The Minimal Elements Required for Clustering of Core Complexes in the 

_Escherichia coli_ Chemosensory Pathway

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TABLE OF CONTENTS

I. Acknowledgements
II. Abstract
III. Introduction
IV. Materials and Methods
   a. Materials
   b. Strains and Plasmids
   c. Cell Growth Conditions
   d. Imaging via Fluorescent Microscopy
V. Results
   a. Strategy for Strain and Plasmid Selection
   b. Determining the Array Binding Parameters for YFP-Fusion Proteins
   c. Use of YFP-Fusion Proteins to Monitor or Modulate Receptor Localization and Array Formation
   d. Determining the Array Binding Parameters of Mutant YFP-CheA-HK-P5 Domains
VI. Discussion
VII. References
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I would also like to mention that the results section, Determining the Array Binding Parameters of Mutant YFP-CheA-HK-P5 Domains, is adapted from a paper¹ that was produced by the Falke lab and published in the fall of 2013. I am an author on this paper and provided the experimental data that is presented in the thesis results section.
Abstract

In the chemotaxis pathway in bacteria, chemotactic stimuli are sensed by thousands of chemosensory core complexes that have localized to the cell poles to form chemosensory arrays. The chemosensory arrays allow for amplified signal transduction that modulates the rotation of the bacteria’s flagella and directs the movement of the cell up an attractant gradient or down a repellent gradient. The three chemosensory proteins that form the core complexes are histidine kinase CheA (CheA-HK), adaptor protein CheW (CheW-AP), and receptor. The chemosensory core complex is the functional unit of the array and consists of a pair of receptor oligomers (each a trimer of dimers) that are bridged by a single CheA-HK dimer in a complex stabilized by two CheW-AP monomers. In the assembled array, the core complexes are bound to one another through a network of interconnected rings formed by the P5 domain of CheA-HK alternating with the structurally homologous CheW-AP protein to yield a hexagonal array of receptor oligomers (Fig. 1). By using chemotactic proteins that are fluorescently labeled, previous in vivo studies have shown that chemosensory complexes localize to the cell poles and can form either tight, point-like clusters or diffuse, cap-like clusters. Point-like, tight clusters are indicative of standard array formation, in which the chemosensory proteins are locked into the tightly-packed hexagonal lattice. Diffuse clusters or caps indicate that the array is not properly formed, allowing the proteins to spread out more freely at the pole. These previous studies have shown that CheA-HK, CheW-AP, and receptors form tight clusters, while receptors in the absence of CheA-HK and CheW-AP form diffuse clusters. The current study seeks to systematically analyze the effects of CheA-HK, its isolated regulatory domain P5, and CheW-AP on array formation in five different modified cell backgrounds lacking one or more of the three core array components. The results confirm the previous observation that tight receptor clusters are observed in the presence of CheA-HK and CheW-AP or in the presence of CheW-AP alone.

The observation that CheW-AP alone can stabilize tight receptor clusters, together with published structural studies of array components, led us to develop a working model
for the minimal architecture required for tight cluster formation. The model draws from the known structure of the hexagonal, interconnected rings formed by CheA-HK and CheW-AP in membrane-bound, native arrays and soluble fragment arrays. The model proposes that tight cluster formation requires just two architectural elements: (i) the receptor oligomers and (ii) hexagonal rings of CheW-AP, and, when present, the P5 domain of CheA-HK that stabilizes the hexagonal packing of the receptors. Notably, the CheA-HK-P5 domain and the CheW-AP protein are homologous and share the same dual SH3-like architecture. The model proposes that the isolated CheA-HK-P5 domain, like CheW-AP, might be able to form hexagonal rings by itself or incorporate into rings with CheW-AP that stabilize tight clusters. The current studies of fluorescent fusion proteins binding to receptor clusters in live cells indicate that the isolated CheA-HK-P5 domain fails to incorporate into clusters in the absence of CheW-AP, but can incorporate together with CheW-AP into tight clusters. These findings provide strong evidence that support a model in which the native hexagonal rings formed by CheA-HK and CheW-AP in wild-type arrays can be formed by the isolated CheA-HK-P5 domain and CheW-AP in the absence of the other CheA-HK domains. To further test this model, mutations were introduced on the surface of the CheA-HK-P5 domain that were predicted to interact with CheW-AP. These mutations were observed to disrupt CheA-HK-P5 incorporation into clusters. Overall, the results strongly suggest that the CheA-HK-P5 domain possesses the full complement of CheA-HK interaction surfaces necessary to form hexagonal rings with receptor and CheW-AP.

