Selective allosteric coupling in core chemotaxis signaling complexes

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Bacterial chemotaxis is mediated by signaling complexes that sense chemical gradients and direct bacteria to favorable environments by controlling a histidine kinase as a function of chemoreceptor ligand occupancy. Core signaling complexes contain two trimers of transmembrane chemoreceptor dimers, each trimer binding a coupling protein CheW and a protomer of the kinase dimer. Core complexes assemble into hexagons, and these form hexagonal arrays. The notable cooperativity and amplification in bacterial chemotaxis is thought to reflect allosteric interactions in cores, hexagons, and arrays, but little is known about this presumed allostery. We investigated allostery in core complexes assembled with two chemoreceptor species, each recognizing a different ligand. Chemoreceptors were inserted in Nanodiscs, which rendered them water soluble and allowed isolation of individual complexes. Neighboring dimers in receptor trimers influenced one another's operational ligand affinity, indicating allosteric coupling. However, this coupling did not include the key function of kinase inhibition. Our data indicated that only one receptor dimer could inhibit kinase as a function of ligand occupancy. This selective allosteric coupling corresponded with previously identified structural asymmetry: only one dimer in a trimer contacts kinase and only one CheW. We suggest one of these dimers couples ligand occupancy to kinase inhibition. Additionally, we found that kinase protomers are allosterically coupled, conveying inhibition across the dimer interface. Because kinase dimers connect core complex hexagons, allosteric communication across dimer interfaces provides a pathway for receptor-generated kinase inhibition in one hexagon to spread to another, providing a crucial step for the extensive amplification characteristic of chemotactic signaling.

transmembrane receptors | bacterial chemotaxis | allosteric coupling | histidine kinases | Nanodiscs

Bacterial chemotaxis is mediated by signaling complexes that sense specific chemicals and direct bacterial cells to favorable chemical environments. They do so by controlling autophosphorylation of a chemotaxis histidine kinase and thereby phosphorylation of the chemotaxis response regulator CheY (reviewed in refs. 1 and 2). The cellular concentration of phospho-CheY determines the pattern of cellular motility and thus directional movement. In *Escherichia coli* and many other bacteria, signaling complexes involve stable noncovalent interactions among transmembrane bacterial chemoreceptors, histidine kinase CheA and coupling protein CheW. In vivo, signaling complexes appear as arrays that can contain tens to thousands of these components (3, 4). In the extensively characterized systems of *E. coli* and *Salmonella enterica*, arrays include several different kinds of chemoreceptors, each able to recognize one or a few specific attractants and repellents.

Chemoreceptors are homodimers organized as a series of helical bundles and coiled coils extending ~300 Å (1, 2). Receptor homodimers interact at their cytoplasmic, membrane-distal tips to form trimers of dimers (5, 6). Because the amino acid sequence of that tip region is essentially identical among the five chemoreceptors of *E. coli*, trimers can contain more than one type of receptor (6). Trimers interact with CheA and CheW to form signaling complexes. The minimal structural and functional core

signaling complex contains two trimers of chemoreceptor homodimers, with each trimer in contact with a monomeric CheW and one of the two protomers in a CheA homodimer (Fig. 1 C and D) (7–10). Using Nanodiscs, individual core complexes can be reconstituted in vitro as water-soluble, active units (Fig. 1A) and their specific features characterized (7, 8, 11, 12). Nanodiscs are small, ~100-nm-diameter, plugs of lipid bilayer with the hydrophobic region shielded from the aqueous environment by a protective belt of membrane scaffold protein (13), thereby rendering that small plug of bilayer water soluble. Nanodiscs made with natural *E. coli* lipids provide a native environment for purified transmembrane chemoreceptors (14, 15). Water-soluble core signaling complexes assembled with purified Nanodisc-inserted receptors, CheA, and CheW activate the kinase ~750-fold, almost as well as complexes formed in vitro with chemoreceptors in their natural membranes (7). As in native arrays, the enhanced activity is controlled by receptors; specifically, it is reduced as a function of receptor ligand occupancy (7).

