Insights into the organization and dynamics of bacterial chemoreceptor clusters through *in vivo* crosslinking studies

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The team signaling model for bacterial chemoreceptors proposes that receptor dimers of different detection specificities form mixed trimers of dimers that bind the cytoplasmic proteins CheA and CheW to form ternary signaling complexes clustered at the cell poles. We used a trifunctional crosslinking reagent targeted to cysteine residues in the aspartate (Tar) and serine (Tsr) receptors to obtain *in vivo* snapshots of trimer composition in the receptor population. To analyze the dynamics of trimer formation, we followed the appearance of mixed trimers when cells expressing Tar were induced for the expression of Tsr and treated with the crosslinker shortly after the onset of induction. In the absence of CheA or CheW, preformed Tar trimers exchanged partners readily with newly made Tsr. Conversely, in the presence of CheA and CheW, receptor trimers seldom exchanged partners, irrespective of the presence or absence of attractants. The C-terminal receptor-coupling domain of the CheA kinase, which contains binding determinants for the CheW protein, was essential for conferring low exchangeability to the preformed trimers of dimers. CheW also was required for this effect, but, unlike CheA, overexpression of CheW interfered with trimer formation and chemotactic behavior. The CheW effect probably occurs through binding interactions that mask the receptor sites needed for trimer formation. We propose that clustered receptors are organized in mixed trimers of dimers through binding interactions with CheA and CheW, which play distinctly different architectural roles. Moreover, once complete signaling teams have formed, they no longer undergo dynamic exchange of receptor members.

chemotaxis | epistasis | receptor clustering | signaling teams | trimer of dimers

Escherichia coli and other motile bacteria monitor their chemical environment with high sensitivity and broad detection ranges and use this information to seek out favorable living conditions. These chemotactic behaviors of bacteria offer tractable models for investigating the molecular basis of biological chemosensing and signal amplification. Indeed, considerable progress has been made in documenting the high-gain signaling properties of bacterial chemoreceptors, but their underlying molecular mechanisms remain elusive (recently reviewed in refs. 1 and 2).

Methyl-accepting chemotaxis proteins (MCPs) are the predominant chemoreceptors in bacteria (3). *E. coli* possesses five MCP-like receptors with different detection specificities; its most abundant types are the serine receptor (Tsr) and the aspartate receptor (Tar) (4). MCPs are integral membrane proteins characterized by a conserved cytoplasmic domain that interacts with the coupling protein CheW and the histidine kinase CheA to form ternary signaling complexes, which communicate with the cell's flagellar motors through protein phosphorylation pathways (1, 2). Tar and Tsr have periplasmic sensing domains that monitor chemoeffector levels through high-affinity binding sites. Changes in ligand occupancy modulate MCP signal output to control the direction of flagellar rotation and elicit appropriate locomotor responses.

MCPs and their associated signaling proteins form supramolecular clusters at the cell pole(s) (5, 6) that are exquisitely sensitive chemical sensors. Concentration changes that alter the ligand occupancy states of only a small fraction of receptor molecules elicit large changes in CheA kinase activity, reflecting an ≈50-fold signal amplification factor (7–9). *In vivo* studies have demonstrated that most of the signal gain originates at the receptor cluster and that it is greatly influenced by interactions between different receptors (8, 10).

Native MCP molecules are homodimers, but the crystal structure of the Tsr-signaling domain revealed a trimer-of-dimers arrangement (11). The principal trimer contact residues are identical in all five *E. coli* MCPs, raising the possibility that mixed trimer formation might be the structural basis for inter-receptor interactions. We found that amino acid replacements at the Tsr trimer contacts invariably abolished Tsr-signaling function, but with different effects on other chemoreceptors (12). Some Tsr defects spoiled Tar function (epistasis) or regained function in the presence of WT Tar (rescue), suggesting that Tsr and Tar molecules might signal collaboratively in "signaling teams" based on a trimer-of-dimers organization (12).

In vivo crosslinking studies have supported the receptor team model (12, 13). Different receptors were shown to crosslink in patterns consistent with the trimer-of-dimers geometry, and null lesions in the trimer contact region abolished that crosslinking (12, 13). In one of our crosslinking approaches, a trifunctional cysteine-targeted reagent [Tris-(2-maleimidoethyl)amide; TMEA] captured what appeared to be the internal (axial) subunits from trimers of dimers (13). In cells expressing different cysteine-bearing receptors, the compositions of mixed crosslinking products reflected random association of receptor dimers into higher-order groups. The presence or absence of CheA and CheW did not influence the extent of receptor crosslinking, suggesting that trimers of dimers may be precursors of the signaling units that form upon recruitment of CheA and CheW.

