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ASSEMBLY AND FUNCTIONAL ARCHITECTURE OF BACTERIAL CHEMORECEPTOR NANOARRAYS

A Dissertation Presented

by

ELIZABETH R. HAGLIN

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 2018

Chemistry
ASSEMBLY AND FUNCTIONAL ARCHITECTURE OF BACTERIAL CHEMORECEPTOR NANOARRAYS

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DEDICATION

To my parents, David and Nadine, and my sister, Anna – for showing me that with passionate perseverance, a scientific and artistic brain truly can coexist as one.

To my future husband, Bob Adolf – for steadfast encouragement, love, and inspiration, and for being my most valued critic.

To my best friend, Benni – for being my companion in solitude and entering my life when I needed you the most. You will forever be loved and missed.
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I owe my remaining sanity to the generous support of my family and friends. First, thanks to my parents and sister for inspiring me to fight hard for what I want, and helping me never lose sight of my true self (that being equal parts 6-year old Princess and 16-year old
Goth). I am fortunate to have amassed a number of extraordinary friends and compatriots at UMass. Thanks for sharing fears, tears, beers, and cheers with me. Each and every one of them has impacted my life and overall happiness for the better.

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ABSTRACT

ASSEMBLY AND FUNCTIONAL ARCHITECTURE OF BACTERIAL CHEMORECEPTOR NANOARRAYS

FEBRUARY 2018

ELIZABETH R. HAGLIN, B.S., SIMMONS COLLEGE
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Directed by: Professor Lynmarie K. Thompson

Transmembrane chemotaxis receptors are found in bacteria in extended hexagonal arrays stabilized by the membrane and by cytosolic binding partners, the kinase CheA and coupling protein CheW. Models of array architecture and assembly propose receptors cluster into trimers-of-dimers that associate with one CheA dimer and two CheW monomers to form the minimal "core unit" necessary for signal transduction. Reconstructing in vitro chemoreceptor ternary complexes that are homogenous, functional, and exhibit native architecture remains a challenge. Here we report that His-tag mediated receptor dimerization with divalent metals is sufficient to drive assembly of native-like functional arrays of a receptor cytoplasmic fragment. Our results indicate receptor dimerization initiates assembly and precedes formation of ternary complexes with partial kinase activity. Restoration of maximal kinase activity coincides with a shift to larger complexes, suggesting that kinase activity depends on interactions beyond the core unit. We hypothesize that achieving maximal activity requires building core units into hexagons and/or coalescing hexagons into the extended lattice. This discovery may also address a previously observed density-dependent transition between signaling states. To further test this, we implemented a paramagnetic relaxation enhancement (PRE) based
solid-state NMR approach to obtain long-range (≥ 20 Å) distance constraints across the trimer of dimers interface. Overall, the work presented here shows that minimally perturbing His-tag mediated dimerization promotes assembly of chemoreceptor arrays with native architecture, and thus enabled us to gain insights into the mode of array assembly and the role of the core functional unit.
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CHAPTER 1

BACTERIAL CHEMOTAXIS AND OVERVIEW OF THIS STUDY

1.1. Introduction

As gatekeepers of the cell, membrane proteins are crucial players in cellular homeostasis. Many bacteria and archaea rely on transmembrane proteins that govern signal transduction pathways to sense and adapt to their environment. One such pathway, the chemotaxis system, enables motile bacteria to track chemical gradients of attractants or repellents by transmitting information about the extracellular chemical environment to the flagellar motor in order to alter swimming direction. The membrane proteins responsible for this process, called chemoreceptors or methyl-accepting chemotaxis proteins (MCPs), regulate a histidine kinase CheA. CheA phosphorylates a response regulator protein CheY, which in turn controls the rotation of the flagellar motor. Chemoreceptors are typically anchored in the membrane and form stable ternary complexes with CheA and a coupling protein CheW. Together, these ternary complexes cluster into exquisitely sensitive and highly ordered polar arrays. Organized as a hexagonal lattice comprised of trimers of receptor dimers at each vertex, the architecture is remarkably conserved among bacteria and archaea (Briegel et al., 2009, 2012, 2015). The chemosensory proteins of *Escherichia coli* and *Salmonella typhimurium* are the most widely and thoroughly studied, due the limited number of components that retain a high degree of sophistication, and have therefore served as a model system for both chemotaxis and transmembrane signal transduction research alike. Despite the breadth of structural and functional knowledge available today, there are still a number of open
questions including how arrays assemble and influence receptor activity and how signals are propagated through the receptor cytoplasmic domain (Parkinson et al., 2015).

In this work, we present a novel *in vitro* assembly method that can be used to trigger formation of functional and native-like arrays of the aspartate chemoreceptor cytoplasmic fragment (CF). This method makes it possible to follow the time course of assembly in a manner that could not previously be done with cryo-electron tomography (ECT) and with other assembly methods. The results provide an alternative view of the minimal unit for kinase activity and of a previous proposal that ligand-induced receptor expansion controls kinase activity. We initiated an NMR-based approach that sets the stage to obtain long-range distance constraints for the receptor trimer of dimers to test this proposal and to further define the receptor structure within the native array. Overall, this study provides a platform to follow assembly and test a proposed signaling-related conformational change.

This chapter first describes how chemoreceptors and their component proteins work together to achieve chemotaxis. This is followed by a discussion of the structural features of chemoreceptors and the remarkable arrays they form. We then outline methods of assembly needed to generate *in vitro* chemoreceptor complexes and how those have and continue to be used to test mechanisms of signal propagation. Lastly, the key objectives and findings of this study are discussed.
1.2. Background

1.2.1. Chemotaxis signal propagation, adaptation, and amplification

Motile bacteria such as *E. coli* have four to six flagella driven by reversible rotary motors that are powered by the flux of ions (Berg, 2003). The direction of flagellar rotation dictates cellular movement. Cells swim forward with counter clockwise (CCW) rotation, which forms a flagellar bundle to propel the cell forward. Conversely, clockwise (CW) rotation causes the bundle to fall apart, leading to tumbling in place. Chemoreceptors localized at the cellular poles detect gradients of attractants (amino acids and sugars) or repellents (metal ions and acids) that bind to the periplasmic ligand-binding domain. Mutational (Ames and Parkinson, 1988), cysteine-crosslinking (Chervitz and Falke, 1995), EPR (Ottemann, 1999), and NMR (Murphy et al., 2001; Isaac et al., 2002) studies indicate that ligand binding induces a 2 Å piston-like motion of one transmembrane helix relative to the others within the dimer. It remains unclear how this signal then travels down the length of the cytoplasmic domain to the membrane-distal signaling subdomain to inhibit the kinase CheA. Since the signaling mechanism is known in this region, the work presented here focuses on the cytoplasmic domain. Together with a coupling protein CheW, ternary complexes exhibit kinase-on and kinase-off output states. As illustrated in Figure 1.1, attractant binding shifts the receptors to the kinase-off state, which in turn slows the flux of CheA phosphoryl groups (yellow circles labeled “P” in Figure 1.1) to the response regulators CheY and CheB. Phospho-CheY carries the signal to flagellar motor proteins, which subsequently induce CW rotation, leading to the cell tumbling in place.
Figure 1.1. The chemoreceptor signaling pathway in E. coli. For simplicity, the full-length receptors (MCPs) are shown as single homodimers (monomers colored gray and black). In their native architecture, receptors form trimers of dimers in complex with the kinase CheA (blue when active) and CheW (cyan) (see Figure 1.3). Components shown in red reduce CheY-P levels and promote counter clockwise (CCW) flagellar rotation and a swimming response; those in green enhance CheY-P and lead to clockwise (CW) rotation and cellular tumbling. The small circles on receptor dimers indicate unmodified (white) or methylated (green) adaptation sites. The fully methylated receptor can be mimicked through mutations of the four sites to Glu (Q) in intact receptors or cytoplasmic fragments (CF).

The exquisite sensitivity and large dynamic range of signal detection (10^{-7}–10^{-3} M) (Adler, 1969) is partly mediated through a sensory adaptation that effectively resets
the ON–OFF equilibrium to a pre-stimulus level. This is accomplished by covalent modification of several glutamyl residues located in the methylation subdomain of the receptor (Figure 1.2, white and green circles) (Springer et al., 1979). Ligand binding shifts the receptor to the kinase-off state and also increases the rate of methylation by the methyltransferase CheR, causing an immediate (~sec) decrease in tumbling frequency. Adaptation then occurs in minutes, when methylation shifts the receptor back toward the kinase-on state and resets the ligand affinity (Springer et al., 1979). Likewise, a methylesterase CheB—activated by the kinase-on state via phosphorylation by CheA—hydrolyzes the receptor glutamyl ester to glutamic acid, resulting in a shift toward the kinase-off state. Both CheR and CheB are recruited to the receptor by a penta-peptide (NWETF) sequence on the flexible C-terminal tail (Wu et al., 1996).

In the cell, signal transmission is amplified across interconnected and extended arrays of chemoreceptors. Remarkably, attractant binding to one receptor can regulate roughly 35 kinases within the array (Sourjik and Berg, 2002). Moreover, the attractant response is highly cooperative with Hill coefficients ($n_H$) of 10–27 observed (Li and Weis, 2000; Sourjik and Berg, 2004; Han and Parkinson, 2014), depending on the preparation, receptor type, and methylation state. This indicates a high level of cross talk between receptors and/or CheA and CheW, and suggests that receptors operate in concert as allosteric arrays. Large polar patches of ternary complexes have been widely reported in cells by fluorescence imaging (Maddock and Shapiro, 1993; Gestwicki et al., 2000; Sourjik and Berg, 2000; Kentner and Sourjik, 2006) and electron cryotomography (ECT) (Zhang et al., 2007; Briegel et al., 2012; Liu et al., 2012). Furthermore, chemoreceptor arrays exhibit similar architecture among a diverse set of species, indicating a universally
conserved mechanism of receptor clustering (Briegel et al., 2009, 2015). Given the cooperativity and arrangement, it’s likely that interactions within the arrays are critical for function.

1.2.2. Structural features of chemotaxis receptors and their complexes

A considerable amount of structural information has been collected on soluble fragments and full-length forms of the chemotaxis proteins. Both X-ray and NMR structures have been deposited in the Protein Data Bank (PDB) for chemotaxis proteins and protein fragments originating from a number of species. We therefore have a broad understanding of many structural features for each of the chemosensory proteins.

Chemoreceptors are intertwined transmembrane homodimeric proteins that are rod-shaped and predominantly α-helical. In addition to high-resolution x-ray structures of chemoreceptor fragments, electron microscopy has shown that each intact receptor dimer is approximately 380 Å long (Wadhams and Armitage, 2004) and oriented perpendicular to the membrane (Weis et al., 2003). As shown in Figure 1.2A, there are three functional elements: (1) a periplasmic sensing domain for ligand binding that connects to four transmembrane helices, (2) a cytoplasmic, membrane-proximal HAMP domain (found in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases (Aravind and Ponting, 1999)), and (3) a cytoplasmic signaling domain comprised of methylation, flexible bundle, and protein interaction subdomains.

The x-ray crystal structures of the *E. coli* Tsr receptor cytoplasmic domain (Kim et al., 1999) and *Thermatoga maritima* TM1143 (Park et al., 2006), combined with mutagenesis studies (Falke and Kim, 2000) have shown that the signaling domain is predominantly a continuous four-helix, anti-parallel, coiled-coil with a hairpin turn at the
membrane-distal tip. The Tsr crystal structure revealed a trimer of receptor dimers (Figure 1.2B) architecture, which was further confirmed in vivo through cross-linking (Studdert and Parkinson, 2004; Parkinson et al., 2005). Remarkably, the five E. coli chemoreceptors share identical trimer contacts, allowing the lower abundance receptors (Tap, Trg, and Aer) to form mixed trimers with the higher abundance aspartate (Tar) and serine (Tsr) receptors (Gestwicki and Kiessling, 2002; Studdert and Parkinson, 2004; Gosink et al., 2006).

Chemoreceptors bind CheA at their membrane-distal tips. CheA is a large histidine kinase consisting of five domains (P1–P5, domain organization shown in Figure 1.3) that are connected by flexible linkers (Bilwes et al., 1999; Boukhvalova et al., 2002; Griswold et al., 2002; Wang et al., 2012a, 2012b). The P1 domain contains the histidine phosphorylation site. The response regulators CheY and CheB dock at the P2 domain. Dimerization of CheA is mediated through the P3 domain. P4 contains the active site for ATP binding and phosphorylation of P1. Lastly, P5 binds the receptor and CheW. Interestingly, P5 and CheW are structurally highly homologous. CheW plays a critical role in coupling CheA to the receptor to facilitate assembly and signaling, yet its precise function remains unclear.
Figure 1.2. Structure of dimeric chemoreceptors. (A) A ribbon model showing the predominantly $\alpha$-helical structure of an intact chemoreceptor dimer is was based on crystal structures of the Tsr cytoplasmic domain (PDB: 1QU7 (Kim et al., 1999)), several periplasmic domain crystal structures, and a solution NMR structure of Tsr HAMP (PDB: 2ASW) (courtesy of L.K. Thompson). Domain organization and functional regions are indicated and include: (1) periplasmic sensing domain, (2) HAMP domain, and (3) cytoplasmic signaling domain, comprised of three subdomains. The methylation sites within the methylation subdomain are shown in magenta. (B) A structural model of the cytoplasmic side of the trimer of dimers. This model (provided by A. Briegel, B. Crane, and G. Jensen) was generated by docking crystal structures of Tsr (1QU7) and ternary complex fragments (PDB 3UR1) into EM density (Briegel et al., 2012).

Electron cryotomography has shown that chemoreceptor arrays can occupy membrane surface areas ranging from 12,000 to 144,000 nm$^2$ in different species (Briegel
et al., 2009). The array features are remarkably similar: hexagonally packed lattices retain a 12 nm center-to-center spacing in at least 14 species of bacteria (Briegel et al., 2009) and archaea (Briegel et al., 2015). As previously discussed, chemoreceptor dimers associate in the membrane into trimers of dimers that are further constrained through direct contacts with CheA (Wang et al., 2012a; Li et al., 2013; Piasta et al., 2013) and CheW (Vu et al., 2012; Li et al., 2013; Pedetta et al., 2014) at the membrane-distal tip, thus forming a ternary complex. As depicted in Figure 1.3, the trimers of dimers reside at the vertices within a hexagonal lattice, surrounding a ring of alternating CheA regulatory domains (P5) and the structurally homologous CheW (Bilwes et al., 1999; Griswold et al., 2002), which form a stabilizing “baseplate”.

**Figure 1.3.** Receptor core units and hexagonal arrays. A core signaling unit is comprised of two trimers of receptor (MCP) dimers (gray), one CheA dimer (blue) and four CheW monomers (cyan). Each CheA monomer has five domains (P1–P5); the two monomers in the dimer are shown in two shades of blue. A cross section at the receptor tip that is in line with P3-P5-CheW is rotated 90° to show the top down view (center). Core units combine to form the proposed architecture of extended hexagonal array (right) with 12 nm center-to-center spacing (black line). The existence of a CheW-only ring (and two extra CheW in core unit) is currently in debate and discussed in Chapters 3–4.

Due to their highly ordered nature, chemoreceptor arrays are well suited for ECT, and advances in the technique—including subvolume averaging—have enabled
incredible three-dimensional images of both near-native cells and well-ordered \textit{in vitro} assemblies at macromolecular resolution (\(\approx 4\) nm) (Briegel and Jensen, 2017). Such images have been used to create structural models of the array by docking crystal structures into the EM density. In addition to biochemical and high-resolution structural data, these structural models have significantly improved our understanding of the hexagonal lattice and its components. For example, a crystal structure of alternating CheW and CheA-P5 rings bound to receptor fragments (PDB 3UR1) with some rotations of its components, was fit into EM density, uncovering both the organization of CheA/W rings with respect to the trimer of dimers, and how CheA-P3 domains link the neighboring rings together (Briegel et al., 2012). In parallel, Liu and coworkers published a \(\approx 3.2\) nm resolution electron density map imaged from \textit{in vivo} arrays with nearly identical hexagonal architecture (Liu et al., 2012), which also revealed the existence of CheW-only rings (Figure 1.3). CheW-only rings have been also been observed by ECT for \textit{in vitro} arrays of cytoplasmic fragments assembled onto monolayers (Cassidy et al., 2015) and are proposed to provide array stability. However, these findings are in contrast to other studies, which found excess CheW disrupts both kinase activity and trimer formation (Studdert and Parkinson, 2005; Cardozo et al., 2010). The existence of CheW-only rings is particularly interesting given the discrepancies in the field regarding its role and stoichiometry in the core unit and the arrays.

\subsection{1.2.3. Assembly of receptor complexes}

Chemotaxis by bacteria has been recognized for more than 135 years (Engelmann, 1881), and has been the focus of intense studies for several decades (Adler, 1966). Methodologies have expanded from \textit{in vivo} to a variety of \textit{in vitro} systems as a way to
probe the molecular mechanisms in simplified and controlled environments. A widely used *in vitro* preparation consists of native membrane vesicles containing intact chemoreceptors (Borkovich et al., 1992; Li and Weis, 2000; Falke and Hazelbauer, 2001; Lai et al., 2005; Erbse and Falke, 2009; Amin and Hazelbauer, 2010). The disadvantages of these preparations include protein impurities and lack of control over receptor packing (Lefman et al., 2004) and orientation (Erbse and Falke, 2009). Additionally, recent ECT studies have shown a high degree of heterogeneity upon *in vitro* reconstitution with CheA and CheW to form signaling complexes. Array sizes varied from single trimers (inverted and outward facing in vesicles) to individual and linked hexagons, and hexagonal patches exhibiting both 9 nm and 12 nm center-to-center spacing (Briegel et al., 2014a). While some native functions are at least partly preserved (ligand binding, kinase inhibition, receptor adaptation), the challenges posed by the heterogeneity of these samples led to the development of improved assembly methods that recapitulate biological functionality using purified components in an environment that mimics the native membrane.

Intact chemoreceptor trimers of dimers reconstituted into soluble nanodiscs form complexes with CheA and CheW that exhibit ligand control of kinase and methylation activities (Boldog et al., 2006). These ~10 nm nanodiscs are essentially plugs of lipid bilayers surrounded by an amphipathic membrane scaffold protein (Denisov et al., 2004), into which inactive, detergent-solubilized intact receptors will insert with random orientation and regain their function. The nanodisc dimensions prevent incorporation of more than one trimer of dimers, and maximal kinase activity is observed when receptors incorporated at levels of ≥ 5 dimers/nanodisc are reconstituted with CheA and CheW. It is proposed that kinase activity occurs in core units consisting of two trimers of dimers.
(each in a separate nanodisc) that bind one CheA dimer and two CheW monomers (Li and Hazelbauer, 2011). This 6:1:1 stoichiometry of the core unit is consistent with some studies, but not others. Moreover, nanodisc assemblies are heterogeneous, with receptors in both orientations, and they lack the native array.

