Attractant Regulation of the Aspartate Receptor−Kinase Complex: Limited Cooperative Interactions between Receptors and Effects of the Receptor Modification State†

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ABSTRACT: The manner by which the bacterial chemotaxis system responds to a wide range of attractant concentrations remains incompletely understood. In principle, positive cooperativity between chemotaxis receptors could explain the ability of bacteria to respond to extremely low attractant concentrations. By utilizing an in vitro receptor-coupled kinase assay, the attractant-dependent response curve has been measured for the *Salmonella typhimurium* aspartate chemoreceptor. The attractant chosen, α-methyl aspartate, was originally used to quantitate high receptor sensitivity at low attractant concentrations by Segall, Block, and Berg [(1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8987−8991]. The attractant response curve exhibits limited positive cooperativity, yielding a Hill coefficient of 1.7−2.4, and this Hill coefficient is relatively independent of both the receptor modification state and the mole ratio of CheA to receptor. These results disfavor models in which there are strong cooperative interactions between large numbers of receptor dimers in an extensive receptor array. Instead, the results are consistent with cooperative interactions between a small number of coupled receptor dimers. Because the in vitro receptor-coupled kinase assay utilizes higher than native receptor densities arising from overexpression, the observed positive cooperativity may overestimate that present in native receptor populations. Such positive cooperativity between dimers is fully compatible with the negative cooperativity previously observed between the two symmetric ligand binding sites within a single dimer. The attractant affinity of the aspartate receptor is found to depend on the modification state of its covalent adaptation sites. Increasing the the level of modification decreases the apparent attractant affinity at least 10-fold in the in vitro receptor-coupled kinase assay. This observation helps explain the ability of the chemotaxis pathway to respond to a broad range of attractant concentrations in vivo.

The chemotatic response to the chemoattractant aspartate in *Escherichia coli*, *Salmonella typhimurium*, and related bacteria is initiated by the aspartate receptor. The aspartate receptor is a member of a family of methyl-accepting taxis receptors that share a high degree of sequence homology in their cytoplasmic domains and are able to mediate chemo-, thermo-, osmo-, photo-, and pH-taxis in many prokaryotic organisms (*1*−*12*). This taxis receptor family belongs to a large receptor superfamily that regulates two-component pathways in both prokaryotes and eukaryotes (*13*, *14*).

The aspartate receptor is a stable homodimer of two 60 kDa subunits that binds the attractant aspartate in the periplasm and propagates a signal across the lipid bilayer, thereby regulating a cytoplasmic phosphorylation pathway that controls the swimming state of the flagellar motor. Considerable information has been obtained regarding the structure of the receptor and the mechanism of transmembrane signaling (*15*−*30*). Moreover, it has been shown that the receptor serves as the structural framework for the signaling complex formed by the assembly of the cytoplasmic pathway components upon the cytoplasmic domain of the receptor (*31*, *32*). This signaling complex consists of the receptor cytoplasmic domain, the histidine kinase CheA, the aspartate kinase CheY, and other pathway components. The aporeceptor stimulates the CheA kinase autophosphorylation rate, while attractant ligand binding downregulates this rate approximately 100-fold (*33*, *34*). The aspartate kinase CheY binds to CheA where it catalyzes the transfer of phosphate from the active site histidine of CheA to a specific aspartate in the active site of CheY (*35*, *36*). The resulting P_i-CheY then dissociates from the signaling complex and diffuses to the flagellar motor where it docks and alters the swimming activity of the cell (*37*). The cytoplasmic domain of the receptor also possesses four covalent adaptation sites that serve to modulate receptor-coupled kinase activity (*38*, *39*). These glutamate side chains at positions 295, 302, 309, and 491 can be methylated by the methyltransferase enzyme CheR and demethylated by the methylesterase CheB, which is activated by phosphotransfer from CheA (*40*, *41*). The relative rates of receptor methylation and demethylation reactions determine the steady-state level of receptor modification (*42*). In the wild-type receptor, positions 295 and 309 begin as Gln side chains, which are rapidly deamidated by CheB in vivo to yield Glu side chains that can be methylated by CheR (*43*).