In the present study, we extended the use of TMEA-based crosslinking to analyze the dynamics of receptor trimers under different cellular conditions. Competitions between cysteine-marked Tar and unmarked Tsr molecules demonstrated that TMEA-based assays are reliable indicators of trimer-of-dimers formation and that the crosslinker mainly captures subunits from different dimers. Exchange assays in which homogeneous populations of Tar reporter molecules were challenged by inducing expression of cysteine-marked Tsr molecules showed that trimers continue to exchange members in the absence of either CheA

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Abbreviations: Tar, aspartate receptor; Tsr, serine receptor; Tsr·C, Tsr-S366C; Tar·C, Tar-S364C; MCP, methyl-accepting chemotaxis protein; IPTG, isopropyl β-D-thiogalactopyranoside; TMEA, Tris-(2-maleimidoethyl)amine.

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or CheW, whereas in the presence of both proteins, preformed trimers did not exchange partners with newly made receptors. These findings indicate that trimers of dimers are probably important building blocks of chemoreceptor-signaling clusters. Moreover, their interactions with CheA and CheW slow dimer–trimer exchanges in the receptor array and establish connections between trimer units that may engender signal amplification through receptor–receptor communication.

Materials and Methods

Bacterial Strains and Plasmids. All strains were derivatives of *E. coli* K12 strain RP437 (14) and carried the following genetic markers relevant to the current study: UU1596 [*tar-S364C* Δ(*tsr*)*7028* Δ(*trg*)*100*]; UU1598 [*tar-S364C* Δ(*tsr*)*7028* Δ(*trg*)*100* Δ(*tap-cheB*)*2241*)]; UU1603 [*tar-S364C* Δ(*tsr*)*7028* Δ(*trg*)*100* Δ(*tap-cheB*)*2241* Δ(*cheA*)*1643*]; UU1604 [*tar-S364C* Δ(*tsr*)*7028* Δ(*trg*)*100* Δ(*tap-cheB*)*2241*]; UU1610 [*tar-S364C* Δ(*tsr*)*7028* Δ(*trg*)*100* Δ(*tap-cheB*)*2234*] *cheW-Q109 a.m.*]; and UU1613 [*tar-S364C* Δ(*tsr*)*7028* Δ(*trg*)*100* Δ(*tap-cheB*)*2234* Δ(*cheA-cheW*)*2167*].

Parental plasmids derived from pACYC184 (15), which confers chloramphenicol resistance, were pRR31 [$nahR/p_{nahG}$ cloning vector (12, 16)] and pCS12 (p_{nahG} *tsr*; optimal complementation at 0.6 μM sodium salicylate). Parental plasmids derived from pBR322 (17), which confers ampicillin resistance, were pCJ30 (18), pRR48 ($lacI/p_{tac}$ cloning vectors), pPA770 [p_{tac} *cheW*; optimal complementation at 25 μM isopropyl β-D-thiogalactopyranoside (IPTG); pKJ9 [p_{tac} *cheA*; optimal complementation at 0 μM IPTG (19, 20)], and pRR53 (p_{tac} *tsr*; optimal complementation at 80 μM IPTG).

TMEA Crosslinking. Cells were grown at 30°C to mid-log phase in tryptone broth (21), harvested by centrifugation, and resuspended at $OD_{600} = 2$ in 10 mM potassium phosphate (pH 7) and 0.1 mM EDTA. Cell suspensions (0.5 ml) were incubated for 5 min at 30°C and then treated with 50 μM TMEA (Pierce) for 20 sec at 30°C. Reactions were quenched by the addition of 10 mM *N*-ethylmaleimide. Cells were pelleted and then lysed by boiling in 50 μl of sample buffer (22). Lysate proteins were analyzed by SDS/PAGE as described in refs. 13 and 23 and visualized by immunoblotting with an antiserum that reacts with the highly conserved MCP-signaling domain (24).

