Cooperative Signaling among Bacterial Chemoreceptors†

Run-Zhi Lai,‡ Josiah M. B. Manson,§ Arjan F. Bormans,‡ Roger R. Draheim,‡ Ngoc T. Nguyen,‡ and Michael D. Manson*,‡

Department of Biology, 3258 TAMU, Texas A&M University, College Station, Texas 77843, and Department of Chemistry, Texas A&M University, College Station, Texas 77843

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ABSTRACT: Four chemoreceptors in *Escherichia coli* mediate responses to chemicals in the environment. The receptors self-associate and localize to the cell poles. This aggregation implies that interactions among receptors are important parameters of signal processing during chemotaxis. We examined this phenomenon using a receptor-coupled in vitro assay of CheA kinase activity. The ability of homogeneous populations of the serine receptor Tsr and the aspartate receptor Tar to stimulate CheA was directly proportional to the ratio of the receptor to total protein in cell membranes up to a fraction of 50%. Membranes containing mixed populations of Tar and Tsr supported an up to 4-fold greater stimulation of CheA than expected on the basis of the contributions of the individual receptors. Peak activity was seen at a Tar:Tsr ratio of 1:4. This synergy was observed only when the two proteins were expressed simultaneously, suggesting that, under our conditions, the fundamental "cooperative receptor unit" is relatively static, even in the absence of CheA and CheW. Finally, we observed that inhibition of receptor-stimulated CheA activity by serine or aspartate required significantly higher concentrations of ligand for membranes containing mixed Tsr and Tar populations than for membranes containing only Tsr (up to 10^2-fold more serine) or Tar (up to 10^4-fold more aspartate). Together with recent analyses of the interactions of Tsr and Tar in vivo, our results reveal the emergent properties of mixed receptor populations and emphasize their importance in the integrated signal processing that underlies bacterial chemotaxis.

Motile bacteria migrate toward or away from certain chemicals in a behavior called chemotaxis (reviewed in refs *1*−*4*). In *Escherichia coli*, these chemicals are recognized by one of four closely related chemoreceptors that localize to the cell membrane. The Tar receptor mediates attractant responses to L-aspartate and maltose, as Tsr does to L-serine. Trg mediates responses to ribose, glucose, and galactose, and Tap is responsible for chemotaxis toward di- and tripeptides. The coupling factor CheW connects the histidine protein kinase CheA to the cytoplasmic domain of these receptors (*5*) at their membrane distal tip. Occupancy of ligand-binding sites in the periplasmic domain of the receptors modulates the autophosphorylation activity of CheA. Phosphorylated CheA, in turn, transfers its phosphoryl group to the response regulator CheY. Binding of phospho-CheY to FliM in the flagellar motor increases the probability of a reversal from counterclockwise (CCW) rotation of the flagellum to clockwise (CW). The balance between the activities of CheA and the CheY phosphatase, CheZ, determines the CCW:CW ratio of flagellar rotation. CCW rotation leads to smooth swimming, and CW rotation of one or more flagella induces a tumble.

CheA activity is also modulated by the methylation state of the receptor to which it is coupled. Methyl groups are added by a methyltransferase, CheR, and removed by a methylesterase, CheB. CheB is much more active when it is phosphorylated by CheA. Increased levels of methylation at four specific glutamyl residues bias a receptor toward CheA stimulation, whereas decreased levels of methylation cause a receptor to be less active in stimulating CheA. Adjustments in the relative rates of methylation and demethylation allow the receptor ensemble to maintain nearly the same baseline level of CheA activity at any constant concentration of attractant or repellent. Tar and Tsr are both synthesized with the first and third methylatable glutamyl residues as glutaminyl residues to produce the QEQE form of the receptors, in which Q functionally mimics a methylated glutamyl residue (E^m). Phospho-CheB then deamidates these two glutaminyl residues to generate the EEEE form of the receptors. All of the in vitro studies described here were performed with the QEQE forms of Tar and Tsr.

E. coli chemoreceptors localize to cell poles (*6*) in clusters that contain all four receptors (*7*). Formation of these clusters requires CheW and CheA. In addition, at least some of the CheB, CheR, CheY, and CheZ proteins in the cell are associated with these patches (*6*, *8*, *9*), although none of them are required for patch formation or maintenance. Receptor clustering has also been observed in other bacteria (*10*, *11*).

The crystal structure of the cytoplasmic domain of the Tsr receptor indicates that it, and presumably the other receptors, exists as a trimer of dimers (*12*). Genetic studies also provide evidence for the existence of such trimers (*13*). It has been proposed that arrays of such trimers of dimers connected by CheW and CheA can form higher-order lattices within a patch (*14*, *15*).

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* To whom correspondence should be addressed. Telephone: (979) 845-5158. Fax: (979) 845-2981. E-mail: mike@mail.bio.tamu.edu.

‡ Department of Biology.

§ Department of Chemistry.

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Close physical association of receptors suggests that they interact in chemotaxis signaling. *E. coli* can detect submicromolar concentrations of some attractants (*16*), and gradients can be sensed over 5 orders of magnitude (*17*, *18*). Also, the inhibitory signal initiated by ligand binding can be amplified up to 35 times more than expected from shutting off individual receptors (*19*). Thus, it seems to be crucial to consider the activity of the chemoreceptor patch as a whole (*14*, *20*−*24*) and to take relatively long-range interactions among receptors into account (*13*, *25*, *26*).

We report here the results from in vitro receptor-coupled kinase assays designed to examine such higher-order interactions. We find that receptors Tar and Tsr combine synergistically to stimulate CheA to levels that cannot be attained by either receptor in isolation. Mixed receptor populations also show decreases of several orders of magnitude in their sensitivities to aspartate and serine. Finally, we present evidence that the interactions among receptors that lead to these phenomena are stable for hours, both within cells and in inner-membrane preparations.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. E. coli strain RP3098 [Δ(*flhD-flhB*)4] (*27*), a derivative of *E. coli* K12 strain RP437 (*28*), was used for high-level expression of chemoreceptors and CheA. Strain BL21(λDE3) (Novagen) was used to produce CheY for purification. The λDE3 derivative of BL21 [F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm*] contains a prophage that encodes the T7 RNA polymerase gene under the control of the *lacUV5* promoter (*29*, *30*).