**Introduction**

The chemotaxis pathway is a signaling system that enables bacteria to migrate toward attractant gradients or away from repellent gradients. In *Escherichia coli*, the chemosensory core complex is the minimal functional unit of the chemosensory array that can produce receptor-mediated kinase activity. The core complex consists of a histidine
kinase CheA (CheA-HK) and an adaptor protein CheW (CheW-AP) that are bound to the cytoplasmic tips of transmembrane chemoreceptors.\(^5\) CheA-HK is a homodimer in which each identical subunit is divided into five domains, P1-P5, where P5 is the regulatory domain that is bound to CheA-HK through CheW-AP.\(^6\) When a core complex senses an attractant or repellent molecule, the catalytic P4 domain transfers a phosphate from ATP to a His sidechain on the P1 substrate domain. P2 binds CheY, a response regulator protein, which positions CheY to receive the phosphate group from P1. Phosphorylated CheY diffuses to the motor where it modulates the switching of the flagella between its two rotational and swimming states, CW (tumbling) and CCW (smooth swimming). By alternating the rotation of the flagella, the bacteria cell is able to migrate in a series of steps and tumbles up or down a gradient. Other chemotactic proteins, CheR-methyltransferase (CheR-MT) and CheB, control the methylation state of the receptor adaptation sites, allowing the pathway to adapt to the changing background level of stimulus as it moves in the gradient.\(^5\)

Thousands of chemosensory core complexes join together at the poles of cells to form chemosensory arrays that sense chemotactic stimuli.\(^2\) Using fluorescent microscopy and fluorescently labeled chemotactic proteins, previous studies have been able to visualize these clusters in live cells, which appear as point-like, tight clusters or cap-like, diffuse clusters.\(^7\) The formation of the native, hexagonal chemosensory array yields tight polar clusters, whereas diffuse clusters or caps suggest that native arrays are not being formed, allowing the receptors to spread throughout the polar region. The localization of thousands of core complexes to the cell poles and the formation of native arrays allow the cell to amplify and transmit signal to downstream proteins and the flagella in order to direct the cell’s movement in a cooperative manner. Despite the integral role of chemosensory arrays in signal transduction, the protein-protein interactions essential for stabilization of the array architecture is still not fully understood.\(^3\) A preliminary model for chemosensory protein interactions was first outlined when a crystal structure of the
cytoplasmic domain of the serine receptor, Tsr, revealed a trimer of receptor dimers connected at the signaling tip.\textsuperscript{8,9}

\begin{figure}
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\caption{A) Electron density model of the chemosensory array created from cryo-EM, crystallography, and cross-linking studies. Three core complexes form this array. Green: His kinase CheA-HK. Blue: adaptor protein CheW-AP. Beige: serine receptor. B) 2-D geometric depiction of the chemosensory array assembled in a hexagonal lattice. The serine receptor (beige) forms trimers of dimers, which sit at each of the corners of the hexagon.\textsuperscript{10}}
\end{figure}

Around the same time, it was proposed that CheA-HK and CheW-AP were required for receptor cluster formation, since reduced clustering occurred in the absence of CheA-HK and CheW-AP.\textsuperscript{11,7} A crosslinking study echoed a similar theory by showing that the binding of CheA-HK and CheW-AP to receptor complexes stabilized array formation.\textsuperscript{12} Combining the trimer of receptor dimers model with crystallographic and cryo-EM evidence for the role of CheA-HK and CheW-AP in cluster formation led to the current model of the chemosensory array (Fig. 1). In this model, the cytoplasmic tips of the trimers of receptor dimers bind to CheA-HK through CheW-AP and are connected by the dimeric CheA-HK, forming a hexagonal lattice arrangement.\textsuperscript{13} However, more recent studies have challenged this model by showing that CheA-HK and CheW-AP enhance receptor clustering, but only CheW and receptor are required for tight cluster formation.\textsuperscript{3} The CheR-methyltransferase (CheR-MT) protein, one of the adaptation enzymes, binds to the C-terminus of receptors and can be used to visualize the clustering state of the receptor population. By attaching a yellow fluorescent protein (YFP) tag to CheR-MT and
transforming it into a cell strain lacking CheA-HK and CheW-AP, the study was able to show receptor localization in diffuse clusters, which is in contrast to the tight clusters that were observed when CheR-MT was introduced to wild-type cells containing CheA-HK and CheW-AP. When a YFP-CheW-AP fusion protein was transformed into a strain lacking CheA-HK and CheW-AP, the clusters resembled the wild-type, tight clusters. Since tight clusters indicate array formation, the result suggested that YFP-CheW-AP alone was able to stabilize the receptor array, and that CheA-HK dimers were not required for cluster formation.

The finding that CheW-AP alone can stabilize tight receptor clusters, along with other structural studies of the array components, has led to the development of a new working model for the architecture required for tight cluster formation. The model proposes that tight cluster formation requires receptor oligomers and hexagonal, interconnected rings of CheW-AP. When the P5 domain of CheA-HK is present, the model suggests that CheW-AP and CheA-HK-P5 can also stabilize the hexagonal packing of the receptors in order to produce tight clusters. Since CheA-HK-P5 and CheW-AP are homologous proteins, the model proposes that isolated CheA-HK-P5, like CheW-AP, might be able to form hexagonal rings by itself. However, the results of the current study indicate that CheA-HK-P5 fails to incorporate into clusters in the absence of CheW-AP but can incorporate with CheW-AP into tight clusters when CheW-AP is present. These findings provide strong evidence for a model in which the native hexagonal rings formed by CheA-HK and CheW-AP in wild-type arrays can also be formed by CheA-HK-P5 and CheW-AP in the absence of the other CheA domains. To further test this model, mutations were introduced on the surface of the CheA-HK-P5 domain that were predicted to disrupt protein interactions with CheW-AP. As expected, the mutations were observed to disrupt CheA-HK-P5 incorporation into clusters. Overall, the results strongly suggest that CheA-HK-P5 is able to form interconnected, hexagonal rings with receptor and CheW-AP.

