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ACS chemical biology

Chemical Probes of Bacterial Signal Transduction Reveal That Repellents Stabilize and Attractants Destabilize the Chemoreceptor Array

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ABSTRACT The signal transduction cascade responsible for bacterial chemotaxis serves as a model for understanding how cells perceive and respond to their environments. Bacteria react to chemotactic signals by migrating toward attractants and away from repellents. Recent data suggest that the amplification of attractant stimuli depends on receptor collaboration: occupied and unoccupied chemoreceptors act together to relay attractant signals. Attractant signal transmission, therefore, depends on the organization of the chemoreceptors into a lattice of signaling proteins. The importance of this lattice for transducing repellent signals was unexplored. Here, we investigate the role of inter-receptor communication on repellent responses in *Escherichia coli*. Previously, we found that multivalent displays of attractants are more potent than their monovalent counterparts. To examine the importance of the chemoreceptor lattice in repellent signaling, we synthesized ligands displaying multiple copies of the repellent leucine. Monomeric leucine and low-valency leucine-displaying polymers were sensed as repellents. In contrast, multivalent displays of leucine capable of binding multiple chemoreceptors function not as potent repellents but as attractants. Intriguingly, chemical cross-linking studies indicate that these multivalent ligands, like monovalent attractants, disrupt the cellular chemoreceptor lattice. Thus, repellents stabilize the intrinsic chemoreceptor lattice, and attractants destabilize it. These results indicate that signals can be transmitted with high sensitivity via the disruption of protein–protein interactions. Moreover, our data demonstrate that repellents can be transformed into attractants merely by their multivalent display. These results have implications for designing agonists and antagonists for other signaling systems.

Motile bacteria possess a sensing mechanism that allows them to integrate various stimuli to seek out attractants and avoid repellents. This robust and highly sensitive signal transduction system has long served as a model for receptor-mediated signaling (*1, 2*). To transmit signals from the periplasm to the cytoplasm, a two-component phosphorelay system is used that depends upon methyl-accepting chemotaxis proteins (MCPs or chemoreceptors). MCPs exist as homodimers, and *Escherichia coli* harbor five types. Tar and Tsr are high-abundance MCPs that comprise ~90% of the chemoreceptors (*3, 4*). Tar senses the attractants maltose and aspartate and the repellent Ni^{2+}, whereas Tsr transduces an attractant response to serine and a repellent response to either pH changes or leucine (*5*). The low-abundance MCPs Aer, Tap, and Trg relay attractant responses to O_2 levels, dipeptides, and sugars (glucose, galactose, and ribose), respectively (*6*). Together, the chemoreceptors can mediate responses to dozens of disparate stimuli (*7–9*).

Structural and biochemical studies suggest that the MCP homodimers are organized into higher-order complexes that include the scaffold protein CheW and the histidine kinase CheA (*10–13*). Upon chemoeffector binding, MCP homodimers transmit a signal to CheA. CheA in turn modulates phosphorylation of the response regulator CheY; the relative levels of CheY and phospho-CheY influence the direction of flagellar rotation (*3*). Attractant binding leads to decreased phospho-CheY levels that bias the organism toward counterclockwise flagellar rotation, ultimately leading to an increase in "running" or straight, coordinated swimming. Repellents promote clockwise flagellar rotation, which increases a cell's propensity to "tumble". Tumbling allows a bacterium to randomly reorient its direction of

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Received for review October 7, 2007 and accepted January 2, 2008.

Published online February 15, 2008

10.1021/cb700211s CCC: $40.75

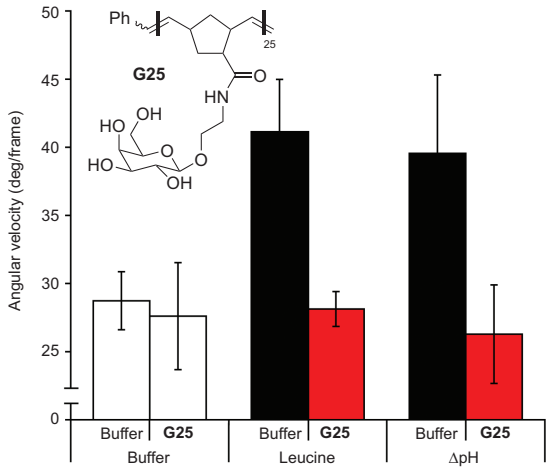


Figure 1. Motile wild-type *E. coli* cells (AW607) were treated either with buffer or with a solution of galactose-bearing polymer with a degree of polymerization of ~25 (G25) at 50 μM. After 5 min (a period sufficient to allow for adaptation and therefore chemoreceptor methylation), buffer, leucine (10 mM final concentration), or buffer with NaOH (final pH of 8.6) was added. Data points are the average angular velocity of 8 s intervals. Angular velocities were taken within 45 s of ligand addition. Error bars are 2σ uncertainties based on averages of four or more ~8 s videos.

swimming and therefore move away from the repellent source. In addition to controlling levels of phospho-CheY, the kinase CheA also regulates the amount of cellular phospho-CheB, an enzyme that adjusts chemoreceptor methylation levels (*14*). Receptor methylation completes an adaptation feedback loop that returns receptor output to prestimulus levels shortly after attractants or repellents are detected (*15*). Together, these processes underlie temporal sensing.