Exchange Assay. Plasmids expressing Tsr-S366C (Tsr·C) from an inducible promoter were transferred to host strains expressing Tar-S364C (Tar·C) from its native chromosomal promoter. Cells were grown in tryptone broth to mid-log phase and then induced for expression of Tsr·C. In experiments involving coexpression of CheA or CheW from a compatible plasmid, the cells were grown from the outset in the presence of the CheA or CheW inducer. Samples were withdrawn from the cultures at different times after Tsr·C induction and treated with TMEA as described above. Crosslinked products were resolved by SDS/PAGE and detected by immunoblotting, using ^{35}S-Protein A to detect the anti-Tsr Ab. Gels were scanned with a PhosphorImager (Molecular Dynamics), and bands corresponding to the various two-subunit crosslinking products (Tar·C∼Tar·C, Tar·C∼Tsr·C, and Tsr·C∼Tsr·C) were individually quantified with IMAGEQUANT software (Molecular Dynamics). The relative amounts of Tar·C and Tsr·C in two-subunit products were determined for each sample by assuming that (*i*) the Ab recognized Tar·C and Tsr·C equally well, and (*ii*) all two-subunit products originated from interdimer crosslinking (see *Results*). Therefore, the fraction of Tar·C subunits (p) = ([Tar·C∼Tar·C] + 0.5 [Tar·C∼Tsr·C])/([Tar·C∼Tar·C] + [Tar·C∼Tsr·C] + [Tsr·C∼Tsr·C]). The fraction of Tsr·C subunits (q) is $1 - p$, and the expected fraction of Tar·C∼Tsr·C products, if the two types of dimers mix randomly, is $2pq$. By using the measured proportions of Tar·C and Tsr·C subunits, we defined an "exchange factor" as the ratio of the observed to the expected fraction of Tar·C∼Tsr·C product. Exchange factors can range from 0, indicative of no mixing, to 1, representing completely random mixing.

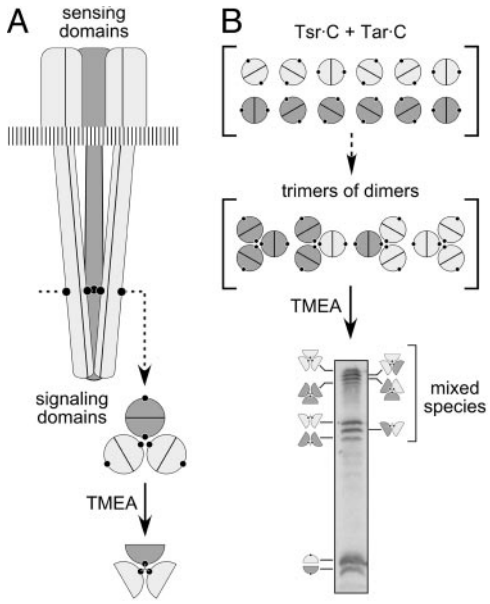

Fig. 1. TMEA crosslinking assay for receptor trimers of dimers. (*A*) Schematic representation of a mixed trimer of dimers formed between one Tar·C dimer (dark gray) and two Tsr·C dimers (light gray). Shown are a cross-section at the level of the cysteine-reporter sites (black dots) and the TMEA product predicted to arise from crosslinking the internal subunits of a trimer of dimers. (*B*) Example of a typical TMEA experiment. UU1613 (CheA$^-$ CheW$^-$ Tar·C) cells carrying pCS12-Tsr·C were induced with sodium salicylate, treated with TMEA, and analyzed by SDS/PAGE as described in *Materials and Methods*. The cartoon labels for the gel indicate the subunit compositions of the various crosslinking products.

Results

Competition Test of Trimer-Forming Ability. The trifunctional crosslinker TMEA is thought to trap the axial subunits of receptor dimers at the trimer interface (Fig. 1*A*). Cells expressing two different receptors that each carry an appropriate cysteine reporter, e.g., Tsr·C (marked serine receptor) and Tar·C (marked aspartate receptor), yield a variety of two- and three-subunit TMEA-crosslinking products (13) (Fig. 1*B*). In addition to pure products with only one type of receptor subunit, the cells produce mixed products containing different receptor subunits. We propose that mixed crosslinking species arise from mixed trimers whose composition is dictated by the relative expression levels of the component chemoreceptor dimers (Fig. 1*B*). Accordingly, both pure and mixed three-subunit products must arise through dimer–dimer crosslinking events. Our interpretation also assumes that two-subunit products represent incompletely crosslinked subunits from trimers of dimers. Clearly, mixed two-subunit products (Tar·C∼Tsr·C) can only originate from interdimer interactions, because Tar and Tsr do not seem to form heterodimers (12, 25). However, the provenance of pure two-subunit products (Tar·C∼Tar·C and Tsr·C∼Tsr·C) is ambiguous; they could conceivably represent intradimer crosslinking events.