Plasmid pDM011 is a pET24a(+) derivative containing the *cheY* gene expressed from a T7 promoter. Plasmid pKJ9 carries an isopropyl β-D-thiogalactopyranoside (IPTG)[1] inducible *cheA* gene (*31*). Plasmid pCJ30, a derivative of the ColE1 plasmid pBR322, confers Amp^r and carries a *tac* (IPTG inducible) promoter preceding a multiple-cloning site (*32*). Plasmid pBAD18, also a derivative of pBR322, confers Amp^r and has the *araBAD* promoter preceding a multiple-cloning site (*33*). Plasmid pLC112, a derivative of the P15A plasmid pACYC, confers Cam^r and has the *nahG* (salicylate inducible) promoter preceding a multiple-cloning site (*13*). Plasmid pBAL03 was constructed from pACYC and pBAD18. It bears the P15A origin, confers Cam^r, and has the *araBAD* promoter preceding a multiple-cloning site. The wild-type *tar* and *tsr* genes were introduced into these plasmids to allow for control of their expression by different inducers and, in the case of the *araBAD* promoter, for repression by the addition of the anti-inducer fucose (*33*−*35*). Table 1S of the Supporting Information provides a complete list of strains and plasmids used in this study.

Purification of CheY and CheA. CheY was purified according to the method of Matsumura et al. (*36*), with minor modifications. Transcription of *cheY* was induced from plasmid pDM011 with 500 μM IPTG in a 1 L Luria broth (LB) (*37*) culture of strain BL21 (λDE3) grown at 30 °C. CheY was eluted from a Cibacron blue column, dialyzed, and then concentrated by ultrafiltration before being loaded onto a Superose 12 column. CheA was purified by the method of Hess and Simon (*38*). CheA expression from the pKJ9 plasmid in strain RP3098 was induced at 30 °C by adding IPTG to a final concentration of 500 μM to a 1 L LB culture. The purities of CheY and CheA were estimated from densitometric scans (ImageMax scanner) of Coomassie blue-stained 16 and 12% acrylamide−SDS gels, respectively. The total protein concentration was determined using the Bradford assay (*39*).

Preparation of Inner Membranes Containing Overexpressed Receptors. Cultures were grown in 1 L of LB at 30 °C. Receptor synthesis from plasmid-borne *tar* and *tsr* genes was induced in strain RP3098 by addition of the desired concentration of the relevant inducer at the appropriate time. Cells were harvested at an OD_{600} of ∼1.0 by centrifugation at 4500*g*, using a Beckman JA10 rotor.

The preparation of inner membranes was based on the method of Osborn and Munson (*40*). Spheroplasts were made from the harvested cells and lysed by osmotic shock. Cell debris was removed by centrifugation at 1200*g*. Membranes were pelleted at 17700*g*, washed, and resuspended in a 25% (w/v) sucrose solution. Membranes were fractionated on a sucrose step gradient [4 mL of 55% sucrose, 8 mL of 45% sucrose, 8 mL of 40% sucrose, and 10 mL of 30% sucrose (w/v)] in a Beckman SW28 rotor operated at 4 °C and 140000*g* for 12 h. The band at the interface of the 30 and 40% sucrose layers contained the highest percentage of receptor to total protein. This fraction was dialyzed twice against 2 L of TE buffer [10 mM Tris and 1 mM EDTA (pH 8.0)], then pelleted by centrifugation at 210000*g*, and resuspended in TE buffer containing 15% glycerol (v/v). The ratio of receptor to total protein was determined by quantitative scanning densitometry of Coomassie-stained 12% SDS gels using the ImageMax scanner and NIH imager software. The total protein concentration was determined by the Bradford assay (*39*).

In Vitro Receptor-Coupled Phosphorylation Assay. The receptor-coupled phosphorylation assay was performed using a modified version of the procedure of Borkovich and Simon (*41*). Five picomoles of CheA and 20 pmol of CheW were mixed and incubated at 4 °C overnight in a total volume of 2.5 μL to maximize formation of CheW−CheA dimer−CheW complexes. Then, 500 pmol of CheY, 0.5 μL of 20× phosphorylation buffer [1 M Tris-HCl, 1 M KCl, and 100 mM $MgCl_2$ (pH 7.5)], 0.5 μL of 40 mM DTT, and an aliquot of an inner-membrane preparation containing 20 pmol of receptor were added to the CheA/CheW mix to yield a total volume of 8 μL. For assays involving chemoeffectors, 1 μL of a solution containing ligand at the desired concentration was added. The mixtures were incubated for 4 h at room temperature to allow ternary receptor−CheA−CheW complexes to assemble. Reactions were initiated by addition of 1 μL of [γ-^{32}P]ATP (a mixture of 3.3 μM radioactive ATP with 10 mM unlabeled ATP at a 1:1 ratio). Reactions were halted after 20 s by the addition of 40 μL of 2× SDS−PAGE sample buffer, and samples were subjected to 16.5% acrylamide SDS−PAGE. Gels were dried and analyzed using a phosphorimager (Fuji BAS 2000). Phospho-CheY levels were calibrated with reference to densitometric scans of a dilution series of [γ-^{32}P]ATP spots made with the same batch of [γ-^{32}P]ATP used in the assay from that day.

The effect of the *E. coli* membrane per se on the in vitro CheA activity was tested in parallel assays in the presence of (A) no added membranes, (B) receptor-free membranes,

[1] Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside.

(C) receptor-containing membranes, and (D) mixtures of receptor-containing and receptor-free membranes. No significant difference in CheA activity was seen between assays A and B, and for an equal concentration of receptor, the same large increase in CheA activity was observed in assays C and D (data not shown).

Data Analysis. Ligand-dependent inhibition of receptor-coupled CheA kinase activity was analyzed as described previously (*42*). The titration curves were fitted with the Hill equation, using KaleidaGraph version 3.6. The analysis yielded two parameters: the half-maximal inhibitory concentration (IC_{50}) and the Hill coefficient for binding cooperativity (n_H). Standard deviations of the mean for the IC_{50} and n_H values were calculated ($n \geq 3$).

The distribution of CheA kinase activity stimulated by simultaneously expressed Tar and Tsr was fitted with a Beta Distribution, Beta(a,b) (*43*), using MATLAB version 7.0. The Beta distribution is designed to fit a set of bounded (between 0 and 1) data. Because the CheA activity stimulated by Tsr is twice that of Tar, the left end point is lower than the right end point. Thus, a background activity corresponding to the expected contribution of Tsr and Tar, assuming they do not interact synergistically, was subtracted from the CheA activities actually measured at each Tar:Tsr ratio that was examined. The transformed data were then fitted with the Beta function. Finally, the background values were added back to yield the curve shown. The symmetry properties of the distribution were determined from the skew of the fitting curve.

Receptor Activity Simulation Program. In our model of receptor interactions, we made the simplifying assumption that the functional unit is a trimer of dimers. Although we do not now think that this molecular structure is preserved in ternary receptor−CheA−CheW complexes, we propose that receptors are delivered to the functional signaling assemblages as trimers of dimers. Thus, the association of receptors in trimers of dimers will be a major factor in identifying the nearest neighbors of a receptor dimer within the actual signaling array. This controversial point is examined in detail in the Discussion.