In an effort to prepare homogenous in vitro complexes that restore the native array architecture, Weis and coworkers developed templating vesicles to serve as a membrane mimetic for soluble chemoreceptor cytoplasmic fragments (CF) that do not assemble into functional ternary complexes without the vesicles (Shrout et al., 2003). This approach, which has also been extended to lipid monolayers (Cassidy et al., 2015), utilizes the high-affinity interaction between the CF N-terminal hexahistidine-tag and a Ni$^{2+}$-chelating phospholipid (DOGS-NTA-Ni$^{2+}$). Recent ECT shows these templating-vesicle complexes exhibit native-like hexagonal array architecture (Briegel et al., 2014a). Alternatively, CF can also be assembled into functional arrays using the molecular crowding agent PEG (Fowler et al., 2010). The PEG-mediated arrays exhibit a double layer array of chemoreceptors flanked by two CheA/W baseplates—so-called “sandwich” arrays, that retain the canonical 12 nm hexagonal spacing. While not native for the membrane-bound aspartate receptor, some other chemotactic species exhibit cytoplasmic-only sandwich arrays. These have been imaged in Vibrio cholerae (Briegel et al., 2016), Rhodobacter sphaeroides (Briegel et al., 2014b), and Methanobacterium formicicum (Briegel et al., 2015).

1.2.4. Current views and hypotheses for signaling mechanisms

The mechanism of signal transduction within the periplasmic and transmembrane domains is widely thought to involve a 2 Å piston motion (Ames and Parkinson, 1988;
Ottemann, 1999; Falke and Hazelbauer, 2001; Murphy et al., 2001; Isaac et al., 2002). Thus, efforts are now focused on understanding signaling mechanisms within the cytoplasmic domain. Although CF arrays cannot be used to probe the effects of ligand binding, since the periplasmic, transmembrane, and HAMP domains have been removed, they are quite useful for investigating mechanisms of signal propagation through the cytoplasmic domain. Therefore, in vitro CF assembly methods have proven to be a powerful approach to probe chemotaxis signaling mechanisms in these regions, including signaling state dependent dynamics (Koshy et al., 2014; Kashefi and Thompson, 2017) and receptor conformational changes (Vaknin and Berg, 2007; Sferdean et al., 2012).

The ligand-induced expansion model put forth by Sferdean, Thompson and coworkers is rooted in an observed density dependence of signaling output (Besschetnova et al., 2008). Besschetnova assembled cytoplasmic fragments (CF) of the aspartate receptor into arrays at the lowest (4E) and highest (4Q) levels of covalent modification. Increasing the receptor density—controlled by altering available lipid surface area per CF—resulted in a cooperative increase in kinase activity and FRET efficiency, which indicated a decrease in interdimer receptor distances, for CF4E. Moreover, low CF4E surface concentrations in the presence of CheA and CheW exhibited both decreased kinase activity and increased methylation activity, consistent with the inverse activity properties of the native receptor. Sferdean showed that reconstituted intact receptors retain similar ligand affinities at both low and high receptor densities, indicating receptors do not dissociate at low densities (Sferdean et al., 2012). From these combined results, it was proposed, as illustrated in Figure 1.4, that receptors undergo a signaling-
related conformational change in which the trimers of dimers are expanded in the kinase-off state, and more compact in the kinase-on state (Sferdean et al., 2012).

**Figure 1.4.** Proposed ligand-induced expansion model for chemotaxis signaling. This model is based on observed density-dependent kinase activity and methylation for vesicle-assembled CF4E, combined with evidence for density-independent ligand affinity for reconstituted intact receptors (Besschetnova et al., 2008; Sferdean et al., 2012).

Alternatively the observed density-dependent signaling could be a result of scattered core units when assembled at low densities (with an excess of vesicle surface area) that gain full activity upon formation of larger array units (eg a hexagon) when assembled at high densities. Ultimately, it remains unclear whether the density experiments are an effect of crowding or can be attributed to an increase in array size.

### 1.3. Objectives

Current views of the assembly mechanism for arrays are based solely on the variety of complexes observed by ECT in images of reconstituted receptor complexes
While these images are suggestive of the proposed progression from trimers of dimers to core complexes to hexagons, methods are needed to directly monitor the time course of assembly of ternary complexes into hexagonal arrays and to determine when the system achieves each function.

**Figure 1.5.** Tomographic evidence of small units lead to proposed model of assembly. (A) Smaller structures include (1) two linked trimers of dimers and (2) single hexagons containing six trimers of dimers. Hexagons are linked together in (B, 3) and are presumed to combine to form a hexagonal array patch (C, 4). Adapted from (Briegel and Jensen, 2017).

To address this, Chapter 3 describes a novel mode of assembling *in vitro* chemoreceptor arrays that mimic native structure and function. We show that soluble cytoplasmic fragments (CF) of the aspartate chemoreceptor fused with N-terminal His-tags dimerize upon addition of divalent metals, and form arrays with native architecture, stoichiometry, and kinase activity. The metal-mediated His-tag dimerization strategy provides a new approach to stabilize multi-protein assemblies for analysis of protein mechanisms within their native complexes.

Chapter 4 describes the use of this method to monitor the kinetics of assembly and gain insight into the mechanism and the minimum functional unit. ECT is best suited to detect large and highly ordered complexes, and thus cannot be used to detect small and/or less ordered species and to quantify the distribution of species during assembly of the
array. Therefore, we turned to size exclusion chromatography (SEC) to probe size changes over time. Other *in vitro* assembly methods for CF complexes are incompatible with SEC; vesicles are significantly larger than the proteins and would therefore mask the size changes that occur during assembly of the complexes, and the high concentrations of PEG8000 required for assembly would foul the column. The metal-mediated assembly, however, was perfectly suited for SEC analysis to track the process of assembly and probe array formation in parallel with kinase activity. We find that complete binding of CheA and CheW formation does not immediately yield full kinase activity. This suggests an alternative to the ligand-induced expansion model: rather than modulating an expansion/contraction transition of the receptor, high density assembly conditions may lead to higher activity by causing core units to coalesce into larger complexes.

Lastly, Chapter 5 lays the groundwork to obtain NMR distance constraints for the receptor trimer of dimers (which is absent in crystal structures of complexes and distorted in crystal structures of CF alone) and to test whether the trimer expands in the kinase-off state. We initiated a paramagnetic relaxation enhancement (PRE) based solid-state NMR approach to obtain long-range (≥ 20 Å) distance constraints across the trimer of dimers interface. By incorporating a nitrooxide spin label with 75% efficiency, we are able to observe PRE effects in the membrane-proximal region of CF that are consistent with predictions based on the structural model.

In summary, the work presented here uses a novel His-tag mediated assembly tool to provide new insights into the mode of chemoreceptor array assembly and the role of the core functional unit, and lays the groundwork to further interrogate signaling mechanisms in the context of the native array.
CHAPTER 2

MATERIALS AND METHODS

2.1. Proteins

2.1.1. Expression Plasmids and Cloning

The *E. coli* expression strain BL21(DE3) was used to overproduce all proteins used in this study. TEV-cleavable His-tagged CheA, CheW, and CheY plasmids were constructed by Aruni P. K. K. Mudiyanselage as previously described (Kashefi and Thompson, 2017). Expression plasmids for the aspartate chemoreceptor Tar cytoplasmic fragment (CF), containing residues 257-553 with an N-terminal hexahistidine-tag, encode glutamine at all four primary methylation sites (pHTCF4Q) or glutamic acid (pHTCF4E) and were previously constructed (Wu et al., 1996). A CF4E mutant (pCF4E.S487C) was generated previously (Mudiyanselage et al., 2013) and used in this study for spin labeling and NMR (see section 2.8.2 and Chapter 5). For protein production, all CF plasmids were co-transformed into BL21(DE3) with pCF430 (encoding lacI and tetR).

To generate a TEV-protease (tobacco etch virus)-cleavable His-tagged version of pHTCF4Q, the recognition and cleavage sequence for TEV-protease (ENLYFQG) was inserted directly following the N-terminal His6-tag of pHTCF4Q using site-directed mutagenesis and polymerase chain reaction (PCR). Forward (5’-GAAAAACCTGTATTTTCAGGGCGGA TCCCCTATGCAACG-3’) and reverse (5’-GCCCTGAAAATACAGGTTTTCGATGGGTGGGTGATG-3’) overlapping primers were designed to include the TEV-protease recognition sequence and were purchased from Eurofins Genomics. The PCR reaction was done in a thermocycler (Bio-
Rad MJ Mini), and reagents, including Phusion DNA polymerase, dNTPs, and DpnI were purchased from New England Biolabs. The PCR product (pTEVCF4Q, ampR) was subjected to DpnI digestion and transformed into DH5αF’ for plasmid purification. Following sequence verification (Genewiz), pTEVCF4Q was co-transformed with pCF430 (encoding lacIq and tetR) into BL21(DE3) for protein expression.

2.1.2. Tar cytoplasmic fragment (CF)

BL21(DE3) E. coli cells expressing H6CF (pHTCF4Q, ampR) or H6TEV-CF (pTEVCF4Q, ampR) co-transformed with pCF430 (tetR) were grown in Luria-Bertani (LB) broth with ampicillin (100 µg/mL) and tetracycline (10 µg/mL) at 37°C until the optical density at 600 nm reached approximately 0.6. The temperature was decreased to 15°C for induction with 1 mM IPTG for 16–18 h. Cells were pelleted, resuspended in lysis buffer [75 mM K2HPO4 (pH 7.5), 500 mM NaCl, 5 mM imidazole, and 1 mM EDTA], and lysed with a microfluidizer at 16K psi. PMSF (1 mM) was added every hour following cell lysis to limit proteolysis. Cell debris was separated by centrifugation and the supernatant applied to a HisTrap FF Ni2+-NTA affinity column (GE Healthcare) equilibrated with 10 column volumes of 75 mM K2HPO4 (pH 7.5), 500 mM NaCl, and 5 mM imidazole. The column was washed with 5 column volumes of 75 mM K2HPO4 (pH 7.5), 500 mM NaCl, and 50 mM imidazole, before elution with 75 mM K2HPO4 (pH 7.5), 500 mM NaCl, and 500 mM imidazole. Eluted protein was verified by SDS-PAGE and fractions were pooled prior to treatment with 5 mM EDTA to chelate any Ni2+ stripped from the column, followed by dialysis against 75 mM K2HPO4 (pH 7.5) 75 mM KCl with 7 kDa molecular weight cutoff SnakeSkin tubing (Thermo Scientific) to remove EDTA and imidazole and to exchange high NaCl concentrations for low KCl.
concentrations. Typically half of the purified H₆TEV-CF was subjected to His-tag removal by TEV-protease cleavage.

For isotopic labeling, BL21(DE3) cells harboring pCF4E.S487C (amp<sup>R</sup>) and pCF430 (tet<sup>R</sup>) were grown in M9 minimal media using natural abundance glucose and (¹⁵NH₄)₂SO₄ as the carbon and nitrogen sources. Starter cultures were prepared from single colonies grown on LB amp/tet plates that were inoculated into ~5 mL LB broth supplemented with ampicillin (100 µg/mL) and tetracycline (10 µg/mL), and grown until ~0.6 OD<sub>600</sub> at 37°C. This was used to inoculate 1 L minimal media, which was typically grown overnight at 30°C with 200 rpm shaking until OD<sub>600</sub> ~ 0.7–0.9. Protein production was induced with 1 mM IPTG for 5 h at 25°C. Cells were harvested and purification as described above, with the exception that 2 mM TCEP was included in all buffers except the final dialysis buffer for protein storage.

2.1.3. TEV-cleavable His-tagged CheA, CheW, and CheY

Plasmids encoding TEV-cleavable His-tagged CheA (pTEVcheA, kan<sup>R</sup>), CheW (pTEVcheW, kan<sup>R</sup>), and CheY (pTEVcheY, kan<sup>R</sup>) were expressed in BL21(DE3) and grown at 37°C in LB broth supplemented with 50 µg/mL kanamycin. At an optical density at 600 nm of ~0.7–0.9, 1 mM IPTG was added to induce expression for 3 h before the cells were harvested and purified with HisTrap affinity chromatography as described for CF (section 2.1.2). A different buffer system for CheA, CheW, and CheY purification was used: lysis buffer [75 mM Tris-HCl (pH 7.4), 100 mM KCl, and 1 mM EDTA], equilibration buffer [75 mM Tris-HCl (pH 7.4), and 100 mM KCl], wash buffer [75 mM Tris-HCl (pH 7.4), 100 mM KCl, and 10 mM imidazole], and elution buffer [75 mM Tris-HCl (pH 7.4), 100 mM KCl, and 250 mM imidazole]. Following elution,
fractions containing protein were verified by SDS-PAGE, pooled and treated with 5 mM EDTA, then dialyzed as for CF into 75 mM Tris-HCl (pH 7.4) and 100 mM KCl prior to His-tag removal.

2.1.4. TEV-protease

The plasmid pRK793 (amp<sup>R</sup>) encoding N-terminally His-tagged TEV-protease (a gift from D. Waugh, Addgene plasmid 8827) (Kapust et al., 2001) was expressed in BL21(DE3)-RIL (amp<sup>R</sup>). Cells were grown at 37°C in LB broth containing 150 µg/mL ampicillin and 50 µg/mL chloramphenicol. When an optical density of 0.6 was reached, protein production was induced at 30°C with 1 mM IPTG for 4 h. The protein purification protocol and buffer system for CheA, CheW, and CheY was also used here (see section 2.1.3).

2.1.5. His-tag cleavage

Following purification, CheA, CheW, CheY, and H<sub>6</sub>TEV-CF were incubated with TEV-protease at a 50:1 His-tagged protein:TEV-protease molar ratio, and the mixture was shaken at 4°C overnight and then 25°C for 3 h. Complete cleavage was confirmed by a gel shift observed by SDS-PAGE. Cleaved proteins were separated from TEV-Protease by passage through the equilibrated HisTrap column, collected, and concentrated with 10 kDa centrifugal concentrators (Amicon). Protein concentrations were measured with a BCA assay (Thermo Scientific), and proteins were frozen in liquid nitrogen and stored at -80°C.
2.2. Lipid Vesicles

A mixture of DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and the nickel-chelating lipid DOGS-NTA-Ni$^{2+}$ (1,2-dioleoyl-sn-glycero-3-{$[N-(5-amino-1-carboxypentyl)-iminodiacetic acid]succeyl}$) (Avanti Polar Lipids) were combined in chloroform at a 1.5:1 DOPC:DOGS-NTA-Ni$^{2+}$ molar ratio. Lipids were dried into a thin film with a stream of N$_2$ gas and left under vacuum for 1 h before being rehydrated with 1× PKB [phosphate kinase buffer: 50 mM K$_x$H$_y$PO$_4$ (pH 7.5), 50 mM KCl, and 5 mM MgCl$_2$] and vortexed extensively (30 s vortex, 5 min rest, repeated three times) to form multilamellar vesicles, followed by five cycles of 3-minute freezing/thawing. Freeze/thaw cycles were accomplished as follows: the lipids were dipped in liquid nitrogen for 20 s to freeze, left on the bench for 3 min, and then held for 3 min in a 37°C water bath. Extrusion was then performed with an extrusion apparatus (Avanti Polar Lipids) using a 100 nm diameter pore size polycarbonate membrane. High-concentration stocks at 3 mM total lipid (1.8 mM DOPC and 1.2 mM DOGS-NTA-Ni$^{2+}$) were prepared and a final [lipid]$_{total}$ of 725 µM was used for assembly. The lipid concentrations were optimized in parallel with CheA and CheW concentrations to maximize kinase activity under conditions under which the available vesicle surface area could accommodate all CF as hexagonal arrays (See Appendix).

2.3. Complex Assembly

Preparation of ternary complexes was performed as previously described for vesicles (Shrout et al., 2003) and PEG (Fowler et al., 2010) with some modifications. Briefly, vesicle samples were prepared by combining the following (in order): autoclaved water, 1 mM PMSF dissolved in 100% ethanol, phosphate kinase buffer (PKB) from 5x
stock, 12 µM CheA, 24 µM CheW, 30 µM CF (H₆CF, H₆TEV-CF, or ΔH₆CF), 725 µM vesicles. PEG-assembled samples were made by combining the following (in order): autoclaved water, 1 mM PMSF, PKB, 12 µM CheA, 20 µM CheW, 50 µM CF (H₆CF, H₆TEV-CF, or ΔH₆CF), 7.5% w/v PEG8000 (from a 40% w/v stock), 4% w/v d-trehalose (from a 40% w/v stock). Metal-assembled samples were prepared under protein and buffer conditions equivalent to those optimized for vesicle-assembly, except that vesicles were replaced with metal salts (NiCl₂•6H₂O, ZnCl₂ anhydrous, CuCl₂•2H₂O, MnCl₂•4H₂O, and CoCl₂•6H₂O, all obtained from Sigma-Aldrich) prepared at ~1 mM in autoclaved Milli-Q H₂O. Metal stock concentrations were verified before use (see section 2.5). All metal-mediated assembly experiments outside of the metal titration (see section 3.2.1) used metal concentration conditions that produced the largest quantity (based on sedimentation) of maximally active complexes (NiCl₂ was 180 µM, ZnCl₂ was 300 µM, and CoCl₂ was 360 µM). For the mixed NiCl₂ and PEG-mediated CF4E assembly (see section 3.2.7), 180 µM NiCl₂, 7.5% w/v PEG, and 4% w/v d-trehalose were used; protein concentrations matched those of PEG-mediated assembly).

Once combined, samples were incubated in a 25°C water bath for 4 h or overnight before use. For kinetic experiments, biochemical assays or SEC-MALS were performed at time points immediately following addition of all complex components.

2.4. Biochemical Assays

2.4.1. CheA kinase activity

Kinase activity was measured using an enzyme-coupled ATPase assay that couples NADH oxidation to consumption of ATP (Nørby, 1988; Shrout et al., 2003).
Briefly, activity measurements were conducted immediately following a 100-fold dilution of assembled complexes into PKB containing 55 µM CheY, 2.2 mM phosphoenolpyruvate, 4 mM ATP, 250 µM NADH, and 20 units of PK/LDH enzyme (Sigma-Aldrich). New CheY preparations were tested at both 55 µM (1X) and 110 µM (2X) to confirm the rates were unchanged and excess CheY was available so that phosphorylation of CheA was rate limiting. The background activity of CheY under identical conditions in the absence of the complex was subtracted before calculation of kinase activity. The kinase activity (inverse seconds) was determined from the linear change in absorbance at 340 nm \( \frac{d[ATP]}{dt} = -6220 \left( \frac{dA_{340}}{dt} \right) \) over 1.5 min. Total activity is based on the full amount of CheA in the sample (12 µM), which is divided by the amount of CheA in the complex (quantified by sedimentation, as described in 2.4.2) to compute the specific activity of CheA in complexes with CF.