To test these ideas, we expressed unmarked Tsr molecules to different extents in cells containing a fixed, chromosomally encoded level of Tar·C and followed the pattern of Tar·C crosslinking products upon TMEA treatment. The cells lacked the CheA and CheW proteins to increase the exchangeability of

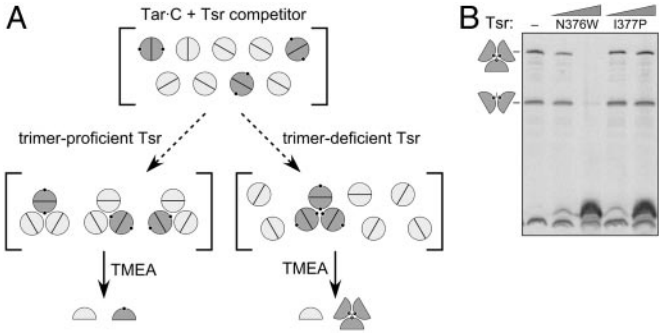

Fig. 2. Crosslinking competition assay for trimer-of-dimer interactions. (*A*) Experimental rationale. Tar·C dimers (dark gray) are coexpressed with a stoichiometric excess of unmarked Tsr dimers (light gray). Trimer-proficient Tsr dimers should form mixed trimers with Tar·C and eliminate TMEA crosslinking products. Trimer-deficient Tsr molecules should not interfere with the formation of Tar·C-crosslinking products. (*B*) Example of a competition experiment. UU1613 (Tar·C CheA$^-$ CheW$^-$) cells carrying pCJ30 (no Tsr control), pCS12-N376W (trimer-proficient Tsr), or pCS12-I377P (trimer-deficient Tsr) were induced with 0 or 1.2 μM sodium salicylate, treated with TMEA, and analyzed by SDS/PAGE as described in *Materials and Methods*.

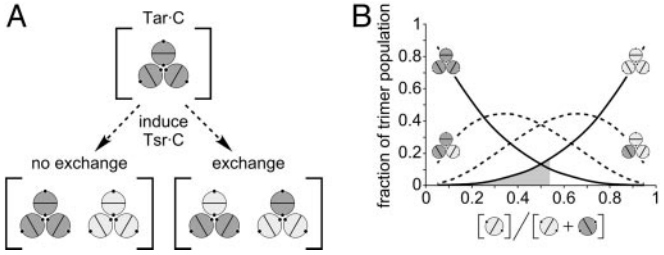

Fig. 3. Assay for assessing dimer–trimer exchangeability. (*A*) Experimental rationale. Tsr·C expression is induced in cells containing preformed Tar·C trimers. At short induction times, pure trimers should predominate in the absence of dimer exchanges, whereas mixed products will predominate if trimers are highly dynamic. (*B*) Predicted proportions of various trimer species at different ratios of Tar·C and Tsr·C dimers, assuming random dimer mixing. Exchange assay experiments were typically analyzed at relatively low Tsr levels (shaded region).

dimers between trimers (see below) and also lacked the MCP-modifying enzymes, CheR and CheB, to simplify the gel patterns. Our working model predicts that an excess of unmarked, trimer-proficient Tsr molecules will form mixed trimers of dimers, thereby reducing the number of pure Tar·C trimers and, consequently, their ability to yield crosslinking products (Fig. 2*A*). Indeed, we found that WT Tsr and trimer-proficient Tsr mutants (e.g., N376W) were effective competitors of Tar·C crosslinking (Fig. 2*B*). At high relative expression levels, they not only blocked formation of three-subunit Tar·C products but two-subunit products as well. This result indicates that pure two-subunit TMEA products originate predominantly from interdimer crosslinking, presumably within trimers-of-dimers. We also tested Tsr mutants (e.g., I377P) with previously demonstrated defects in trimer formation, as assessed by direct crosslinking tests of mutant Tsr·C reporter molecules (13). Our model predicts that trimer-deficient Tsr molecules should not reduce the extent of Tar·C crosslinking (Fig. 2*A*) and this result is what we found (Fig. 2*B*). This finding substantiates the trimer-formation phenotypes of mutant receptors inferred from direct crosslinking tests and excludes the possibility that "trimer-deficient" lesions simply alter the accessibility of the TMEA-reporter site. Moreover, these results demonstrate that we can assess the trimer-forming ability of unmarked receptor molecules through their effectiveness as crosslinking competitors.

Time-course experiments that followed the Tar·C crosslinking pattern after turning on expression of the Tsr competitor showed that in cells containing both CheA and CheW, there was a several-generation delay between the onset of full Tsr expression and the time at which the competition effect was apparent (data not shown). This observation suggested that preformed Tar·C trimers could not readily exchange partners with newly formed Tsr molecules in the presence of CheA and CheW and prompted us to investigate this issue by using a more direct assay.

