded rectangles) are arrayed on CFs (circles), which are either in a kinase-stimulating state (open) or in an inhibitory (shaded) state. The NS-CFs are marked with stars.
Parameters generated from the analysis of cooperative CheA activity and binding in WT-CF/S461L-CF mixtures

| WT-CF | S461L-CF | S461L-CF | S461L-CF |
|-------|---------|---------|---------|
| SCF   | TCSCF   | S461L-CF | S461L-CF |
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| SCF   |         |         |         |
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Conclusions—Signaling proteins in bacterial chemotaxis must simultaneously perform several functions to sense and adapt to the different stimuli that are detected through homologous receptors. These demands are met with a dynamic signaling array, which, as this study demonstrates, assembles and remodels through mechanisms that involve competing and cooperative interactions. The unifying features of membrane receptor phenomena suggest that these mechanisms may be general and that they can be investigated effectively by template-directed self-assembly.

Here template-directed assembly of membrane-associated signaling proteins has been shown to be a useful tool to determine the factors that regulate CheA, the central kinase of bacterial chemotaxis. The detailed analysis of this complex system is facilitated through the use of purified proteins, which are assembled under relatively well defined conditions. That the ligand binding domain and transmembrane segments of the receptor are not present, although seemingly a drawback, does allow a focused examination of the contributions made by the cytoplasmic domain under conditions that resemble the environment of this domain in the intact receptor. The good correspondence between the effects of known signaling mutations in vivo (5, 25), and the effects reported here, serves to validate the approach.

The proteins are assembled on the exterior of vesicles, which naturally leads to the expectation that the local membrane curvature of template-assembled proteins is opposite to the curvature experienced by the cytoplasmic domains of receptors in the cell. The influence of membrane curvature, when probed using vesicles of different radii (curvature), are generally modest. The effects that are observed provide a means to assess the strength of protein interactions.4 Several groups have recently focused attention on the factors that influence receptor cluster formation (37–41).

By varying the protein/lipid ratio in a controlled manner, we believe that template-directed assembly can be used to address these issues.

APPENDIX

Models of Competitive CF Interactions—The bridging (B) and overlapping site (OS) models were developed to help distinguish the salient features of receptor complex formation based on CheA (A) activity and recruitment (fB) data. The kinase (Act) and fB data in Fig. 6 were analyzed as a function of the binary mixture composition of the CFs (C) in wild type (stimulating) and two mutant (nonstimulating) forms. Both the specific kinase activity of complex-bound CheA and the fraction of CheA bound to templated CF were used in this analysis. The kinase activity of CheA not bound to the vesicle templates (1 – fB) was assumed not to contribute significantly to the overall activity, because of the large increase in activity (~200-fold) that accompanied complex formation (26). In both the B and OS models, Act and fB are given by Equations A1 and A2,

\[
\text{Act} = \frac{\text{Act}^*\text{[C]}^* + \text{Act}^*\text{[C]}^A}{[\text{C}^*] + [\text{C}^A]} \quad (\text{Eq. A1})
\]

\[
f_B = \frac{[\text{C}^*] + [\text{C}^A]}{[\text{A}]_{\text{TOT}}} \quad (\text{Eq. A2})
\]

where C* and C represent the stimulating and nonstimulating CFs, respectively. [C*A] and [C*A] generically represent the bound and stimulated CheA in complex with CF, where CheW is also present, although the precise stoichiometry of the active complex is left unspecified. Act* and Act* are the specific activities of CheA bound to 100% WT-CF and 100% NS-CF templates, and [A]TOT is the total CheA concentration. These equations and the mass conservation and equilibrium binding equations were used to express Act and fB as a function of adjustable parameters (the model-dependent equilibrium association constants) and the known total protein concentrations quantities ([A]TOT, [W]TOT, and [C]TOT). In these experiments, the total CF concentration, [C]TOT, was held constant, and the concentrations of activating (C*) and inhibiting (C*) CFs were varied as shown in Equation A3,

4 A. Asinas, T. Besschetnova, D. J. Montefusco, A. L. Shrout, and R. M. Weis, unpublished observations.
Functional Interactions in Signaling Complexes

The mass conservations in the overlapping site model are shown in Equations A4–A7,

\[ [C^*]_{\text{TOT}} = [C^*] + [C^*W] + [C^*A] \quad (\text{Eq. A4}) \]

\[ [W]_{\text{TOT}} = [W] + [C^*W] + [C^*A] \quad (\text{Eq. A5}) \]

and the expressions for the equilibrium association constants are shown in Equation A8,

\[ K_{C\text{w}} = \frac{[C^*W]}{[C^*][W]} \quad K_{C\text{w}} = \frac{[C^*W]}{[C^*A]} \quad K_{C\text{a}} = \frac{[C^*A]}{[C^*][A]} \quad (\text{Eq. A8}) \]

These equations were used to express [CW] and [CA] in terms of [W], [A], [C^*], and [C^*]. In the OS model, the known association between W and A was not treated explicitly (33); it was accommodated in the dependence of \(K_{C\text{a}}\) on \([W]_{\text{TOT}}\) by allowing \(K_{C\text{a}}\) to adopt different values at the two different values of \([W]_{\text{TOT}}\) (4 and 15 μM) used in the experiments. With this in mind, the [CW] and [CA] are given by Equations A9 and A10,

\[ [C^*W] = K_{C\text{w}}[C^*][W] \quad [C^*A] = K_{C\text{w}}[C^*][A] \quad (\text{Eq. A9}) \]

\[ [C^*A] = K_{C\text{w}}[C^*][A] \quad [C^*A] = K_{C\text{a}}[C^*][A] \quad (\text{Eq. A10}) \]

Substitution of Equations A9 and A10 into the conservation equation for \([C^*]_{\text{TOT}}\) (Equation A4) gives an equation for \([C^*]\) in terms of \([C^*]_{\text{TOT}}\). For the OS model, this equation is given in Equations A11 and A12,

\[ [C^*]_{\text{TOT}} = [C^*] + K_{C\text{w}}[C^*][W] + K_{C\text{a}}[C^*][A] \]

\[ = [C^*]\left(1 + K_{C\text{w}}[W] + K_{C\text{a}}[A]\right) \quad (\text{Eq. A11}) \]

\[ [C^*] = \frac{[C^*]_{\text{TOT}}}{\left(1 + K_{C\text{w}}[W] + K_{C\text{a}}[A]\right)} \quad (\text{Eq. A12}) \]

The analogous equation for \([C^*]\) is shown in Equation A13,

\[ [C^*] = \frac{[C^*]_{\text{TOT}}}{\left(1 + K_{C\text{w}}[W] + K_{C\text{a}}[A]\right)} \quad (\text{Eq. A13}) \]

These equations were used to express \([CW]\) and \([CA]\) in terms of \([W]\) and \([A]\). The mass conservations in the overlapping site model are shown in Equations A8, A12, and A13 are introduced into the CheW mass conservation equation (Equation A6) to give Equation A14, which depends on \([W]\) and \([A]\).

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