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Although the chemotaxis pathway is well-characterized on a structural level (*44*−*53*), fundamental questions remain about the mechanism of signaling through the pathway. The pathway is able to sense attractant concentrations spanning more than 5 orders of magnitude and thus exhibits an impressive dynamic range (*54*). At low attractant concentrations, the chemotaxis system is extremely sensitive and can respond to just a few molecules of attractant (*55*). It is unclear how a few molecules of attractant could regulate the entire receptor population since previous studies have concluded that the receptor is an independent dimer in which the two symmetric aspartate binding sites exhibit negative cooperativity, or half-of-sites binding (*15*, *56*−*58*). One hypothesis is that the dimers are clustered in positively cooperative arrays, wherein the binding of the attractant to a single dimer can downregulate the kinase activities of many nearby dimers (*59*−*61*). Alternatively, cooperative interactions could occur either at a later step in the chemotaxis pathway or at a combination of steps in a multiplicative manner to produce the observed sensitivity (*60*).

Cooperative interactions between the chemotaxis receptors themselves are plausible since they have been observed to cluster together with other components of the ternary signaling complex at the poles of *E. coli* cells (*62*). There are also reports of receptor cytoplasmic domain fragments associating to form higher-order oligomers in vitro (*63*, *64*). In the crystal structure of the serine receptor cytoplasmic domain, the dimeric domain forms a trimer of dimers, although it is unclear whether this interaction occurs in the full-length membrane-bound receptor when assembled into the signaling complex (*16*). There is evidence that clustering of receptors is essential for the adaptive methylation of minor receptors which are present in low copy number and lack the adaptation enzyme (CheR and CheB) binding site present on the aspartate and serine receptors (*40*, *59*, *65*−*70*). Moreover, a mathematical model invoking receptor cooperativity among many neighboring receptors in large receptor arrays has been proposed to explain the sensitivity of the chemotactic response (*61*, *71*). Finally, higher-order receptor clustering has been suggested in other receptor systems such as tyrosine kinase-linked receptors, indicating that clustering may be a shared feature of receptor signaling systems (*72*).

The study presented here examines whether aspartate receptor-coupled kinase activation responds to varying concentrations of attractant in a cooperative manner, as would be expected if receptor dimers are clustered together in positively cooperative arrays. The approach utilizes the reconstituted receptor−kinase signaling complex in an in vitro assay for attractant-triggered kinase regulation (*33*, *34*). The results reveal the existence of limited positively cooperative interactions in the receptor-coupled kinase response, but the high cooperativity expected for large arrays of tightly coupled receptors is not detected. The observed limited positive cooperativity is only weakly dependent on CheA concentration. Furthermore, examination of receptors with different adaptation site modifications reveals only a weak dependence of cooperativity on the receptor modification state, while the attractant affinity is more strongly dependent on the modification state. Together, these results suggest that positive cooperativity in the receptor-coupled kinase reaction accounts for only part of the chemosensory sensitivity observed at low attractant concentrations. However, the strong dependence of attractant modulation on modification state has important implications for the dynamic range of chemosensing.

MATERIALS AND METHODS

Materials. The plasmid pSCF6 and its mutated variants, which were used to express the *S. typhimurium* aspartate receptor under control of its native promoter, have been previously described (*28*), as have the plasmids and strains used to produce purified CheA, CheW, CheY, and CheR (*19*). The strain used for receptor expression and characterization in in vitro studies was RP3808 *[*Δ*(cheA-cheZ)-DE2209 tsr-1 leuB6 his-4 eda-50 rpsL136 [thi-1* Δ*(gal-attl)DE99 ara-14 lacY1 mtl-1 xyl-5 tonA31 tsx-78]/mks/*]. Tests of in vivo receptor function were carried out in strain RP8611 [Δ*tsrDE7028* Δ*(tar-tap)DE5201 zbd::Tn5* Δ*(trg)-DE100 leuB6 his-4 rpsL136 [thi-1 ara-14 lacY1 mtl-1 xyl-5 tonA31 tsx-78] CP362* of G. Hazelbauer via F. Dahlquist]. Both strains of *E. coli* were provided by J. S. Parkinson (University of Utah, Salt Lake City, UT) (*73*). The enzyme substrate [γ-^{32}P]ATP (6000 Ci/mmol) was obtained from NEN. Mutagenic oligos and sequencing primers were synthesized by Gibco-BRL. Kunkel mutagenesis reagents (T7 DNA polymerase, T4 DNA ligase, and deoxynucleotide triphosphates) were purchased from Bio-Rad. The reagent α-methyl D,L-aspartate (greater than 99% purity and containing no detectable aspartic acid as assayed by thin-layer chromatography) was purchased from Sigma. All other materials were reagent grade and were purchased from commercial suppliers.