Elucidating the key architectural features of the chemosensory array is not only an important area for bacterial chemotaxis research but also for clinical and diagnostic
applications. New, broad-spectrum antibiotics could be designed to inhibit array assembly or stability and block bacterial migration to wounds. Such antibiotics would likely have minimal toxic effects in humans and animals, since they would target chemosensory proteins that are evolutionarily conserved in chemotactic bacteria and do not exist in cells of high-order organisms.\textsuperscript{5} Another promising application is biosensor development. By designing a biological transducer to recognize a specific analyte, such as a drug or toxin, the concentration of the analyte could be detected with high sensitivity and dynamic range. Thus, further understanding of array architecture has broad implications.

**Materials and Methods**

**Materials**

Reagent grade chemicals were obtained from Sigma unless otherwise noted. The YFP-fusion expression plasmids employed (YFP-CheR-MT (pDK20), YFP-CheW-AP (pDK12), YFP-CheA-HK (pDK28), and YFP-CheA-HK-P5 (pDK36)), were gifts from the Parkinson and Sourjik labs.\textsuperscript{7} The cell strains employed (Table 1; RP437, RP9535, UU1607, UU2734, UU2612, and UU2682) were gifts from the Parkinson lab.\textsuperscript{14}

**Strains and Plasmids**

The YFP-fusion plasmids were previously constructed using the following procedure.\textsuperscript{7} The desired target gene (CheA-HK, CheR-MT, or CheW-AP) and the yellow fluorescent protein (YFP) gene were amplified via PCR, using primers with a GGGSV linker. A second round of PCR produced fragments with the target protein attached to YFP via the GGGSV linker. The YFP-fusion sequences were then cloned into the expression vector, pDK4, near the pTRC promoter to form YFP-CheA-HK (pDK28), YFP-CheR-MT (pDK20), and YFP-CheW-AP (pDK12). YFP-CheA-HK-P5 (pDK36) was formed by isolating the P5
domain of YFP-CheA-HK, amplifying it via PCR, and cloning it back into the pDK4 expression vector.

A few features of the YFP-fusion plasmids make them particularly desirable for experimental use. The pTRC promoter in the YFP-fusion plasmid enables expression of YFP-fusion proteins when induced by Isopropyl-β-D-1-thiogalactopyranoside (IPTG). This feature allows for controlled expression of the YFP-fusion proteins during experiments. The pDK4 vector contains an ampicillin resistant gene, which allows for selective uptake of YFP-fusion plasmids into cells growing in media containing ampicillin. Ampicillin resistance increases the likelihood that the YFP-fusion plasmids will be the only plasmids taken up and incorporated into cells’ genomes, while control over protein expression allows for optimal imaging of chemoreceptor clusters once the YFP-fusion proteins are expressed.

Six *Escherichia coli* cell strains, RP437, RP9535, UU1607, UU2734, UU2612, and UU2682, were used in this study. All strains were derived from the wild type strain, RP437, and were obtained from the Parkinson lab. Each modified strain lacked one or more of the CheA-HK, CheW-AP, and receptor genes, which code for the chemosensory proteins that form the core complex (Table 1). For ease of comprehension, strains may also be referred to as +A+W+R (RP437), -A-W-R (UU2682), -A-W+R (UU1607), +A+W-R (UU2612), +A-W+R (UU2734), and −A+W+R (RP9535) from this point on.

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*Table 1.* Description of cell strains used in experiments. Pluses and minuses indicate the presence or absence, respectively, of CheA-HK, CheW-AP, and receptor genes in cell strains.
**Cell Growth Conditions**

A YFP-fusion plasmid was transformed into each of the six *E. coli* strains previously described and grown overnight at 37° C on media plates containing ampicillin (100 μg/mL). One colony was selected for each strain and grown overnight at 30° C in 4 ml of VBC-HMLTT Minimal Essential Media (20 mM lactate, 40 μg/mL D,L-histidine, 20 μg/mL L-leucine, 1 μg/mL thiamine, 0.75% glycerol, 100 μg/mL ampicillin). After overnight growth, the samples underwent a 1:10 dilution with new minimal media and were placed in a shaking incubator at 30° C for about four hours or until an optical density of 0.4-0.9 was reached. The cells were then induced with Isopropyl β-D-1-thiogalactopyranoside (50 μM IPTG), prompting the expression of the YFP-fusion protein. After induction, the cells were placed back into the shaking incubator at 30° C for two hours until they achieved mid-log growth. An aliquot of 9 mL was removed from each cell culture and centrifuged at 3200 xg for 5 minutes, producing a compact cell pellet. The cell pellet was resuspended in 700 μL of tethering buffer (10 mM potassium phosphate, 0.1 mM EDTA, 1 mM L-methionine, 10 mM sodium lactate, pH 7.0) and centrifuged again at 3200 xg for 5 minutes. The new cell pellet was resuspended in 50 μL of tethering buffer. Microscopy slides were prepared by adding 20 μL of the resuspended cell sample to a thin agarose pad (0.1% agarose in tethering buffer) and covered with a coverslip. Cells were immediately imaged after slide preparation.