The integration and amplification of responses to chemostimulants are facilitated not by individual receptor−kinase complexes but rather by an assembly or lattice of chemoreceptors. In both bacteria and archaea, chemoreceptors localize at the cell poles (*16, 17*), as do cytoplasmic chemotaxis signaling components such as CheA (*18*). Higher-order MCP arrangement within these polar patches appears to be necessary for proper signal transduction. The close proximity of MCPs within the polar array is also important for adaptation, because low-abundance MCPs require the presence of nearby high-abundance MCPs for controlled methylation or demethylation (*19–21*). Moreover, models (*22–24*) and experimental studies (*25–32*) suggest that chemoreceptors can communicate ligand occupancy states within the lattice to amplify attractant signals.

Multivalent chemoattractants have been used as tools to investigate the role of inter-receptor communication in chemotactic signaling in *E. coli* and other bacteria (*26, 29, 33*). High-valency attractant-bearing polymers can bind multiple MCPs and elicit an enhanced attractant response (*33*). To investigate inter-receptor communication in repellent signaling, we examined chemotactic responses elicited by ligands displaying multiple copies of a repellent.

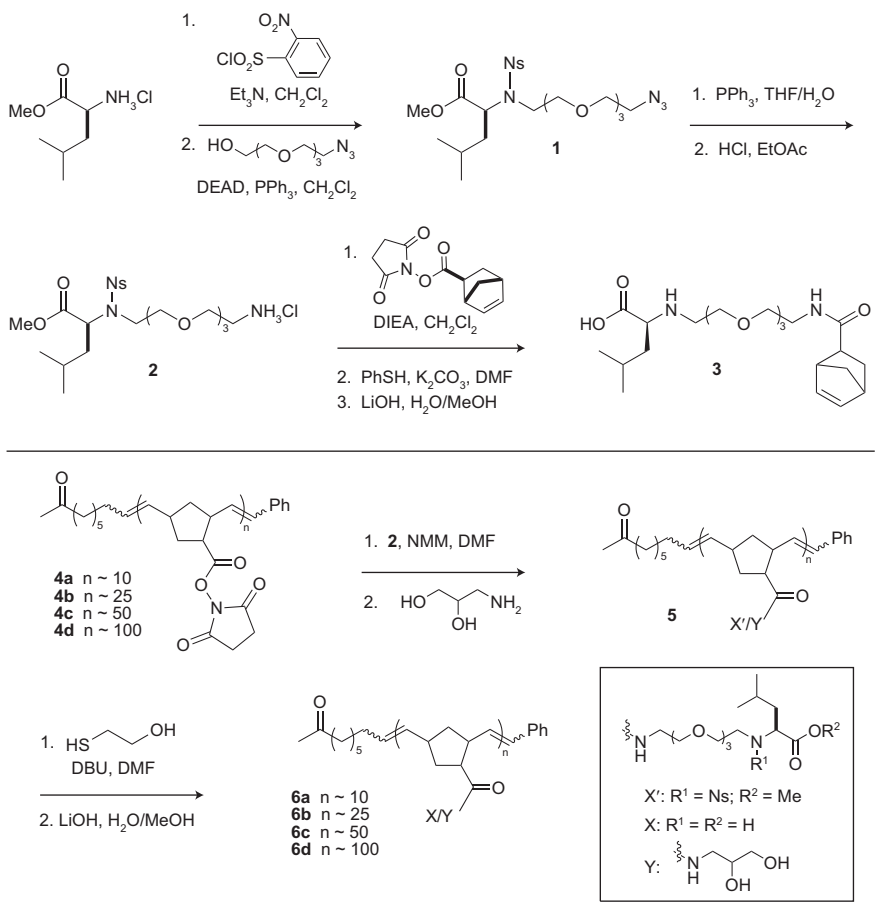
RESULTS AND DISCUSSION

Treatment with Multivalent Attractants That Signal through the Chemoreceptor Trg Abrogates Repellent Responses through Tsr. We found previously that high-valency galactose-bearing polymers targeted at the low-abundance MCP Trg can alter chemoreceptor proximity and potentiate attractant responses (*33*). When *E. coli* cells are exposed to these attractant-bearing polymers and allowed to adapt, responses to other attractants are potentiated (*26*). Similar results were obtained using *Bacillus subtilis* (*29*). These and other data support the involvement of inter-receptor communication in attractant signaling (*27, 31*). To determine whether polymer-induced MCP clustering has a similar effect on repellent responses, we used motion analysis. Running and tumbling behavior can be quantified by tracking the motion of a population of bacteria and measuring their mean angular velocity (*34, 35*). After the addition of an attractant, cells exhibit a decrease in angular velocity that corresponds to an increased propensity to run; repellents cause an increase in the average angular velocity indicative of a bias toward tumbling. Angular velocity measurements are taken immediately after stimulation because the chemotaxis adaptation enzymes soon cause CheA kinase activity (and therefore angular velocity) to return to basal levels. With this caveat, motion analysis can be used to measure either an attractant or a repellent response.