Creation of Receptors Containing Modified Adaptation Sites. Receptors with modified adaptation sites were generated by oligonucleotide-directed site specific mutagenesis of the plasmid pSCF6 according to the method of Kunkel et al. (*74*) with modifications as previously described (*28*). All mutations were confirmed by dideoxy plasmid DNA sequencing using a PCR method and Sequitherm DNA polymerase (Epicenter).

Purification of Membranes Containing the Aspartate Receptor. Mutant and wild-type aspartate receptors were expressed by the pSCF6 plasmid in *E. coli* strain RP3808 and isolated in native membrane vesicles by previously published methods (*18*, *75*). The resulting engineered receptor-containing membranes were resuspended in buffer containing 20 mM sodium phosphate (pH 7.0) with NaOH, 10% v/v glycerol, 0.1 mM EDTA,[1] and 0.5 mM PMSF, then snap-frozen in liquid nitrogen, and stored at −80 °C.

Membrane samples were assayed for total protein by BCA assay (*76*) and calibrated against bovine serum albumin standards (Pierce). After development of the BCA reaction in the presence of 0.1% SDS, absorbance measurements were carried out using a microplate reader (Molecular Devices) (*77*). Protein purity was determined by quantitating the relative total intensities of the receptor and nonreceptor bands on a Coomassie-stained 10% Laemmli SDS−polyacrylamide gel (acrylamide:bisacrylamide ratio of 40:0.2) using a digital camera (Alpha Innotec). Typically, membrane preparations

[1] Abbreviations: SDS, sodium dodecyl sulfate; WT, wild type; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetate; K_D, dissociation constant; α-methyl aspartate, α-methyl D,L-aspartate.

from a 500 mL culture resulted in 5 mg of total membrane protein, of which the aspartate receptor comprised between 5% and 10%.

In Vivo Activity Assays. Chemotaxis swarm assays were performed in *E. coli* strain RP8611 as previously described (*18*, *78*). Controls using an empty vector without the receptor or a vector carrying the wild-type receptor (pSCF6) were performed to determine the relative swarm rates of receptorless cells and cells possessing the native receptor. Swarm rates were determined on minimal media agar plates with or without 100 μM aspartate. Aspartate was used in these assays rather than α-methyl aspartate, as this aspartate analogue is nonmetabolizable and thus does not yield a self-generated attractant concentration gradient. Aspartate-specific swarm rates of wild-type or mutant receptors were determined by subtracting the (−)-aspartate swarm rate from the (+)-aspartate swarm rate to correct for pseudotaxis (*79*), and the resulting rates were then normalized to the wild-type rate for comparison. All cells containing mutant receptors exhibited swarm rates essentially equivalent to that of the wild-type receptor. This demonstrates that all adaptation site mutants were able to be effectively deamidated by the CheB enzyme present in strain 8611, thus restoring native receptor signaling characteristics (data not shown).

In Vitro Activity Assays. The chemotaxis proteins CheA, CheW, CheY, and CheR were overexpressed, produced in *E. coli*, and isolated as previously described (*18*). The in vitro receptor-coupled kinase assay was performed essentially as described with the following modifications (*18*, *33*, *34*). Unless otherwise specified, receptor-containing membranes were combined with purified chemotaxis components to yield the final monomeric concentrations of 2 μM aspartate receptor, 2 μM CheW, 0.5 μM CheA, and 10 μM CheY. The mixtures were preincubated either in the presence or in the absence of α-methyl aspartate at room temperature (22 °C) for 45 min to allow formation of the receptor signaling complex. This incubation time was sufficient to provide maximal kinase activity for each receptor that was tested (data not shown). The receptor-coupled kinase reaction was initiated by the addition of 100 μM [γ-^{32}P]ATP to a reaction mixture containing membrane-bound receptor, CheW, CheA, and CheY in 50 mM Tris (pH 7.5) with HCl, 50 mM KCl, and 5 mM $MgCl_2$. After 10 s, the reaction was quenched with 2× Laemmli sample buffer supplemented with 25 mM EDTA to prevent further phosphorylation and dephosphorylation reactions (*80*). The production of [γ-^{32}P]CheY was found to be linear with time for at least 20 s for standard reaction mixtures containing either the wild-type (QEQE) or the fully modified (QQQQ) receptor (data not shown), indicating that the measured 10 s time points sampled the initial linear rate of even the fastest receptor-coupled kinase reactions. The initial stoichiometries of the components have also been shown to yield receptor-regulated CheA autophosphorylation as the rate-determining step in the phosphotransfer reaction. [^{32}P]P_i-CheY, resulting from rapid phosphotransfer from [^{32}P]P_i-CheA, was resolved on a 15% Laemmli SDS−polyacrylamide gel (acrylamide:bisacrylamide ratio of 40:1.25) containing 22% urea. Gels were dried immediately following electrophoresis, and the relative initial rate of the phosphotransfer reaction was determined by quantitation of [^{32}P]P_i-CheY production between 0 and 10 s in each reaction by phosphoimaging (Molecular Dynamics).