Control of kinase activity by receptor ligand occupancy exhibits notable signal amplification. In vivo it can be as much as 35-fold (16), implying that occupancy of one receptor can inhibit up to 35 kinase active sites. Such amplification is thought to involve allosteric spread of inhibition from a single ligand-occupied receptor to multiple kinases through the network of physical contacts in core signaling complexes and their arrays (1). We were interested in investigating allosteric influences in the simplest structural and functional unit, the core signaling complex. An experimental approach was suggested by studies in vivo (17) and in vitro (18) documenting that the presence of two kinds of receptors altered sensitivity and cooperativity of attractant responses mediated by each receptor type, indicating that chemoreceptors were affected by their neighbors, presumably through allosteric interactions. Thus,

Significance

The notable cooperativity and signal amplification of bacterial chemotaxis are thought to arise from allosteric interactions in its multicomponent, membrane-bound signaling complexes. However, little is known about this presumed allostery. By characterizing signaling in isolated core signaling complexes, assembled using Nanodisc-inserted transmembrane chemoreceptors, we found that core complexes were not unified allosteric units. Instead, allosteric coupling among its components was selective and asymmetric. This observation was unanticipated and has significant implications for understanding the central coupling of ligand-occupied chemoreceptors to chemotaxis histidine kinases. More widely, our observations provide an example of unconventional asymmetry within an allosteric unit. In addition, we deduced allosteric coupling between protomers of the dimeric kinase, identifying a crucial link in the allosteric spread of ligand-induced kinase inhibition.

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Fig. 1. The core chemotaxis signaling complex and its components. (A) Cartoon of an isolated, water-soluble core signaling complex, assembled with two Nanodisc-inserted trimers of chemoreceptor homodimers, one CheA homodimer, and two CheWs (7). (B) Domain organization of the dimer of the chemotaxis histidine kinase CheA. The two protomers of the dimer are distinguished by shading for one and a prime on the domain names of the other. P1 carries the autophosphorylated histidine, P2 binds substrate proteins CheY and CheB, P3 is the four-helix bundle dimerization domain, P4 is the catalytic domain, and P5 is the receptor-binding domain and a structural homolog of CheW. (C and D) Molecular models, derived from fitting X-ray structures to tomographic densities, of interactions among components of the core signaling complex modified from figure P1 in ref. 10. (C) View parallel to the membrane. (D) view from the membrane toward CheA and CheW.

we characterized ligand control of kinase activity in Nanodisc-based, isolated core complexes in which both trimers contained two kinds of receptors, each recognizing a different ligand.

Results

Preparation of Nanodiscs with Mixed Chemoreceptor Compositions.

We assembled Nanodiscs containing the *E. coli* aspartate chemoreceptor Tar, the serine receptor Tsr, or both. Each receptor carried a carboxyl-terminal affinity tag, either six histidines or biotin attached to a cysteine via a sulphydryl-reactive biotinylation reagent. Both receptors were in the intermediate signaling state generated by two glutamines and two glutamates at the four major sites of adaptational modification. Receptor-containing Nanodiscs carrying a single receptor type were separated from empty discs using an affinity column for the relevant affinity tag. Discs carrying both Tar and Tsr were separated from empty discs and from discs containing only one receptor type by two sequential affinity columns, one specific for the affinity tag on one receptor and the other specific for the affinity tag on the other receptor (Fig. 2). Most preparations of receptor-containing discs were fractionated by molecular sieve chromatography to enrich for discs containing approximately three chemoreceptor dimers per disc, the number required for formation of trimers of receptor dimers capable of forming signaling complexes (7, 8). We prepared Nanodiscs containing only Tar (100% Tar), only Tsr (100% Tsr), Tar and Tsr at a 2:1 ratio (66.7% Tar, 33.3% Tsr), or Tar and Tsr at a 1.15:1.85 ratio (39% Tar, 61% Tsr). The two-affinity-tag purification procedure for discs with mixed receptors meant that each disc would carry both tags, ensuring that each disc contained at least one Tar dimer and one Tsr dimer. Thus, every receptor