2.4.2. Binding assay

The amount of bound protein was determined with a sedimentation assay carried out at 25°C in a benchtop ultracentrifuge (Beckman TLX, TLA 120.2 rotor, 125,000g, 30 minutes). Typically, a 35 µL aliquot of sample (in either assembled ternary complexes or individual proteins) was centrifuged. Following sedimentation, the supernatant was carefully removed and placed into a clean Eppendorf tube to minimize contamination of free protein in the pellet containing the bound fraction of proteins. The pellets were slowly resuspended to the original volume in autoclaved Milli-Q H₂O and vortexed to homogenize resuspension. Aliquots of Total (free plus bound protein, before sedimentation), Supernatant (free protein), and Pellet (bound protein) were run on SDS-PAGE (12.5% acrylamide) and stained with Gel-code Blue (Pierce Chem. Co.). Gels
were imaged by densitometry with a Gel Doc EZ Imager (Bio-Rad) and the integrated intensities were analyzed with ImageJ software. (Schneider et al., 2012) The bound concentrations were computed as \( \frac{I_{\text{Pellet}}}{I_{\text{Total}}} \times [\text{Protein}]_{\text{Total}} \) for each protein to be quantified. For the excess CheA and CheW conditions used in this study, quantification of bound protein by the pellet was more reproducible than \( I_{\text{Total}} - I_{\text{Supernatant}} \). [For future studies, we suggest making the Pellet gel sample from the entire resuspended pellet. This might further increase reproducibility, by making it unnecessary to achieve a complete and homogeneous resuspension of the pellet.] Gels included two additional lanes of the Total sample diluted 6x and 12x such that a calibration curve from three intensities for known concentrations could be calculated. The y-intercept was then subtracted from each integrated intensity to correct for the gel background intensity and more accurately estimate the fraction of protein bound in a complex.

2.4.3. Methylation assay

Methylation assays were carried out on various complexes following overnight assembly. Vesicle- and Ni(II)-mediated assemblies were prepared normally (section 2.3), while NiCl\(_2\) + PEG-mediated assemblies contained: 180 \( \mu \)M NiCl\(_2\), 7.5% PEG8000, 4% D-trehalose, 50 \( \mu \)M CF, 12 \( \mu \)M CheA, and 20 \( \mu \)M CheW. Methylation was initiated upon the addition of 6 \( \mu \)M CheR (prepared previously by Guoyong Li) and 10 \( \mu \)M s-adenosyl-L-methionine (SAM, Sigma Aldrich). Aliquots were removed after 0.1 and 4 h, quenched by the addition of gel-loading buffer, and analyzed by SDS-PAGE. Quantification was done with ImageJ (Schneider et al., 2012).
2.5. Inductively Coupled Plasma Optical Emission Spectroscopy

Metal concentrations of the ~1 mM aqueous metal stocks (NiCl$_2$, ZnCl$_2$, CuCl$_2$, MnCl$_2$, CoCl$_2$) and the amounts of metal bound to H$_6$CF (alone or in complex) were measured using a PerkinElmer Optima DV4300 inductively coupled plasma optical-emission spectroscopy (ICP-OES) instrument. Metal standards (TraceCERT by Sigma-Aldrich) were used to produce a calibration curve for each metal at 0.1, 0.2, 0.75, 1, 2, and 10 ppm. Metal stocks were diluted 100-fold and protein-metal samples were diluted 10-fold into MilliQ water that was also used to blank the instrument. Protein samples were prepared by overnight incubation of 30 µM H$_6$CF (alone or with 12 µM CheA and 24 µM CheW) with the metals at the optimal concentrations that produced active complexes (180 µM NiCl$_2$, 300 µM ZnCl$_2$, or 360 µM CoCl$_2$) in a 25°C water bath. Aliquots of these samples were then subjected to ultracentrifugation (60k rpm for 30 minutes at 25°C) to separate large metal-protein complexes from unbound protein and metal in the supernatant. Bound protein and metals in the pellet were carefully resuspended to the initial volume in MilliQ water. The entire resuspended pellet was used for ICP-OES analysis so that any chunks of protein pellet were still part of the sample measured.

2.6. Size Exclusion Chromatography with Multi-Angle Light Scattering

Size exclusion chromatography and multi-angle light scattering (SEC-MALS) was used to monitor metal-mediated complex formation. The SEC is coupled to an 18-angle static light scattering detector (DAWN HELEOS-II), a dynamic light scattering detector (WyattQELS), and a differential refractometer (Optilab T-rEX, Wyatt Technology). Proteins were injected onto a TSKgel G2000SW$_{XL}$ column maintained at
4°C and pre-equilibrated overnight with PKB buffer (pH 7.0) kept on ice. Typical injections were 75 µL of 0.5-3 mg/mL protein. Data was collected every second for 30 minutes at a flow rate of 0.5 mL/min. Protein elution was monitored at A$_{280}$ and A$_{214}$, in addition to static and dynamic light scattering. The accompanying ASTRA VI software was used to determine molar mass, peak polydispersity, and UV peak areas for all individual peaks and the entire elution to determine % loss in the pre-column 0.22 µm filter.

2.7. Electron Cryo-Tomography (ECT)

All ECT presented in this study was done by our collaborators Ariane Briegel and Wen Yang at the NeCEN Facility run by the University of Leiden. Samples were prepared by mixing protein assemblies (see section 2.3) with a bovine serum albumin-treated 10 nm colloidal gold solution (Cell Microscopy Core, Utrecht University, Utrecht, The Netherlands). After brief vortexing, 3 µL mixtures were applied to freshly plasma-cleaned R2/2 copper Quantifoil grids (Quantifoil Micro Tools). Blotting and plunge freezing in ethane were done in approximately 1 second with a Leica EMGP (Leica microsystems, Wetzlar, Germany), set at 20°C and 95% humidity. Grids were stored in liquid nitrogen until imaging. Data was collected on a Titan Krios transmission electron microscope from Thermo Fisher Scientific (formerly FEI; Hillsboro, Oregon) with a field emission gun operating at 300 kV. The microscope was equipped with a Gatan (Pleasanton, CA) image filter and a Gatan K2 Summit direct electron detector. Data acquisition was done using Tomography 4.0 (Thermo Fisher Scientific) in batch mode. Tilt series for PEG and Ni(II)-assembled H$_6$CF ternary complexes were collected at a nominal magnification of 42,000x and 33,000x, respectively. A discontinuous tilt scheme
was used for imaging, taking images from 0° to −60° followed by 0° to +60° with a 2° tilting increment. The cumulative dosage for each tilt series was 80 e/Å². Defocus was set to -8 µm. The tilt series for Ni(II)-assembled H₆CF (without CheA or CheW) and PEG-assembled ΔH₆CF complexes were collected using the same tilt scheme but at a defocus of -300 nm with volta phase plate (VPP) (Thermo Fisher Scientific). The VPP was heated to 225°C and activated for 80s before each tilt series aiming to generate an approximate phase shift of 90° (Danev et al., 2014). An extra 5s conditioning time was applied between each tilt image. All images were acquired using the low-dose routine integrated in the Tomography software. Drift correction and tilt series alignment were performed with software package IMOD (Kremer et al., 1996; Mastronarde, 1997). Tomograms were reconstructed using both weighted back-projection (WBP) and 9 iterations of simultaneous iterative reconstruction (SIRT). PEET was used for subvolume averaging (Nicastro et al., 2006). Visualization and image analysis were carried out with Image J and Chimera (Pettersen et al., 2004; Schneider et al., 2012).

2.8. Solid-State Nuclear Magnetic Resonance

2.8.1. Paramagnetic spin labeling

The nitrooxide paramagnetic spin label MTSL [(1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl)methanethiosulfonate] was purchased from Toronto Research Chemicals, immediately solubilized in 100% acetonitrile to a stock concentration of 100 mM, and stored at −20°C wrapped in parafilm and aluminum foil to prevent evaporation and light damage.
To prepare the CF4E.S487C for labeling, dimers were first reduced with a 5 mM TCEP treatment for 30 min in a 25°C water bath. Approximately 2 mL protein was injected per 5 mL HisTrap desalt column (GE Healthcare) equilibrated with 75 mM K$_2$H$_2$PO$_4$ (pH 7.4) and 50 mM KCl using a 5 mL syringe. For larger protein volumes, multiple 5 mL desalt columns were used in series. Initially, the flow through was collected in 0.5 mL fractions and the protein was eluted at 2.0–4.5mL (as monitored by A$_{280}$ and SDS-PAGE). This elution profile was highly reproducible for a 2 mL injection volume into a single 5 mL column, and generally imparted a 1.3-fold dilution. It took at least 1.5 hours following the TCEP removal desalt column to identifying the protein-containing fractions with SDS-PAGE so this step was eliminated once the reproducibility of the desalt elution profile was confirmed. A number of spin labeling protocols were tested, including binding the protein to a Ni$^{2+}$-affinity column and flowing an 800 µM MTSL solution (25 mL of 75 mM K$_2$H$_2$PO$_4$ (pH 7.4), 50 mM KCl and 200 µL of 100 mM acetonitrile-dissolved MTSL) through the column in a loop overnight, and incubation of 200–800 µM CF with 20-fold excess MTSL in 75 mM K$_2$H$_2$PO$_4$ (pH 7.4) and 50 mM KCl for times and temperatures ranging from 4–72 hours and 4–25°C. We found that the least dimers formed when the protein was eluted from the desalt column for TCEP removal directly into a tube containing a 20-fold molar excess of MTSL to minimize dimer formation. This solution was then left gently stirring overnight at 4°C. MTSL was subsequently removed with another desalt column, and the eluted protein concentration was measured by BCA assay, since MTSL absorbs light at 280 nm (a 1 mM solution of MTSL in water is ~0.7 at 280 nm, but does not react with the BCA assay reagents).
2.8.2. NMR Sample preparation

Each NMR sample was assembled as 14 mL of the vesicle-mediated complex and incubated at 25°C overnight. The kinase activity and sedimentation were measured prior to packing the NMR rotor to confirm successful assembly. Total kinase activities were typically 5–7 s⁻¹ and protein binding stoichiometries were ~ 6 CF:1 CheA:2 CheW. The samples were pelleted by ultracentrifugation for 2.5 h at 25°C with 24,000 rpm in a Beckman Ti70 rotor. The pellet was packed into a 50 µL restricted volume 4 mm Bruker rotor by first transferring the pellet to a 200 µL flame-sealed pipette tip with a spatula. The tip was placed into a test tube, stabilized with Kimwipes, and gently centrifuged (a 5 second pulse up to ~6k rpm) to move the pellet to the tip base. The flame seal was removed with a razor and inserted into the rotor and centrifuged as before. The top plastic insert and cap were then used to estimate the level to pack the rotor, and subsequently used to seal it tightly. Approximately 45 mg of assembled complex were packed into each rotor. The mass of CF in each sample was calculated as (total volume of complex) × (CF concentration) × (packed sample weight)/(total pellet weight), resulting in ~286 nmol of labeled CF for both MTSL-labeled and non-labeled U-¹⁵N-CF4E.S487C NMR samples.

2.8.3. Spectroscopy and data analysis

All NMR experiments were done on a 14.1 T Bruker Avance III spectrometer (¹H = 600 MHz, ¹³C = 150 MHz, ¹⁵N = 60 MHz) in a 4 mm E-free HCN probe. Sample temperature was estimated by a calibration of the chemical shift changes of TmDOTP (Zuo et al., 1998) dissolved in DMPC vesicles at ionic strengths similar to protein samples; heating due to decoupling and MAS was counterbalanced with VT gas flow.
cooling, for an effective sample temperature of ~10–15°C. A one-dimensional (1D) sequence for cross-polarization/magic angle spinning (CP/MAS) was used with 11.1 kHz MAS, a contact time of 1.5 ms, and a recycle delay of 1 s. The 90° pulses were 2.8 µs and 5.6 µs for ¹H and ¹⁵N, respectively, with SPINAL-64 ¹H decoupling at 92.5 kHz. Each 1D spectrum contained the combined FIDs for 9600 scans (collected in 2400 scan blocks), for a total experiment time of ~3 hr.

Topspin 3.2 was used for all data processing. Chemical shifts were referenced to adamantane at 40.5 ppm (relative to DSS at 0 ppm), and no line broadening was applied. Spectra for with or without spin label were overlaid in the Topspin multiple display feature without additional scaling; the difference spectrum was likewise created using the Topspin, but the intensity was scaled up 2-fold for the final comparison in Figure 5.6.
CHAPTER 3

METAL-MEDIATED HIS-TAG DIMERIZATION FOR ASSEMBLY OF CHEMORECEPTOR ARRAYS

This chapter describes the use of divalent metals to initiate chemoreceptor His-tag dimerization and drive assembly of native-like arrays \textit{in vitro}. Most of this chapter was published in the article “His-tag-mediated dimerization of chemoreceptors leads to assembly of functional nanoarrays” in \textit{Biochemistry} (Haglin et al., 2017).

3.1. Introduction

Many fundamental biological processes are carried out by multi-protein complexes (Alberts, 1998; Marsh and Teichmann, 2015). To fully understand how these processes occur within the cell, methods are needed both for monitoring such processes \textit{in vivo} and for isolation and/or reassembly of such complexes for \textit{in vitro} structure–function analysis. Isolation and reassembly of complexes often requires modifications or deletions of portions of the protein components: for instance, deletion of transmembrane domains of membrane proteins may be needed to prepare homogeneous complexes for structural or biophysical studies. Typically, such truncations also delete stabilizing interactions, and it becomes difficult to reassemble complexes with native-like architecture and activity. We have developed a novel strategy for the stabilization of assemblies with native structure and activity for \textit{in vitro} mechanistic studies.

Bacterial chemotaxis receptors are an ideal system for understanding the molecular details of signal transduction by membrane proteins and their complexes. Chemotaxis receptors are dimeric transmembrane proteins anchored in the inner–
membrane in large patches at the poles of the cell, which are responsible for sensing and responding to environmental stimuli. Upon ligand binding to the receptor periplasmic domain, a signal is transmitted down the ~300 Å length of the receptor to control the autophosphorylation activity of an associated histidine kinase, CheA, which plays a central role in the signal transduction pathway. In addition to forming a complex with CheA and an adapter protein CheW, receptors form large clusters (a membrane surface area of 53000 nm$^2$ for *E. coli* (Maddock and Shapiro, 1993; Zhang et al., 2007; Briegel et al., 2009) that enable extraordinary levels of cooperativity and sensitivity (Sourjik and Berg, 2002, 2004). The native system is built of trimers of receptor dimers that each form one vertex of a hexagon within the membrane and are stabilized at the membrane-distal tip of the receptor by hexagonal rings of alternating CheA and CheW (Briegel et al., 2012; Liu et al., 2012; Cassidy et al., 2015). It is challenging to prepare homogeneous samples of functional chemoreceptor complexes *in vitro* for structure–function analysis. Complexes of the intact membrane-bound receptor with CheA and CheW are not homogeneous, in part due to the inability to control orientation of the receptor within membrane vesicles (Sferdean et al., 2012; Briegel et al., 2014a) or nanodiscs (Boldog et al., 2006). In principle, the membrane orientation issue can be addressed by using a cytoplasmic fragment of the receptor, but this truncated construct does not form functional complexes with CheA and/or CheW in solution. Presumably, this is because the missing transmembrane and periplasmic domains are needed to provide protein–protein interactions that stabilize the receptor dimer and geometrical alignment within the membrane that promotes assembly of the array.
In an elegant combination of membrane mimetics and nano-scale assembly tools, Weis and coworkers developed templating vesicles that promote the assembly of membrane-associated multi-protein complexes from soluble component proteins. This technology harnesses the high affinity interaction between histidine imidazole groups and the Ni(II)-NTA functional group originally developed for immobilized metal affinity chromatography protein purification (Hochuli et al., 1987). The N-terminally His-tagged cytoplasmic fragment (CF) of the aspartate chemoreceptor binds to vesicles containing lipids with nickel-chelating headgroups (DOGS-NTA), which enables binding of CheA and CheW into ternary complexes with kinase and methylation activity that mimic native signaling states (Shrout et al., 2003; Besschetnova et al., 2008). Molecular crowding agents such as PEG8000 provide an alternate means of driving assembly of functional complexes, with no requirement for membrane vesicles (Fowler et al., 2010). Both assembly methods result in extended hexagonal arrays with native-like architecture (Briegel et al., 2014a), and PEG-mediated assembly forms sandwich lattices remarkably similar to cytoplasmic chemoreceptor arrays seen in some bacterial species (Briegel et al., 2014a, 2016).

Here we report a novel method for assembling functional arrays \textit{in vitro}. We show that adding divalent metal salts to ternary mixtures of His-tagged CF, CheA, and CheW promotes receptor dimerization and assembly into active complexes with native stoichiometry and architecture. This dimerization strategy may prove to be valuable for assembly of other complex systems, particularly given the widespread use of polyhistidine-tags as a convenient and effective affinity tag capable of facilitating a variety of applications.
Cornelissen, van Hest, and coworkers discovered that metal-ion-induced interactions between His tags could be used to stabilize a capsid assembly that is otherwise unstable in the absence of a high negative charge from either encapsulated RNA or low pH. By the simple addition of divalent metals to the His-tagged cowpea chloric mottle virus capsid protein, capsid structures with native particle size form at neutral pH and can be used to encapsulate other proteins or drugs (Minten et al., 2011; Van Eldijk et al., 2016). Their model suggests the metal promotes His-tag-to-His-tag interactions that stabilize the large oligomeric capsid structure. This chapter describes a similar phenomenon that we demonstrate is a metal-mediated dimerization. His-tag mediated dimerization provides a new approach to stabilize multi-protein assemblies for analysis of protein mechanisms within their native complexes.

3.2. Results and Discussion

3.2.1. Divalent metals restore chemoreceptor ternary complex formation and function

Assembling aspartate chemoreceptor cytoplasmic fragments (CF) into functional arrays with CheA and CheW has thus far been accomplished with templating vesicles (Shrout et al., 2003), lipid monolayers (Cassidy et al., 2015), and with molecular crowding agents (Fowler et al., 2010). Under conditions similar to those used to form functional complexes with templating vesicles, we have discovered that some divalent metals are sufficient to restore function, with no requirement for vesicles. A series of metal titrations reveal the conditions needed to restore both kinase activity of CheA (measured with an enzyme-coupled ATPase assay, using the native phosphoryl acceptor protein CheY) and ternary complex formation (measured with sedimentation assays).
Five divalent metal chlorides were tested (NiCl$_2$, ZnCl$_2$, CoCl$_2$, CuCl$_2$, and MnCl$_2$) at concentrations ranging from 0 to 540 µM, while keeping protein concentrations constant at levels that promote maximal incorporation of CF into functional complexes (30 µM CF plus excess CheA (12 µM) and CheW (24 µM)). As shown in Figure 3.1, NiCl$_2$ promotes active complex formation at the lowest concentrations, with maximum kinase activity reached at 180 µM NiCl$_2$. The optimal ZnCl$_2$ concentration for kinase activity is 300 µM, and the total kinase activity is 90% of the highest observed for NiCl$_2$. While CoCl$_2$ also produced active complexes in this titration series, the highest kinase activity (reached at 360 µM CoCl$_2$) is only 50% of the optimal NiCl$_2$ sample. Thus the relative efficiency of these metals for promoting assembly of functional complexes is NiCl$_2$ > ZnCl$_2$ > CoCl$_2$.