Data Analysis. All attractant titrations of receptor-coupled kinase activity with varying concentrations of attractant were best fit by equations for two binding models. The first equation tested was for a single, independent-site model

$$R = M\left[1 - \left(\frac{[A]}{K_D + [A]}\right)\right] + m \quad (1)$$

where R is the normalized receptor-regulated CheA kinase rate, M is the maximal rate, m is the background CheA kinase rate at saturating attractant concentration, [A] is the attractant concentration, and K_D is the apparent dissociation constant for attractant binding to the receptor−kinase complex. The second equation that was tested was the Hill equation for a cooperative system of multiple sites:

$$R = M\left[1 - \left(\frac{[A]^H}{K_D{}^H + [A]^H}\right)\right] + m \quad (2)$$

where H is the Hill coefficient.

Titration curves were plotted and fit using KaleidaGraph 3.0 software for Macintosh (Synergy Software, Reading, PA) which employs a Levenberg−Marquardt algorithim to determine the best fit. Indicated error ranges represent the standard deviation of the mean when $n \geq 3$.

RESULTS

In Vitro Assay for Receptor-Coupled Kinase Activity. To determine the effect of attractant on aspartate receptor activity in the signaling complex, a receptor-coupled kinase assay was utilized to provide a sensitive and reproducible measure of receptor-mediated kinase regulation (*28*, *33*, *34*). To carry out this assay, isolated *E. coli* membranes containing overexpressed wild-type or mutant aspartate receptor were combined with purified CheW coupling protein, purified CheA histidine kinase, and purified CheY aspartate kinase. The components were then incubated for 45 min, which allowed the assembly reaction to reach completion in all mutant and wild-type samples; then [γ-^{32}P]ATP was added to start the kinase reaction, and the formation of a ^{32}P excess of CheY ensured that (i) receptor-regulated CheA autophosphorylation was the rate-limiting step in the phosphotransfer reaction that forms [^{32}P]P_i-CheY and (ii) the sampled time points were in the initial linear range of [^{32}P]P_i-CheY formation for all reactions.

The attractant that was utilized was the aspartate analogue α-methyl aspartate, which has previously been shown to signal through the aspartate receptor (*81*). This same attractant was utilized in the original experiments that detected the high sensitivity of the chemotaxis pathway at low attractant concentrations (*55*). Thus, if receptor cooperativity is responsible for sensitivity, it should be possible to detect this cooperativity utilizing α-methyl aspartate as a regulatory ligand. Moreover, the apparent dissociation constant for the binding of α-methyl aspartate to the aspartate receptor ($K_D \sim 20$ μM; see below) is significantly higher than that of aspartate ($K_D \sim 1$ μM) (*58*). As a result, α-methyl aspartate provides higher-quality titration curves than aspartate in the receptor-coupled kinase assay where the receptor concentration is in the low micromolar range.

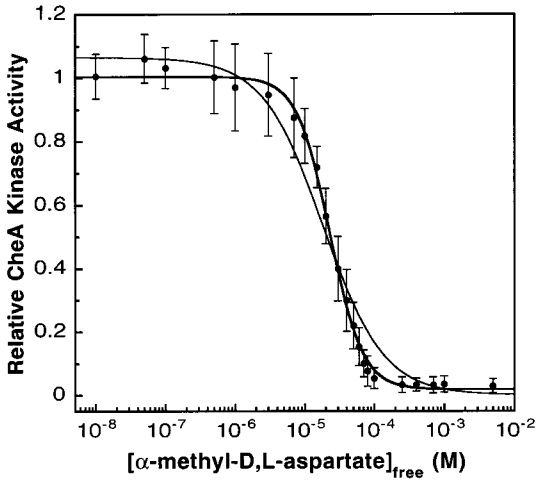

FIGURE 1: Effect of the chemoattractant α-methyl D,L-aspartate on the activity of the wild-type aspartate receptor in the in vitro receptor-coupled phosphorylation assay. The relative CheA kinase activity was determined at different attractant concentrations, and the resulting data were fit by a multisite Hill model (bold line) and a single-site model (fine line). The best-fit Hill analysis yielded an apparent dissociation constant K_D of 23 ± 4 μM and a Hill coefficient H of 1.8 ± 0.1. Error bars were determined by averaging six or more independent experiments carried out at 22 °C. Reactant and buffer concentrations were 1 μM receptor dimer, 0.5 μM CheA monomer, 2 μM CheW, 10 μM CheY, 100 μM ATP, 5 mM $MgCl_2$, 50 mM KCl, and 50 mM Tris (pH 7.5) with HCl.