**Imaging via Fluorescent Microscopy**

After aliquoting cells onto microscopy slides, the cells were imaged using a Nikon TE-2000-E microscope with a 60x oil immersion objective, CFP/YFP/RFP dichroic mirror, and CoolSNAP ES camera with an exposure time of 600 ms. A mercury lamp provided excitation light with a wavelength of 490-500 nm and emissions of 520-560 nm.
Results

Strategy for Strain and Plasmid Selection

Previous studies have explored the effects of YFP-fusion proteins on receptor localization and clustering in certain cell strains. Studies have shown that YFP-CheA-HK does not localize in strains lacking CheW-AP or in receptorless strains [Δ(Tar, Tsr, Tap, Trg)]. Another study showed that YFP-CheR-MT did not localize in a receptorless strain but did localize and form tight, polar clusters in a strain with only CheA-HK, CheW-AP, and Tsr. YFP-CheR-MT also localized in a strain with only Tsr, although the clusters were more diffuse, and many of the cells displayed polar caps. Diffuse clusters and polar caps indicate that YFP-CheR-MT was binding to receptor at the poles, but arrays were not forming. The same study also tested the effects of YFP-CheW-AP, YFP-CheA-HK, and YFP-CheA-HK-P5 on cluster formation in wild-type cells (RP437) and the strain with only Tsr. Results showed that YFP-CheW-AP and YFP-CheA-HK-P5 formed tight, polar clusters in wild-type cells and the Tsr strain. YFP-CheA-HK formed tight, polar clusters in wild-type cells but did not localize or form clusters in the Tsr strain. In summary, previous studies have examined YFP-CheA-HK, YFP-CheW-AP, and YFP-CheA-HK-P5 in wild-type cells and Tsr-only cells, YFP-CheA-HK and YFP-CheR-MT in receptorless strains, and YFP-CheR-MT in a strain with only Tsr and a strain with only CheA-HK, CheW-AP, and Tsr.

The current study attempted to construct a more comprehensive and systematic approach for testing the effects of YFP-fusion proteins on receptor localization and clustering. This was done in two ways: a) The YFP-fusion proteins that were selected for testing consisted of all major proteins that form the chemosensory array (CheA-HK, CheW-AP, and CheA-HK-P5) and CheR-MT. b) The strains that were selected for testing consisted of all reasonable combinations of the presence or deletion of receptor, CheA-HK, and CheW-AP genes (+A+W+R, -A-W-R, -A-W+R, +A+W-R, +A-W+R, and -A+W+R). This
experimental design allowed for the hypothesis of the study to be tested and to expand the experiments of previous studies. For example, by transforming YFP-CheA-HK into -A+W+R and -A-W+R and analyzing the cluster formation, it could be determined if CheA-HK was able to bind to receptor and stabilize the formation of an array without CheW-AP. By transforming YFP-CheW-AP into -A+W+R and -A-W+R, it could be determined if CheW-AP was able to bind to receptor and stabilize the formation of an array without CheA-HK by forming a ring with other CheW-AP proteins.

![Figure 2](image)

**Figure 2.** Measuring the fluorescent intensity of a cell. After imaging cells with a 60x objective, the array fluorescence (inside red circle, $F_A$), cytosolic fluorescence (gold circle, $F_C$), and the background fluorescence (blue circle, $B$) are each measured over a 2x2 pixel area.$^{10}$

**Determining the Array Binding Parameters for YFP-Fusion Proteins**

While fluorescent microscopy images can be a useful, qualitative depiction of receptor localization and clustering, it can also be helpful to have a quantitative representation. The degree to which a YFP-fusion protein binds to an *in vivo* receptor cluster can be described by an incorporation parameter. To determine this parameter, cells
are first imaged using a 60x objective. The fluorescent intensity of the array, cytosol, and background of a cell is measured over a 2x2 pixel area (Fig. 2). Applying Equation 1 to the measured intensities corrects for fluctuations in the expression levels of the YFP-fusion proteins that can occur in cells.

\[ A_{\text{Intensity}} = \frac{(F_A - B)}{(F_C - B)} \]  
\text{(Eq. 1)}^{10}

Corrected fluorescent intensity, \( A_{\text{Intensity}} \), for an array in a given cell, where \( F_A \) = array fluorescence, \( F_C \) = cytosolic fluorescence, \( B \) = background fluorescence.