To test whether repellent signals are amplified, we treated *E. coli* with a galactose-bearing polymer (**G25**) that signals via the chemoreceptor Trg (Figure 1). After cells were allowed to adapt fully (5 min), various repellents were added, and the mean angular velocity of individual cells was assessed. For cells pretreated with

buffer alone, the addition of a repellent (either 10 mM leucine or an increase in pH, both of which are sensed by Tsr) prompted an increase in average angular velocity. In contrast, *E. coli* pretreated with the galactose-bearing polymer and allowed to adapt and then stimulated with repellent (high pH or leucine) did not exhibit repellent responses (Figure 1). Thus, pretreatment of cells with a multivalent ligand targeting the chemoreceptor Trg impaired repellent signaling through the receptor Tsr.

Design and Synthesis of a Monovalent Leucine-Derived Repellent. Our initial results suggest that polymer-induced perturbation of the MCP lattice is deleterious to repellent signaling. To investigate further the role of the chemoreceptor lattice in repellent signaling, we sought to generate multivalent ligands that display an *E. coli* repellent. We had several design criteria: first, the repellent derivative must maintain its ability to bind to the target chemoreceptor; second, the multivalent ligands must be accessible via chemical synthesis; and third, the linker that tethers the repellent moiety to the polymer backbone must not abrogate biological activity. Of known repellents, L-leucine seemed most amenable to derivatization. Tso and Adler (*9*) had reported previously that *N*-acetyl leucine and leucine ethyl ester are inactive, but the *N*-methyl derivative of leucine is a repellent. Their results suggest that a protonated amine group is critical for the binding of leucine to Tsr and that secondary amines are tolerated. We therefore designed our leucine moiety **3** (Scheme 1, top) with an *N*-alkyl linker.



Scheme 1. Synthetic route to repellent-bearing compounds

To determine whether relevant *N*-alkyl leucine derivatives function as repellents, we synthesized norbornene derivative **3** (Scheme 1, top). We converted leucine methyl ester into an *o*-nitrobenzenesulfonamide (Ns) group to facilitate secondary amide formation. A Mitsonobu reaction afforded the protected amine **1**. The terminal azide was converted to the amine via the Staudinger reduction (*36*), a chemoselective reaction that resulted in azide reduction while leaving the nosyl nitro group intact. The product, amine (**2**), was used in a coupling reaction with norbornene succinimidyl ester to afford the expected amide. The nosyl group was removed with thiophenol and potassium carbonate, and the methyl ester was hydrolyzed by saponification with lithium hydroxide. This sequence afforded leucine derivative **3**.

To test whether leucine derivative **3** functions as a repellent, we performed motion analysis experiments (Figure 2). Compound **3** and leucine elicited similar concentration-dependent increases in mean angular velocity, indicating that compound **3** is an effective repellent. The augmentation of mean angular velocity exhibited by leucine derivative **3** is within standard error of that observed for leucine. Thus, the *N*-alkyl substituent in **3** has little influence on repellent activity.

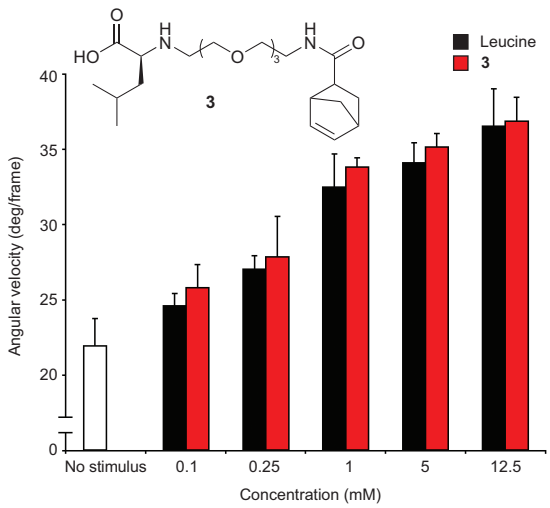


Figure 2. Response of wild-type *E. coli* (AW607) to leucine (black) and compound 3 (red). Error bars are 2σ uncertainties based on averages of four or more 6–8 s videos.