trimer in the 66.7% Tar and 33.3% Tsr preparation would be expected to have two Tar dimers and one Tsr dimer (Tar₂Tsr₁). For the Tsr-in-the-majority preparation we aimed for a complementary 1:2 ratio. However, differential efficiency of Nanodisc incorporation for the two chemoreceptors meant that the ratio achieved was 1.15 Tar:1.85 Tsr. At this ratio, and given that detergent-solubilized receptors seem to incorporate into Nanodiscs as independent dimers (7, 11), the three-dimer per disc population would be 83% Tar₁Tsr₂ and 17% Tar₂Tsr₁.

Effects of Heterologous Chemoreceptor Neighbors. Isolated, soluble core signaling complexes contain one CheA dimer, two CheWs, and two trimers of chemoreceptor dimers, each trimer in an independent Nanodisc (7). We used each of the four receptor-containing Nanodisc preparations described above to assemble soluble core signaling complexes with the corresponding receptor compositions. The receptor-Nanodisc preparations were incubated with kinase CheA and coupling protein CheW to form signaling complexes. These were assayed directly for chemoreceptor-mediated control of kinase activity by measuring initial rates of CheY phosphorylation. Measurements could be performed without separating signaling complexes from residual free CheA because the kinase activity of CheA incorporated into isolated core signaling complexes is ~750-fold greater than that of free CheA (7). Thus, free CheA would make negligible contributions to the measured kinase activity.

We determined activity for signaling complexes assembled with each receptor composition in the presence of the Tar ligand aspartate and of the Tsr ligand serine at concentrations from zero to saturation. Fig. 3 shows normalized kinase activity plotted as a function of ligand concentration and fitted to a modified Hill equation (*Materials and Methods*). The fits generated numerical values for operational ligand affinity expressed as ligand concentration at half-maximal kinase inhibition ($[Asp]_{1/2}$ or $[Ser]_{1/2}$), cooperativity among binding sites for the respective ligands expressed as a Hill coefficient (n), and the proportion of CheA activity independent of the ligand-occupied receptor expressed as the proportion of kinase activity persisting at saturating ligand (A_S) (Table 1). In core signaling complexes

Fig. 2. Preparation of mixed chemoreceptor Nanodiscs. Affinity-tagged, detergent-solubilized, and purified chemoreceptors Tar and Tsr were mixed at an experimentally determined ratio and used to prepare and isolate Nanodiscs containing at least one of each receptor. The figure illustrates the procedure for Tar carrying a six-His tag (Tar-6H) and Tsr carrying biotin coupled by a disulfide bond to a carboxyl-terminal cysteine (Tsr-S-S-Biot). For some preparations, the affinity tags were reversed, but the order of the columns remained the same.

Table 1. Parameters derived from fitting data to a Hill relationship

Receptor composition		+Asp			+Ser		
Tar (%)	Tsr (%)	<i>n</i>	<i>Asp</i> _{1/2}	<i>A_s</i>	<i>n</i>	<i>Ser</i> _{1/2}	<i>A_s</i>
100	0	1.5 ± 0.3	5.5 ± 0.5	0			
67	33	1.2 ± 0.1	13 ± 4	0.15 ± 0.02	0.80 ± 0.07	65 ± 9	0.57 ± 0.07
39	61	0.97 ± 0.2	32 ± 13	0.45 ± 0.03	0.89 ± 0.08	44 ± 3	0.25 ± 0.04
0	100				0.82 ± 0.03	31 ± 1	0.033 ± 0.03

none of these ideas was consistent with the data. We considered more complicated models, but these involved postulating features of the core complex, such as nonlinear coupling, that were not suggested by experimental evidence.