Our receptor activity simulation program (Program 1S in the Supporting Information) uses a Monte Carlo approach to determine the distribution of trimers of dimers of Tsr and Tar. A full description of the program is provided in the Supporting Information. Each dimer is considered to be a homogeneous receptor unit. There are four trimer types, and the probability of formation of each is stored in a table. The user can specify the number of each type of receptor homodimer in the population. The program chooses a random sample of three receptor dimers from the population. If they form trimers as specified by the probability table, they are removed from the uncombined dimer population. This selection and combination process continues until there are no uncombined receptors left. The calculated distribution of receptor trimers is then written to a file for the user to analyze. Any value of CheA-stimulating activity for a given trimer type (Tar−Tar−Tar, Tar−Tar−Tsr, Tar−Tsr−Tsr, and Tsr−Tsr−Tsr) can be assigned. We assigned the same total activity to the two mixed trimers, since each contains an equal number of Tar−Tsr and Tsr−Tar dimer interfaces.

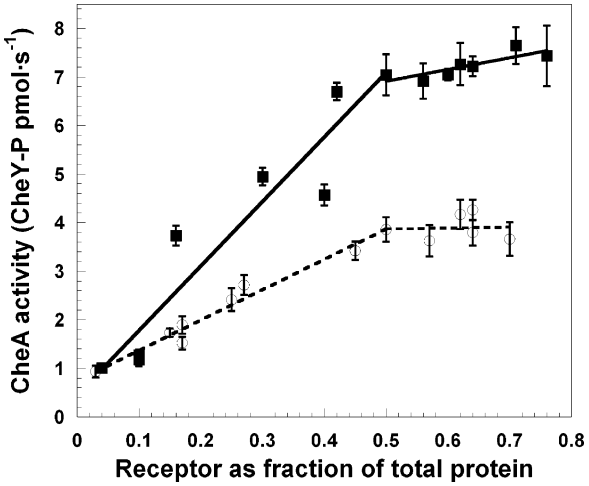

FIGURE 1: Effect of the receptor as a fraction of total membrane protein membrane on CheA activity. CheA activity stimulated by receptors expressed at different levels was determined using the in vitro receptor-coupled CheY phosphorylation assay. CheA activity is expressed as the amount of phospho-CheY produced per second. The total amount of receptor present was the same in each assay. The fraction of receptor to total protein was measured as described in Materials and Methods. The data for Tar and Tsr are shown as empty circles and filled squares, respectively. Each point is the mean value from three independent measurements with a given receptor preparation. The error bars show the standard deviation of the mean, for which $n = 3$.

RESULTS

Dependence of CheA Activity on Receptors as a Fraction of Total Membrane Protein. Before we could evaluate interactions among different receptors, we first had to determine the interactions among receptors of one type. To examine how the receptor fraction of total membrane protein affects the ability of Tar and Tsr to stimulate CheA kinase activity, inner membranes were prepared in which receptors made up 3−75% of the total protein. An equal total amount (20 pmol) of receptor was used in each assay. Both Tar and Tsr showed a striking improvement in their ability to stimulate CheA with an increasing receptor fraction (Figure 1). The linear fits of the data obtained with each receptor were done separately for activities measured at receptor levels that were ≤50% of total protein and ≥50% of total protein because there was an obvious discontinuity at this point. There was considerably more scatter in the Tsr data. Equations 1−4 define the lines that were obtained

$$\nu_{Tar} = 0.062P_a + 0.7 \quad P_a \leq 50 \tag{1}$$

$$\nu_{Tar}' = 0.002P_a + 3.8 \quad P_a \geq 50 \tag{2}$$

$$\nu_{Tsr} = 0.133P_s + 0.5 \quad P_s \leq 50 \tag{3}$$

$$\nu_{Tsr}' = 0.024P_a + 5.7 \quad P_s \geq 50 \tag{4}$$

where ν_{Tar} and ν_{Tsr} represent CheA kinase activity supported by Tar and Tsr, respectively, expressed as picomoles of phospho-CheY produced per second, and P_a and P_s represent Tar and Tsr, respectively, as a proportion of the total protein.

The lines for Tar and Tsr ($P \leq 50$) intercept the ordinate at ∼0.7 and ∼0.5 pmol of phospho-CheY/s, respectively. For comparison, the CheA activity in the absence of receptor is ∼0.1 pmol of phospho-CheY/s under our assay conditions.

Table 1: CheA Activity Stimulated by Mixtures of Tar-Containing and Tsr-Containing Membranes[a]

	CheA activity (pmol of CheY-P/s)		
Tar:Tsr ratio	Tar membranes	Tsr membranes	mixed membranes
1:2	0.9 ± 0.2	3.3 ± 0.6	4.4 ± 0.7
1:1	1.4 ± 0.2	2.5 ± 0.2	3.3 ± 0.4
2:1	1.8 ± 0.3	1.6 ± 0.3	2.9 ± 0.4

[a] The fractions of receptor to total protein were 27 and 30% for Tar-containing and Tsr-containing membranes, respectively. The same volumes of membrane preparations used to assay the CheA activities supported by individual receptors were combined for the assays of mixed membranes.

This extrapolation suggests that, even at extreme dilution in the membrane, receptors can stimulate CheA activity by ~5-fold. The 2-fold steeper slope of the Tsr line for receptor levels of ≤50% of total protein is consistent with reports in the literature (*5*, *44*) and the results of this study which show that Tsr is approximately twice as effective as Tar at stimulating CheA.

Since receptor activities depended strongly on their expression level, it seemed possible that the range over which CheA stimulation would increase linearly with total receptor amount might vary at different receptor expression levels. This possibility was tested by determining CheA activity as a function of receptor concentration with membrane preparations in which Tar or Tsr was present as a different fraction of total protein. CheA activity increased linearly with Tar or Tsr concentration up to 80 pmol/10 μL of reaction mix with all preparations tested (see Figure 1S of the Supporting Information). We accordingly selected 20 pmol of receptor per reaction, which is well within the linear range, as our standard assay condition.

Tar and Tsr Expressed in Separate Cells Do Not Interact in Mixed Membrane Preparations. Membrane preparations containing Tar or Tsr at 27 and 30% of the total protein, respectively, were mixed at ratios of 2:1, 1:1, and 1:2 while holding the total amount of receptor constant at 20 pmol. These samples were then subjected to the in vitro receptor-coupled CheA assay. The activity supported by the mixed receptor populations was within ±16% of the sum of the activities expected for the individual receptors assayed separately (Table 1). It should be noted that we do not know whether our membranes fuse under the conditions of our assay, but the results obtained suggest that, if they do, the fusion has no functional consequence.