Interestingly, the sedimentation trends for CF, CheA, and CheW with each metal titration track mostly with the respective onset of activity (Figure 3.2). For example, NiCl$_2$-assembled complexes show both activity and sedimentation beginning at low NiCl$_2$ concentrations, and ZnCl$_2$-assembled complexes show nearly zero sedimentation until 180 µM ZnCl$_2$, where activity is first observed. Furthermore, maximal sedimentation of CF, CheA, and CheW is reached for most cases under conditions that give maximal activity, and the CoCl$_2$ titration reaches activity and sedimentation of only 50% of the optimal NiCl$_2$ sample. Thus three divalent metals restore the ability of the chemotaxis receptor CF to assemble with CheA and CheW into sedimentable complexes that activate the kinase CheA.

Interestingly, NiCl$_2$ concentrations higher than 180 µM are clearly detrimental to kinase activity, and sedimentation of CF increases an additional 15%, while CheA and CheW sedimentation remain constant (Figure 3.2). In contrast, ZnCl$_2$ and CoCl$_2$ cause
only a modest decrease in activity at high concentrations (and no significant additional sedimentation).

Neither CuCl$_2$ nor MnCl$_2$ restored formation of active complexes. Kinase activity throughout the titration is similar to that of the proteins with no metal ($< 1$s$^{-1}$). Furthermore, all samples with CuCl$_2$ and MnCl$_2$ also had very low protein sedimentation of $<15\%$, again similar to levels for the no-metal control, with the exception of CuCl$_2$ and CheA. At high [CuCl$_2$], nearly all of CheA sedimented (data not shown). Since there is no appreciable kinase activity or sedimentation of CheW and H$_6$CF, we can conclude that CuCl$_2$ is most likely causing aggregation of CheA.
Figure 3.1. Three divalent metals promote kinase. All experiments use excess CheA and CheW, under conditions optimized for maximal incorporation of CF into complexes with maximal kinase activity (30 µM H₆CF, 12 µM CheA, 24 µM CheW). Additions of NiCl₂ (filled circles), ZnCl₂ (filled triangles), or CoCl₂ (filled diamonds) each promote kinase activity, with NiCl₂ promoting the highest activity, followed by ZnCl₂ and then CoCl₂. Both CuCl₂ (open circles) and MnCl₂ (open triangles) do not promote activity: activity comparable to the no-metal control is observed at all metal concentrations. As a positive control, PEG-mediated samples were run in parallel, with typical total kinase activities ~11 s⁻¹. Arrows denote maximally active conditions used for further studies, including metal:protein stoichiometry measurements listed in Table 3.1. Activities are averages of four to eight replicates measured on two or three days; error bars indicate ± one standard deviation. For this plot, measurements on each day were averaged, and the averages for all days were then combined, with propagation of the errors. Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.
Figure 3.2. Divalent metals that promote kinase activity also lead to protein sedimentation. Quantification of H$_6$CF, CheA, and CheW sedimentation for (A) NiCl$_2$, (B) ZnCl$_2$, and (C) CoCl$_2$ titration samples shown in Figure 3.1. All samples contain 30 µM H$_6$CF, 12 µM CheA, and 24 µM CheW, but sedimentation is typically less than 100%, particularly for CheA and CheW which are added in excess to drive assembly. Approximate stoichiometries of the functional complexes were estimated using averages of the sedimented concentrations for all sample conditions with maximal activity (gray shaded region): 6:1.3:2.9 for Ni(II)-mediated assembly, 6:1.2:2.7 for Zn(II)-mediated assembly, and 6:0.9:2.7 for Co(II)-mediated assembly. Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.
3.2.2. Stoichiometry of metal bound to receptor

Measurements of bound metals indicate that the active complexes have metal:protein stoichiometries close to 1:1 (Ni(II) and Co(II)) and 3:1 (Zn(II)). The metal ion concentrations bound to CF were determined using inductively coupled plasma optimal-emission spectroscopy (ICP-OES), in samples of active complexes (prepared with CheA and CheW) as well as CF alone. Metals producing active complexes lead to sedimentation of both CF alone and CF with CheA and CheW (with the exception of CoCl$_2$ + CF alone, which does not sediment). Samples were prepared with each metal at the optimal concentration based on the activity data (Figure 3.1, arrows) and incubated for long assembly times that maximize kinase activity (greater than 4 h). The bound protein (CF alone or in complex with CheA and CheW) and metal were separated from free protein and metal by ultracentrifugation. Resuspended pellets were then analyzed for metal content by ICP-OES, and protein content by SDS-PAGE (via comparison to a CF standard measured with a BCA assay). Unfortunately we were unable to implement sulfur quantification by ICP-OES, which would have enabled measurement of a more accurate metal:protein ratio as the number of sulfur atoms per CF is known. As listed in Table 3.1, CF alone and CF in complex have nearly identical stoichiometries for Ni(II) and Co(II), which suggests that metals bind similarly to CF in both cases. Moreover, the calculated stoichiometries are both close to one metal ion per CF monomer. In contrast, Zn(II) exhibited both a higher and a more variable stoichiometry of 3.9 Zn(II) per CF alone and 3.1 per CF in complexes. Thus, for all three metals, we can conclude that a large excess (6–12 equiv) is necessary to drive assembly to maximally active CF ternary
complexes, but only a fraction of metal is directly involved in the assembly. All further studies were done on Ni(II)-assembled samples at 180 µM NiCl₂.

**Table 3.1. Stoichiometry of metal binding to CF measured by ICP-OES.**

<table>
<thead>
<tr>
<th>Metal added</th>
<th>Metal bound to CF alone</th>
<th>Metal bound to CF in complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni(II)</td>
<td>1.2 ± 0.04</td>
<td>1.1 ± 0.05</td>
</tr>
<tr>
<td>Zn(II)</td>
<td>3.8 ± 0.16</td>
<td>3.1 ± 0.12</td>
</tr>
<tr>
<td>Co(II)</td>
<td>ND</td>
<td>1.1 ± 0.15</td>
</tr>
</tbody>
</table>

*Data are averages ± one standard deviation for the number of metal ions bound per CF monomer from 10 independent ICP-OES measurements of each metal. Metal added at concentrations found to give maximally active complexes (Figure 3.1, arrows). 30 µM CF in assembly. 30 µM CF, 12 µM CheA, and 24 µM CheW in assembly. CoCl₂ with CF alone does not sediment, and thus, metal content could not be measured.

### 3.2.3. Receptor His-tag is essential for metal-mediated assembly

We hypothesized that the His-tag could be involved in the divalent metal-mediated assembly of functional chemoreceptor complexes, perhaps via stabilization of CF dimers or oligomers. To test this, we engineered a variant of the CF construct (henceforth called H₆CF for the sake of clarity) by inserting the recognition and cleavage sequence for TEV-protease after the His-tag (H₆TEV-CF). TEV-protease cleavage of H₆TEV-CF yields ΔH₆CF, with the native CF sequence plus a single glycine at the N-terminus. For each version of CF (H₆CF and H₆TEV-CF with His-tags, and ΔH₆CF with no His tag, as shown in Figure 3.3A) we tested the assembly of ternary complexes with CheA and CheW by three *in vitro* methods (vesicles, PEG, and Ni(II)) and measured both the kinase activity and protein stoichiometry of sedimented complexes.
The three proteins together in buffer with no vesicles, PEG, or divalent metal show low kinase activity (Figure 3.3B, None) and <15% sedimentation (Figure 3.2). We had previously observed variable levels of kinase activity for H₆CF, CheA, and CheW assembled in the absence of vesicles, PEG, or added metals, and realized H₆CF may contain variable amounts of Ni(II), stripped from the NTA-affinity column during purification (Sprules et al., 1998). We incorporated a 5 mM EDTA treatment step into all purifications, following elution of protein from the column, and this EDTA is removed in the subsequent dialysis step. Thus the low level of activity and sedimentation observed in the absence of vesicles, PEG, or metal (Figure 3.3B, None) is not due to adventitious Ni(II), but presumably reflects the low stability of these complexes formed with the truncated CF.

As shown in Figure 3.3B, both H₆CF and H₆TEV-CF complexes are functional with all assembly methods, indicating that the TEV insertion does not interfere with assembly of functional CF complexes. As anticipated, removing the His-tag results in no kinase activity and background levels of protein binding for both vesicle and Ni(II) assembly methods, demonstrating that the His-tag is required for metal-mediated assembly.

The native stoichiometry of chemoreceptor arrays is thought to be 6:1:2 receptor:CheA:CheW (Liu et al., 2012; Cassidy et al., 2015). As shown in Figure 3.3C, all active complexes of the different constructs assembled with vesicles, PEG, or Ni(II) display near-native stoichiometries. The variation in CheW (ranging from 2.1 to 3.0) may be due to weak staining of CheW resulting in less accurate quantification. Overall, the stoichiometries are consistent with the native-like architecture of PEG arrays, which have
high CheA occupancy observed by electron cryotomography (Briegel et al., 2014a). Thus PEG mediates assembly via molecular crowding that does not require the His-tag; both vesicle and Ni(II) assembly require the His-tag to mediate assembly of complexes with comparable kinase activity and protein stoichiometry.
Figure 3.3. The His-tag is required for metal-mediated assembly. (A) Cartoon depicting constructs tested to determine the role of the His-tag (H₆, red box) in metal-mediated assembly of active complexes. (B) Specific activity of CheA kinase assembled with CheW and His-tagged receptor (black for H₆CF or gray for H₆TEV-CF) or non-His-tagged receptor (striped for ΔH₆CF). Activities are averages of four to eight replicates measured on two to four days; error bars indicate ± one standard deviation. (C) Stoichiometries for active complexes determined as the ratio of proteins in the sedimented complexes. The ~25–28 μM sedimented CF was set to 6 for calculations of the relative amounts of bound CheA (blue) and CheW (cyan), based on ratios of band intensities in SDS-PAGE of sedimented complexes. Horizontal solid and dashed lines correspond to native 6:1:2 molar stoichiometries. Stoichiometries are averages of two to four replicates measured on two to four days; error bars indicate ± one standard deviation. Error bars are either large or missing for CheW stoichiometries in PEG complexes, due to gel distortions from PEG in the CheW molecular weight range. Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.
3.2.4. Metal-mediated complexes form native-like arrays

Chemoreceptor complexes with CheA and CheW form an extended lattice of hexagonal arrays located within the bacterial inner membrane that is responsible for ensuring a sensitive and integrated response to chemical gradients. Because ternary complex formation and kinase activity have been observed in the absence of arrays, (Boldog et al., 2006; Swain et al., 2009; Greenswag et al., 2015) we used electron cryotomography (ECT) to assess the structural features and homogeneity of the metal-mediated complexes. ECT images of both Ni(II)- and Zn(II)-mediated H₆CF ternary complexes prepared under conditions leading to maximal activity reveal clear high-contrast hexagonal lattices (Figure 3.4A and Figure 3.5). ECT results show that Zn(II)-mediated complexes are less abundant and the patches are smaller compared to Ni(II) or PEG. This may be due to less rigorous optimization for Zn(II) assemblies. Perhaps excess Zn(II) incorporation into the proteins (3–4 Zn per H₆CF compared to 1 Ni per H₆CF, Table 3.1) may somehow perturb extended array formation. Despite their small size, subvolume averaging for Zn(II) complexes was still achieved. With 12 nm center-to-center spacing between hexagons, both metal-mediated arrays are indistinguishable from in vivo arrays and from our other in vitro assemblies formed with templating-vesicles and PEG-mediated crowding (Briegel et al., 2014a). Interestingly, metal-mediated arrays also form the sandwich-like structures seen with PEG (Figure 3.4B). This architecture is likely necessary to stabilize the membrane-proximal ends of the long CF in the absence of organization by a membrane.
Figure 3.4. Ni(II)-mediated functional complexes form native-like arrays. Tomographic slices of (A) Ni(II)-mediated H$_6$CF and (B) PEG-mediated H$_6$CF show both top down patches of hexagonal arrays (white arrows) and side view sandwich-like structures (black arrows) made from two layers of CF with overlapping N- and C-termini that are sandwiched between baseplates of CheA and CheW on the outside. Scale bars are 50 nm, and insets show enlarged subvolume averages of the hexagonal array with the canonical 12 nm center-to-center spacing (dotted lines in A and B insets are 12 nm) that is identical to in vivo arrays (Briegel et al., 2012, 2014a). Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.
**Figure 3.5.** Zn(II)-mediated complexes form small native-like arrays. Tomographic slice showing Zn(II)-mediated H₆CF ternary complexes assembled under maximally active conditions. Native-like hexagons (white arrows) with 12 nm center spacing and side view sandwich architecture (black arrows) indicate similar structural features as Ni(II) and PEG arrays. Scale bar is 50 nm. Inset shows subvolume average of single hexagons; scale bar is 10nm. Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.

To further investigate the structural similarities between arrays assembled by these methods, we measured the baseplate distances between CheA/CheW layers for three assemblies that formed sandwiches. Figure 3.6, a plot of the intensity of the 3D volume of the side view for Ni(II)-mediated H₆CF, PEG-mediated H₆CF, and PEG-mediated ΔH₆CF sandwiches (examples in left panels of Figure 3.6), reveals identical distances of 33 nm between baseplates. Given an approximate H₆CF receptor length of ~21.8nm (145 residues, assuming all alpha helical) from the N-terminus to the membrane-distal tip, a 33 nm sandwich thickness (Figure 3.6) predicts that CF’s from
each side of the sandwich overlap with each other in the central ~10.6 nm region. This may be consistent with the disorder (low electron density) observed in the central ~1/3 of the sandwich, as seen in a side view of the isosurfaces of electron density calculated by subvolume averaging of tomograms for Ni(II) and PEG-mediated H$_6$CF (Figure 3.7). Remarkably, the similarity in baseplate distances indicates that the overlap interactions that stabilize the sandwich assembly do not depend critically on what stabilizes the complexes (metal or PEG) or on the sequence of the N-termini (H$_6$CF vs. ΔH$_6$CF) that are present in the overlap region. Ultimately, the similarity in the architecture of the PEG-assembled H$_6$CF and ΔH$_6$CF arrays indicates that the ECT resolution is not sufficient to detect whatever differences in structure and dynamics account for the 2-fold difference in kinase activity (Figure 3.3B) between these samples.
Figure 3.6. Estimating baseplate distances using tomographic 3D volume intensities. (Left panels) Five individual sandwiches for Ni(II)-mediated H₆CF (red), PEG-mediated H₆CF (blue), and PEG-mediated ΔH₆CF (green) ternary complexes were selected and centered at the identical 3D dimension. For each sandwich structure, 20 slices (corresponding to 7 nm of depth) were integrated into one image to enhance the contrast. Intensities for each image were measured based on the gray value and plotted along the distance measured in pixels (right) in ImageJ. The dip in intensity corresponds to the baseplates composed of CheA and CheW, revealing identical sandwich lengths of 33 nm for all samples. Scale bars are 20 nm. Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.
**Figure 3.7.** Ni(II) and PEG assembled H$_6$CF complexes have similar electron densities that overlap with native chemoreceptor arrays. Isosurface representation for H$_6$CF assembled with Ni(II) (isosurface in yellow for panel A and B) or PEG (isosurface in purple for panel C and D). The cyan mesh represents the density of *in vivo* hexagonally packed chemoreceptor arrays from *E. coli* (EMDB ID: 2158) (Briegel et al., 2012). Side view shows sandwich architecture for two layers of trimers-of-dimers (B, D). Top down view (A, C) show two layers of receptor hexagons illustrating that trimers of dimers are vertically aligned in the sandwich. In both Ni(II) (A) and PEG (C) samples, the hexagonal packing of receptors closely fit with the electron density map of the native intact chemoreceptor array. Overlay of maps was done manually in Chimera; density thresholds were chosen to highlight the receptor array similarity between different arrays. Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.
3.2.5. Receptors dimerize and oligomerize with metal

The results presented above suggest that metal binds to the His-tag of H₆CF and somehow stabilizes formation of the functional, native-like array with CheA and CheW. Within the native architecture, what interactions could be stabilized by metal bridging two His-tags? The N-terminal His-tags are likely to be near each other within the H₆CF dimer. In contrast, His-tags on two different dimers are not adjacent in the trimer-of-dimers (as shown in Figure 3.8A, they are ~50 Å apart in the ECT-derived structural model, Protein Data Bank entry 3JA6 (Cassidy et al., 2015)) and His-tags on the two H₆CF layers are not likely to be adjacent in the sandwich (they are on opposite sides of the ~10 nm overlap region, Figure 3.8B). Thus, it seems likely that metal binding bridges two His-tags within a H₆CF dimer, and stabilization of the dimer drives assembly of the functional array. To investigate this, we monitored the formation of metal-mediated complexes of H₆CF using size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS).
Figure 3.8. Approximate His-tag-to-His-tag distances in chemoreceptor structural models. (A) In the ECT-derived model containing the *Thermatoga maritima* receptor trimer of dimers (PDB code 3JA6 (Cassidy et al., 2015)), the residues corresponding to the end of the CF His-tag are shown as red spheres on each monomer. Within a single dimer, His-tags are 10–15 Å apart, while across the trimer interface can be from 50–65 Å, depending on the rotation of the helices. (B) Another structural model was made by docking the *E. coli* serine receptor dimer (PDB 1QU7 (Kim et al., 1999)) into ECT density of sandwiched PEG arrays. In this model, the His-tags (red spheres) are more than 80 Å apart across the receptor membrane proximal overlap region. Coordinates for this model were a gift from Ariane Briegel.