Synthesis of Ligands That Present Multiple Copies of Leucine. Given that monovalent ligand **3** elicits a repellent response, we generated a leucine-based multivalent ligand (Scheme 1, bottom). We employed a general postpolymerization modification strategy for the synthesis of defined multivalent arrays via the ring-opening metathesis polymerization (ROMP) (*37*). The polymers presenting succinimidyl ester groups can undergo reaction with amine-bearing epitopes to form biologically active multivalent ligands. Polymer backbones (**4a–d**) were synthesized as previously reported using a ruthenium carbene catalyst known to afford well-defined polymers $[(H_2IMes)(3\text{-Br-py})_2(Cl)_2Ru{=}CHPh]$ (*38, 39*). In this way, diverse ligands can be assembled rapidly. To modify the polymer, we took advantage of our previous results demonstrating that different moieties can be conjugated readily to the backbone in controlled ratios; biologically active ligands as well as groups that act as spacers or solubilizing agents can be appended (*40, 41*). Specifically, the leucine-derived ligand **2** was conjugated to the backbone in the appropriate stoichiometry to provide a mole fraction (χ) of ligand within 0.68–0.71, as ascertained by integration of the characteristic aromatic peaks of the nosyl groups in the ^{1}H NMR spectrum. The remaining electrophilic sites were blocked by the addition of a 5-fold excess of 3-amino-1,2-propanediol, which afford the protected multivalent displays (**5a–d**). An added benefit of using the amine-containing diol to cap any remaining succinimidyl ester groups is that the final polymeric products (**6a–d**) have excellent water solubility. The target ligands were generated by nosyl protecting group removal (*42, 43*) and methyl ester saponification to yield the desired multivalent leucine derivatives (**6a–d**).

High-Valency Leucine-Bearing Polymers Are Sensed as Attractants. We tested the effects of the multivalent leucine derivatives on *E. coli* chemotaxis. In studies with multivalent attractants (*33*), we found that short oligomers incapable of clustering the chemoreceptors (e.g., a 10-mer) could function as attractants. They were, however, no more potent than the monovalent attractants. In contrast, higher valency attractants that can cluster the chemoreceptors were extremely potent attractants. Therefore, we compared a lower valency ligand displaying repellent groups, **6a**, to higher valency compounds (**6b–d**). The low-valency leucine ligand **6a** elicited a repellent response as predicted from previous observations. Threshold levels and response curves for oligomer **6a** were similar to those of leucine and monovalent repellent **3** (Figure 3). Because our initial results indicated that preclustering the Trg receptor with the **G25** ligand impaired subsequent repellent signaling, we hypothesized that high-valency repellent-bearing polymers added to cells might also impair repellent signaling. Intriguingly, higher valency leucine-bearing compounds (**6b–d**) not only impaired repellent signaling but were sensed as attractants. Indeed, treatment with multivalent ligands **6b–d** elicited significant decreases in mean angular velocity (Figure 3). The compounds that afforded an attractant response all have a degree of polymerization ≥25 and are capable of binding multiple chemoreceptors (*26, 33*). Thus, increasing the valency of the leucine-bearing polymers changes them from a repellent into an attractant.

To test whether the activity of the synthesized leucine-bearing compounds is mediated via Tsr, motion analysis studies were conducted with compounds **3** and **6d** in a Δ*tsr* strain (RP5700). Improvements in the sensitivity of chemotaxis assays have shown that leucine, once thought to be sensed solely as a repellent by Tsr, can give rise to a weak attractant response at low concentrations mediated through Tar (*44, 45*). It was therefore important to ascertain which MCP mediates the response to leucine-substituted polymers. In the Δ*tsr* strain, the addition of leucine (10 mM) appears

to cause a subtle decrease in angular velocity, which is consistent with a Tar-mediated attractant response. With the Tsr deletion strain, the addition of compound **3** (10 mM) or **6d** (2.5 mM) resulted in angular velocity levels that were within error of those of nonstimulated cells (Supplementary Figure S1). These observations indicate that the monovalent *N*-alkyl leucine derivative does not promote any chemotactic response in the Δ*tsr* strain. Similarly, treatment of the deletion strain with multivalent leucine derivative **6d** fails to elicit a chemotactic response. These data reveal that the both repellent (monovalent **3**) and attractant (multivalent **6d**) responses are mediated by Tsr. Thus, the observed attractant response in wild-type cells arises through the receptor that senses leucine as a repellent.

Multivalent Leucine Derivatives Disrupt the Chemoreceptor Lattice. A recent study indicates that attractant-induced changes in MCP interactions can be visualized by chemical cross-linking (*32*). In the majority of untreated cells, the MCPs localize to the poles. When cells are stimulated with high concentrations of attractant (95% receptor occupancy), subsequent cross-linking with paraformaldehyde results in a greater proportion of both *E. coli* and *B. subtilis* cells displaying diffuse MCP staining. One explanation for these differences is that the ability of cross-linking agents to bridge the MCPs is diminished by attractant binding. The decrease in the efficiency of MCP cross-linking within the lattice is manifested as an increase in the number of cells exhibiting diffuse staining, and this change can be quantified. In contrast to what is observed upon attractant treatment, the addition of the repellent leucine (1 mM) results in an increase in the number of cells exhibiting localized MCP staining. Given the differences in repellent and attractant treatments we sought to determine whether our high valency leucine-bearing polymers would promote the type of diffuse MCP staining seen with monovalent attractants.