Instead, we considered a model based on structure of the core complex that was determined experimentally by a combination of electron tomography and X-ray crystallography (9, 10). In that structure, among the dimers in the receptor trimers of dimers there are dimer-specific contacts with CheA and with CheW. Through its P5 domain (Fig. 1*B*), each CheA protomer contacts only one of the three dimers in the trimer of receptor dimers, and each CheW contacts a single, different dimer (Fig. 1*C* and *D*) (9, 10). This suggested that only the dimer in direct physical contact with CheA P5 or alternatively only the dimer in direct contact with CheW had the ability to inhibit the kinase activity of the trimer-associated CheA protomer. However, there was an additional issue. Inhibition from the physically interacting receptor dimer might be limited to the CheA protomer associated with that dimer. Alternatively, it might spread to the other CheA protomer via allosteric coupling, perhaps through the P3 dimerization domain (Fig. 1*B*). The two extremes would be (i) no allosteric coupling between CheA protomers, or (ii) 100% allosteric coupling. Fig. 4 compares the predictions of the respective extreme models to the experimentally determined kinase activity insensitive to saturating aspartate or insensitive to saturating serine. Inhibition is substantially greater than predicted by no allosteric coupling but less than predicted by 100% coupling. We conclude that the two protomers of the CheA dimer in a core signaling complex are allosterically coupled but that coupling is incomplete. Coupling might be more efficient when core complexes organize as extended arrays (*Discussion*).

Discussion

This study provided two principle insights into allosteric influences in bacterial chemotaxis signaling complexes: (i) control of kinase activity by chemoreceptors is selective and asymmetric among the dimers in a trimer of receptor dimers; and (ii) the two protomers of the dimeric kinase are allosterically coupled. These insights, their implications, and related observations are considered in the following paragraphs.

Structural and Functional Asymmetry in Core Signaling Complexes. In the deduced structural organization of core signaling complexes *in vivo* (9, 10), only one dimer in a trimer physically contacts the CheA P5 domain, and only one, a different dimer, physically contacts CheW (Fig. 1*C* and *D*) (9, 10). Our studies of isolated, mixed-receptor core signaling complexes provide a functional correlate for this structural asymmetry. Analysis of our data indicated that receptor occupancy-induced inhibition of kinase activity in the core signaling complex occurred through only one chemoreceptor dimer in the trimer of dimers. We suggest that this functional asymmetry among the three dimers is the result of the physical asymmetry of the receptor trimer of dimers in signaling complexes. The relevant physical asymmetry is likely direct contact with the CheA P5 domain, in which only a single dimer in the trimer participates. Alternatively, it could be contact

with CheW because CheW interacts directly with its companion CheA P5 domain. In either case the combination of structural and functional asymmetry among the dimers of a chemoreceptor trimer of dimers reinforces the significance of each observation and provides a strong argument that chemoreceptor trimers are asymmetrically coupled to the kinase they control. Functional asymmetry, in which only one of the three dimers in the trimer is able to inhibit the kinase as it is occupied by ligand, had not been anticipated by previous observations. Incorporating the notion of functional asymmetry into comprehensive views and computational representations of the chemotaxis signaling system could yield new and unanticipated insights into the molecular mechanisms of this sophisticated signaling process.

Coupling of kinase inhibition generated by ligand occupancy to only one receptor dimer among the three in the trimer implies that there is no functionally significant communication among the dimers of a trimer for ligand occupancy-driven kinase

Fig. 4. Allosteric coupling between protomers of the CheA dimer in isolated core signaling complexes. For mixed-receptor core signaling complexes at the indicated receptor ratios, the observed percentage of kinase activity insensitive to saturating ligand (Obs, black bars) is compared with values predicted if only the receptor in physical contact with CheA P5 (or CheW) could inhibit kinase, the identity of that receptor is determined by the receptor ratios, and allosteric coupling between CheA protomers were 0 (0, open bar) or 100% (100, open bar). With 100% coupling, kinases not inhibited by saturating ligand would be those with neither CheA protomer contacting a ligand-occupied chemoreceptor dimer. This value is (% unoccupied receptor in signaling complex preparation)². With 0 coupling, every kinase protomer not contacting a ligand-occupied chemoreceptor would remain active at saturation, which is (% unoccupied receptor in signaling complex preparation). Predictions for effects of saturating serine are adjusted for the 3.3% kinase activity not inhibited by serine-saturated Tsr (Table 1). (A) 66.7% Tar and 33.3% Tsr preparation. (B) 39% Tar and 61% Tsr preparation.