Sequentially Coexpressed Tar and Tsr Do Not Interact. *E. coli* chemoreceptors localize to the cell poles in the absence of CheA and CheW, although they do not form tight clusters. Thus, the overall dynamics of receptor interactions in the presence and absence of CheA and CheW are still not clear, although they have been the subject of intensive investigation (*6*, *13*, *25*, *45*). To investigate the stability of receptor interactions under our admittedly nonphysiological conditions of overexpression, we produced Tar and Tsr sequentially in the same cells. Synthesis of one receptor was induced for 2−3 h with 0.2% arabinose from a plasmid in which the gene was transcribed from the *araBAD* promoter control. Then, 0.4% fucose was added to block all subsequent expression of that receptor (*33*−*35*). After 10 min, synthesis of the second receptor was induced with IPTG or salicylate, as appropriate, and the cells were allowed to grow for an additional 2−3 h, for a total of 5 h. The membranes prepared from these cells were designated Tar//Tsr if Tar was expressed first and Tsr//Tar if Tsr was expressed first. The results of receptor-coupled CheA kinase assays conducted with these membranes are shown in Figure 2.

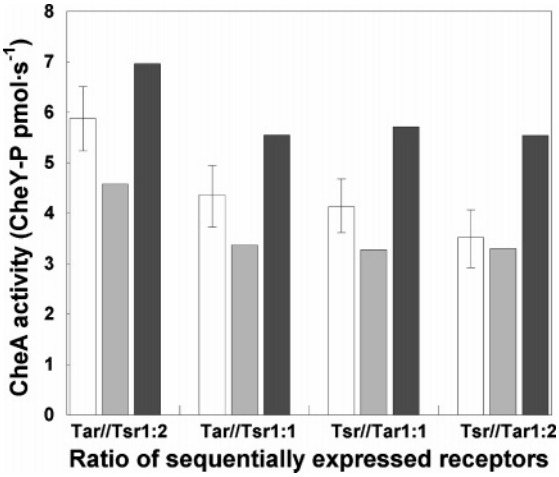

FIGURE 2: CheA-stimulating activity of membranes containing Tar and Tsr expressed sequentially in the same cells. CheA kinase activities stimulated by Tar//Tsr and Tsr//Tar membranes (see the text for definitions) at different ratios of the receptors (indicated below the histogram) are shown as white bars. Values of ν_L (light gray bars) and ν_H (dark gray bars) were calculated from eq 5 and eq 6 or 7, respectively. The fractions of receptor relative to total protein were 72.4% for a Tar//Tsr 1:2 ratio, 61.5% for a Tar//Tsr 1:1 ratio, 59.9% for a Tsr//Tar 1:1 ratio, and 68.2% for a Tsr//Tar 1:2 ratio.

As in the experiments carried out with the mixed membrane preparations, interpretation of these results requires an estimate of the activities expected if there is no functional interaction among the receptors. Because of the strong dependence of receptor-stimulated CheA activity on the fraction of Tar and Tsr in the membrane, we set up two boundary conditions. The lower one assumed that Tar and Tsr do not interact in any way so that the baseline activity for each receptor can be estimated from the fraction of that receptor only. On this basis, the total activity of the mixed population is given by eq 5

$$\nu_L = [(0.062P_a + 0.7)P_a + (0.133P_s + 0.5)P_s]/(P_a + P_s) \quad (P_a \le 50 \text{ and } P_s \le 50) \quad (5)$$

where ν_L is the activity expected if there is no functional interaction and P_a and P_s have the same meanings as in eqs 1−4. The upper boundary assumes that the total receptor fraction should be used to calculate the activity expected from each receptor. The activity of the mixed population is then given by eq 6 (for fractions of ≤50%) or eq 7 (for fractions of ≥50%)

$$\nu_H = \{[0.062(P_a + P_s) + 0.7]P_a + [0.133(P_a + P_s) + 0.5]P_s\}/(P_a + P_s) \quad (P_a + P_s \le 50) \quad (6)$$

$$\nu_H = \{[0.002(P_a + P_s) + 3.8]P_a + [0.024(P_a + P_s) + 5.7]P_s\}/(P_a + P_s) \quad (P_a + P_s \ge 50) \quad (7)$$

where ν_H is the activity expected if the fractions of the two receptors are combined to calculate the receptor fraction used to determine the contribution of each receptor to overall activity.

The values of ν_L and ν_H set the lower and upper limits, respectively, for the receptor-coupled CheA activity expected if there is no positive or negative cooperativity between Tar and Tsr. As seen in Figure 2, the CheA activities supported by the Tar//Tsr and Tsr//Tar membranes fall between the values of ν_L and ν_H for each preparation. We conclude that there is no significant functional interaction between Tar and Tsr when they are expressed sequentially in the same cells.

Tar and Tsr Expressed Simultaneously Interact Synergistically. The results described in the previous paragraphs demonstrate that receptors overexpressed separately, in time or space, in the absence of CheA and CheW do not interact functionally when CheA and CheW are subsequently added. We wanted to extend our analysis to ask whether Tar and Tsr exhibit different behavior in mixed populations when they are synthesized, and presumably inserted into the membrane, at the same time. To this end, membranes were prepared from cells in which Tar and Tsr were expressed together at different ratios.

Tar and Tsr, encoded by compatible plasmids, were induced together in cultures that had reached an OD_{600} of 0.1. The IPTG concentrations that were used were chosen on the basis of the gene which was under control of the *tac* promoter. This approach is valid because RP3098 is a *lacY* strain in which the level of induction is proportional to the concentration of IPTG over a fairly wide range. Synthesis of the other receptor was induced with a saturating concentration of its inducer, either salicylate or arabinose.

Membranes were prepared from these cells and used in the in vitro CheA assay. All the samples were normalized to the CheA kinase activity contributed by 1% receptor to total protein to correct for the large effect of receptor fraction on activity. The normalized activities measured with membranes in which Tar and Tsr were simultaneously expressed were all significantly higher than the ν_H values (Figure 3). The data set was fit with a Beta distribution ($a = 2$, $b = 4$). The curve is skewed toward the Tar side, with a peak when the [Tsr]/[Tar + Tsr] ratio is ~0.2. The activities for mixed Tsr-containing and Tar-containing membranes or membranes from cells in which Tsr and Tar were expressed sequentially all fell between the calculated values for ν_L and ν_H and are shown for comparison.