To probe for changes in chemoreceptor organization, *E. coli* were treated with compound **6c** (1 mM per leucine epitope) and then exposed immediately to paraformaldehyde. This procedure yielded an increase in the number of cells exhibiting diffuse MCP staining (Figure 4). This increase was similar to that resulting from treatment with the attractant serine (1 mM). In all cases, the proportion of cells exhibiting diffuse staining returns nearly to unstimulated levels after adaptation. This observation suggests that the initial effects of attractants

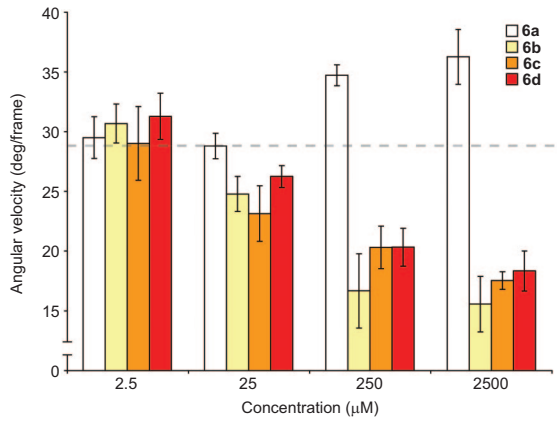


Figure 3. Response of wild-type *E. coli* (AW607) to leucine-substituted polymers of different valencies. Bacteria were treated with various concentrations of ligand 6a **(white),** 6b **(yellow),** 6c **(orange), or** 6d **(red). The angular velocity of unstimulated cells is depicted by the dashed line. Motion analysis was performed on at least four videos of 6–8 s duration. Videos were recorded within 45 s of stimulant addition. Error bars are given in 2σ uncertainties.**

on the chemoreceptor lattice are not due to changes in receptor methylation that accompany adaptation. Because perturbations of the chemoreceptor lattice are detected only when the cross-linking agent is added immediately after stimulation, we conclude that they are reporting on the features of the lattice present during signal transmission. These data indicate that repellents stabilize the signaling array and that attractants (including multivalent leucine derivatives) destabilize it. Thus, like monovalent attractants, high-valency leucine-substituted polymers that elicit attractant chemotactic responses disrupt the signaling lattice.

Chemotactic Stimuli Alter the Cellular Lattice of Signaling Proteins. Our findings indicate that both attractants and repellents transduce signals by influencing the lattice of signaling proteins. Evidence is mounting that attractant binding perturbs protein–protein interactions within the signaling lattice. For example, in cells producing chemoreceptor variants with cysteine residues at select positions, disulfide formation between receptors is altered in the presence of attractant (*30, 46*). Fluorescence anisotropy studies (*47, 48*) of chemoreceptor dimers in live cells indicate that attractant binding causes them to move apart whereas repellents promote receptor clustering. We have focused on the influence of attractant and repellent binding on the

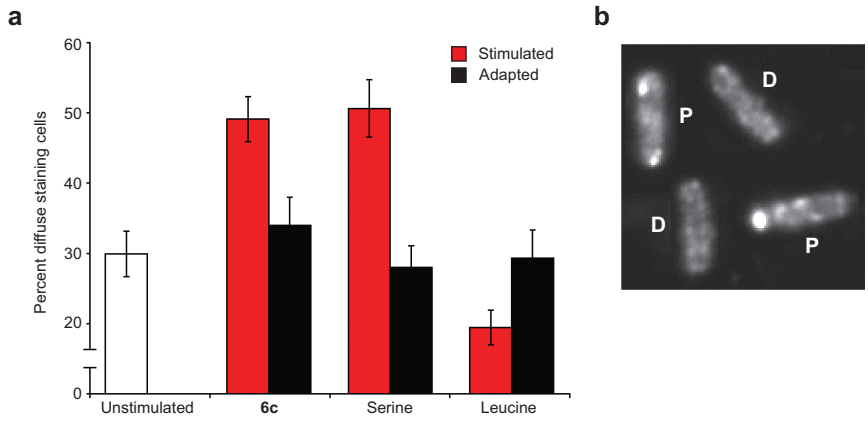


Figure 4. Percentages of *E. coli* cells exhibiting diffuse staining when treated with chemostimulants at 1 mM. a) Cells labeled "stimulated" (red) were fixed immediately after chemostimulant addition. Adapted cells (black) were treated with 1 mM stimuli 5 min prior to fixing. b) Example of cells displaying polar (P) or diffuse MCP staining (D).

entire signaling assembly, the chemoreceptor array. Our previous cross-linking data indicated that when either *E. coli* or *B. subtilis* cells are stimulated with high concentrations of attractants, the number of cells exhibiting diffuse versus polar MCP staining increases (*32*). In this study, we found that, unlike attractants, repellents stabilize the larger array.