Dimer Exchange Assay of Trimer Dynamics. We know that receptor trimers of dimers can form in either the absence or presence of CheA and CheW (13). However, because these proteins bind to receptor molecules to form ternary-signaling complexes, they might influence the dynamic properties of trimer associations. To assess CheA- and CheW-dependent effects on trimer dynamics, we measured the ability of preexisting trimers to exchange members with newly made receptors. The experimental logic is outlined in Fig. 3. Strains carrying a constitutively expressed, chromosomally encoded Tar·C reporter and an IPTG-inducible Tsr·C plasmid were grown to mid-log phase and then induced for Tsr·C expression. Samples were taken at different times after induction, treated with TMEA, and analyzed for crosslinking products. If trimers of dimers are highly dynamic, i.e., readily able to exchange partners with one another, then the composition of trimers at any moment would depend entirely on the relative cellular levels of the two receptor types, as depicted in Fig. 3*B*. In this case, many of the newly made Tsr·C molecules should be found in mixed trimers, which would yield mixed crosslinking products (Fig. 3*A*). However, if preformed Tar·C trimers are less dynamic, more of the newly made Tsr·C molecules should be found in pure crosslinking products (Fig. 3*A*). The difference in the two exchange patterns should be most obvious at short induction times, when the levels of Tsr·C are relatively low (Fig. 3*B*, shaded region). Thus, to assess the exchangeability of newly made Tsr·C dimers with the preexisting Tar·C population, we compared the measured levels of mixed crosslinking products with those predicted by random mixing at the same relative Tsr·C expression level (Fig. 3*B*).

Contributions of CheA and CheW to Trimer Dynamics. The appearance of mixed crosslinking products proved slower in cells containing both CheA and CheW than in cells lacking one or both of these proteins (Fig. 4). The differences between strains are apparent in the three-subunit products but more obvious in the two-subunit products. In both cases, pure crosslinking products were relatively more abundant in the *cheA*$^+$*cheW*$^+$ cells, whereas the mixed crosslinking products were relatively more prominent in the other genetic backgrounds. To quantify the differences between strains, we defined their "exchange factor" as the ratio of the observed to predicted levels of the Tar·C~Tsr·C product (see *Materials and Methods* for calculation). After 10 min of Tsr·C expression, the strains lacking CheA, CheW, or both exhibited relatively high exchange factors, ranging between 0.77 and 0.95, indicative of nearly free exchange of new and old receptor molecules (Fig. 4). In contrast, the strain containing both CheA and CheW showed an exchange factor of 0.35, indicating that the newly made Tsr·C receptors were not freely exchanging with the preexisting Tar·C population (Fig. 4).

To better assess the exchangeability of trimers of dimers in the presence of CheA and CheW, we induced Tsr·C expression for 30 min and then added chloramphenicol to stop further protein synthesis. After harvesting the cells and resuspending them in buffer containing chloramphenicol, we followed TMEA crosslinking over longer time periods. Surprisingly, the patterns did not change for up to 3 h under these conditions (data not

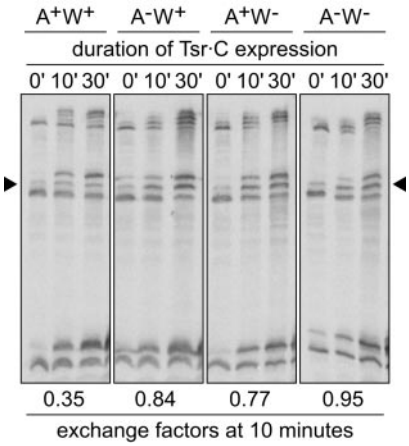

Fig. 4. The contribution of CheA and CheW to dimer–trimer exchangeability. Tsr·C expression was induced with 150 μM IPTG from plasmid pRR53 in strains UU1604 (Tar·C CheA$^+$ CheW$^+$), UU1603 (Tar·C CheA$^-$ CheW$^+$), UU1610 (Tar·C CheA$^+$ CheW$^-$), and UU1613 (Tar·C CheA$^-$ CheW$^-$). At 0, 10, and 30 min after induction, cells were treated with TMEA and analyzed by SDS/PAGE as described in *Materials and Methods*. Pointers indicate the mixed two-subunit product (Tar·C~Tsr·C) used to determine the exchange factors (see *Materials and Methods*).

shown). Moreover, we observed identical crosslinking patterns when attractants (10 mM serine, 10 mM aspartate, or both) were included in the incubation buffer (data not shown). We conclude that receptors synthesized in the presence of both CheA and CheW assemble into an exchange-resistant complex based on trimers of dimers and that attractant stimuli do not alter the low exchangeability of preassembled receptor trimers.