Attractant Concentration Dependence of Receptor-Coupled Kinase Activity. To quantitate the ligand binding affinity and cooperativity associated with α-methyl aspartate-triggered kinase regulation, the receptor-coupled kinase assay was utilized to determine relative initial rates of P_i-CheY formation at different concentrations of this attractant. The resulting plot of CheA activity versus attractant concentration is displayed in Figure 1 for the wild-type aspartate receptor. Two models were employed to fit the data, a single-site binding model (fine line) and a multisite Hill model (bold line). The Hill model provided a significantly better fit than the single-site model. The apparent dissociation constant and Hill coefficient of the best-fit Hill model were 23 ± 4 μM and 1.8 ± 0.1, respectively. Notably, the observation that the Hill coefficient is significantly larger than unity indicates that the attractant-triggered regulation of the receptor−kinase complex involves positive cooperativity between interacting receptors.

CheA Concentration Dependence of Limited Receptor Cooperativity. It has been observed that the dimeric CheA molecule helps to stabilize patches of receptor observed at the poles of *E. coli* cells and may help mediate interactions between receptor dimers (*59*, *62*). To test whether the newly detected receptor−receptor cooperativity is sensitive to CheA concentration, the mole ratio of CheA to wild-type receptor was varied in the receptor-coupled kinase assay. While the concentrations of receptor and other components were held constant, the CheA concentration was varied over a 16-fold range to yield CheA to receptor monomer ratios from 0.0625:1 to 1:1. As the CheA to receptor mole ratio increased from 0.0625:1 to 0.25:1, the rate of P_i-CheY production increased proportionally, but further increases in the CheA concentration had no effect, indicating that the receptor complex was saturated with CheA (data not shown). Table 1 shows that throughout the range of mole ratios that were investigated, little or no change was observed in the apparent K_D and Hill coefficient for α-methyl aspartate regulation of CheA kinase activity. Thus, the observed positive cooperativity between receptors was relatively constant as the CheA concentration rose from unsaturating to saturating levels.

Table 1: Effect of CheA Concentration on the Attractant Dependence of Receptor-Coupled Kinase Activity[a]

CheA:receptor mole ratio	attractant apparent K_D (μM)	Hill coefficient
0.0625:1	22 ± 2	2.0 ± 0.2
0.125:1	26 ± 2	2.4 ± 0.2
0.25:1	23 ± 4	1.8 ± 0.1
1:1	16 ± 5	2.0 ± 0.3

[a] Apparent K_D values and Hill coefficients determined by titrating the attractant α-methyl D,L-aspartate into the receptor-coupled kinase assay as indicated in Figure 1 and Materials and Methods. The CheA:receptor mole ratio is defined as the ratio of total dimer concentrations for the two proteins.

Effect of the Receptor Modification State on Attractant Affinity and Receptor Cooperativity. To investigate the effect of the receptor modification state on the attractant dependence of receptor-coupled kinase activity, the four adaptation sites of the aspartate receptor were mutated from their wild-type state (QEQE) to give amidation states ranging from fully unmodified (EEEE) to fully amidated (QQQQ). The resulting mutants were all functional and able to mediate wild-type chemotaxis in the in vivo swarm assay (see Materials and Methods) as expected, since CheB rapidly deamidates glutamine at the adaptation sites and thereby ensures that the mutant and wild-type receptors will be equivalent in vivo (*43*). When the receptors are not exposed to CheB, however, the amidation of the adaptation site glutamates is stable and closely mimics the regulatory effects of the native methyl esterification triggered by CheR (*82*−*84*). Membranes containing the modified receptors were isolated from a bacterial strain (RP3808) lacking the modification enzymes CheR and CheB, thus yielding a homogeneous population of modified receptors. Figure 2 shows that the level of receptor-coupled kinase activity detected in the in vitro assay increased with an increasing level of amidation of the adaptation sites, and that 5 mM α-methyl aspartate inhibited kinase activity to levels near those observed in the absence of receptor. These results are in agreement with the increasing kinase activity previously observed when the adaptation sites of the *E. coli* aspartate receptor were methylated (*34*, *82*).