Our studies showed previously that oligomeric attractants are more potent than the corresponding monomeric attractants. Indeed, there are many experiments in which the addition of antibodies or a multivalent ligand to a receptor initiates signal transduction (*49, 50*). The common interpretation of such data is that receptor clustering elicits signal transmission. In contrast, we found that the addition of multivalent ligands can decrease the localization of signaling receptors. Although the binding of multivalent ligands can promote the clustering of proximal receptors, it can disrupt the preexisting chemoreceptor array. Thus, multivalent ligand binding can disrupt endogenous protein interactions. With regard to bacterial chemotaxis, our findings suggest that the most potent attractants will be those that exert the greatest perturbation upon the resting state of the chemoreceptor lattice. In the context of a receptor array, multivalent ligands should be more disruptive, because to achieve multivalent binding, multiple transmembrane receptors must reorganize. If multivalent binding disrupts the chemoreceptor lattice and if this perturbation results in an attractant response, any multivalent ligand, whether it displays attractant or repellent groups, should function as an attractant. Our results support this model. These results underscore the importance of considering not only the interactions promoted by ligand binding but also pre-existing protein–protein interactions.

In light of their stabilizing influence on the chemoreceptor array, it is interesting to note that the repellent sensing occurs at much higher threshold levels than attractant sensing ($<$10 nM for the attractant L-aspartate and $>$10 μM for the repellent Ni^{2+}) (*9, 44*). These data suggest that the chemoreceptor array is tuned to respond sensitively to attractants but not to repellents. The ability of the system to detect small changes in attractant concentration may stem from the ability of a single molecule of attractant to cause the disruption of multiple local protein–protein interactions within the assembly. It is notable that *E. coli*, which is relatively fast-growing, responds sensitively to attractants. This attractant sensitivity is consistent with the physiological need to identify nutrient sources; avoiding repellents should be less crucial. It would be interesting to determine whether slow-growing bacterial species will be more attuned to repellents.

The *E. coli* chemotaxis system, like many signal transduction systems initiated by extracellular signals, consists of a network of interacting proteins. Disrupting resting-state interactions present in unstimulated signaling complexes could be a general mechanism for activating cellular responses. Indeed, our results reveal striking and unexpected similarities in the responses of the chemotaxis system in *E. coli* and some mediated through G-protein coupled receptor (GPCR) signal transduction systems in eukaryotes. In this study, we show that chemotactic repellents can be made into attractants when multivalent repellent ligands cluster their target receptors. For some GPCRs, treatment with antibodies (*51*) or multivalent ligands (*52–54*) can elicit an agonist response. In addition, disparate signaling outcomes with monovalent and multivalent ligands have been observed in epidermal growth factor signaling (*55*). The parallels between the chemotactic signaling in *E. coli* and eukaryotic signaling are surprising considering how

much they deviate in terms of the signaling components and overall functions. Thus, multivalent display of antagonists may prove to be a general method of creating potent agonists.

METHODS

General Synthetic Procedures and Materials. All moisture- and oxygen-sensitive reactions were carried out in flame-dried glassware under a nitrogen atmosphere. Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification. Methylene chloride (CH_2Cl_2) and *N*,*N*-diisopropylethylamine (DIEA) were distilled from calcium hydride, tetrahydrofuran (THF) was distilled from sodium/benzophenone, methanol (MeOH) was distilled from magnesium, and water (H_2O) was purified with a MilliQ filtration system (Millipore). Dimethylformamide (DMF) was rendered amine-free with Dowex 50WX8-200 cation exchange resin, H^+ form, 1 g L^{-1}. Analytical thin layer chromatography (TLC) was used to monitor reactions and was performed on 0.25 mm precoated Merck silica gel 60 F_{254}. Compounds were visualized with ultraviolet light (254 nm) or charring with ninhydrin stain (0.3 g of ninhydrin, 100 mL of *n*-butanol, 2 mL of AcOH) or potassium permanganate stain (1.5 g of $KMnO_4$, 10 g of K_2CO_3, 2.5 mL of 5% aqueous NaOH, 150 mL of H_2O). Flash chromatography was performed using silica gel 60, 230−450 mesh (Sorbent Technologies). PD-10 desalting columns (Sephadex G-25 Medium) were purchased from Amersham Biosciences. Microcon centrifugal filter devices (YM-3, 3000 MWCO) were purchased from Millipore Corporation. ^1H and ^{13}C NMR spectra were recorded on Bruker AC-300 or Varian Inova-500 spectrometers. Chemical shifts (δ) are reported in ppm relative to TMS or residual solvent peaks in the Supporting Information.