The corrected array fluorescent intensity, \( A_{\text{Intensity}} \), is then normalized by Equation 2 to produce \( A_{\text{Bound}} \), which is the array incorporation parameter for the YFP-fusion protein in a particular cell strain relative to the wild-type strain.

\[ A_{\text{Bound}} = \frac{(I_E - I_0)}{(I_{\text{Wt}} - I_0)} \]  
\text{(Eq. 2)}^{10}

Normalized incorporation parameter, \( A_{\text{Bound}} \), for a cell, where \( I_E \) = corrected fluorescent intensity for a YFP-fusion protein in an experimental strain, \( I_0 \) = corrected fluorescent intensity for a YFP-fusion protein in the -A-W-R control strain, \( I_{\text{Wt}} \) = corrected fluorescent intensity for a YFP-fusion protein in the wild-type control strain.

The corrected fluorescent intensity for a YFP-fusion protein in wild-type is the positive control \( (I_{\text{Wt}}) \) and has a binding parameter of 1, because all YFP-fusion plasmids exhibit binding in wild-type cells. The corrected fluorescent intensity for a YFP-fusion protein in -A-W-R is the negative control \( (I_0) \) and has a binding parameter of 0, because no YFP-fusion plasmids exhibit binding in this strain. By normalizing to a positive control and a negative control, an incorporation parameter can be determined for a YFP-fusion protein in a given strain that is relative to that protein’s incorporation in wild-type and lack of incorporation in -A-W-R. Using this quantification method, binding parameters were obtained for CheR-MT, CheW-AP, CheA-HK, and CheA-HK-P5 in the six cell strains that were mentioned earlier (Fig. 4).
Use of YFP-Fusion Proteins to Monitor or Modulate Receptor Localization and Array Formation

**Figure 3.** Use of *in vivo* YFP-fusion proteins to monitor and modulate localization and clustering in various cell lines. A-X. Localization of YFP-CheR-MT (pDK20; A, E, I, M, Q, and U), YFP-CheW-AP (pDK12; B, F, J, N, R, and V), YFP-CheA-HK (pDK28; C, G, K, O, S, and W), and YFP-CheA-HK-P5 (pDK36; D, H, L, P, T, and X) in +A+W+R (RP437; A-D), -A-W-R (uu2682; E-H), -A-W+R (uu1607; I-L), +A+W-R (uu2612; M-P), +A-W+R (uu2734; Q-T), and −A+W+R (RP9535; U-X).
**Figure 4.** The *in vivo* binding of YFP-fusion proteins to arrays in various cell lines. A-D. Quantification of array binding and normalization to the positive control, +A+W+R (RP437), and negative control, -A-W-R (uu2682), for each YFP-fusion protein: YFP-CheR-MT (pDK20; A), YFP-CheW-AP (pDK12; B), YFP-CheA-HK (pDK28; C), and YFP-CheA-HK-P5 (pDK36; D). The pluses and minuses under each bar indicate the presence or absence, respectively, of CheA-HK, CheW-AP, and receptor in the cell line used. Error bars indicate standard error for a measurement of 45 cells in three separate experiments. Asterisks above bars indicate significant changes in array binding (p < 0.05).

**YFP-CheR-MT**

YFP-CheR-MT was tested in each of the six strains used in this study (+A+W+R, -A-W-R, -A-W+R, +A+W-R, +A-W+R, and -A+W+R) in order to analyze receptor localization and clustering in cells via fluorescent microscopy as summarized in Fig. 3. YFP-CheR-MT was observed in tight clusters in wild-type cells and -A+W+R cells (Fig. 3A and U, respectively). Diffuse clusters were observed in -A-W+R cells (Fig. 3I) and +A-W+R cells (Fig. 3Q). No localization or cluster formation occurred in cells lacking receptor (Fig. 3E).
and M). Figure 4A quantifies the relative levels of YFP-CheR-MT incorporation into clusters. Similar array incorporation was observed in wild-type cells and -A+W+R cells. About 1.5 times as much incorporation occurred in -A-W+R cells and about 2.5 times as much incorporation occurred in +A-W+R cells in comparison to wild-type cells. No incorporation occurred in receptorless cells.

**YFP-CheW-AP**

YFP-CheW-AP was tested in each of the six strains used in this study (+A+W+R, -A-W-R, -A-W+R, +A+W-R, +A-W+R, and -A+W+R), and its incorporation into polar clusters was observed via fluorescent microscopy (Fig. 3). YFP-CheW-AP was observed in tight clusters in wild-type cells, -A+W+R cells, and -A-W+R cells (Fig. 3B, V, and J, respectively). Tight clusters occurred in most +A-W+R cells, but inclusion bodies occurred in a subset of +A-W+R cells (Fig. 3R). No localization or cluster formation occurred in cells lacking receptor (Fig. 3F and N). Quantitative analysis of YFP-CheW-AP incorporation into arrays showed similar incorporation parameters for wild-type cells, -A+W+R cells, and -A-W+R cells (Fig. 4B), while ~1.7 times as much incorporation occurred in +A-W+R cells. No incorporation occurred in receptorless cells.