CheA Domains Needed to Render Trimers Exchange-Resistant. The CheA kinase has a multidomain architecture and functions as a homodimer (Fig. 5). The CheA phosphorylation site (H48) is located in the N-terminal P1 domain. P1 interacts with the ATP-binding domain (P4) in the opposing subunit during autophosphorylation (26, 27). Receptors most likely regulate CheA activity through allosteric control of P1–P4 encounters in the ternary-signaling complex. The C-terminal P5 domain of CheA,

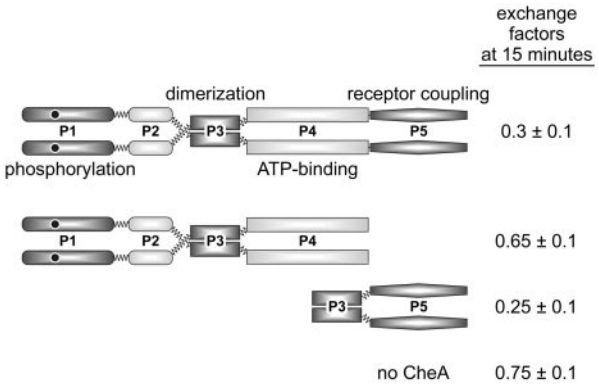

Fig. 5. CheA domains needed to render trimers exchange-resistant. Exchange assays were performed as described in *Materials and Methods* with UU1603 (Tar·C CheA$^-$ CheW$^+$) cells carrying pCS12-Tsr·C and a compatible plasmid: pKJ9 (CheA), pKJ9-I561am (CheAΔP5), pJZ11 (CheAΔP1-P2-P4), and pCJ30 (no CheA control). Different IPTG concentrations were used to ensure comparable expression levels of the various CheA-related proteins (determined by immunoblotting with polyclonal anti-CheA antiserum; data not shown): pKJ9 (5 μM), pKJ9-I561am, and pJZ11 (20 μM). The exchange factor values are averages and SDs for three independent experiments.

although not essential for autophosphorylation (28), binds to CheW (29, 30) and is essential for receptor coupling control (28). Two other CheA domains mediate dimerization (P3) and binding interactions (P2) with CheB and CheY, the response regulator targets of CheA-generated phosphoryl groups.

To determine which CheA domain or combination of domains was responsible for reducing trimer exchanges with the pool of newly made receptor dimers, we performed exchange experiments like those described above (see Fig. 3) in cells deleted for *cheA* but containing plasmids that provided various CheA fragments (Fig. 5). Exchange factors were high in the absence of CheA or in the presence of CheA molecules (at normal stoichiometric levels) lacking only the P5 receptor-coupling domain (Fig. 5). This result demonstrates that the P5 domain is essential for CheA-mediated reduction of trimer dynamics. Conceivably, the P5 domain alone could confer exchange resistance through its interactions with CheW and receptors, but we could not test this hypothesis directly because CheA fragments containing just the P5 domain are not sufficiently stable (31). However, a P3–P5 fragment consisting of the CheA dimerization and receptor-coupling domains produced low exchange factors characteristic of full-length CheA, demonstrating that the P3 and P5 domains are sufficient for exchange resistance (Fig. 5). Until we contrive to test a monomeric P5 domain, we cannot exclude the possibility that the low-exchange state requires dimeric P5 domains to bridge CheW and receptor trimers into stable structures. In any event, these findings permit us to conclude that the CheA autophosphorylation and phosphotransfer activities play no role in trimer dynamics.

CheW Stoichiometry Effects on Trimer Formation. It has long been known that overexpression of CheW inhibits chemotaxis in otherwise WT cells (29, 32, 33). A simple explanation for this behavior is that CheW binds to both receptors and CheA, and at high expression levels the preponderance of one-partner binding interactions (CheW–receptor; CheW–CheA) outcompetes the two-partner binding interactions (receptor–CheW–CheA) needed to form the ternary signaling complex (34). This scenario predicts that, by sequestering CheA molecules, high CheW levels also should increase trimer dynamics. To test this possibility, we conducted receptor exchange experiments in cells containing normal amounts of CheA and varying levels of CheW (Fig. 6*A*). With no CheW, the exchange factor was high (0.74), whereas at normal CheW levels, the exchange factor was low (0.42). These results are consistent with previous exchange experiments and indicate that CheW plays an essential role in reducing dimer–trimer exchangeability. However, CheW overexpression did not cause the predicted increase in dimer exchange between trimers, but rather a drastic decline in all receptor crosslinking products (Fig. 6*A*). This result could mean that high levels of CheW block trimer formation or, less likely, that CheW interferes with the crosslinking chemistry itself.