Leucine Monomer Synthesis: *N*-[2-(2-[2-(2-Azidoethoxy)-ethoxy]-ethoxy)-ethyl]-*N*-(2-nitrobenzenesulfonyl)-leucine Methyl Ester (1). L-Leucine methyl ester hydrochloride (1.00 g, 5.50 mmol) was dissolved in CH_2Cl_2 (500 mL). To the solution was added triethylamine (1.15 mL, 8.26 mmol) and 2-nitrobenzenesulfonyl chloride (1.83 g, 8.26 mmol), and the reaction was stirred at ambient temperature for 2 h. The reaction mixture was washed with 5% citric acid (2 × 50 mL). The organic layer was dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (CH_2Cl_2) to yield *N*-(2-nitrobenzenesulfonyl)-leucine methyl ester as a yellow crystalline solid (1.730 g, 95%).

Triphenylphosphine (467 mg, 1.78 mmol) was dissolved in CH_2Cl_2 (5 mL). The solution was cooled to 0 °C and diethyl azodicarboxylate (280 μL, 1.78 mmol) was added dropwise over 30 min. Triethyleneglycol azidoalcohol (*56*) (325 mg, 1.48 mmol) and *N*-(2-nitrobenzenesulfonyl)-leucine methyl ester (490 mg, 1.48 mmol) were each dissolved in CH_2Cl_2 (5 mL) and added dropwise to the solution. The reaction mixture was stirred at 0 °C for 10 min then warmed to RT and stirred for 12 h. The reaction mixture was washed with 5% sodium hydroxide (2 × 15 mL) and brine (15 mL). The organic layer was dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (1:1 → 2:3 hexane/ethyl acetate) to afford azide **1** as a viscous yellow oil (602 mg, 76%).

***N*-[2-(2-[2-(2-Aminoethoxy)-ethoxy]-ethoxy)-ethyl]-*N*-(2-nitrobenzenesulfonyl)-leucine methyl ester hydrochloride (2).** Triphenylphosphine (1356 mg, 0.517 mmol) was combined with azide **1** (250 mg, 0.470 mmol) in wet THF (4.5 mL). The reaction mixture was stirred for 36 h. The solution was concentrated under reduced pressure, and the residue was dissolved in EtOAc. Concentrated HCl (50 μL) was added to form the HCl salt, and the mixture was extracted with H_2O. The aqueous layers were brought to pH 7 with NaOH (1 M), diluted to 500 mL, and freeze-dried. The product was dissolved in EtOAc, and the salts were removed by filtration. The filtrate was concentrated under reduced pressure to give **2** as a viscous, amber oil (183 mg, 72%).

Leucine Monomer (3). *exo*-Norbornene succinimidyl ester (53.5 mg, 0.228 mmol) and amine **2** (148.0 mg, 0.273 mmol) were dissolved in CH_2Cl_2 (2.5 mL). Diisopropylethylamine (DIEA) (79 μL, 0.46 mmol) was added, and the reaction mixture was stirred for 2 h. The solution was diluted with EtOAc (10 mL) and washed with 1 M aqueous HCl (3 × 5 mL), sat. $NaHCO_3$ (10 mL), and brine (10 mL). The organic layer was dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (3% MeOH/$CHCl_3$) to yield the protected monomer as a viscous yellow oil (109 mg, 77%).

The protected monomer (93.2 mg, 0.149 mmol) was then combined with potassium carbonate (61.8 mg, 0.447 mmol) in anhydrous DMF (1.5 mL). Thiophenol (18.4 μL, 0.179 mmol) was added under nitrogen. After 1 h, a saturated aqueous solution of sodium bicarbonate was added, and the solution was extracted with ether (3 × 5 mL). The organic layers were dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography ($CHCl_3$ then 5% MeOH/$CHCl_3$) to afford a pale yellow oil. The oil (47.6 mg, 0.108 mmol) was stirred with lithium hydroxide monohydrate (6.8 mg, 0.16 mmol) in 2:1 H_2O/MeOH (3 mL) for 18 h. The reaction was quenched with Amberlyst 15, filtered, and concentrated under reduced pressure to afford **3** as a white solid (14.7 mg, 23% over two steps).

Leucine-Substituted Polymer Synthesis. All polymer lengths were generated using the representative procedure used to produce the 100-mer.

***N*-(2-Nitrobenzenesulfonyl)-leucine Methyl Ester-Substituted Polymer (5d).** The succinimidyl ester-substituted polymer **4d** (*39*) (n = 100, 1.0 mg, 0.042 μmol) was combined with leucine derivative **2** (1.7 mg, 3.2 μmol) and *N*-methylmorpholine (1.4 μL, 13 μmol) in DMF (40 μL), and the mixture was allowed to stand at ambient temperature for 24 h. To the solution was added 3-amino-1,2-propanediol (2.0 mg, 21 μmol), and the reaction mixture was allowed to stand for 24 h. The mixture was purified by size exclusion chromatography (PD-10 column, H_2O as the eluent) and concentrated under reduced pressure to yield the *N*-(2-nitrobenzenesulfonyl)-leucine methyl ester-substituted polymer (**5d**).