The Receptor Stoichiometry Affects the Response to Attractants. Another measure of the functional interaction between receptors is how their CheA-stimulating activities are inhibited by attractant ligands. When Tar and Tsr are expressed together, each should stimulate some fraction of CheA activity, and the Tar or Tsr portion of the activity should be inhibited by the addition of aspartate or serine, respectively. Titration of receptor-coupled CheA activity with these attractants is shown in Figure 4. For simultaneously expressed, mixed receptor populations, neither serine nor aspartate inhibited CheA activity entirely. Therefore, maximal inhibition by an attractant was defined by the activity corresponding to the plateau value reached at the highest concentrations of that attractant. The titration curves were fitted with the Hill equation (*42*), which yielded two parameters: a half-maximal inhibitory concentration (IC_{50})

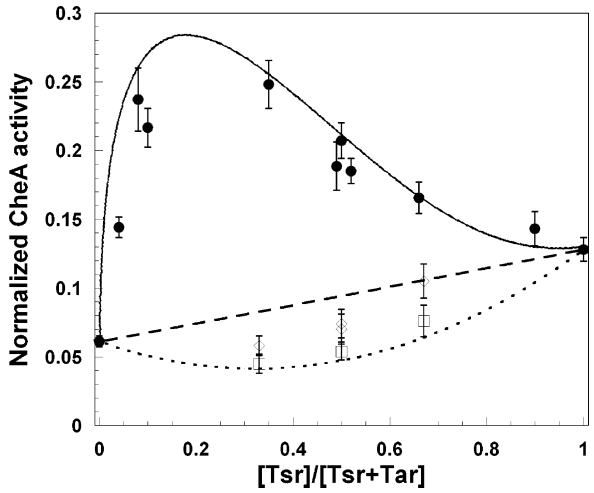

FIGURE 3: Coexpressed Tar and Tsr interact synergistically to stimulate CheA. CheA kinase activities (●) stimulated by membranes containing Tar and Tsr coexpressed at the ratios indicated were measured in the in vitro receptor-coupled CheA kinase assay. Values of ν_L (···) and ν_H (− − −) were calculated using eq 5 and eq 6 or 7, respectively. Data for mixed Tar−Tsr membranes (□) from Table 1 and data for membranes containing sequentially expressed receptors (◇) from Figure 2 are included for comparison. CheA activity was normalized to the average contribution of each receptor in the reaction.

and a binding cooperativity Hill coefficient (n_H). The IC_{50} and n_H values for both serine and aspartate were significantly different for membranes containing mixed receptor populations and for membranes containing Tar or Tsr alone (Table 2). The IC_{50} for serine increased from 2.6×10^{-4} to $\sim 3 \times 10^{-2}$ M (~10^2-fold), and the corresponding change with aspartate was from 7.6×10^{-6} to $>6 \times 10^{-2}$ M (~10^4-fold). The values of n_H decreased from ~2 to ~1. Neither of these effects was seen with mixtures of Tar-containing and Tsr-containing membranes or with membranes containing sequentially expressed Tar and Tsr (Table 2).

DISCUSSION

E. coli cells sense and adapt to concentrations of chemoeffectors that range over 5 orders of magnitude (*46*), and they can migrate purposefully even in very shallow chemical gradients (*16*). To understand this and other comparable signal transduction systems fully, the basis of their exquisite sensitivity and plasticity must be determined. One possibility is that different receptors interact synergistically. In vitro and in vivo studies have shown high (*25*, *47*) or low (*42*) receptor cooperativity in response to ligands, depending on the conditions that are used. Our results show that Tar and Tsr, at least in their QEQE configurations, cooperate to stimulate CheA kinase activity and that mixed populations of these two receptors give dramatically altered responses to attractant ligands compared to pure Tar or Tsr populations. Such synergistic interactions may increase the sensitivity of the system and expand the range of chemoeffector concentrations over which the cells respond.

The Amount of Receptor Affects CheA Kinase Activity. Our results confirm that the fraction of receptors relative to total protein in the cell membrane affects their behavior. Receptors expressed at low levels are apt to be more randomly scattered and isolated than receptors expressed at higher levels. We see a linear increase in Tar-stimulated and Tsr-stimulated

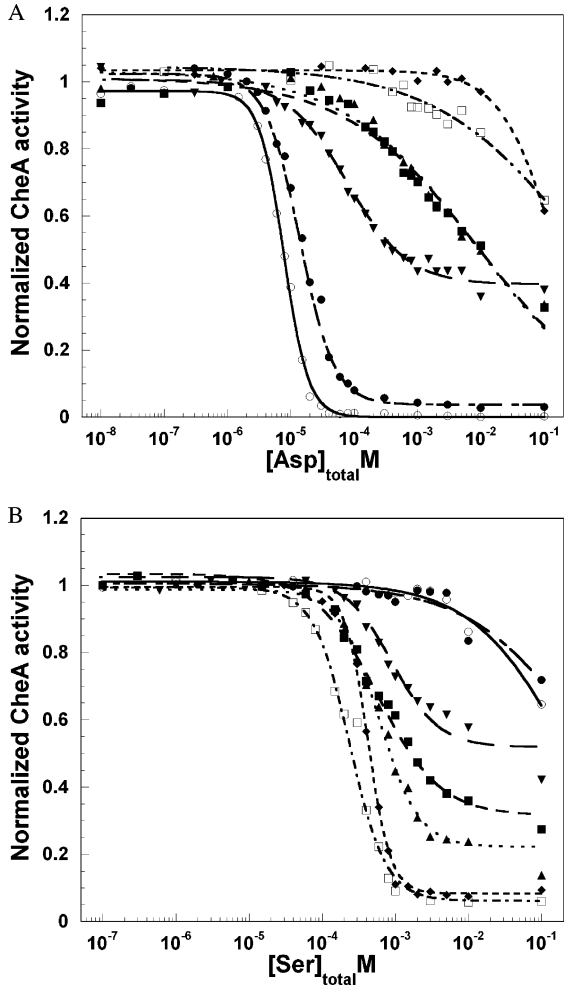

FIGURE 4: Effect of receptor stoichiometry on attractant-mediated inhibition of receptor-coupled CheA kinase. CheA activity was measured in the in vitro receptor-coupled assay. Normalized CheA activity was determined for a series of aspartate (A) and serine (B) concentrations. A best-fit curve was calculated using the Hill equation. The different mixtures are indicated as follows: Tar only (○), Tsr only (□), 10:1 Tar:Tsr (●), 2:1 Tar:Tsr (▼), 1:1 Tar:Tsr (■), 1:2 Tar:Tsr (▲), and 1:10 Tar:Tsr (◆). Note that inhibition of heterologous receptors (Tsr by aspartate and Tar by serine) may obscure the expected plateau for the inhibition of cognate receptors (Tar by aspartate and Tsr by serine) at the highest concentrations of attractants.