To compare the CheW overexpression effects on trimer formation/detection and on chemotaxis, we examined trimer formation by a single receptor reporter (Tar·C) at different CheW levels (Fig. 6*B*) and tested the chemotactic ability of a *cheR*$^+$ *cheB*$^+$ version of the same strain (Fig. 6*C*). At the highest CheW level tested, only 7% of the receptor subunits became crosslinked (Fig. 6*B*), and chemotactic ability was completely blocked (Fig. 6*C*). At lower CheW levels, the severity of the effects on trimers paralleled those on chemotaxis, consistent with the idea that trimer formation underlies chemotactic ability. In similar experiments, overexpression of CheA to levels that impaired chemotactic ability did not reduce trimer formation (data not shown), indicating that CheA and CheW play different structural roles in receptor clustering.

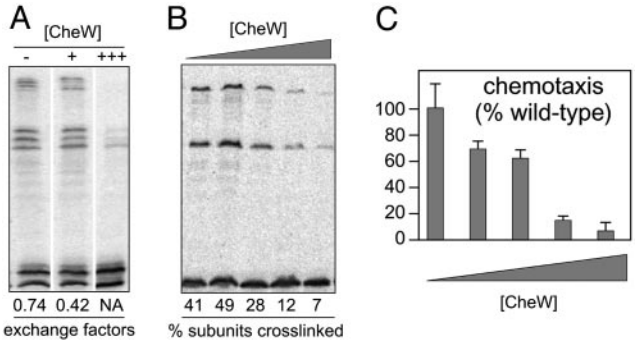

Fig. 6. Effects of CheW expression level on trimer exchangeability, trimer formation, and chemotactic behavior. (*A*) Exchange assay at different CheW levels. Exchange factors were determined with UU1610 (Tar·C $CheA^+$ $CheW^-$) carrying pCS12-Tsr·C and either pCJ30 (no CheW control) or pPA770 (CheW) induced at 25 or 100 μM IPTG. (*B*) Tar·C trimer formation at different CheW levels. UU1598 (Tar·C) cells carrying pPA770 (CheW) were grown in the presence of various IPTG levels (from left to right: 0, 25, 50, 75, or 100 μM), treated with TMEA, and analyzed by SDS/PAGE as described in *Materials and Methods*. Percentage crosslinking values are the measured proportions of Tsr·C material in two- and three-subunit products. (*C*) Chemotaxis at different CheW expression levels. The chemotactic behavior of strain UU1596 (Tar·C $CheR^+$ $CheB^+$) carrying pPA770 (CheW) was assessed by colony size on tryptone soft agar plates containing 50 μg/ml ampicillin and IPTG concentrations of 0, 25, 50, 75, or 100 μM. Plates were scored after 8.5-h incubation at 32.5°C. Note that the host strains in *B* and *C* also carry a chromosomal copy of the *cheW* gene, controlled by its native promoter.

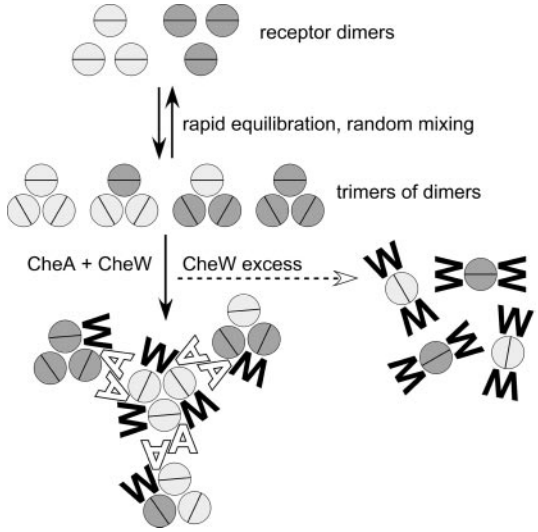

Fig. 7. Model for assembly and architecture of receptor signaling teams. See text for details.

Discussion

TMEA-Based Assays of Trimer Formation. The competition assay described here supports our contention that the principal targets of TMEA crosslinking are the axial subunits in trimers of dimers. Accordingly, two-subunit crosslinking products are reliable indicators of dimer–dimer interactions. The competitive ability of mutant Tsr receptors correlated well with their trimer-forming ability assessed previously by direct crosslinking. Thus, the competition assay should be a useful tool for assessing trimer formation by receptors that do not bear cysteine reporters.