Complex formation of H$_6$CF alone provided the best means of assessing the effect of metal on the H$_6$CF monomer–dimer equilibrium, because the CF dimer cannot be resolved from CheA (see Chapter 4). We anticipate that binding of Ni(II) to the H$_6$CF His-tag is similar in the presence and absence of CheA and CheW, because ICP-OES indicates 1:1 binding in both cases. As discussed above, incubation of H$_6$CF alone with NiCl$_2$ results in sedimentable aggregates. Apparently, these aggregates can become too large to pass the 0.22 µm precolumn filter, as the total eluted protein was observed to
decrease over the time course of the experiment (Table 3.2). Figure 3.9A shows the overlaid chromatograms from the time course series, all scaled to the same total integrated peak area. The amount of aggregate lost in the precolumn filter (dashed line in Figure 3.9B) was calculated from the decrease in area with each injection. To quantify the fraction of monomer, dimer, and oligomer, elution time ranges were set at the points of minimum \( A_{280} \) between peaks (Figure 3.9A, gray vertical lines at 10, 11.7, 12.8, and 15.5 min), and these peak areas were used to calculate the percentage of \( H_6\text{CF} \) in monomer, dimer, and oligomer forms (Figure 3.9B).

**Table 3.2.** Amount of Ni(II)-induced \( H_6\text{CF} \) aggregation increases with time.\(^a\)

<table>
<thead>
<tr>
<th>Incubation time (^b)</th>
<th>Total Elution UV Peak Area (A.U.) (^d)</th>
<th>Aggregate (%) (^e)</th>
<th>Scaling factor (^f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No NiCl(_2)</td>
<td>0.0268</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>&lt;1 min (^c)</td>
<td>0.0260</td>
<td>3.0</td>
<td>1.03</td>
</tr>
<tr>
<td>30 min</td>
<td>0.0214</td>
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<td>1.25</td>
</tr>
<tr>
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<td>0.0174</td>
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<td>1.54</td>
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<tr>
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<td>0.0144</td>
<td>46.3</td>
<td>1.86</td>
</tr>
<tr>
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<td>2.11</td>
</tr>
<tr>
<td>4 h</td>
<td>0.0079</td>
<td>70.5</td>
<td>3.39</td>
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</tbody>
</table>

\(^a\)30 \( \mu M \) \( H_6\text{CF} \) with 180 \( \mu M \) NiCl\(_2\).

\(^b\)Aliquots were injected from a single large volume sample, to prevent sample-to-sample variation, except for a separate “No NiCl\(_2\)” sample of 30 \( \mu M \) \( H_6\text{CF} \) alone.

\(^c\)The minimum amount of time required to add NiCl\(_2\) to \( H_6\text{CF} \) and inject it onto the SEC.

\(^d\)Measured across total elution time (10–15.5 min) using ASTRA VI.

\(^e\)Calculated as \((1−[(\text{Total Peak Area})/(\text{“No NiCl\(_2\)” Total Peak Area})])\times100\). Data also plotted in Figure 3.9B.

\(^f\)Calculated as \((\text{“No NiCl\(_2\)” Total Peak Area})/(\text{Total Peak Area})\). Used to scale chromatograms presented in Figure 3.9A.
SEC-MALS of H$_6$CF incubated with 180 µM NiCl$_2$ (Figure 3.9) demonstrates that Ni(II) stabilizes the H$_6$CF dimer. Prior to the addition of NiCl$_2$, the monomer:dimer ratio is approximately 10:1 (Figure 3.9, red) based on the integrated UV peak area ratio of 5.1:1 (area of the dimer peak divided by two gives dimer concentration). Strikingly, within the minimal time to add metal to the protein and inject it onto the column (<1 min), there is a significant decrease in the monomer:dimer ratio to 3.7:1 (Figure 3.9, orange), which corresponds to a 2.7-fold decrease in this ratio. At remaining time points, both monomer and dimer concentrations continue to decrease in parallel, due to a significant increase in the aggregate fraction (Figure 3.9B, Table 3.2). The system is not at equilibrium, but the monomer to dimer ratio remains in the range of 6–8:1, consistent with Ni(II) stabilization of H$_6$CF dimers.

Figure 3.9. Ni(II) stabilizes the H$_6$CF dimer, which in turn favors larger oligomers. (A) SEC-MALS chromatograms of 30 µM H$_6$CF incubated with 180 µM NiCl$_2$, colored by rainbow according to the increasing incubation time. Vertical lines indicate elution time bounds used to distinguish the monomer (12.8–15.5 min), dimer (11.7–12.8 min), and oligomers (10–11.7 min). Although identical volumes (75 µL) of the same sample were injected, some H$_6$CF aggregate was caught in the pre-column filter. Chromatograms are scaled to the same total area to account for aggregated protein (B, dashed line). (B) The peak areas of the monomer (circle), dimer (triangle), and oligomers (square) were used to calculate the amount of H$_6$CF in each state for all incubation times. The percent H$_6$CF in aggregates larger than the 0.22 µm pre-column filter (dashed line, listed in Table 3.2) was calculated from the loss of area under the SEC elution curve. Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.
3.2.6. Ni(II) promotes partially reversible H$_6$CF aggregation and fibril formation

Chemoreceptors have been observed to form alternate non-native structures in the absence or with insufficient quantities of their cytoplasmic binding partners, CheA and CheW. In particular, so-called “zippers” with interdigitation of the receptor cytoplasmic tips have been reported for overexpressed receptors \textit{in vivo} (Lefman et al., 2004; Zhang et al., 2007), reconstituted intact receptors (Weis et al., 2003), and CF assembled on templating vesicles (Montefusco et al., 2007). A more detailed analysis of the overexpressed intact receptors revealed hexagonal packing with 9 nm center-to-center spacing that is distinct from that of the wild-type 12 nm arrays observed in both membrane-bound and sandwich architectures (Briegel et al., 2014a). Thus we expected that the Ni(II)-induced aggregation in the absence of CheA and CheW would produce a similar zipper-like structure. Instead, ECT images (Figure 3.10A) demonstrate these conditions yield a mixture of nonspecific aggregates and fibrils with uniform 7 nm widths but varying lengths (40–105 nm). Therefore we have identified yet another structure formed by CF in the absence of CheA and CheW: double-stranded fibrils. Interestingly, these fibrils and aggregates are at least partially reversible: addition of CheA and CheW after pre-incubation of H$_6$CF with NiCl$_2$ can produce complexes with kinase activity that is 80% of that of normal complexes assembled by adding NiCl$_2$ last (Figure 3.10B).
Figure 3.10. Ni(II) promotes partially reversible H₆CF aggregation and fibril formation. (A) Tomographic slice shows that H₆CF incubated with NiCl₂ without CheA or CheW forms non-specific aggregates (black arrows) and double-stranded fibrils (white arrows) 7 nm in width. Scale bar is 50 nm. (B) Onset of kinase activity comparing normal assembly to pre-incubation of NiCl₂ with H₆CF before assembly reveals that aggregation induced by pre-incubation is partially reversible. For normal assembly (filled circles), 180 µM NiCl₂ was added last to initiate assembly. Pre-incubation (open circles) of NiCl₂ with H₆CF for 15 minutes was done prior to addition of CheA and CheW to initiate assembly. Activities are averages of four replicates measured on two days; error bars indicate ± one standard deviation. Pre-incubation activity is plotted as % of maximum normal assembly activity on the same day. Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.

3.2.7. PEG and metal promote assembly of CF4E kinase-off state

The CF4E version of the aspartate receptor has four glutamic acid residues, as does the native non-methylated state. It forms complexes with CheA and CheW when assembled on vesicles, but crowding of the complexes is needed to force CF4E into a kinase-on state (Besschetnova et al., 2008). CF4E also does not form arrays under PEG-mediated assembly conditions (no significant sedimentation or kinase activity). The sandwich architecture of metal and PEG assemblies places the added charge of the glutamic acid in CF4E in the overlap region thus, effectively doubling the negative charge in that region. Thus electrostatic repulsion is likely to hinder formation of
sandwich arrays. However, we observed significant sedimentation of CF4E (63%) under conditions for Ni(II)-mediated assembly of complexes. On the other hand, only 11% sedimentation occurs in the absence of CheA and CheW, presumably because electrostatic repulsion opposes the metal-induced aggregation observed for CF4Q. Since Ni(II) and PEG promote assembly of sandwiches by different mechanisms, we hypothesized that combining NiCl₂ and PEG together would increase the level of complex formation and that the resulting complexes would be in the kinase-off/methylation-on signaling state.

To test these hypotheses, we prepared complexes of CF4E with NiCl₂ or NiCl₂ + PEG and measured the both kinase activation and methylation (Figure 3.11; methylation upon incubation with CheR and SAM results in a gel shift due to faster migration). Both conditions yielded sedimentable complexes with native-like stoichiometries (Table 3.3). To assess the signaling state of these complexes, we compared their kinase and methylation activities to those of NiCl₂-assembled CF4Q complexes representing the kinase-on/methylation-off state and vesicle-assembled CF4E.A411V (Ames and Parkinson, 2006) representing the kinase-off/methylation-on state. Remarkably, all CF4E complexes exhibit some degree of methylation and no kinase activity, leading us to conclude they are in the kinase-off/methylation-on state.

Importantly, the methylation assay (gels shown in Figure 3.11B and C) was done on the full assembly sample and thus reflects methylation of both complexed and free CF4E. Free CF4E is not significantly methylated, as previously shown (Shrout et al., 2003) and as indicated by the results for Ni(II)-assembled CF4E in the absence of CheA or CheW (predominantly free, since only 11% sedimentation, and only 9% of total CF
methylated after 4 h). We therefore assumed that all methylation occurs on the complexed CF4E and report the %methylated-CF divided by the % sedimented-CF in Table 3.3 to indicate the percent methylation of complexed CF4E under each assembly condition. As hypothesized, the addition of PEG to NiCl2 improves complex formation (63% sedimentation with NiCl2 versus 93% for NiCl2 + PEG), but it is possible that the higher CF4E concentrations used for the PEG assembly may account for some of the increase in complex formation. The total methylation of CF4E complexes at 4 hours is comparable (80-90%) for all of the metal assembly conditions. There are differences in the methylation at 0.1 hour, which may reflect a difference in methylation rates; additional experiments and controls are needed to investigate this possibility.

**Figure 3.1.** NiCl2 and NiCl2 + PEG promote assembly of CF4E in the methylation-on and kinase-off state. Kinase activity (A) and methylation (B, C) of CF4E assembled under various conditions. Control samples are NiCl2-assembled CF4Q for the kinase-on/methylation-off state, vesicle-assembled CF4E.A411V for the kinase-off/methylation-on state. An additional control for the methylation assay is NiCl2 CF4E (–SAM, s-adenosyl-l-methionine) that lacks the substrate for methylation. All CF4E complexes assembled with NiCl2 (with or without CheA and CheW) and NiCl2 + PEG exhibit little to no kinase activation (A) but do show methylation in the gel shift assay (C, quantified in Table 3.3). Gels (B, C) were cropped to show only CF, methylated-CF (meth-CF), and CheR for clarity.
Table 3.3. Sedimentation, stoichiometry, and % methylation of CF4E complexes

<table>
<thead>
<tr>
<th>Assembled Complex&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sedimented CF&lt;sup&gt;f&lt;/sup&gt;</th>
<th>Methylation&lt;sup&gt;i&lt;/sup&gt; of CF&lt;sup&gt;0.1 h&lt;/sup&gt;</th>
<th>Methylation&lt;sup&gt;i&lt;/sup&gt; of CF&lt;sup&gt;4 h&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>CF (µM)</td>
<td>Method&lt;sup&gt;e&lt;/sup&gt;</td>
<td>A/W (%)</td>
<td>Stoichiometry&lt;sup&gt;g&lt;/sup&gt; (%)</td>
</tr>
<tr>
<td>4Q</td>
<td>Ni(II)</td>
<td>+</td>
<td>25.6</td>
</tr>
<tr>
<td>4E.A411V</td>
<td>Vesicle</td>
<td>+</td>
<td>27.7</td>
</tr>
<tr>
<td>4E&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ni(II)</td>
<td>–</td>
<td>3.2</td>
</tr>
<tr>
<td>4E</td>
<td>Ni(II)</td>
<td>+</td>
<td>19.0</td>
</tr>
<tr>
<td>4E&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Ni(II) + PEG</td>
<td>+</td>
<td>46.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Corresponding kinase and methylation data presented in Figure 3.11.
<sup>b</sup>Protein concentrations 30 µM CF, 12 µM CheA, and 24 µM CheW, except as noted.<sup>c,d</sup>
<sup>c</sup>No CheA or CheW present in this 30 µM CF4E sample.
<sup>d</sup>Sample contains 50 µM CF, 12 µM CheA, and 20 µM CheW.
<sup>e</sup>Ni(II)-mediated complexes contain 180 µM NiCl<sub>2</sub>; Vesicle-mediated complexes contain 725 µM lipid (290 µM DOGS-NTA-Ni<sup>2+</sup> and 435 µM DOPC); Ni(II) + PEG-mediated complexes contain 180 µM NiCl<sub>2</sub>, 7.5% w/v PEG8000, and 4% w/v D-trehalose.
<sup>f</sup>Quantified from sedimentation assay (see section 2.4.2).
<sup>g</sup>Calculated stoichiometries of CF:CheA:CheW from pellet lane ratios in sedimentation assay (see section 2.4.2).
<sup>h</sup>Not applicable as no CheA or CheW is present.
<sup>i</sup>Quantified from gel shown in Figure 3.11. %Methylated-CF = [(I<sub>meth-CF</sub>)/(I<sub>CF</sub> + I<sub>meth-CF</sub>)]/ %Sedimented-CF.
<sup>j</sup>Corrected values indicate the sedimented free CF4E is predominantly methylated, but it accounts for only 9% of the total CF4E.

The sandwich architecture exhibited by PEG and Ni(II)-mediated arrays have thus far only been observed for CF4Q arrays. Is it possible for CF4E assembled with Ni(II) and PEG to retain the sandwich architecture despite electrostatic repulsion? To answer this, we returned to ECT to image Ni(II) + PEG-mediated CF4E arrays. Previous ECT of CF4E was limited to vesicle-assembled arrays, which tend to have lower resolution due to vesicle curvature (Briegel et al., 2014a). Ideal chemoreceptor candidates for ECT are thin, flat, and highly ordered (Briegel and Jensen, 2017).
While the high concentrations of PEG led to thicker blots on the ECT grids (Wen Yang, personal communication), the first look at Ni(II) + PEG-mediated CF4E complexes (Figure 3.12) clearly indicates the presence of sandwich arrays that retain the 12 nm hexagonal array spacing. Unfortunately the collected tomograms show a tendency of these arrays to form thick clumps that lack enough order to assign specific structures. Moreover, an error in data acquisition may have contributed to lower quality tomograms (Wen Yang, personal communication). However, a few small yet distinctive structures were visible, including a single hexagon (Figure 3.12A, B) and a sandwich (3.12C).

**Figure 3.12.** CF4E assembled with Ni(II) and PEG exhibit hexagon and sandwich architecture. Tomographic slices show predominately low-order aggregation due to PEG and non-ideal experimental conditions with the exception of a single hexagon cluster (white arrow in A that is zoomed-in in B) and a sandwich structure (C) from elsewhere in the tilt series. Scale bars indicate 50 nm in A and 20 nm in B and C.
This newfound ability to image CF4E in sandwiches provides a unique opportunity to investigate structural changes of signaling and receptor methylation state in the context of the array. For example, how does the disordered region observed for CF4Q (Figure 3.7) compare to CF4E? Does the added electrostatic interaction alter the structural stability in the predominately disordered membrane proximal region? Additionally, the ligand-induced expansion model predicts this region would exhibit the largest conformational change in which the trimers of dimers are spread farther apart in the kinase-off/methylation-on state (Sferdean et al., 2012). Until now, preparing CF complexes in this signaling state were limited to: (1) CF4E on low-density vesicles, (2) CF4E.A411V on high-density vesicles, or (3) CF4Q.A411V on high-density vesicles, PEG, or metal. It’s been shown that vesicle assemblies are limited in resolution due to curvature, so we imaged CF4Q.A411V on PEG or metals to probe the structure of the kinase-off/methylation-on state. Unfortunately no measurable differences were observed compared to CF4Q (data not shown). Recent work from Briegel and coworkers reported no conformational changes for various signaling-related mutants of the Tsr receptor, but did observe distinct differences in the associated CheA density, particularly for locked-off mutants (Briegel et al., 2013).

Moving forward, we propose that investigating the structure, disorder, and CheA density of CF4E arrays assembled into sandwiches in the kinase-off/methylation-on state may provide new insights into signaling mechanism. However, we note that experimental conditions will need to be optimized to minimize thickness on ECT grids (perhaps by decreasing PEG concentrations) and to maximize array size (perhaps by increasing PEG or protein concentrations). Alternatively, CF4E with Ni(II) alone avoids PEG, but further
optimization may be needed to fully form sedimentable complexes as current conditions only obtain 63% complexation (Table 3.3).

3.2.8. Metal-assembled complexes are stable after removing metal

We have demonstrated that metal-mediated assembly involves metal binding to the His-tag to stabilize CF dimers. Assuming excess metal is needed to drive the metal binding equilibrium, can the metal be removed without disrupting the array? To investigate this, we monitored activity and complexation over time of Ni(II)-mediated H₆CF complexes following the addition of the metal chelator EDTA. Ni(II) has a much higher affinity for EDTA than for hexahistidine-tags on proteins [EDTA Kᵋ ≈ 4×10⁻¹⁹ M, 6His-tagged protein Kᵋ ≈ 10⁻⁶ M (Knecht et al., 2009)]. Therefore, we assume that adding stoichiometric EDTA (180 µM) will chelate the 150 µM unbound Ni(II) (180 µM total Ni(II) – 30 µM H₆CF-bound Ni(II)) immediately. It’s unclear, however, how quickly the Ni(II) bound to H₆CF will be removed by the EDTA. To limit dilution effects, a small amount (2.5% of the total complex volume) of a highly concentrated EDTA stock was added to assembled Ni(II)-mediated complexes. We then measured the kinase activity and sedimentation after 30 min, 2 h, and 24 h, all kept at 25°C (Figure 3.13).

Remarkably, Ni(II)-mediated complexes do not immediately fall apart following the addition of EDTA. In fact, complexes remain 90% intact after 2 h, and ~70% after 24 h (Figure 3.13B), suggesting the arrays are kinetically stable. Perplexingly, we see a larger decrease in kinase activity: 88% activity was retained after 2 h, which dropped to 46% after 24 h. Without the addition of EDTA, metal-mediated arrays are ~90% active after 4 days, but this long-term stability may partially be a result of storing the complexes
at 4°C (not shown). Combined, these results suggest that metal removal causes both a loss of complexes and a decrease in activity of the remaining complexes.