**YFP-CheA-HK**

YFP-CheA-HK was tested in each of the six strains used in this study (+A+W+R, -A-W-R, -A-W+R, +A+W-R, +A-W+R, and -A+W+R), and its localization in clusters was observed via fluorescent microscopy (Fig. 3). Tight clusters were observed in wild-type cells and -A+W+R cells (Fig. 3C and W, respectively). No localization to clusters was detected in cells lacking receptor or CheW-AP (Fig. 3G, K, O, and S). Quantitative analysis of YFP-CheA-HK incorporation into arrays showed slightly more incorporation in -A+W+R cells in comparison to wild-type cells (Fig. 4C).
**YFP-CheA-HK-P5**

YFP-CheA-HK-P5 was tested in each of the six strains used in this study (+A+W+R, -A-W-R, -A-W+R, +A-W-R, +A-W+R, and -A+W+R), and its localization in clusters was investigated via fluorescence microscopy (Fig. 3). Like YFP-CheA-HK, tight clusters were observed in wild-type cells and -A+W+R cells (Fig. 3D and X, respectively). No localization to clusters occurred in cells lacking receptor or CheW-AP (Fig. 3H, L, P, and T). Quantitatively, YFP-CheA-HK-P5 showed similar incorporation into arrays in wild-type and -A+W+R cells (Fig. 4C). No binding occurred in receptorless cells or cells lacking CheW-AP.

**Determining the Array Binding Parameters of Mutant YFP-CheA-HK-P5 Domains**

*Selection of a library of tryptophan and alanine mutants for TAM-IDS analysis:*

In order to test whether the CheA-HK-P5 domain possesses the normal CheW contacts observed in crystal structures of the native ring, we employed the Tryptophan and Alanine Mutagenesis to Identify Docking Sites (TAM-IDS) approach. The method was used to generate both a bump and a hole at the selected positions on the CheA-HK-P5 surface that contacts CheW-AP at interface 1. Trp substitutions at a critical protein-protein interface typically perturb binding and/or function, except in cases where they can rotate out of the contact region. Interfacial alanine mutations are often less perturbing, unless the native residue is essential. For the present study, four positions were selected on the surface of CheA-HK-P5 surface to target for Trp and Ala substitutions (Fig. 5A). Three of the positions (N630, V634, and L650) were predicted by the crystal-structure based threaded model to be within the packed area of CheA-HK-CheW-AP interface 1, whereas an additional position distal to the interface (I617) was selected as a negative control. The
chosen mutations were introduced at each target position in the YFP-CheA-HK-P5 construct via site-directed mutagenesis.

**Figure 5.** Quantifying the effects of Trp and Ala mutations on the incorporation of YFP-CheA-HK-P5 into live cell arrays: *in vivo* TAM-IDS. A) Model of the four Trp mutations on CheA-HK-P5 selected for TAM-IDS analysis. Notably, all common conformers of N630W, V634W, and L650W are predicted to clash with the blue adaptor protein, while none of the I617W are predicted to clash. B) CheA-HK-P5 was fused to YFP and its ability to stably incorporate into the polar arrays of live cells lacking intrinsic CheA-HK was quantified by fluorescence microscopy as previously described.10 The resulting array binding parameter ranges from unity for the native incorporation exhibited by the wild-type YFP-CheA-HK-P5 domain to zero for the negative control domain YFP-CheA-HK-P2. The standard error is shown for each binding reaction, where n = 30 cells or more that were quantified in three separate experiments. Asterisks indicate statistically significant losses of array incorporation relative to YFP-CheA-HK-P5 (p < 0.05). For consistency with our other studies employing the *S. typhimurium* kinase *in vitro*, the indicated position labels are for *S. typhimurium* residues. The corresponding residues (*italicized*) on the *E. coli* domain employed in this *in vivo* experiment are: kI617
Analyzing contacts between CheA-HK-P5 and CheW-AP using TAM-IDS in live cells.

The abilities of the mutant YFP-CheA-HK-P5 constructs to assemble into arrays in cells lacking endogenous CheA-HK were assessed. Using the quantitative method to evaluate array binding, the corrected array brightness of each cell in a population was first measured. Then the corrected array brightness was averaged and normalized over the population to generate a relative binding parameter ranging from 0 to 1, where 0 corresponds to the negligible array binding of a negative control (YFP-CheA-HK-P2 domain), while 1 represents the normal level of binding seen for the wild-type YFP-CheA-HK-P5 domain (see Determining the Array Binding Parameters for YFP-Fusion Proteins for a more detailed version of the quantitative method).