The attractant dependence of receptor-coupled kinase activity was determined using the in vitro assay for each modification state as shown in Figure 3, with the exception of EEEE which yielded too little kinase activity for accurate characterization. Each modification state yielded an α-methyl aspartate dependence that was well-approximated by the multisite Hill model, while the single-site model was inadequate. The resulting best-fit apparent K_D values and Hill coefficients are summarized in Table 2. The apparent K_D for α-methyl aspartate increased 10-fold as the modification state of the receptor increased from QEEE to QQQQ, while the Hill coefficient increased only 1.5-fold over the same range. Overall, the results confirm that the 10-fold effect of adaptation site modification on aspartate affinity previously observed in direct binding experiments is maintained in the attractant-mediated kinase regulation of the receptor−kinase

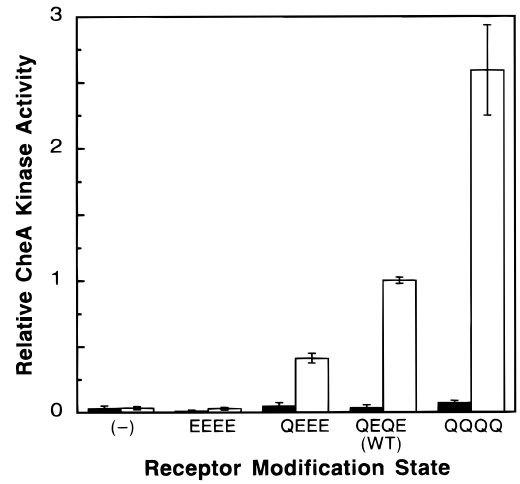

FIGURE 2: Effect of adaptation site modification on maximum CheA kinase activity. For each reconstituted receptor−kinase complex possessing adaptation sites that were modified by amidation as indicated, the CheA kinase activity was measured in the receptor-coupled kinase assay and normalized to that for the wild-type complex measured in parallel. Filled bars indicate the activity in the presence of 5 mM α-methyl D,L-aspartate, and open bars indicate the kinase activity in the absence of attractant. Also shown is the CheA kinase activity observed for membranes lacking aspartate receptor. Experimental conditions were as described in the Figure 1 legend.

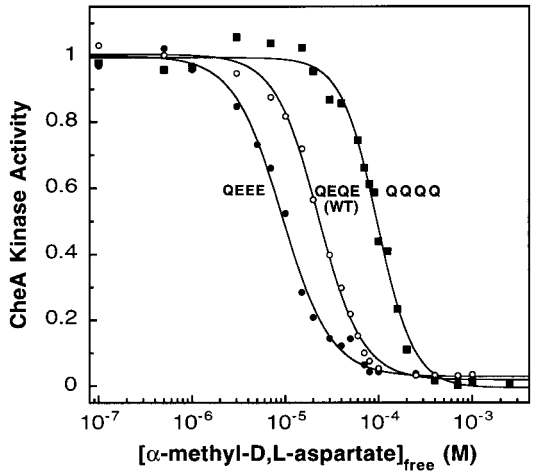

FIGURE 3: Effect of α-methyl D,L-aspartate on the receptor-coupled kinase activation of the QEEE, QEQE (wild-type), and QQQQ receptors. For each indicated receptor, CheA kinase activity was measured in the receptor-coupled kinase assay at different α-methyl D,L-aspartate concentrations, and the resulting data were best fit by a multisite Hill model. The maximal activity was normalized to unity for ease of comparison. Best-fit apparent K_D values and Hill coefficients are summarized in Table 2. Each point is the average of three determinations, and for simplicity, error bars (typically less than 20% of the signal) were omitted. Experimental conditions were as described in the Figure 1 legend.

complex (*82*). In contrast, adaptation site modification has a significantly smaller effect on positive cooperativity between receptors in the receptor-coupled kinase assay.