Our results clearly indicate the arrays are kinetically stable for a few hours once assembled. We cannot, however, distinguish whether these are metal-bound complexes (if metal does not immediate dissociate) or metal-free complexes (EDTA strips out metal faster than complex dissociation). In the case of metal-free kinetically stable complexes, this suggests that arrays can be stabilized by protein–protein interactions alone.
Figure 3.13. EDTA causes slow disassembly and loss of kinase activity of Ni(II)-mediated H$_6$CF complexes. Total kinase activity (A) and sedimentation (B) was measured 30 min, 2 h, and 24 h following the addition of 180 µM EDTA to Ni(II)-assembled complexes and incubated at 25°C. Activity and sedimentation data are normalized to the Ni(II) control sample prior to addition of EDTA (total activity of 8.7 s$^{-1}$ and sedimentations of 23 µM H$_6$CF, 4.4 µM CheA, and 7.8 µM CheW).

3.3. Summary and Conclusions

In this chapter we’ve demonstrated a novel method for triggering assembly of the aspartate chemoreceptor cytoplasmic fragment (CF) into nanoarrays through metal-mediated His-tag dimerization and compared these to other in vitro native-like arrays. Functional complexes of CF, CheA, and CheW form upon addition of specific divalent
metals and assemble into an extended hexagonal lattice that exhibits the widely conserved (Briegel et al., 2009, 2011), native 12 nm center-to-center spacing (Figure 3.4). Intact chemoreceptor arrays are normally stabilized by a combination of anchoring in the membrane and cytosolic binding of CheA and CheW in rings at the membrane-distal cytoplasmic tip of the receptor. In current structural models of the complex, the long (∼200 Å) cytoplasmic domain has protein–protein interactions with itself, CheA, and CheW only at its membrane-distal end, so the constraints imposed by its transmembrane and periplasmic domains are likely critical to the stability of the array. The necessary membrane-proximal stability to form CF arrays can be achieved with a membrane mimetic (templating vesicles) (Shrout et al., 2003) or with a sandwich architecture produced with PEG (Fowler et al., 2010) or divalent metals (this work). Importantly, all three in vitro preparations with CF yield high CheA occupancy and native stoichiometry (Figure 3.3C), although PEG arrays have previously been shown to lack the alternating hexagons of CheA observed for in vivo arrays (Briegel et al., 2014a). Measured CheW stoichiometries of 2 monomers per 6 CF monomers in these assemblies provide further evidence of CheW-only rings that are proposed to provide structural integrity to the array (Liu et al., 2012; Cassidy et al., 2015).

In contrast to the non-native “zippers” that form with cytoplasmic tips interdigitated in the center in the absence of sufficient CheA and CheW, “sandwiches” have receptors oriented in the opposite direction: cytoplasmic tips interact with CheA/CheW rings on both surfaces of the sandwich, forming hexagonal arrays with the canonical 12 nm center-to-center packing. Although sandwiches are not the native architecture for the E. coli aspartate receptor used in this study, similar cytoplasmic
arrays exhibiting both 12 nm hexagonal lattices and sandwich architectures are observed for cytoplasmic chemoreceptors (lacking transmembrane domains) from a variety of bacteria and archaea, including *Vibrio cholerae* (Briegel et al., 2016), *Rhodobacter sphaeroides* (Briegel et al., 2014b), and *Methanobacterium formicicum* (Briegel et al., 2015).

The observed metal dependence of assembly is consistent with the proposed His-tag dimerization mechanism. The relative efficiency of the divalent metals in promoting active complex formation, Ni(II) > Zn(II) > Co(II), follows the relative affinities of these metals for peptides containing multiple His residues (Sovago et al., 2016). This is consistent with preliminary X-ray absorption spectroscopy (XAS) data (Hsin-Ting Huang and Michael J Maroney, unpublished observations) suggesting that the Ni(II) bound to H₆CF is coordinated by 6 ligands, including multiple histidines. Importantly, knowing Ni(II) is 6-coordinate and therefore paramagnetic suggests it may be possible to obtain distance constraints with paramagnetic relaxation enhancement NMR experiments involving comparison to the diamagnetic Zn(II)-assembly that has identical array architecture by ECT (Figure 3.5). This could provide critical structural information in the overlap region of the sandwich where electron density is lost in ECT (Figure 3.6).

Assembly of large multiprotein complexes *in vitro* that retain native structure and function is a challenging but essential step in understanding how protein machines operate in the cell. His-tag-mediated dimerization is a means of stabilizing protein dimers that is much less perturbing than fusion to a protein dimerization motif. For example, fusion to a leucine zipper (LZ) introduces 30–60 residues, which is more perturbing than a polyhistadine-tag and can interfere with assembly of native complexes. Interestingly,
fusion of a 45-residue LZ to the chemotaxis receptor CF used in our study restored kinase activation but perturbed the assembly of native complexes with CheA and CheW. The LZ-CF assembled into sandwich-like particles in the opposite orientation (two CF layers on the outside of a middle layer of CheA and CheW) that prevented formation of the native array (Francis et al., 2002, 2004; Wolanin et al., 2006). This comparison of LZ and His-tag-mediated dimerization of the same system demonstrates that using the least perturbing means of stabilization protein interactions can be critical to assembling native complexes. This approach has significant potential for application to other multiprotein systems, for the stabilization of functional complexes with native architecture. His-tags are widely used and they are easily introduced at many locations within a recombinantly expressed protein. This should make it possible to drive dimerization at a known dimer interface with minimal perturbation to structure or function, for straightforward and accurate \textit{in vitro} studies of the mechanisms of key processes in the cell.
CHAPTER 4

INSIGHTS INTO THE MODE OF CHEMORECEPTOR ARRAY FORMATION 
AND KINASE ACTIVATION

This chapter reports on the assembly mechanisms that govern formation of hexagonally packed chemoreceptor arrays in complex with CheA and CheW. The metal-mediated assembly technology (described in Chapter 3) enabled the first experimental observation of the early stages of assembly. Most of this chapter was published in the article “His-tag-mediated dimerization of chemoreceptors leads to assembly of functional nanoarrays” in Biochemistry (Haglin et al., 2017).

4.1. Introduction

As discussed in the previous chapter, structure–function analyses of protein assemblies in vitro that mimic native systems require a high degree of homogeneity in isolating or reassembling such multiprotein complexes. This is particularly challenging when proteins must be altered (i.e. truncation or mutation) to facilitate in vitro preparations. Moreover, the shear size of large protein assemblies such as chemoreceptor arrays can impart an additional challenge to perform accurate and precise biophysical studies. However, it remains unclear whether reassembly of the full array is necessary to understand the architecture and molecular mechanisms of chemotaxis signal transduction. An in vitro reconstitution of intact receptors as trimers of dimers into nanodiscs revealed that the core unit involved in the chemoreceptor array is an independent signaling unit capable of all chemosensory functions, including ligand binding, transmembrane signaling, adaptation modification, and kinase activation/control (Boldog et al., 2006; Li
Additionally, the nanodisc-assembled core units contained a stoichiometry of six receptors to one CheA and one CheW. This is at odds with the mounting evidence for CheW-only rings seen by ECT (Liu et al., 2012; Cassidy et al., 2015), and the stoichiometries we measured for three in vitro native-like arrays (see Chapter 3), which indicate native stoichiometry is 6:1:2. Thus, when and how does the additional CheW get incorporated into the array if it is not present in the core unit?

To answer these questions, we must first understand how the array assembles. The current model (Liu et al., 2012; Briegel et al., 2014a; Briegel and Jensen, 2017) predicts that array formation starts with assembly of trimers of dimers that subsequently form core units by binding CheA and CheW. Core units then merge into hexagons that grow into a full array through incorporation of additional core units. This model is based on ECT data showing the existence of linked trimers of dimers, single hexagons, linked hexagons, and patches of hexagons in preparations of isolated native membranes reconstituted with overexpressed receptor and excess CheA and CheW. This ECT study did not follow the time course of assembly. ECT cannot detect disordered species, including all of the separate protein components, and also cannot quantify the ordered species present in a sample. A complementary approach is needed to follow the time course of array assembly.

As presented in this chapter, we used our novel metal-mediated assembly method to investigate chemoreceptor array formation. Advantages of this method include the ability to trigger the assembly in solution and monitor the kinetics of the onset of activity and the formation of complexes. Moreover, by eliminating the vesicle and PEG components needed for vesicle-mediated and PEG-mediated assembly, we were able to
use SEC-MALS to separate and follow the components and complexes, and deduce when CheA and CheW are incorporated into the array. Lastly, we correlated the extent of kinase activity with array size, leading to a proposal that allosteric effects within the array contribute to the overall signaling output.

4.2. Results and Discussion

4.2.1. Kinetics of kinase activity and binding

To understand the mechanism of metal-mediated formation of functional complexes, we investigated the assembly kinetics by measuring the onset of kinase activity and sedimentation immediately after combining the H₆CF, CheA, CheW, and 180 µM NiCl₂. Assembly with either vesicles (Montefusco et al., 2007) or PEG (unpublished observations) requires incubation at 25°C for at least 4 h before maximal activity is reached. Although the NiCl₂ assembly activity similarly achieves maximal activity after 4 hours (Figure 4.1), the sedimentation profile shows nearly complete binding (80-90%) of all three proteins within the first 30 min. Note that complex formation may continue during the 30 min centrifugation, whereas the kinase activity measurements take < 2 min; therefore complete binding occurs at 30–60 min. Thus, the kinase activity is only 40–60% when the CheA and CheW incorporation is maximal, which suggests that initially the three proteins bind quickly but further changes are needed to reach maximal activity.
Figure 4.1. Onset of kinase activation and protein binding of Ni(II)-mediated H$_6$CF ternary complex assembly. Time course of kinase activity (black circles) and sedimentation of H$_6$CF, CheA, and CheW (black, dark blue, and cyan bars, respectively) combined with 180 µM NiCl$_2$. The zero time point is before NiCl$_2$ addition. Activity and sedimentation data are normalized to their respective maxima, achieved at 4 h (maximum total activity of 7.6 s$^{-1}$, and maximum sedimentations of 27.0 µM H$_6$CF, 5.8 µM CheA, and 13.6 µM CheW). Activities and sedimentation percentages are averages of two (sedimentation) and four (activity) replicates measured on two days; error bars indicate ± one standard deviation. Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.

4.2.2. Ternary complex and array size increases during assembly

We returned to SEC-MALS to monitor complex formation with NiCl$_2$. The three component proteins and their complexes can be partially resolved via SEC, with the largest complexes and proteins eluting first, but SEC does not yield accurate molecular weights from variably shaped proteins like the rod-shaped H$_6$CF. The MALS data enable the determination of hydrodynamic properties for better estimation of MW. However, MW estimation is not accurate for overlapping peaks or high-MW complexes that elute near the void volume (10 min on the SEC-MALS used in this study, details in Chapter 2) and are highly polydisperse. Separate injections of the individual proteins demonstrate they are well resolved and that MALS estimates of the molecular masses are reasonably
close to the actual values (Figure 4.2 and 4.3): the 32.7 kDa H$_6$CF elutes at 13.5 min with an apparent MW of 34 kDa (black), the 142.7 kDa CheA dimer elutes at 11.5 min with an apparent MW of 139 kDa (blue), and the 18.1 kDa CheW monomer elutes at 16 min with an apparent MW of 18 kDa (cyan). These proteins were injected at the concentrations used for metal-mediated assembly (30 µM H$_6$CF, 12 µM CheA, and 24 µM CheW). The H$_6$CF peak observed at 12 min with an apparent MW of 73 kDa (black) corresponds to the 65.4 kDa dimer, but is hidden under the CheA peak once all proteins are combined. When injected together at the same concentration as the individual injections, ternary mixtures exhibit apparent molecular weights uniformly increased by 1.3-fold (Figure 4.4). It is not immediately clear why all the MW estimates increase, yet the elution times exhibit varied changes: CheW elutes faster which is consistent with a MW increase, while CheA and CF either don’t change, or elute slower, respectively, neither of which is expected for an increase in MW. One possibility is that an increase in peak overlap makes MW estimations less reliable. The two peaks for H$_6$CF and CheW move together slightly, which could indicate a possible interaction between the two proteins in solution or on the column. There is also a small shoulder at 10.8 min, which is likely to be a small amount of ternary complex formation, consistent with the <15% sedimentation observed under these conditions for an identical sample (0 time in Figure 4.1, which has no Ni(II)).
**Figure 4.2.** SEC chromatograms of separately injected ternary components. H$_6$CF (black), CheA (blue), and CheW (cyan) were injected at concentrations used for assembly (30 µM, 12 µM, and 24 µM, respectively), as well as a single injection of all three in solution without NiCl$_2$ (red). Molecular masses estimated from multi-angle light scattering (MALS) are indicated and are reasonably consistent with the molecular weights of dimeric CheA (142.7 kDa), dimers and monomers of H$_6$CF (32.7 kDa), and CheW (18.1 kDa). All chromatograms are normalized to a value of 1 for the largest peak in the series. Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.

**Figure 4.3.** Accurate molecular masses are estimated from SEC-MALS of individual protein injections. Chromatograms for separate 75 µL of each protein at assembly conditions (30 µM H$_6$CF, 12 µM CheA, and 24 µM CheW) correspond to data in Figure 4.2. Molecular weights corresponding to each protein are averages across each elution peak. All chromatogram peak heights are normalized to an A$_{280}$ value of 1 for the CheA. Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.
Figure 4.4. Apparent molecular mass estimations from SEC-MALS are higher for ternary mixtures. As in Figure 4.3, molecular weights corresponding to each protein are averages across each elution peak. All chromatogram peak heights are normalized to an $A_{280}$ value of 1 for the CheA.

The first changes observed by SEC during complex assembly involve primarily $H_6CF$. The difference between the no NiCl$_2$ and $< 1$ min with NiCl$_2$ injections (Figure 4.5 and 4.6, red and orange) is a decrease in $H_6CF$ peak height and a corresponding increase in the intensity of a high MW species that elutes at 11.1 min. The latter may consist of only $H_6CF$ oligomers, because there is no significant change in the intensities of the CheA and CheW peaks. Next, incorporation of CheA and CheW into complexes with $H_6CF$ appears to be nearly complete in 30 min, based on both the sedimentation data (Figure 4.1) showing nearly complete binding at 30–60 min, and the decrease in the intensities of the CheA and CheW SEC peaks (Figure 4.5 and 4.6, yellow) with no further changes at longer time points. CheA and CheW are presumably incorporated into a complex with $H_6CF$ that elutes as a large SEC peak at 11.1 min. While the elution time is the same as that of the small shoulder seen immediately after the addition of NiCl$_2$ (Figure 4.5 and 4.6, orange), this peak is highly polydisperse and thus likely contains a
mixture of species with different molecular weights. At longer time points (1–4 h, green to blue to violet, Figure 4.5 and 4.6), a second peak emerges at 10.5 min (indicated with arrows) at an even higher MW and the H₆CF peak continues to decrease to nearly zero intensity. These changes coincide with the assembly reaching maximum kinase activity, as shown in Figure 4.1. The H₆CF, CheA, and CheW peaks remaining at 4 h are consistent with the assembly conditions: large amounts of free CheA and CheW because these are added in excess, and only ~20% free H₆CF, consistent with sedimentation analysis that typically shows ~10–15% free H₆CF after assembly.

Figure 4.5. SEC chromatograms of H₆CF, CheA, and CheW incubated with NiCl₂ reveal assembly features. Chromatograms are colored by rainbow according to increasing incubation time. A high MW complex of H₆CF, CheA, and CheW (11 min elution time) that forms in low yield in the absence of NiCl₂ (0 min sedimentation in Figure 4.1, and red chromatogram also shown in Figure 4.2) and in high yield at early incubation times (30 and 60 min in Figure 4.1, yellow & green here) sediments but does not have high kinase activity. The arrow indicates a shift to an even higher MW complex eluting at 10.5 min near the void volume at 10 min elution time. All chromatograms are normalized to a value of one for the largest peak in the series. Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.

This experiment was repeated on a second and newer column to test the reproducibility of the SEC results. In comparing the series in Figure 4.5 and 4.6, some
interesting differences are apparent. Most notably, a shoulder between CheA and H$_6$CF that likely corresponds to H$_6$CF dimer can been seen in the new column, indicating improved separation. Moreover, it disappears sometime between 1–30 min of assembly, which is consistent with rapid dimer incorporation into oligomers (Figure 3.9) and arrays. CheA and CheW take longer to fully incorporate (mostly complete after 1 h, with a small additional incorporation of CheA at 2 h) compared to binding of CheA and CheW in the previous experiment, which was complete after 30 min (Figure 4.5, yellow). Another difference is the absence of any complex before the addition of nickel (red), and the fact the initial small amount of complex formed at <1 min with little or no CheA and CheW (11 min, orange) is resolved from the dominant complex peak at 10.8 min (yellow and green). Finally, the larger complex that appears at long times is better resolved from the initial complex (arrows in Figures 4.5 and 4.6), likely due to improved separation on this column. While the variations in these results indicate that assembly does not follow an identical pathway, it’s clear that CheA/W incorporation occurs prior to assembly of the full size extended array.
Figure 4.6. Replicate SEC-MALS assembly experiment. Chromatograms of H$_6$CF, CheA, and CheW incubated with NiCl$_2$, colored and labeled as in Figure 4.4. The proteins were assembled under identical conditions but injected onto a new column, resulting in slight variations in elution times. All chromatograms are normalized to a value of one for the largest peak in the series.

Lastly we must report an unfortunate reality of the SEC-MALS experiments: for reasons that remain unclear, the two SEC columns used for these studies acquired a CF-specific protease at some point following injection of ternary mixtures. Any variant of CF (including CF4E, CF4Q.A411V, H$_6$TEV-CF, and CF4Q prepared by Xuni Li and Maryam Kashefi) experienced nearly complete proteolysis on the column and eluted predominantly with the buffer components at 22 min. None of the CF variants tested exhibited proteolysis in an SDS-PAGE, and were fully capable of forming active complexes (data not shown). It is particularly striking that no other proteins experience proteolysis. CheA, CheW, BSA, and many other proteins remain completely intact. The SEC-MALS is maintained by the Institute of Applied Life Sciences core facility and used by many researchers; none have reported proteolysis problems. Future experiments will need to avoid SEC-MALS or find a way to prevent proteolysis on a new column.
4.2.3. Model of chemoreceptor array formation

Characterization of the metal-mediated assembly of CF arrays by activity assays, sedimentation assays, and SEC-MALS allows us to propose a model for the mechanism of this assembly (Figure 4.7) and yields new insights into the assembly and properties of native chemoreceptor arrays. The fast (< 1 min) initial increase in the dimer fraction (reported in Chapter 3, Figure 3.7) indicates that assembly begins with the stabilization of CF dimers, most likely by metals bridging two His-tags. Subsequently CheA and CheW are incorporated with metal-stabilized CF dimers into sedimentable complexes. However, binding of CheA and CheW is complete within 30–60 min, forming sedimentable complexes smaller than the fully active complexes that form in 4 h (Figures 4.1, 4.5, and 4.6). While our data cannot distinguish the exact size of these initial smaller complexes, Figure 4.7 shows one possibility that includes full binding of CheA and CheW into a sandwich of two core units, each containing 12 CFs (two trimers of dimers), bridged by one CheA dimer, and also containing four CheW monomers. It is unclear what changes occur between 1–4 h to yield larger complexes with higher activity that ultimately have the canonical hexagonal architecture. In one SEC-MALS series (Figure 4.5) there was a small increase in the level of CF binding, while in another series (Figure 4.6) there was a small increase in the level of binding of all three proteins. We propose that building to larger complexes with maximal activity likely involves assembly of core units into hexagons that coalesce to form larger arrays. This final assembly phase also involves binding of additional CF, and in some cases of CheA, and/or CheW, to unoccupied sites in the coalesced array, as it may include rearrangement of CheA and CheW into the native pattern that maximizes CheA binding in alternating hexagons of CheA/W (Briegel
et al., 2014a) and only CheW. In any case, it appears that protein–protein interactions beyond those in the core unit are required for full activity.