Figure 5B shows the in vivo array binding parameter for the Ala and Trp mutants at each of the selected positions. The introduction of Trp substitutions at each of the three crystallographic interface 1 positions (N630W, V634W, L650W) greatly reduced stable array incorporation of the YFP-CheA-HK-P5 domain, a result consistent with the idea that the isolated, monomeric P5 domain forms the same CheW-AP contacts previously observed for dimeric CheA-HK in structural studies of the CheA-HK-P5-CheW-AP ring. Bulky Trp substitutions at these positions would be expected to disrupt the packing interactions required for normal interface 1 assembly, resulting in the observed loss of incorporation. Both Trp and Ala substitutions at position L650 caused virtually total loss of cluster incorporation, indicating that the native Leu side chain is essential for interface 1 assembly, tolerating neither bulky substitution nor truncation. By contrast, Trp and Ala substitutions at control residue I617 had considerably smaller effects on cluster incorporation, as expected due to its location largely outside the packing region of the crystallographic interface. Andrew Natale of the Falke lab observed that the same three Trp substitutions positions (N630W, V634W, L650W) that disrupt YFP-CheA-HK-P5 binding to clusters in
*vivo* also inhibit the binding of full-length CheA-HK to arrays *in vitro*, although the inhibition is smaller due to the multiple interactions of dimeric CheA-HK with the lattice.\(^1\)

**Discussion**

**YFP-CheR-MT**

YFP-CheR-MT localization to polar clusters in the different cell backgrounds was similar to the results of previous studies in the Sourjik lab,\(^2\) although those studies used a smaller set of test strains. Like the previous experiments, YFP-CheR-MT did not bind in receptorless strains, it formed tight clusters in the strain with CheA-HK and CheW-AP, and it formed diffuse, cap-like clusters in the strain lacking CheA-HK and CheW-AP. Because CheR-MT always binds to receptor when it is present, the absence of clusters in strains lacking receptor was not surprising. The tight clusters in the strain containing both CheA-HK and CheW-AP indicated that arrays were forming. For the strain lacking CheA-HK and CheW-AP, the results indicated that receptors were still localized at the cell poles but arrays were not forming, since the clusters were diffuse. Together these findings confirmed that CheA-HK and CheW-AP were able to stabilize the array, as they do in the native lattice architecture, yielding tight, polar clusters in live cells.

YFP-CheR-MT was also examined in a strain lacking only CheA-HK and in a strain lacking only CheW-AP. Previous studies have not examined either of these cell backgrounds for YFP-CheR-MT. YFP-CheR-MT was observed in tight clusters in a strain containing CheW-AP but lacking CheA-HK, indicating that CheW-AP alone can stabilize receptor arrays in the absence of CheA-HK. However, in a strain containing CheA-HK but lacking CheW-AP, diffuse, polar caps were observed, indicating that YFP-CheR-MT was localizing and binding to receptor in the cell, but no arrays were forming in the absence of CheW-AP. This suggests that full-length CheA-HK requires the presence of CheW-AP to form the array, which is in agreement with prior findings.\(^11,7,3\)
**YFP-CheW-AP**

Like the Sourjik lab’s studies with YFP-CheW-AP, tight clusters formed in wild-type cells and in cells lacking both CheA-HK and CheW-AP. These results indicate that YFP-CheW-AP is able to incorporate into fully formed arrays in wild-type cells, and that YFP-CheW-AP alone is sufficient to stabilize an array-like distribution of receptors. The latter is an interesting finding, because it suggests that CheW-AP molecules can fill in where CheA-HK is absent in the interconnecting rings, presumably yielding the native hexagonal array of receptor oligomers within the tight cluster.

YFP-CheW-AP was also examined in receptorless strains, a strain lacking CheA-HK, and a strain lacking CheW-AP. Previous studies have not examined the two latter experimental conditions for YFP-CheW-AP. As expected, YFP-CheW-AP did not bind in receptorless strains but did form tight receptor clusters containing only CheW-AP, providing further evidence that CheW-AP is able to stabilize the array structure when CheA-HK is absent. Interestingly, in a strain lacking CheW-AP, YFP-CheW-AP appears to be capable of forming an array-like structure with CheA-HK in most cells, but in some cells the presence of bright caps suggests that arrays are not forming. Perhaps the steric hindrance of the YFP tag on the CheW-AP fusion protein can sometimes complicate its incorporation between full-length CheA-HK proteins to link the individual core complexes into an array.

**YFP-CheA-HK**

Similar to the Sourjik lab’s results with YFP-CheA-HK, tight clusters were observed in wild-type cells and no cluster formation was detected in cells lacking both CheA-HK and CheW-AP. The tight clusters in wild-type cells indicate that YFP-CheA-HK is able to incorporate into native arrays as expected. Since no clusters were observed in the strain lacking both CheA-HK and CheW-AP, it follows that YFP-CheA-HK requires CheW-AP to form native receptor clusters.\textsuperscript{11,7,3}
YFP-CheA-HK was also examined in receptorless strains, a strain lacking CheA-HK, and a strain lacking CheW-AP. YFP-CheA-HK did not bind in receptorless strains and formed tight clusters in the strain lacking CheA-HK. This suggests that CheW-AP is able to form an array with YFP-CheA-HK in the absence of endogenous CheA-HK. No localization to clusters was detected in the strain lacking CheW-AP, echoing earlier findings in this study and prior studies that CheA-HK requires CheW-AP to form clusters.