CheA activity up to receptor fractions of 50% of total inner-membrane protein (Figure 1), suggesting that formation of ternary complexes and/or larger patches of receptors is favored as the receptor fraction increases. However, *E. coli* chemoreceptors expressed at normal levels localize to the cell poles, although not in tight clusters (*6*). This tendency could bring receptors together even at physiological levels of synthesis to create high local population densities (*48*).

Assemblages of Overexpressed Receptors Are Relatively Stable. Receptor-stimulated CheA activities measured when membranes from cells expressing either Tar or Tsr were mixed were virtually the same as those seen when Tar and Tsr were expressed sequentially in the same cells (Table 1 and Figure 2). In contrast, when Tar and Tsr were expressed at the same time, they exhibited strong interactions that were seen both as synergistic stimulation of CheA activity (Figure 3) and as an increase in the amount of aspartate or serine required to inhibit CheA activity (Figure 4 and Table 2). In the case of sequential expression, the receptors had several hours in vivo, the entire time (24 h) of membrane preparation, and the 4 h incubation with CheA and CheW in vitro to interact and switch partners. However, no significant synergistic interactions were observed under these conditions.

Structural studies (*12*) and genetic (*13*) and chemical-cross-linking (*45*) analyses have suggested that receptors interact, through their cytoplasmic domains, to form trimers of dimers. One explanation for the lack of synergy between sequentially overexpressed Tar and Tsr is that they remain in relatively homogeneous patches so that trimers composed of the two types of receptors are unlikely to interact. Another possibility is that synergy requires association of Tar and Tsr in trimers of dimers that must be established during the initial assembly of the receptors into the membrane.

Electron micrographs of negatively stained inner membranes highly enriched for the QEQE from of Tsr (R. S. McAndrew, unpublished results) reveal that, in the absence of CheA and CheW, Tsr is detected as only trimers of dimers. In the presence of CheA and CheW, hexagonal closely packed lattices of receptors appear, and trimers can be caught in the act of joining, or leaving, the lattices. Also, in crude preparations containing both inner and outer membranes, the 35 C-terminal residues are removed from ~50% of the population of QEQE Tsr (R.-Z. Lai, unpublished results). We propose that endogenous proteases retained in these preparations remove these flexible tails, which remain unresolved in the crystal structure of the QQQQ form of Tsr (*12*). Within trimers of dimers, only half of the tails may be accessible, with the tail of one subunit of a dimer pointing outward from the trimer axis and the tail of the other subunit pointing toward the center of the trimer, where it is protected. The finding that the 50% truncation is highly reproducible and that incubation at room temperature for 24 h does not lead to a significant increase in truncation suggests that these trimers of dimers are quite stable.

We realize that this interpretation may seem to be at odds with conclusions drawn from in vivo chemical cross-linking experiments that suggested that Tar and Tsr can exchange among trimers in the absence of CheA and CheW (*45*). The explanation may lie with different levels of receptor expression, which were high in the protease protection and electron microscopy analyses and were at physiological levels in the cross-linking experiments. Clearly, a concerted and carefully controlled study will be required to resolve the issue, but the failure of sequentially expressed Tar and Tsr to exhibit functional interactions is at least consistent with the stability of trimers of receptor dimers in the absence of CheA and CheW.

Coexpressed Tar and Tsr Stimulate CheA Synergistically. Sourjik and Berg (*25*) recently reported that the increased level of expression of Tar in the presence of Tsr increases CheA activity in vivo. Our in vitro data are consistent with their results and support the idea that a mixture of receptors enhances receptor-dependent stimulation of CheA. In particular, a Tar−Tsr dimer pair may support more CheA activity than a Tar−Tar or Tsr−Tsr dimer pair.

We imagine two possible reasons for the enhanced activity of a mixed dimer pair. First, a ternary complex formed by a Tar−Tsr dimer pair may have higher specific activity than a complex formed by a Tar−Tar or Tsr−Tsr dimer pair. Second, a Tar−Tsr pair might have a higher affinity for CheW and/or CheA than a Tar−Tar or Tsr−Tsr pair. We

Table 2: Ligand Inhibition Parameters Estimated from the Hill Equation

[Tsr]/[Tar + Tsr]	% Tar[a]	% Tsr[a]	ν_{max}[b]	ν_{min}[c] for Ser	ν_{min}[c] for Asp	IC_{50} for Ser (μM)	n_H	IC_{50} for Asp (μM)	n_H
1.0	0	30	0.13 ± 0.01	0.01 ± 0.004	0.08 ± 0.01	260 ± 13	1.8 ± 0.1	≥9000	n/a[g]
0.92	2.8	32	0.14 ± 0.01	0.02 ± 0.01	0.05 ± 0.01	540 ± 28	2.4 ± 0.2	≥60000	n/a[g]
0.90	2.8	26	0.15 ± 0.02	0.01 ± 0.003	0.09 ± 0.02	420 ± 18	2.9 ± 0.3	$\geq 1.4 \times 10^5$	n/a[g]
0.89	3.2	26	0.15 ± 0.01	0.02 ± 0.003	0.06 ± 0.01	370 ± 20	2.3 ± 0.2	$\geq 7 \times 10^5$	n/a[g]
0.67	15	30	0.17 ± 0.01	0.02 ± 0.01	0.06 ± 0.02	730 ± 35	1.3 ± 0.1	1400 ± 100	0.7 ± 0.1
0.52	29	31	0.21 ± 0.01	0.04 ± 0.02	0.07 ± 0.02	800 ± 95	0.9 ± 0.1	1500 ± 330	0.9 ± 0.2
0.49	29	28	0.19 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	760 ± 49	1.0 ± 0.1	760 ± 48	1.0 ± 0.1
0.35	35	19	0.25 ± 0.02	0.1 ± 0.03	0.09 ± 0.03	1500 ± 133	0.9 ± 0.1	75 ± 6	0.8 ± 0.04
0.10	20	2.2	0.22 ± 0.02	0.12 ± 0.02	0.01 ± 0.003	≥30000	n/a[g]	17.2 ± 0.7	1.9 ± 0.1
0.082	20	1.8	0.24 ± 0.02	0.15 ± 0.01	0.01 ± 0.002	≥20000	n/a[g]	15.4 ± 0.7	1.6 ± 0.1
0.037	21	0.8	0.14 ± 0.01	0.09 ± 0.02	0.01 ± 0.01	≥20000	n/a[g]	10.8 ± 0.4	1.7 ± 0.1
0	25	0	0.06 ± 0.004	0.04 ± 0.01	0	≥14000	n/a[g]	7.6 ± 0.3	2.1 ± 0.1
0.53[d]	27	30	0.05 ± 0.01	0.01 ± 0.003	0.03 ± 0.01	270 ± 20	2.1 ± 0.3	8.0 ± 0.5	2.0 ± 0.4
0.53[e]	29	32	0.07 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	280 ± 25	1.7 ± 0.3	8.2 ± 0.4	2.3 ± 0.3
0.47[f]	32	28	0.07 ± 0.01	0.01 ± 0.002	0.04 ± 0.01	310 ± 22	1.8 ± 0.4	8.1 ± 0.5	2.1 ± 0.3