**Figure 4.7.** Model of array assembly by Ni(II)-mediated His-tag dimerization. Cartoon representation showing progression of assembly starting with CF monomers (side view: gray bars and top down: gray circles) rapidly forming dimers upon Ni(II) (black dots) binding to CF His-tags (red line). CheA dimers (blue) and CheW monomers (cyan) bind to stabilized H$_6$CF dimers to capacity within 30–60 min. Binding of a CheA dimer and four CheW drive assembly of a pair of trimers-of-receptor dimers into a core unit, that is likely further stabilized as a sandwich. At longer time points (4 h and beyond), the assemblies form hexagons and larger arrays with alternating CheA/CheW and CheW-only rings. Reprinted (adapted) with permission from (Haglin et al., 2017). Copyright 2017 American Chemical Society.

### 4.3. Summary and Conclusions

In this chapter we used our novel *in vitro* metal-mediated His-tag dimerization method to trigger formation of arrays and investigate the early stages of how these proteins assemble into the intricate geometry of the hexagonal array. While the architectural features of the fully formed array are known (Briegel et al., 2012; Liu et al., 2012), the mode of assembly has not yet been clearly established given the difficulty of obtaining snapshots in the context of forming the full array. Thus our metal-assembly provided a unique and critical opportunity to probe the mode of assembly *in vitro*. Moreover, the insights from this study may shed light on how these remarkable arrays form in the cell.
Briegel and others (Briegel et al., 2014a) proposed the following model for \textit{in vivo} array assembly: (1) receptors dimerize, (2) three receptor dimers form a trimer of dimers, (3) pairs of receptor trimers of dimers are bridged by a CheA dimer and bind two CheW to form the core unit, (4) three core units coalesce to form hexagons containing CheA and CheW, and (5) the array grows through binding of additional core units or of additional CheA/W-filled hexagons. Our data support the importance of the initial receptor dimerization in the membrane, since metal-mediated stabilization of the CF dimer is sufficient to drive array assembly (Chapter 3). We see no evidence for formation of discrete trimers of CF dimers in the absence of CheA and CheW (Lai et al., 2005), and instead observe that CF dimers form various oligomers and large aggregates, consistent with prior observations \textit{in vivo} (Zhang et al., 2007). We propose that in the absence of a stabilizing membrane or membrane mimetic, CheA and CheW are required to mediate assembly of receptor dimers into trimers of dimers. These are then bridged by CheA dimers into core units that also bind four CheW. This would satisfy all of the receptor binding sites (2 receptor/CheA interfaces, 4 receptor/CheW interfaces, and 6 receptor/receptor interfaces) and thus prevent non-native interactions that lead to aggregation both \textit{in vitro} and \textit{in vivo}. Our data support incorporation of 4 CheW into the core unit because there is little additional CheW binding upon assembly of larger arrays (Figure 4.4). Two of the four CheW in the core unit are weakly bound (lacking the CheA/CheW protein-protein interaction), and thus would likely be lost during purification of core complexes with full-length receptors in nanodiscs (Li and Hazelbauer, 2011) (which contained two CheW per core unit). These weakly bound CheW are more likely to be retained under excess CheW conditions as in our CF
assembly conditions. It seems likely that the additional two weakly bound CheW in the core unit would increase the rate of assembly and, as previously suggested (Liu et al., 2012), increase the stability of the array. Finally, we propose that assembly of core units into hexagons is required to achieve maximal kinase activity.

Several important differences are expected between the His-tag mediated assembly of arrays of receptor fragments and in vivo assembly of membrane-bound chemoreceptor arrays. Studies of assembly of CF arrays cannot provide insights into the effects of the missing periplasmic, transmembrane, and HAMP domains, or the effects of ligand binding (Li and Weis, 2000). Assembly of core units, hexagons, and arrays likely occurs faster in vivo due to alignment of receptors in the membrane facilitating encounters by 2D diffusion within the plane of the membrane rather than by 3D diffusion. Interestingly, although the cellular concentrations of the E. coli chemotaxis array proteins have been shown to vary significantly with strain and growth conditions, the protein ratios in the cell remain consistent at 2.9 receptors to 1 CheA to 1.2 CheW (Li and Hazelbauer, 2004). This excess CheA and CheW is similar to the conditions of our His-tag mediated assembly of CF arrays, containing a ratio of 2.5 CF to 1 CheA to 2 CheW. Although in vitro assembly does not duplicate the crowded conditions in the cell that alter protein diffusion rates and affinities, it may be possible to investigate whether excess CheA and CheW promote assembly as previously suggested (Briegel et al., 2014a).

In conclusion, we have demonstrated that kinetic analysis with this new assembly method provides the first experimental evidence that initial binding of CheA and CheW into ternary complexes proposed to be the “core unit” does not yield high activity, and
that activity reaches its maximum upon formation of larger arrays. His-tag mediated
dimerization provides a new approach to trigger assembly of native-like chemoreceptor
complexes that avoids the use of PEG and vesicles which interfere with SEC-MALS
analysis. This novel assembly method may prove useful for kinetic analysis of the
assembly of other multi-protein complexes.
5.1. Introduction

Transmembrane homodimeric chemoreceptor proteins are responsible for signal transmission in bacterial chemotaxis. These predominantly α-helical proteins relay ligand binding events from the periplasmic domain to the cytoplasmic subdomains via a 2 Å piston displacement of the transmembrane signaling helix that is coupled to the conserved HAMP domain (Miller and Falke, 2004; Hazelbauer et al., 2008). The signaling output properties of chemoreceptors are characteristic of a two-state model in which kinase-on and kinase-off outputs are in an equilibrium that shifts depending on ligand occupancy and receptor methylation state. The latter is a mode of adaptation governed by the enzymes CheR and CheB that covalently modify receptors at specific sites. Upon high levels of methylation, affinity for ligand decreases which restores prestimulus kinase activity. It is not yet clear what molecular mechanisms translate the signal through the cytoplasmic subdomains to ultimately control the activation or inhibition of the kinase.

One proposal is that trimers of receptor dimers undergo a conformational change upon ligand binding that alters the activity of CheA. Changes in the spacing between FRET pairs in the membrane proximal region of Tar and Tsr were correlated to an expansion with attractant bound (and subsequent kinase inhibition) and a contraction with repellant bound (with kinase activation) (Vaknin and Berg, 2007). Additionally, Besschetnova and coworkers observed an apparent receptor density-dependent transition between the kinase-on and kinase-off states of receptor cytoplasmic fragments assembled...
on vesicles (Besschetnova et al., 2008). High density receptor packing would disfavor dissociation or expansion, and low density reconstituted intact receptors were shown not to dissociate (Sferdean et al., 2012). Combined, these data led to a proposed model of ligand-induced expansion for signal transmission. Given that receptors are anchored at their membrane-distal cytoplasmic tips into a lattice of rings of CheA and CheW, this model predicts the largest conformational change occurs in or near the membrane due to a tilting or straightening of the receptors in the kinase-off or kinase-on state, respectively.

In light of the findings presented in Chapter 4, we must also consider the possibility that the density-dependent activity could be a result of assembly into hexagons. We found that kinase activity increases as complexes become larger than the minimal core unit. Therefore the proposed low-density, kinase-off state may be smaller units that are widely dispersed on the vesicles that coalesce into more functional arrays upon restricting the two-dimensional surface area. ECT of such samples has observed some hexagons (Ariane Briegel, personal communication), but it is not possible to quantify the fraction of CF that forms hexagons vs. smaller core units. Thus, defining the structural features and interdimer distances in the membrane-proximal region is needed to test the ligand-induced expansion model.

Although significant progress has been made in resolving some structural features of chemoreceptors within the array, the membrane-proximal cytoplasmic region remains elusive. ECT of intact receptors in vivo and receptor fragments assembled into arrays in vitro (including those presented in Chapter 3) all exhibit density at the membrane-distal tip of the receptor that gradually disappears closer to the membrane (Briegel et al., 2012, 2014a; Liu et al., 2012; Cassidy et al., 2015). Consistent with this, Liu and coworkers
reported evidence that the receptor assumes multiple conformations in the membrane-proximal portion, (Liu et al., 2012, supplemental Figure S8). Thus structural restraints in the membrane-proximal region of the chemoreceptor are needed, both to constrain the dimensions of the trimer of dimers and to test the proposed expansion model by comparing arrays assembled in defined signaling states. Such distance constraints would clarify whether the effect of assembly density on kinase activity is due to receptor expansion/contraction or to a coalescing of core units and hexagons into larger and more functional arrays.

A number of NMR methods are available to measure distances such as NOEs (Nuclear Overhauser Effects) and REDOR (Rotational Echo DOuble Resonance). However, the techniques are limited to distance measurements ~5–10 Å. A structural model of the core unit (PDB 3JA6) predicts that dimers are splayed ~20 Å apart in the middle of the methylation subdomain and >60 Å at top of the HAMP domain. Thus we sought an alternative approach to measure longer-range distances across the inter-dimer interface.

Paramagnetic relaxation enhancements (PREs) result from dipolar interactions between unpaired electron spins and surrounding nuclei. These far exceed internuclear dipolar couplings, and can, therefore, affect $^{15}$N nuclei up to 20 Å away from the electron. Unpaired electrons from nitroxide radicals significantly enhance transverse relaxation ($T_2$) of nearby nuclei leading to peak broadening and signal attenuation. In an NMR spectrum, PREs to a nitroxide radical are detectable as a decrease in peak intensities (cartoon representation shown in Figure 5.1) that is directly proportional to the inverse sixth power of the electron–nucleus distance. Therefore, by introducing an
unpaired electron at a specific site within the receptor, structural constraints can be obtained on a length scale consistent with the distances we aim to probe.

**Figure 5.1.** Nitroxide radicals enhance T$_2$ relaxation, leading to signal attenuation. Reduction in peak intensity occurs for resonances in close proximity to a paramagnetic center compared to a diamagnetic center. The reduction corresponds to the PRE, which is proportional to the electron–nuclear distance by the inverse sixth power.

As shown in Figure 5.2, paramagnetic tags are easily introduced into proteins through site-directed spin labeling (Hubbell and Altenbach, 1994a, 1994b) in which a thiol-specific label such as the nitroxide radical MTSL [(1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl)methanethiosulfonate] (Berliner et al., 1982) reacts with a unique cysteine on the protein of interest. The first section of this chapter describes key features of the method and yields of site-directed spin labeling applied to the cysteine mutant S487C of the CF4E receptor. This site was chosen to address a number of criteria: (1) S487 is located near the top of the methylation subdomain (Figure 5.3) in order to probe a distance anticipated to show the largest measurable change upon the proposed expansion (S487 faces the inside of the trimer of dimers, with ~18–20 Å interdimer distance from the S487 hydroxyl protein to the backbone, (2) S487 is predicted to be solvent exposed, leading to improved labeling efficiency, and (3) S487C has previous been shown to retain
60% specific kinase activity and CheA/CheW binding compared to WT CF4E (Mudiyanselage et al., 2013).

Figure 5.2. Structure of the nitroxide spin label (1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl)methanethiosulfonate (MTSL). Introducing it site-specifically is accomplished by reacting with free unique cysteine thiols on a protein. This figure is modified from (Jaroniec, 2012).

Figure 5.3. The location of initial spin label site (S487) is shown on the structural model of the trimer of dimers. (A) Top down and (B) side view are colored with the N-helix in light gray and C-helix in black. The spin label site (red sphere is S487 hydroxyl proton, shown on one monomer for clarity) resides near the top of the methylation subdomain facing the inside of the trimer of dimers (methylation sites shown as magenta spheres).
This chapter presents NMR data comparing spin-labeled and non-spin-labeled CF4E assembled on vesicles. Our results confirm that PREs are detectable in our system and provide a platform, upon further experimental refinement, to obtain the first structural restraints in the membrane-proximal region of chemoreceptors in functional and native-like arrays.

5.2. Results and Discussion

5.2.1. Spin labeling CF4E.S487C yields 75% efficiency and is non-perturbing

Site-directed spin labeling typically utilizes an excess of spin label over protein (typically 5–20 mol spin label/mol protein) that is subsequently removed by gel filtration chromatography or desalting (Hubbell and Altenbach, 1994b). Site-directed spin labeling of cysteines competes with formation of disulfide cross-linked protein dimers, which cannot be spin labeled. Therefore, an important first step to maximize labeling efficiency is to reduce any cross-linked protein prior to the addition of spin label. In solution, CF4E.S487C is approximately 40% dimer (Figure 5.4A), but that is reduced to <1% following incubation with 2 mM TCEP for 30 min at 25°C. Importantly, TCEP is removed immediately before the addition of MTSL, as a reducing environment inhibits successful spin labeling.
Figure 5.4. Non-reducing SDS-PAGE analysis enables quantification of CF4E.S487C dimer fractions. (A) CF4E.S487C in the absence (−) or presence (+) of 2 mM TCEP, added after protein purification. As quantified from ImageJ, treatment with TCEP for 30 min fully reduces all dimers. (B) Removal of TCEP followed by an overnight spin labeling incubation at 4°C, U-^{15}N-CF4E.S487C results in ~25% dimer. Gel samples were prepared by quenching additional dimer formation with 10 mM NEM and gel sample buffer immediately prior to boiling.

Following optimization, we found that eluting CF4E.S487C from the desalt column for TCEP removal directly into a tube containing an excess amount of MTSL led to the most monomer retention by SDS-PAGE. Unfortunately, we were unable to implement an EPR-based method to measure spin label efficiency, as our results were not reproducible. Instead, we used a combined approach: SDS-PAGE was used to quantify monomer and dimer fractions, followed by ESI-MS analysis which was used to distinguish unlabeled CF monomer from MTSL-labeled CF monomer by mass.

With the ultimate goal of PRE measurements by NMR, we isotopically labeled CF4E.S487C with uniform ^{15}N, prior to MTSL-labeling. As seen in Figure 5.4B, approximately 25% dimer is retained following the spin labeling protocol (section 2.8.2). ESI-MS analysis of U-^{15}N-CF4E.S487C + MTSL reported a single mass corresponding to CF + MTSL, indicating 100% labeling yield of the monomer fraction. We therefore conclude a final spin labeling efficiency of 75% was achieved for U-^{15}N-CF4E.S487C.
To monitor the effects of spin labeling on protein function, we measured kinase activity and assembly (with templating vesicles) of CF4E.S487C before and after treatment with MTSL. The results presented in Figure 5.5 clearly indicate normal activity and complex formation (shown in 5.5B for MTSL-labeled U-\(^{15}\)N-CF4E.S487C).

**Figure 5.5.** MTSL-labeled U\(^{15}\)N-CF4E.S487C has normal activity and binding. (A) Spin-labeled and unlabeled U\(^{15}\)N-CF4E.S487C assembled on vesicles with 6 uM CheA and 12 µM CheW exhibit kinase activity similar to typical CF4E activities of ~7 s\(^{-1}\). (B) Sedimentation assay with a non-reducing gel shows the total (T), supernatant (S) and pellet (P) lanes of the MTSL-labeled complexes from (A). Combined, 75% monomeric CF (CF\(_m\)) and 25% dimeric CF (CF\(_d\)) are fully incorporated into complexes with CheA and CheW at a stoichiometry of 6 CF (monomer + dimer): 1.1 CheA: 1.7 CheW. The highest molecular weight band present in T and S lanes may be an impurity, non-functional CF trimers, or CF + CheA cross-links since CheA contains multiple cysteines. However, it not present in complexes, as it is absent in the pellet. Gel samples were prepared by quenching additional dimer formation with 10 mM NEM and gel sample buffer immediately prior to boiling.
5.2.2. Paramagnetic relaxation enhancements (PREs) observed by 1D $^{15}$N NMR

To test for PREs, we assembled spin-labeled and non-spin-labeled U-$^{15}$N-CF4E.S487C into complexes with templating vesicles. Figure 5.6 compares the 1D $^{15}$N cross polarization (CP) spectra of the non-spin-labeled (black) with MTSL-labeled (red) complexes. These spectra were collected using identical parameters (see section 2.8.4) and the samples notably contain precisely the same amount of CF (286 nmol). The largest peak (centered at 121 ppm) corresponds to the bulk of backbone and side chain nitrogen signals. However, some resonance assignments were possible for nitrogens with unique chemical shifts (Table 5.1).

The MTSL-labeled spectrum clearly demonstrates PREs, seen as a reduction in signal intensities for backbone and some side chain signals. To generate a difference spectrum (green), the red spectrum was subtracted from the black spectrum. The intensity of the difference spectrum was multiplied two-fold to facilitate visualization, clearly showing all positive peak differences. This indicates the MTSL-labeled spectrum has a globally lower intensity than the non-labeled spectrum. Notably, the structural model predicts that no Lys are within 20 Å of the hydroxyl proton of S487, which is consistent with the NMR spectra that show no change in the Lys signal intensity (the difference spectrum at 33 ppm is flat). Any free MTSL would presumably cause signal attenuation throughout the protein, including some of the 8 Lys located in the methylation and signaling subdomains of the receptor. Thus the observed difference spectrum is consistent with PREs caused by the MTSL bound specifically to S487C on CF4E.
Figure 5.6. PREs are observed for MTSL-labeled U-{sup}{15}N-CF4E.S487C. The PRE difference spectrum (green) between non-MTSL-labeled (black) and MTSL-labeled (red) kinase-on arrays is consistent with a decrease in signal intensity due to PREs from MTSL bound to S487C. These 1D {sup}{15}N CP spectra were collected as described in section 2.8.4. Resolved nitrogen sidechain and Gly backbone resonances are labeled according to their predicted chemical shifts (also in Table 5.1). The inset table lists the number, residue type, and interface location of resolvable nitrogen signals predicted to be within 20 Å of the hydroxyl proton of S487C. These predictions are from the structural model of the trimer of dimers made by docking crystal structures of receptor fragments into PEG-mediated array EM density (courtesy of Ariane Briegel, and shown in Figure 5.7).