DISCUSSION

The study presented here uses the in vitro receptor-coupled kinase assay to quantitate the attractant dependence of aspartate receptor-regulated kinase activity in the chemosensory signaling complex. Plots of receptor-coupled kinase activity versus attractant concentration provide evidence for cooperative interactions between receptors in the assembled, membrane-bound signaling complex. The attractant concentration dependence could not be modeled as simple binding to a single site; rather, a cooperative multisite Hill model was required. The observed Hill coefficient ranged from 1.7 to 2.4, indicating a moderate level of positive receptor−receptor cooperativity. Such cooperativity is significantly smaller than the cooperativity exhibited by the four tightly coupled O_2 binding sites of hemoglobin, for which the Hill coefficient is approximately 3 (*85*). It follows that the observed positive cooperativity is consistent with local interactions between a few nearby receptor dimers, but disfavors strong, long-range interactions between large numbers of receptors in an extensive, tightly coupled array. Such results rule out models in which attractant binding to a single receptor downregulates the kinase activity associated with dozens of receptors (*61*, *71*), where the Hill coefficient should approach 20 or more. While it could be argued that greater cooperativity between receptors could occur in vivo, in our studies we used overproduced receptors present at up to 50-fold higher densities than in wild-type membranes. Moreover, higher concentrations of cluster-promoting CheA were not observed to increase cooperativity. Thus, the observed level of positive cooperativity likely represents an upper limit on that present in the native chemotaxis pathway. Together, the results suggest that the receptor unit which regulates kinase activity consists of only one or a few receptor dimers.

Table 2: Effect of Adaptation Site Modification on the Attractant Dependence of Receptor-Coupled Kinase Activity[a]

receptor modification state	attractant apparent K_D (μM)	Hill coefficient
QEEE	9 ± 1	1.7 ± 0.1
QEQE (WT)	23 ± 4	1.8 ± 0.1
QQQQ	97 ± 4	2.2 ± 0.2

[a] Apparent K_D values and Hill coefficients determined by titrating the attractant α-methyl D,L-aspartate into the receptor-coupled kinase assay as indicated in Figure 3 and Materials and Methods.

Following submission of this paper, an independent study appeared describing cooperative interactions in the closely related serine receptor (*86*). Comparison of the results for the aspartate and serine receptors reveals qualitative agreement; as in the aspartate receptor, positive cooperativity is observed when the serine receptor is titrated with its ligand in the in vitro receptor-coupled kinase assay. The cooperativity observed in the serine receptor system is, however, significantly higher, yielding a Hill coefficient ranging from 2 to 12 depending on the trial and the methylation state of the receptor. These results suggest that the intrinsic positive cooperativity of the serine receptor may be higher than that of the aspartate receptor. Alternatively, the greater positive cooperativity observed in the serine receptor system could stem from the fact that the receptor overexpression level is 2−5-fold greater than that utilized in the aspartate receptor system, yielding serine receptor densities that are more than 100-fold higher than the native value. Thus, the Hill coefficient and positive cooperativity of the native receptor population could be significantly lower than those of the overexpressed receptor population used in the in vitro receptor-coupled kinase assay.

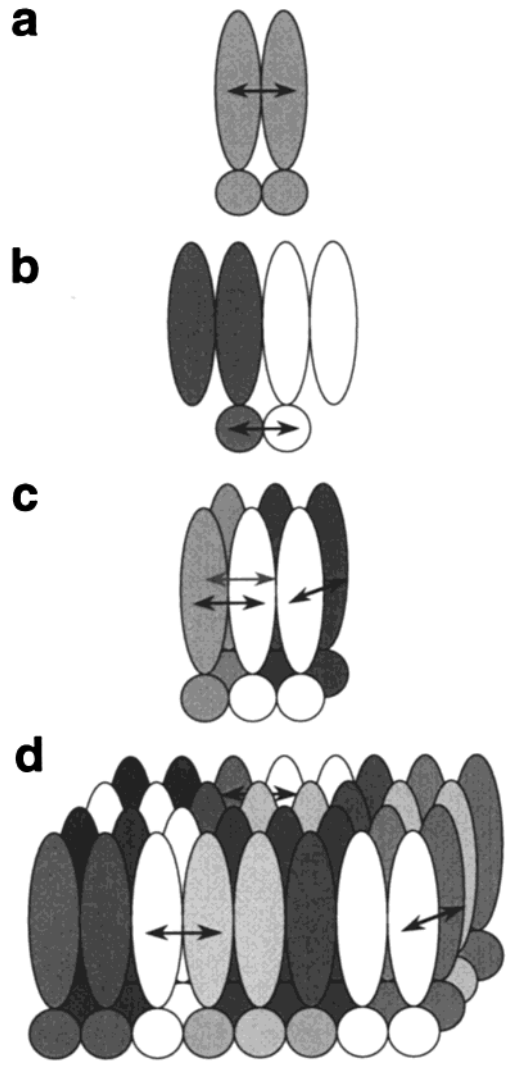

FIGURE 4: Possible causes of the observed limited cooperativity between receptors in the reconstituted signaling complex. The ovals represent individual receptor subunits. The circles represent CheA histidine kinase subunits. Homodimeric taxis receptor pairs are differentiated by shading. (a) Positive cooperativity between the two subunits of a receptor dimer generated by incorporation into the signaling complex. (b) Positive cooperativity between receptor dimers mediated by the dimeric histidine kinase CheA. (c) Positive cooperativity between receptor dimers in a small cluster of receptors such as a trimer of dimers. (d) Limited weak interactions between adjacent receptor dimers in a higher order cluster of receptors.