inhibition. However, we observed allosteric communication among dimers of a trimer in their mutual influences on operational ligand affinity (Table 1). This implies that allosteric coupling in core signaling complexes is selective for different functions. Protein complexes involved in allostery are often considered as a single unit in one of two conformational states. Selective coupling implies that this is not the case for core chemotaxis signaling complexes. Combined with previous observations that chemoreceptor trimers alone do not respond symmetrically to occupancy of their constituent dimers (19), our results suggest that detailed models of chemotaxis signaling should incorporate asymmetry among participating chemoreceptors. Understanding selective allosteric coupling could reveal new features of allostery, applicable not only to the chemotaxis signaling complex but also other multicomponent, allosteric complexes.

Allosteric Coupling Across the CheA Dimer Interface. Analysis of our data indicated that inhibition of a kinase protomer by ligand occupancy of a coupled chemoreceptor dimer substantially inhibited the other kinase protomer. This indicated significant allosteric coupling between the protomers of the kinase dimer in a core signaling complex. The dimer interface is a four-helix bundle, the P3 domain, to which each protomer contributes two α -helices (20). Each P3 half-domain is connected to a respective P4 catalytic domain by a short linker (Fig. 1*B*). This P3–P4 linker has been shown to be central to kinase activity and control, specifically basal autophosphorylation, kinase activation, and initiation of long-range structural and dynamic changes impinging on the active site (21–23). Thus, the P3–P4 linker could be a conduit through which allosteric inhibition passes from an inhibited P4 catalytic domain to the P3 helical bundle, and from P3 to inhibit the P4' catalytic domain of the companion kinase protomer.

Allosteric Communication in Signaling Complex Arrays. Reduced operational affinity and increased ligand-insensitive kinase generated by the presence of heterologous receptors occurs not only in core signaling complexes but also in native arrays of signaling complexes *in vivo* (17) or in complexes assembled *in vitro* using receptor-containing native membranes (18). Thus, the allosteric influences we identified in isolated core signaling complexes are evident in the complete *in vivo* signaling system and its widely used *in vitro* model. We suggest that cooperativity and amplification in the chemotaxis sensory system are intimately linked to these allosteric influences.

Significant in this regard is allosteric communication across the kinase dimer interface. *In vivo*, the kinase-inhibiting effect of receptor occupancy can be amplified as much as 35-fold; that is, occupancy of one receptor dimer can inhibit 35-fold more kinase activity than expected if it inhibited only one kinase active site (17). The mechanism of this amplification is unknown but is widely thought to involve allosteric communication within signaling complex arrays (1). The deduced structure of these arrays (9, 10) identifies potential pathways of protein–protein contact for allosteric communication. In the structure, three core signaling complexes combine to form a hexagonal ring of alternating CheW and CheA P5 domains (Fig. 5). Each ring includes one CheW and one CheA P5 domain from each core complex. The second P5 domain and the second CheW of each core complex are exposed on the periphery of the hexagon, available to be part of hexagons adjacent to the first (Fig. 5). This structural organization suggests two, complementary pathways for the allosteric spread of kinase inhibition from a single, ligand-occupied chemoreceptor dimer. One is around the hexagon of alternating CheW and CheA P5 to affect the respective P4 active sites. Such coupling would generate three inhibited kinase active sites for the three CheA protomers directly in the hexagon. The allosteric coupling we deduced between protomers of a CheA dimer would be a pathway for spreading kinase inhibition to