[a] Amounts of Tsr and Tar are expressed as fractions of the total protein content of the membrane preparations. [b] ν_{max} is the maximum rate of production of picomoles of CheY-P per second, normalized to 1% receptor. [c] ν_{min} is the rate of production of picomoles of CheY-P per second, normalized to 1% receptor, in the presence of a saturating attractant concentration (100 mM). [d] Membranes containing Tar or Tsr were mixed at an ~1:1 ratio before use in the in vitro receptor-coupled kinase assay. [e] Sequentially coexpressed Tar//Tsr at an ~1:1 ratio (see Figure 3) were used in the assay. Tar was expressed first. [f] Sequentially coexpressed Tsr//Tar at an ~1:1 ratio (see Figure 3) were used in the assay. Tsr was expressed first. [g] This parameter could not be determined for the sample.

favor the latter interpretation because the change in CheA-stimulating activity of Tsr in different states of covalent modification is due to the altered affinity of the different forms for CheA and CheW rather than to the specific activity of the ternary complexes once made (*49*).

To model synergy between Tar and Tsr, we developed a computer program that generated an approximation of the CheA activity curve shown in Figure 3. The simulation is based on the existence of trimers that contain both Tar and Tsr dimers. We note that the electron micrographs described above suggest that the integrity of trimers of dimers can be lost in the presence of CheA and CheW and that the actual signaling unit may be an extended hexagonal lattice of receptor dimers. Nonetheless, since the receptors appear to be delivered to these lattices as trimers of dimers (R. S. McAndrew, unpublished results), the distribution of receptors within such arrays will be strongly influenced by the composition of the trimers that serve as building blocks.

The simulation calculates the expected activity of mixed receptor populations based on two parameters. The first parameter is the relative probability of assembling ternary complexes at Tar−Tar, Tsr−Tsr, and Tar−Tsr dimer interfaces. (The difference, if any, in the activity of a Tar−Tsr or Tsr−Tar interface should be irrelevant, since any mixed trimer contains one interface of each type. Thus, only the average activity of the two interfaces need be considered.) The second parameter is the probability of forming the four different trimers of dimers (Tar−Tar−Tar, Tar−Tar−Tsr, Tar−Tsr−Tsr, and Tsr−Tsr−Tsr). The program used to make these calculations is described in Materials and Methods and, in more detail, in the Supporting Information.

Figure 5 shows the best fit to our data generated by the program and also depicts the relative fraction of each of the four possible trimers of receptor dimers at all ratios of Tar to Tsr. The parameters that provided the best fit were as follows: relative CheA activity (equivalent to CheA/CheW affinity) for Tar−Tar, Tsr−Tsr, and Tar−Tsr mixed interfaces = 1:2:21 [we note that this is well within the range of values observed by Shrout and Weis (*49*) using receptors with different states of covalent modification] and relative affinity for trimer formation = 1:100 Tar:Tsr.

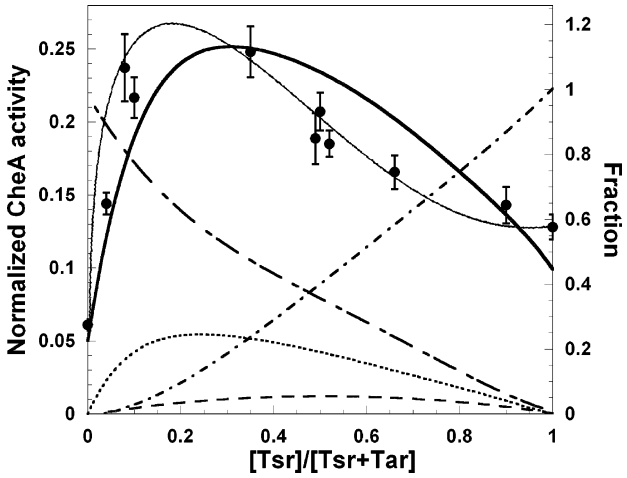

FIGURE 5: Computer-based simulation of CheA activity and receptor trimer formation. The assumptions used in the simulation are described in the text and in the Supporting Information. The thick solid line shows the CheA activity calculated from the simulation program. The thin solid line is the Beta distribution fit for the experimental data from Figure 3. The fractions of trimer types at each ratio of Tar to Tsr are shown as follows: Tar−Tar−Tar (− - −), Tar−Tar−Tsr (•••), Tar−Tsr−Tsr (− − −), and Tsr−Tsr−Tsr (-•-).

Using these parameters, the simulation yielded a peak activity when Tsr comprised 20% of the total receptor population. At this point, ~60% of the trimers are Tar−Tar−Tar, 25% are Tar−Tar−Tsr, 5% are Tar−Tsr−Tsr, and 10% are Tsr−Tsr−Tsr. Although dimers of dimers are presumably an intermediate in the formation of trimers of dimers, a simulation that took this step into account did not yield a curve significantly different from the one generated by the simpler program (J. M. B. Manson, data not shown).

A critical reader can protest that the simulation provides only a rough approximation of the experimental data. We suspect that the biggest factor is the oversimplification of considering only trimers of dimers. Within the larger receptor−CheA−CheW lattices found in our unpublished