**YFP-CheA-HK-P5**

Similar to the Sourjik lab’s experiments with YFP-CheA-HK-P5, tight clusters were observed in wild-type cells, indicating that YFP-CheA-HK-P5 was incorporating into arrays with CheA-HK, CheW-AP, and receptor. However, in the present study no incorporation into polar clusters was detected in cells lacking both CheA-HK and CheW-AP, which is in contrast to the Sourjik lab’s report of tight clusters in this strain.

YFP-CheA-HK-P5 was also examined in receptorless strains, a strain lacking CheA-HK, and a strain lacking CheW-AP, which previous studies have not done. Herein, localization to clusters did not occur in the receptorless strains or in any strain lacking CheW-AP. Tight clusters occurred in the strain lacking CheA-HK but containing CheW-AP. These are the same results that were obtained for YFP-CheA-HK in the corresponding strains.

Overall, the present results for incorporation of the YFP-CheA-HK-P5 domain into tight clusters show the same requirements for receptor and CheW-AP that were observed for full-length YFP-CheA-HK. It follows that the isolated P5 domain possesses all of the CheA-HK surfaces essential for the receptor and CheW-AP contacts needed for tight cluster formation. Moreover, when one of these contacts (CheA-HK-P5-CheW-AP ring interface 1) was perturbed by bulky Trp mutations, cluster formation was disrupted as predicted by the known crystal structure of the contact, providing strong evidence that our YFP-CheA-HK-P5 domain incorporates properly into the CheA-HK-P5-CheW-AP ring. It is curious that in the Sourjik study the YFP-CheA-HK-P5 domain (but not full-length YFP-CheA-HK) was
found to lack the CheW-AP requirement for tight cluster formation. There are at least three possibilities for this discrepancy:

a) The Sourjik lab’s strain that lacks both CheA-HK and CheW-AP also lacks CheB, CheR-MT, CheY, CheZ, Tar, and Tap. The Falke Lab’s strain that lacks both CheA-HK and CheW-AP contains CheB, CheR-MT, CheY, CheZ, Tar, and Tap. It seems unlikely that these strain differences would eliminate the CheW-AP requirement, but this possibility could be tested by repeating the experiment with YFP-CheA-P5 in the identical strain that the Sourjik lab used and in the Falke lab strain, side-by-side.

b) The Sourjik lab’s YFP-CheA-HK-P5 plasmid may not contain the entire N-terminus of the P5 domain. The residue range reported in the Sourjik paper for the P5 domain in the YFP-CheA-HK-P5 construct is shorter than the range defined by the known crystal structure of the domain. The YFP-CheA-HK-P5 construct used in the present study includes the entire P5 domain. A shortened P5 domain may produce different results from the full sequence. This possibility could be tested by obtaining the Sourjik plasmid and sequencing it.

c) The Sourjik lab may have utilized the incorrect YFP-CheA-P5 fusion plasmid. Their findings suggest the possibility that the construct contained CheW-AP rather than P5. The fusion plasmid used in this study has been sequenced and verified as the P5 domain of CheA-HK. This possibility could be tested by obtaining the Sourjik plasmid and sequencing it.

Conclusions

Experiments that were performed in this study and previous studies yielded the same results,\textsuperscript{1,7,3} except for YFP-CheA-HK-P5 in the strain lacking both CheA-HK and CheW-AP, which was observed to form tight clusters in the previous work but in the present study did not exhibit detectable cluster incorporation. New experimental
conditions for observing the effects of YFP-fusion proteins on localization and array formation included the testing of YFP-CheR-MT in -A+W+R and +A-W+R, YFP-CheW-AP in -A+W+R, +A-W+R, and receptorless strains, YFP-CheA-HK in -A+W+R, +A-W+R, and receptorless strains, and YFP-CheA-HK-P5 in -A+W+R, +A-W+R, and receptorless strains. The results indicate that receptor must be present in the strain in order for clusters to form, CheA-HK or CheA-HK-P5 cannot localize to clusters without CheW-AP present, while CheW-AP can localize to and stabilize tight clusters in the absence of CheA-HK.

Overall, the results strongly suggest that the CheA-HK-P5 domain possesses the full complement of CheA-HK interaction surfaces necessary to form hexagonal rings with receptor and CheW-AP. However, while CheW-AP alone can stabilize tight receptor clusters, CheA-HK-P5 requires assistance from CheW-AP and cannot stabilize tight clusters in the absence of CheW-AP. Mutagenesis analysis indicates that bulky Trp substitutions on the surface of CheA-HK-P5 known to contact CheW-AP in crystal structures of the CheA-HK-CheW-AP hexagonal ring disrupt CheA-HK-P5 incorporation, suggesting that CheA-HK-P5 forms a hexagonal ring with receptor and CheW-AP even in the absence of the other CheA-HK domains. Together, the findings are consistent with a simple model in which the formation of a hexagonal ring of alternating CheA-HK-P5 and CheW-AP molecules, or a hexagonal ring of CheW-AP alone, is sufficient to organize receptor oligomers into a hexagonal lattice, yielding the tight clusters observed in fluorescence microscopy. Collaborative cryo-EM studies with the Jensen lab at Caltech will be designed in the future to test this hypothesis.
References