Fig. 5. Suggested pathways for allosteric, conformational spread of kinase inhibition in a signaling complex array. The figure shows diagrammatic cartoon representations of the components of the core signaling complex (*Upper Left*), a core signaling complex in which only two of the five CheA domains, P3 and P5, are shown (*Upper Right*), and a portion of a hexagonal array of core complexes (*Lower*). (*Upper Right*) A single ligand-occupied, kinase-inhibiting chemoreceptor dimer (colored red) in one receptor trimer of the core complex inhibits (thick arrow) the CheA protomer in physical contact (red) and by allosteric communication (thin arrow) inhibits the companion CheA protomer (red). (*Lower*) The postulated allosteric conformational spread of inhibition around the CheA P5/CheW hexagons and from one hexagon to the next across the CheA dimer interface is illustrated with arrows and red kinase components. The figure is a modified version of figure 5 in ref. 10.

adjacent hexagons. This coupling could be across the CheA dimer interface that connects each hexagon to three hexagon neighbors. We suggest that communication through the CheA dimer interface is central to the substantial signal amplification observed in intact cells. It would spread kinase inhibition from one hexagon to its neighbors. A full 35-fold amplification of kinase inhibition would require the allosteric influence of a single occupied receptor dimer to spread outward over several rings of surrounding P5–CheW hexagons in an array, as suggested previously (24). The first level of that array and a pathway of inhibition are diagrammed in Fig. 5. Long-range allosteric coupling would require notably strong connections within and between multiple hexagons. This could be made possible by energetic contributions in the large arrays observed *in vivo* (3) and be at least one important part of functional significance of large arrays. In addition, the allosteric coupling that generates substantial cooperativity among homologous receptors (16) could follow the same pathway by including the kinase-coupled dimer of each trimer in the allosteric network.

Materials and Methods

Genes and Proteins. A gene encoding Tsr-6H, chemoreceptor Tsr carrying a six-histidine affinity tag immediately after the natural carboxyl terminus, was constructed from plasmid pCT1 (25) using a PCR primer 5' to the NdeI site and

a mutagenic primer overlapping the stop codon, which added six histidine codons, a stop codon, and a HindIII site after the final aminoacyl codon, and ligating the PCR product into the original vector cut with NdeI and HindIII. A gene encoding Tar-C, chemoreceptor Tar with a single cysteine added immediately after the natural carboxyl terminus, was constructed by PCR with a mutagenic oligonucleotide that inserted Cys codon TGT between the codon for the carboxyl-terminal residue amino acid and the stop codon. Tsr-6H, Tar-6H (11), and membrane scaffold protein MSP1D1E3(-) (13) were purified as described, as were CheA, CheW, and CheY (26). Tar-C and Tsr-C (27) were purified from membrane vesicles isolated from cells producing the respective chemoreceptors at high levels (12) and for which the receptor content was determined by 7% polyacrylamide gel electrophoresis, staining with Coomassie Brilliant Blue, and comparison with purified Tar-6H and Tsr-6H standards. Such receptor-containing membranes were solubilized with *n*-octyl- β -D-glucoside, mixed with equal-molar *N*-biotinylaminoethyl methanethiosulfonate (Toronto Research Chemicals Inc.), and incubated on ice in the dark for 1 h. After centrifugation for 17 min, 543,000 $\times$ g, 4 °C in a TLA 100.4 rotor, the supernatant was loaded on a column of SoftLink Soft Release Avidin Resin (Promega V2012), the column washed with 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% (wt/vol) glycerol, and 25 mM cholate, and Tar-C-Biotin or Tsr-C-Biotin eluted with that buffer containing 5 mM d-Biotin. Elution with d-Biotin preserved the disulfide-coupled biotin for use in preparation of mixed receptor Nanodiscs.