Figure 4 illustrates several potential sources of the positive cooperativity observed between receptors. (i) Although it is well-established that the two symmetric aspartate binding sites of the isolated dimer exhibit negative cooperativity in aspartate binding (*58*), it is possible that assembly of the receptor−kinase complex converts this negative cooperativity to the positive cooperativity observed in the receptor-coupled kinase assay. Such a conversion is not implausible, since mutation of a single residue at the receptor subunit interface has been shown to shift the intradimer cooperativity from negative to positive, and a dimer can exhibit a Hill coefficient up to 2.0 (*57*). (ii) The dimeric CheA molecule could bridge two receptor dimers, thereby introducing positive cooperativity between adjacent dimeric units. (iii) A recent crystal structure of the isolated cytoplasmic domain revealed that this fragment forms a trimer of dimers that could account for the observed receptor−receptor cooperativity (*16*). However, crystallographic and NMR studies have provided no evidence of dimer−dimer interactions in the isolated periplasmic domain (*15*, *56*). Thus, the physiological relevance of the trimer of dimers is not yet established. (iv) Finally, the observed positive cooperativity could arise from interactions between nearest-neighbor dimers in larger clusters of receptors. The results presented here cannot distinguish between these four possibilities, but do disfavor tight coupling between large numbers of receptors. In models ii−iv, the observed positive cooperativity between dimers is superimposed on the negative cooperativity existing between the symmetric pair of ligand binding sites within a single dimer (*15*, *56*−*58*).

Notably, only minor changes in cooperativity were observed when the modification state of the receptor adaptation sites was increased from QEEE to QQQQ. However, the apparent K_D for attractant binding to the receptor increased 10-fold as the modification level increased, indicating that the affinity of attractant for the receptor−kinase complex decreases with modification (this work; see also refs *82* and *83*). These observations help explain the sensitivity and dynamic range of the chemotaxis pathway. At low background concentrations, the limited positive cooperativity of the receptor−kinase complex will help the pathway respond to small changes in the attractant. As the background level of attractant increases, the modification state of the receptor is increased by methylation of the adaptation sites, which in turn lowers the attractant affinity and adjusts the pathway to the higher attractant background. In a two-state receptor model where there is conformational coupling between attractant binding and kinase activity, the receptor "off" state is favored by attractant binding and the receptor "on" state is favored by attractant removal. Modification of the adaptive sites stabilizes the receptor "on" state and should decrease attractant affinity, as observed. Thus, the observed variation of K_D with the receptor modification state is consistent with a two-state receptor signaling model where the on state activates CheA kinase activity and the off state inhibits the kinase.

The parallel study of the serine receptor revealed a stronger dependence of K_D on the receptor modification state. In this system, the apparent K_D for serine binding increased 10^4-fold as the modification state increased from EEEE to QQQQ (*86*). Moreover, in the serine receptor the measured Hill coefficient for serine regulation increased up to 4-fold as the modification state increased from EEEE to QQQQ. Again, these differences may represent intrinsic contrasts between the aspartate and serine receptors, or may be related to the different overexpression levels of the receptors in the two systems (see above). However, the serine receptor results provide additional support for the importance of receptor covalent modification to sensing a broad range of attractant concentrations.

Although the positive cooperativity observed between receptors may contribute to the sensitivity of the chemotactic response at low attractant concentrations, additional mechanisms are needed to explain the full sensitivity of the chemotaxis pathway. Other possible sources of sensitivity include positive cooperativity between the multiple docking sites for P_i-CheY on the motor, or receptor-mediated regulation of CheZ phosphatase activity. In any case, the degree

of total cooperativity needed in the chemotaxis system is estimated to be large. For example, a Hill coefficient of greater than 11 would be needed in the interaction between P_i-CheY and the motor to achieve the observed chemotactic sensitivity (*60*). A recent experimental study has concluded that strong cooperativity is indeed present in the interaction between P_i-CheY and the switch complex at the flagellar motor, yielding a Hill coefficient of 10 ± 1 (*87*). Such strong cooperativity at the motor switch complex, together with the much weaker cooperativity observed for the receptor population, may be sufficient to fully account for the remarkable sensitivity of the chemotaxis pathway at low attractant concentrations.

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