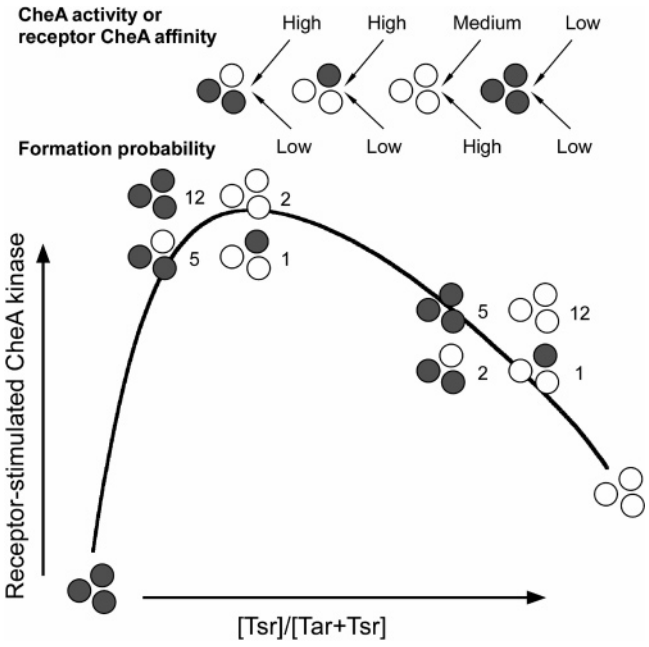

FIGURE 6: Model that explains receptor synergy. Tar and Tsr dimers are represented as filled and empty circles, respectively. Their relative distributions are shown at the ratio that yields peak synergy (1:4 Tsr:Tar) and at the ratio found in wild-type cells in vivo (2:1 Tsr:Tar). The computer-generated curve of predicted CheA-stimulating activities at different Tar:Tsr ratios is the same as in Figure 5. We propose that the composition of a dimer interface determines the CheA activity associated with it, either because of differences in the specific activity of the receptor−CheA−CheW ternary complex or because of differences in the affinity for CheA and CheW. The ratio of 1:2:21 for the activities at Tar−Tar, Tsr−Tsr, and Tar−Tsr (Tsr−Tar) interfaces gave the best fit to the data. The skew of the synergistic peak toward a Tar:Tsr ratio of >1:1 required the assumption that different receptor dimers incorporate into trimers with different affinities. The best fit was achieved with a Tar−Tar−Tar:Tar−Tar−Tsr:Tar−Tsr−Tsr:Tsr−Tsr−Tsr relative probability of formation ratio of 1:1:1:100. However, the curve changed little when the relative probability of formation of Tsr−Tsr−Tsr trimers was varied over a range from 20 to 1000 times the probabilities of formation of the other three trimers of dimers.

electron micrographs and predicted by the polar clustering of receptors observed in vivo (*6*−*8*) and theoretical considerations (*14*, *20*−*23*), more complicated interactions of receptors can occur.

Distribution of Trimers of Dimers at Different Ratios of Tar to Tsr. On the basis of our experimental results and the simulation, we propose that the relationship of global CheA-stimulating activity, as calculated by the computer simulation, and the composition of the trimers of dimers at different ratios of Tar and Tsr will be approximately as shown in Figure 6. Tar−Tsr dimer interfaces support the highest activity because they have the highest affinity for CheA and CheW. Tar−Tar interfaces are the least active, and Tsr−Tsr interfaces support twice the activity of Tar−Tar interfaces. We suggest that Tsr forms trimers of dimers 100-fold more readily than does Tar. This model is presented as a guide to further experiments, not as a definitive explanation, since it does not account quantitatively even for the data presented here.

Biological Significance of Chemoreceptor Crosstalk. The ability of QEQE Tar and Tsr to interact synergistically to increase the overall CheA activity confers no obvious advantage to *E. coli*, since each of these receptors, and presumably their $E^{m}EE^{m}E$ correlates, is capable of stimulating CheA on its own. However, Tar(EEEE) and Tsr(EEEE) have much lower intrinsic CheA-stimulating ability than their QEQE or QQQQ derivatives (*25*). Tar(EEEE) and Tsr(EEEE) are each capable of synergistic enhancement of the in vitro CheA-stimulating activity of the QEQE form of their opposite number (R.-Z. Lai, unpublished results), and presumably also of the $E^{m}EE^{m}E$ forms in vivo. Moreover, the low-abundance receptors Tap and Trg cannot mediate chemotaxis as sole transducers in the cell (*50*, *51*). We intend to test whether they also are capable of synergistic stimulation of CheA activity by Tar(QEQE) and Tsr(QEQE). If so, this ability would permit them to function as actively contributing members of the receptor patch.

The fact that the greatest amount of synergy is seen at a Tsr:Tar ratio of 1:4 rather than at the normal in vivo Tsr:Tar ratio of 2:1 is not particularly troubling. Within an extended lattice, different neighborhoods will presumably show considerable variation in receptor composition. The mechanism of protein synthesis, in which multiple polypeptides are translated from a single mRNA, will contribute to a lack of uniformity, provided that dimers do not readily exchange among trimers. Even if dimers do exchange among trimers in a stochastic fashion, local variations will arise. Thus, within a signaling lattice or a collection of signaling lattices, there will likely be patches enriched for Tar, for Tsr, and possibly for Trg and Tap.

Through interactions among receptor types, attractant ligands sensed by lightly methylated or low-abundance receptors may be able to shut off a significant amount of CheA activity. When added to membranes containing mixtures of Tar(EEEE) and Tsr(QEQE) in ternary complexes with CheA and CheW, saturating concentrations of aspartate inhibit a large fraction of receptor-coupled CheA activity (R.-Z. Lai, manuscript in preparation). Similarly, addition of saturating concentrations of serine to membranes containing Tsr(EEEE) and Tar(QEQE) inhibits a large fraction of receptor-coupled CheA activity. We intend to test whether the same behavior is seen when low-abundance receptors, engineered to bind small-molecule ligands directly (*50*, *51*), are mixed with the QEQE forms of Tar or Tsr and then exposed to attractants sensed by the low-abundance receptors.

A final, and perhaps most important, advantage of synergy is suggested by the data presented in Figure 4. The IC_{50} for aspartate in mixed Tar−Tsr membranes can be up to 10^4-fold higher than it is with a pure population of Tar, and the IC_{50} for serine can be up to 10^2-fold higher than it is with a pure population of Tsr. We speculate that this lower sensitivity, and the decrease in the apparent Hill coefficient from 2 to 1 (Table 2), reflects the different responses of receptors to their ligands depending on their local environment. At the 2:1 ratio of Tsr to Tar found in wild-type cells (*52*), our simulation predicts that ∼25% of the trimers should be Tar−Tar−Tar, ∼10% Tar−Tar−Tsr, ∼5% Tar−Tsr−Tsr, and ∼60% Tsr−Tsr−Tsr (Figure 6). The broad range of attractant concentrations sensed by these different combinations of receptors may augment adaptive methylation to achieve optimal monitoring of the chemical gradients *E. coli* encounters during its normal enteric existence.

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SUPPORTING INFORMATION AVAILABLE

A complete strain and plasmid list (Table 1S), documentation of the linearity of CheA stimulation with receptor concentration at different fractional percents of receptor relative to total membrane protein (Figure 1S), and a detailed description and the simulation program (Program 1S) that we used in Figures 5 and 6. This material is available free of charge via the Internet at http://pubs.acs.org.

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