Nanodiscs containing Tar-6H or Tsr-6H at approximately three receptor dimers per disc were prepared essentially as previously described (12). Discs containing both Tar and Tsr, either Tar-6H and Tsr-C or Tar-C and Tsr-6H, were prepared similarly except that both purified receptors were provided and Nanodiscs were purified by two sequential columns (Fig. 2) in a procedure similar to the isolation of purified signaling complexes (7), but with larger, 10-mL bed-volume columns. These were a Ni column (HisTrap HP; GE Healthcare) equilibrated and washed with 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% (wt/vol) glycerol, and 30 mM imidazole and eluted with the same buffer but with 300 mM imidazole, followed by a column of NeutrAvidin Agarose Resin (Thermo Scientific, catalog no 29204) equilibrated and washed with 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10% (wt/vol) glycerol and eluted with the same buffer containing 50 mM DTT to cleave the disulfide bond with which the biotin was attached to the cysteine-containing receptor. The procedure yielded only discs containing both receptors. Discs containing 67.7% Tar-6H and 33.3% Tsr-C were produced when equal amounts of the two receptors (5 μ M each) were used, and those containing 61% Tsr-6H and 39% Tar-C were generated with a preparation ratio of 2 Tsr:1 Tar. Most preparations were enriched for approximately three receptor dimers per disc by fractionation using molecular sieve chromatography (8). However, in the course of this work we found that patterns of kinase inhibition as a function of ligand occupancy were indistinguishable

whether or not the mixed receptor discs were enriched in this way or used directly for preparation of signaling complexes. For the data used in this publication, one preparation of Tar-6H/Tsr-C-biotin discs and the preparations of Tar-C-biotin/Tsr-6H discs were not fractionated.

Kinase Activation. Kinase activity was assayed essentially as previously described (8, 26). Nanodisc-embedded, size-fractionated receptors (6.25 μ M) or 11.25- μ M unfractionated, Nanodisc-embedded receptors were incubated with 0.31 μ M CheA, 10 μ M CheW, and 25 μ M CheY in 50 mM Tris-HCl (pH 7.5), 10% (wt/vol) glycerol, 100 mM NaCl, 50 mM KCl, and 5 mM MgCl₂. After 1.5 h at room temperature to allow formation of signaling complexes, aspartate (0–100 mM) or serine (0–100 mM) was added. After 15 min at room temperature, phosphorylation was initiated by addition of [γ -³²P]-ATP (Perkin-Elmer, ~63 μ Ci/ μ mol) to 0.4 mM and terminated 15 s later by addition of denaturing SDS sample buffer (8, 26). After addition of ligand and ATP, protein concentrations were 5 or 9 μ M receptor, 0.25 μ M CheA, 8 μ M CheW, and 20 μ M CheY. Samples were submitted to SDS gel electrophoresis on 14% polyacrylamide gels and gels briefly stained, destained, and dried. [³²P] Phospho-CheY was determined by phosphor imaging.

Data Analysis. Data were fit to a modified Hill equation, $A = (A_s - A_0)[1 - L^n / (L_{1/2}^n + L^n)] + A_0$, where A is kinase activity, A_0 is kinase activity in absence of ligand, A_s is kinase activity at saturating ligand, L is ligand concentration, n is the Hill coefficient, and $L_{1/2}$ is ligand concentration at half-maximal inhibition. To compare effects of heterologous neighbors we normalized kinase activity for each receptor composition to activity in the absence of ligand, as identified by the A_0 in the fit of the data before normalization. Normalization was necessary because the limited quantities of signaling complexes that could be assembled made it impractical to isolate those complexes in sufficient amounts to determine kinase activity as a function of ligand concentration yet also quantify CheA in signaling complexes and thus be able to calculate specific activity of CheA in complexes (7). In fact, different compositions of receptor-containing Nanodisc preparation exhibited differential effectiveness for kinase activation. Pilot experiments indicate that efficiency of complex formation is a major contributor to this differential activation. For instance, for the data in Fig. 3, values of A_0 were 100% Tar = 370 $\pm$ 30 nM/s; 66.7% Tar, 33.3% Tsr = 160 $\pm$ 5 (aspartate titrations) and 130 $\pm$ 50 nM/s (serine titrations); 39% Tar, 61% Tsr = 40 $\pm$ 10 (aspartate titrations) and 44 $\pm$ 10 nM/s (serine titrations), and 100% Tsr = 90 $\pm$ 6 nM/s.

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