Leucine-Substituted Polymer (6d). The *N*-(2-nitrobenzenesulfonyl)-leucine methyl ester-substituted polymer **5d** was combined with 2-mercaptoethanol (0.895 μL, 12.8 μmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (1.4 μL, 9.6 μmol) in DMF (100 μL). The reaction mixture was allowed to stand for 24 h at ambient temperature and purified by size exclusion chromatography (PD-10 column, H_2O as the eluent). The solution was concentrated under reduced pressure. The residue was dissolved in dimethyl sulfoxide (50 μL). To this was added 1.6 M aqueous LiOH (20 μL, 31.9 μmol) and MeOH (100 μL), and the reaction mixture was allowed to stand at ambient temperature for 18 h. The solvent was removed under reduced pressure, and the residue was dissolved in H_2O (500 μL) and purified by filtration (Microcon centrifugal filtration device, 5 × 500 μL H_2O). The filtrate was freeze-dried to yield the leucine-substituted polymer as a white solid (0.3 mg, 20% over three steps).

Motion Analysis Video Microscopy. The method of preparation of *E. coli* for motion analysis was similar to that described by Gestwicki *et al.* (*33*). Chemotactic *E. coli* AW607 (wt) or RP5700 (Δ*tsr*) were taken from the outer edge of a 0.3% agar Luria broth (LB) swim plate and grown in LB to an OD_{600} of 0.3. Cells were then washed twice in chemotaxis buffer (10 mM potassium phosphate buffer, pH 7.0, 10 μM ethylene diamine tetraacetic acid (EDTA)) and partially permeablized. This treatment has no effect on chemotaxis but allows for the entry of the multivalent ligands into the periplasm (*33*). Permeablization consisted of incubating the bacteria with 25 μM EDTA for 3 min at RT followed by quenching with 50 μM $CaCl_2$. Bacteria were then diluted with chemotaxis buffer to an OD_{600} of approximately 0.1. Motile *E. coli* cells (4 μL) were placed under a coverslip supported by additional coverslips and allowed to adapt for 2–3 min. Stimulant or buffer (1 μL) was added, and bacterial movement within the first 45 s was recorded (*33*). Bacterial paths were plotted using DataPoint, v0.62 (Glenn A. Carlson and Xannah Applied Science and Engineering), and mean angular velocities were calculated and averaged using Microsoft Excel.

Immunofluorescence Microscopy. Bacteria were prepared as previously described (*32*). *E. coli* strain AW607 (wt) was grown overnight in LB media. Cells (250 μL) were washed twice in phosphate-buffered saline (PBS) and partially permeablized (as above). Cells were then treated with compounds or buffer alone. In the "stimulated" cells, 2% paraformaldehyde in HEPES buffer, pH 7.2, was added immediately after addition of the chemostimulant. In "adapted" cells, bacteria were treated with 2% paraformaldehyde in HEPES (pH 7.2) 5 min after stimulation. Cells were stained for 30 min at 4 °C. Cells were placed on a poly(L-lysine)-treated coverslip for 10 min and then permeablized with methanol. Bacteria were then labeled with anti-Tsr antibody (cross-reactive with the cytoplasmic domains of all MCPs) (*32*) and fluorescein-labeled goat anti-rabbit antibody (1:1000). Cells were viewed with a Nikon microscope using a 60× oil emersion lens. Images were analyzed with Metamorph (Universal Imaging Company, Downington, PA) and Adobe Photoshop.

Cells were scored as having either polar or diffuse localization of MCPs via the same method employed by Lamanna, *et al.* (*32*). Blind scoring was employed: after the images were collected, images were assigned names randomly and individuals scoring the cells as diffuse or polar did not know which number corresponded to which experiment. MCP localization was deduced by comparing the intensity of staining at the poles to that along the lateral edges of the bacterium. A minimum of three random fields of 50 to 100 cells were counted for each experiment, and each experiment was compared with a wild-type, unstimulated control to ascertain any day-to-day variability. Each data set was derived from at least three independent experiments. Standard error of at least three different experiments is reported.

Acknowledgment: We thank J. S. Parkinson for supplying the anti-Tsr antibody and *E. coli* strain RP5700 and J. Adler for supplying *E. coli* strain AW607. We also thank Prof. C. Weise and G.A. Carlson for use of equipment and software. We acknowledge A. L. Anding for assistance with microscopy and E. S. Underbakke for helpful conversations. This research was supported by the NIH (Grant GM55984). M.J.B acknowledges the Molecular Biosciences Training Grant (GM07215) and a Steenbock fellowship for a predoctoral funding. E.M.K. acknowledges the Chemistry Biology Interface Training Grant (T32 GM008505) for a predoctoral fellowship.

Supporting Information Available: This material is free of charge *via* the Internet.

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