Dynamics of Trimers of Dimers Within the Receptor Cluster. We found, using an exchange assay to follow the incorporation of newly synthesized Tsr into mixed trimers with Tar, that trimers formed in the presence of CheA and CheW were remarkably resistant to exchanges. Even after long incubation times, no mixing was observed between pure Tar and pure Tsr trimers, both in the absence and presence of attractants. Several reports demonstrate that chemoattractants cause changes in macroscopic cluster organization (35–37). Our results indicate that these effects do not involve an increase in the dynamic exchange of dimers between trimers, but rather some sort of destabilization of the binding interactions between signaling complexes within the cluster.

Both CheA and CheW were needed to render preformed Tar trimers exchange-resistant. This finding could mean that CheA and CheW together reduce the dynamics of individual trimers of dimers. Alternatively, lowered exchangeability could reflect the CheA/CheW-mediated formation of large clusters, in which the interior trimers might not be available for exchanges with newly made receptors entering at the cluster border. The trimer-level explanation for exchange resistance seems more plausible to us, because attractant-induced dispersal of the cluster should enhance access to its interior, yet attractant stimuli did not render trimers more prone to exchanges.

CheA Domains That Slow Trimer Dynamics. The receptor-coupling domain (P5) of CheA was instrumental in lowering trimer exchangeability. Because the P5 domain contains binding determinants for CheW (29, 30), it seems likely that the CheA–CheW-binding interaction plays a role in this effect, consistent with the fact that neither CheA nor CheW alone could render trimers exchange-resistant. The lower exchangeability produced by the P3–P5 fragment of CheA could mean that P5 is solely responsible for the effect, but because the P3–P5 construct also contains the dimerization domain, we cannot exclude the possibility that a dimeric P5 is actually required.

CheW Levels and Trimer Formation. Although CheW plays an essential role in reducing dimer–trimer exchanges, we also found that high cellular levels of CheW interfered with trimer formation. CheW in excess also impairs chemotactic ability (29, 32, 33); inhibition of receptor trimer formation might account for this effect. If so, then mutant CheW proteins with enhanced or attenuated ability to impair chemotaxis, like those described by Boukhvalova *et al.* (29), should exhibit corresponding differences in their ability to interfere with receptor trimer formation. Preliminary experiments have been consistent with this prediction (C.A.S., unpublished results), lending additional support to the idea that receptor trimers of dimers play functional roles in signaling.

A simple explanation for the trimer-inhibiting effects of CheW is shown in Fig. 7. We propose that CheW binds to the trimer contact region of receptor dimers, with an affinity comparable to that of trimer-forming receptor–receptor interactions. At low CheW stoichiometry, receptor dimers would be expected to have at most one bound CheW molecule, which would allow them to form trimers through their unblocked contact sites. However, at CheW excess all available trimer contacts would be blocked, inhibiting trimer assembly.

Organization and Dynamics of Receptor Clusters. An understanding of the arrangements and the dynamic interactions of the signaling proteins in receptor clusters will undoubtedly contribute to an understanding of the signaling process itself. We propose that trimers of dimers within the receptor cluster do not exchange partners with one another at a detectable rate. Both CheA and CheW, and most likely a binding interaction between them, are required to hinder dimer–trimer exchanges. The P5 domain of CheA, whose structure is similar to that of CheW (38, 39), was essential for this effect but only in conjunction with CheW.

Moreover, high expression levels of CheW, but not CheA, inhibited trimer formation. Thus, CheW and CheA make different and complementary contributions to the integrity and dynamics of receptor-signaling teams. A simple trimer-based model of receptor clusters is shown in Fig. 7. We suggest that monomeric CheW molecules bind to receptors and to the P5-coupling domains of dimeric CheA molecules to form bridging connections between trimers. In this way, trimer and cluster integrity depend on multiple binding interactions involving both CheA and CheW. Attractant stimuli could conceivably destabilize some of these linkages, which might account for reported attractant effects on the formation of periplasmic crosslinks between receptors (37) and on the tightness of receptor packing in polar clusters (35, 36). However, our results argue against attractant-induced disassembly of receptor signaling teams because attractants did not enhance exchanges of dimers between trimers. We conclude that once assembled and bound to CheW and CheA partners, receptor trimers of dimers do not readily dissociate. Thus, attractant stimuli most likely trigger signaling changes in receptor clusters by modulating the conformational interactions within and between trimer-based signaling teams rather than by promoting team diassembly.

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