Table 5.1. Predicted {sup}{15}N chemical shifts for α-helix resonances{a}

<table>
<thead>
<tr>
<th>{sup}{15}N Location</th>
<th>Ser</th>
<th>Thr</th>
<th>Asn</th>
<th>Gln</th>
<th>Gly</th>
<th>Arg</th>
<th>Lys</th>
</tr>
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<tr>
<td>Backbone</td>
<td>114.8</td>
<td>115.3</td>
<td>117.6</td>
<td>118.6</td>
<td>107.3</td>
<td>118.9</td>
<td>119.9</td>
</tr>
<tr>
<td>Side chain</td>
<td>-</td>
<td>-</td>
<td>113</td>
<td>112</td>
<td>-</td>
<td>85 (Ne)</td>
<td>33</td>
</tr>
</tbody>
</table>

{sup}{15}N ≤ 20 Å from S487C

<table>
<thead>
<tr>
<th></th>
<th>Intradimer</th>
<th>Interdimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Arg Ne</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 Arg NH</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 Asn Nδ</td>
<td>0</td>
<td>4 Gln Nε</td>
</tr>
<tr>
<td>7 Gln Nε</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0 Lys Nζ</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>backbone</td>
<td>2 Gly</td>
<td>0</td>
</tr>
<tr>
<td>66 other</td>
<td>8 other</td>
<td></td>
</tr>
</tbody>
</table>

{a}Resonance assignments based on (Wang and Jardetzky, 2002)
Figure 5.7. Predictions of resolved nitrogens within 20 Å of S487C-MTSL. The top down view of the structural model illustrates the unique nitrogens near the spin label site (red sphere, shown on one monomer for simplicity) including intradimer: Gly (cyan), Arg (blue), and Asn (purple), and interdimer: Gln (orange).

The largest signal attenuations observed in the 1D $^{15}$N CP spectra (Figure 5.6) unsurprisingly occur in the backbone region. As previously mentioned, the closest nuclei to the unpaired electron will experience the largest PRE. Beyond backbone nitrogens, there are a number of potentially resolvable side chain nitrogens that are predicted to be within 20 Å of S487 based on the structural model of the Tar trimer of dimers. Figure 5.7 illustrates the nitrogens within 20 Å of S487 with unique chemical shifts (Table 5.1). Most significantly, there are four Gln $\text{N}_\epsilon$ (two on each opposing dimer) predicted to be 18 Å from S487. Since the 1D spectra do not resolve the Gln side chains from Asn (112 vs 113 ppm), we cannot determine whether the PRE observed for the Gln/Asn peak is due to both, or just the intradimer Asn.
5.3. Summary and Conclusion

In this chapter, the feasibility and utility of site-directed spin labeling to obtain paramagnetic relaxation enhancements in functional chemoreceptor arrays was demonstrated. Using thiol-chemistry, a paramagnetic spin label (MTSL) was successfully attached to the unique cysteine S487C of CF4E to a final yield of 75% spin-labeled, as measured by SDS-PAGE and ESI-MS. Together, the kinase activity and sedimentation data show that spin label incorporation does not perturb function or assembly.

The NMR spectra obtained for U-$^{15}$N-CF4E.S487C arrays with and without MTSL confirm our ability to detect PREs, which are observed as signal attenuation of nuclei in close proximity to the unpaired electron of the nitroxide radical. However, these data clearly present a challenge moving forward: the low resolution 1D $^{15}$N CP NMR limits resonance specificity and sensitivity. Our ultimate goal is to obtain interdimer distance restraints in a region of the receptor lacking high-resolution structural information. This is particularly difficult given that the magnitude of PREs (i.e. the signal decrease) drops at the longer distances we aim to measure (Jaroniec, 2012). Using PREs to test the proposed signaling-related expansion (Besschetnova et al., 2008) poses an even greater challenge, as it requires comparing difference spectra of samples prepared in the kinase-on and kinase-off state. At the long-range distances needed to probe the expansion hypothesis, it’s entirely possible the magnitude of PREs is too small to be resolved. Therefore, we have devised strategies to improve specificity and sensitivity in order to test our models and quantitatively measure interdimer distances.

We have devised a combined mixed labeling scheme and site-specific NMR experiments that will further simplify the observed spectra. This approach involves
assembling arrays containing a mixture of cross-linked dimers of U-\(^{13}\)C-\(^{15}\)N-CF4E.S272C and natural abundance MTSL-labeled CF4E.S487C. Cross-linking CF4E dimers at S272C (orange spheres in blue dimers of Figure 5.8) prevents dimer exchange and has been shown to retain ~80% activity (Mudiyanselage et al., 2013). Therefore, this mixed labeling scheme essentially filters out resonances from intradimer PREs given the low \(^{13}\)C and \(^{15}\)N abundance without isotopic labeling of CF4E.S487C. Importantly, we must take into account the distribution of possible trimer of dimer permutations. Assuming a 50:50 random mixture, 12.5% will contain only natural abundance MTSL-labeled CF4E.S487C dimers and 12.5% only isotopically labeled CF4E.S272C. The remaining 75% will be mixed trimers, split equally between one inner MTSL label per trimer (shown in Figure 5.8) and two per trimer. This labeling strategy gives exclusively interdimer PREs and can be used to measure distances in 1D spectra, so extensive signal averaging will enable us to detect small changes from PREs.
**Figure 5.8.** Structural model of the trimer of dimers illustrates proposed approach for interdimer distance measurements. The mixed labeling scheme involves assembling arrays containing natural abundance CF4E.S487C (± MTSL, red sphere) shown in gray and black, and U-^{15}N, 1-^{13}C-Val-CF4E.S272C shown in blue (S272C cross-links, orange spheres). The unique dipeptide VQ490 (Q490 backbone N shown in yellow) is predicted to be 18 Å from the hydroxyl proton of S487C.

While not quantitative at this stage, we have demonstrated measurable PREs to MTSL-labeled S487C, probing the membrane-proximal region of the chemoreceptor cytoplasmic domain in functional complexes. We have designed a feasible strategy to measure quantitative PREs and provide structural constraints in this poorly understood region of the receptor. This work has set the stage for future studies aiming to test the proposed expansion model of signal transmission. Moreover, it will define structural features of the receptor in the context of the array. It is widely applicable to any site that can be mutated to cysteine throughout the receptor, to further constrain the structure.
CHAPTER 6

SUMMARY AND RECOMMENDATIONS

6.1. Summary of Findings

The main objective of this study is to probe the functional architecture and assembly governing bacterial chemotaxis array formation in order to elucidate molecular mechanisms of kinase regulation. To accomplish this, a metal-mediated assembly strategy was developed to recapitulate the activity and structure of native chemoreceptor arrays using aspartate receptor cytoplasmic fragments (CF). In this method, divalent metals including Ni(II), Zn(II), and Co(II) bind to monomeric CF N-terminal His-tags, which sufficiently stabilizes receptor dimers to promote ternary complex formation with CheA and CheW. Activity measurements show that metals promote kinase-on state assemblies of CF4Q, while metals combined with the crowding agent PEG drive assembly of CF4E into the kinase-off/methylation-on state. Furthermore, electron cryotomography (ECT) show these assemblies retain native-like architecture of the canonical 12 nm center-to-center extended hexagonal lattice.

Metal-mediated assembly has enabled a deeper understanding of the relationship between the array and kinase activation. Thus, a number of complementary principles emerge from this work. First, we found that receptor dimerization with metal—observed by size exclusion chromatography (Figure 3.9)—is sufficient to drive assembly of complexes with CheA and CheW. This is consistent with results indicating that cysteine cross-linked receptor dimers form sedimentable complexes with CheA and CheW in the absence of stabilizing agent such as vesicles, PEG, or metal (Haglin, Li, and Kashefi, unpublished observations). We did not, however, observe discrete trimers of receptors.
dimers by SEC. Instead, dimers form large oligomers, aggregates, and fibrils (Figure 3.10) in the absence of CheA and CheW. Remarkably, the metal-induced aggregation is at least partially reversible; kinase activity is restored to aggregated His-tagged CF upon the addition of CheA and CheW. Thus, ternary complex formation is highly favored in the presence of metal.

The second principle that emerges is that allosteric interactions beyond the core signaling unit contribute to fully activating the kinase. Kinetic analysis of the metal-mediated assembly process using kinase activity and sedimentation (Figure 4.1), and SEC (Figure 4.5) indicate CheA and CheW fully incorporate into complexes prior to the onset of full kinase activity. Moreover, a distinct size shift to larger complexes at later assembly times points (1–4 h) coincides with maximal activity. We cannot discern the size of the initially smaller complexes, but they are large enough to sediment and clearly form prior to assembly of the fully extended array. Since no further CheA or CheW binds upon assembly into the extended array and measured stoichiometries are consistently 6:1:2, we conclude that core units contain four CheW in which two are bound strongly to receptor and CheA, and two bound weakly to receptor alone (Figure 4.7). Importantly, our results suggesting important allosteric interactions within the array are directly at odds with reports that single core units prepared with intact receptors reconstituted into nanodiscs are capable of all chemosensory functions to the same degree as native membrane-bound arrays (Boldog et al., 2006; Li and Hazelbauer, 2011; Li et al., 2011). However, we note that their core units are missing extra CheW due to extensive washing. Proposed to provide array integrity as a CheW-only ring (Briegel et al., 2012; Liu et al., 2012), perhaps excess CheW plays a role in preserving optimal array assembly and
symmetry of the alternating CheA/W patterning best observed for in vivo arrays (Briegel et al., 2014a).

Taken together, these outcomes shed light on a correlation between chemoreceptor array size, array integrity, and kinase activity. Previously, it was observed that the density of CF4E on templating vesicles was coupled to both kinase activation and receptor methylation. Concurrently measured FRET efficiencies between receptor C-terminal tails suggested an expanded or dissociated state at lower receptor density (Besschetnova et al., 2008). Additionally, reconstituted intact receptors at multiple densities maintained similar ligand affinity, thus ruling out receptor dissociation (Sferdean et al., 2012). These data lead to a proposed ligand-induced expansion model (Figure 1.4). However, in light of the evidence presented in this study that low kinase activity correlates with smaller arrays, we offer an alternative perspective that could equally describe the observed activity density dependence: at low densities, receptor core units are stably assembled but scattered and activate the kinase with less efficiency; high receptor densities exhibit increased kinase activity as a result of achieving a fully packed hexagonal lattice. The last section of this study established an approach to test these two interpretations. The ligand-induced expansion model predicts the greatest trimer of dimers distance change would occur nearest the membrane. As such, we aimed to collect long-range structural measurements and constrain the trimer of dimers structure in the membrane-proximal region. In comparing kinase-off and kinase-on assembled arrays (either by density or mutation), an observed difference in trimer distances would confirm an expansion whereas no change in distance would suggest core units are scattered at low density.
To measure interdimer distances and test the expansion model, we initiated a paramagnetic relaxation enhancement (PRE) solid-state NMR approach. The data presented in Chapter 5 indicate incorporation of the nitroxide paramagnetic spin label (MTSL) at S487C of CF4E yields high labeling efficiency (75%) and does not perturb activity or array assembly. Furthermore, a comparison of 1D $^{15}$N NMR spectra with or without spin label demonstrates PREs consistent with predictions from the trimer of dimers structural model. Since quantitative distance measurements were not immediately obtainable with a uniform $^{15}$N-labeling scheme, we developed proposals to further improve our sensitivity and specificity, as discussed in section 5.3.

6.2. Recommendations

A number of interesting questions emerge from this study. First, the role of excess CheW in core complexes and the extended array remains unclear due to conflicting observations. On one hand, Studdert and coworkers reported that overexpressing WT CheW or high-receptor affinity mutants of CheW interfered with trimer formation, clustering, and chemotactic ability (Studdert and Parkinson, 2005; Cardozo et al., 2010). Conversely, Maddock et al. found normal polar clustering of chemoreceptors in the absence of CheA, suggesting CheW alone is sufficient to drive assembly of complexes (Maddock and Shapiro, 1993). The repeating protein–protein contacts between CheA/CheW and CheW/Receptor anchor the three components of the core unit and the extended array. These interfaces have been defined through both in vitro (Natale et al., 2013; Piasta et al., 2013) and in vivo (Pedetta et al., 2014) disulfide crosslinking experiments, NMR (Vu et al., 2012; Wang et al., 2012a), and x-ray crystal structures (Briegel et al., 2012; Li et al., 2013). In an ECT-derived model, CheW within the CheW-
only ring interact with adjacent receptors through strong hydrophobic interactions similar to those for CheA-P5 to receptors (Liu et al., 2012). Notably absent from the literature, however, is a detailed study of the CheW/CheW interactions within the functional array. A detailed disulfide mapping of CheW–CheW contacts for arrays would shed light on both the type and strength of these interactions.

We have demonstrated in this study that excess CheW is likely a component of the chemosensory core unit and therefore an integral part of the array as CheW-only rings. Is it possible that by satisfying all available receptor binding sites, excess CheW enhances assembly? One such possibility is that excess CheW helps drive the alternating CheA/W and CheW-only ring patterning observed optimally for in vivo arrays (Briegel et al., 2014a). We could test this by assembling either PEG or metal arrays with both low and high CheW concentrations in the assembly, and analyze the effects on kinase activity, assembly (by SEC), and array structure (by ECT).

While the metal-mediated assembly method has found great use and applicability for bacterial chemotaxis arrays, we ultimately feel it will find a greater use in other large multiprotein complex systems. Given the extraordinarily crowded conditions of the cell (Ellis, 2001), a vast number of important biological processes are performed by protein dimers or protein complexes (Alberts, 1998; Marsh and Teichmann, 2015). Fully understanding these processes is often accomplished through in vitro recapitulations of native systems. Our in vitro strategy is particularly well suited for proteins that dimerize as part of their assembly process, such as many other transmembrane signaling proteins. The mechanism of metal assembly relies on His-tags, which are widely used and easily incorporated into proteins on the termini and within. To conclude, we feel that this work
has begun to unravel integral functional and architectural features of chemoreceptor arrays, and hope that applying this method to other more complex systems can make similar advances.
APPENDIX

UNILAMELLAR VESICLE ASSEMBLY OPTIMIZATION

Template-directed vesicle assembly of chemoreceptor signaling complexes was developed as a simplified approach to study the receptor cytoplasmic fragment (CF) in functional complexes with CheA and CheW (Shrout et al., 2003). Mixed lipid vesicles containing a nickel-chelating lipid (DOGS-NTA-Ni$^{2+}$, henceforth called DOGS for simplicity) were used to guide the assembly of histidine-tagged CF onto the outer leaflet of the lipid bilayer. Preparation of unilamellar vesicles is critical for achieving a known membrane surface area that is fully occupied by hexagonally organized active complexes. Active signaling complexes form under a variety of lipid and protein concentrations, but retaining native-like array hexagonal geometry and protein stoichiometry requires fine-tuning. To address these issues, this appendix describes (1) the implementation of a fluorescence-based assay to measure vesicle lamellarity, and (2) optimization of protein concentrations to achieve maximal kinase activation and native-like stoichiometry (6 CF:1 CheA:2 CheW) on these unilamellar vesicles.

A1. Fluorescence Lamellarity Assay

The preparation of lipid vesicles, as described in Chapter 2 (Section 2.2), includes freeze/thaw cycles following lipid rehydration in order to promote formation of unilamellar vesicles. Prior to including this in the protocol, our vesicle preparations were heterogeneous mixtures of unilamellar, bilamellar, and multilamellar, as seen by ECT (Figure A.1).
Figure A.1. Tomographic slices of binary lipid vesicles containing DOPC and DOGSNTA-Ni$^{2+}$ show extensive lamellae layering. These multilamellar vesicles (MLVs) were extruded to 100 nm (see Chapter 2) but did not undergo cycles of freeze/thaws. ECT kindly provided by Dr. Ariane Briegel.

We employed a fluorescence-based assay to monitor the integrity of our vesicle preparation and determine vesicle lamellarity (McIntyre and Sleight, 1991; Angeletti and Nichols, 1998; Heider et al., 2011). For this experiment, vesicles were prepared as described in Chapter 2 (including freeze/thaw cycles) with the addition of the fluorescent lipid DOPE-NBD (1,2-dioleoyl-sn-glycero-3-phosphethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) such that the final DOPE-NBD concentration was 1% of the total lipid. We assume that the high degree of miscibility of these ternary mixtures of lipids (DOGS + DOPC + DOPE-NBD) promotes equally dispersed NBD on the inner and outer leaflets. Vesicles were diluted to ~100 µM lipid for a final NBD concentration of ~1 µM, and placed in a fluorometer that is setup to perform a time-based experiment (~10 minutes) with NBD excitation and emission wavelengths at 460 nm and 535 nm, respectively. As shown in Figure A.3, the first 200 seconds of recording was sufficient to determine initial intensity and photo bleaching effects. Then, the membrane impermeable NBD fluorescence quencher dithionite was added to a 1000-fold excess over the NBD
(for example, 1 mM dithionite added to 1 µM DOPE-NBD that is at 1% concentration of the 100 µM vesicles), and fluorescence levels recorded for an additional 200 seconds. The drop in fluorescence was then used to estimate the number of lamellae present in the vesicle population. Following quenching, the remaining fluorescence of predominantly unilamellar vesicles should be ~50% of the initial intensity, originating from the interior bilayers of the vesicles (Figure A.2 and A.3). Quenching to less than 50% of initial fluorescence may indicate the presence of leaky vesicles. Quenching to levels between 50 and 100% may indicate the presence of multilamellar vesicles.

Figure A.2. Cartoon representation of outer leaflet quenching of DOPE-NBD. NBD labeled lipids are colored in red. Upon addition of dithionite (S$_2$O$_4^{2-}$), only inner leaflet DOPE-NBD lipids retain fluorescence. Reprinted (adapted) with permission from (Heider et al., 2011). Copyright 2011 American Chemical Society.

To solubilize the vesicles, Triton X-100 was added above its critical micelle concentration of 0.22 mM (Tiller et al., 1984) and at least a 4-fold excess over total lipid concentration (for example, 400 µM Triton X-100 added to 100 µM vesicles). The return to baseline fluorescence intensity indicates complete vesicle disruption and full quenching of the inner leaflet DOPE-NBD.
Figure A.3. Relative fluorescence intensity changes upon outer leaflet quenching. This plot of relative fluorescence intensity is from a population of vesicles showing fractional intensity loss as a result of quenching the outer leaflet NBD with dithionite. A 100 µM solution of 59% DOPC, 40% DOGS-NTA-Ni\(^{2+}\), and 1% DOPE-NBD was placed into a cuvette and fluorescence measured over time (excitation at 460 nm and emission at 535 nm). At approximately 200 s, addition of sodium dithionite (Na\(_2\)S\(_2\)O\(_4\)) quenches NBD fluorescence leading to a drop in relative intensity to ~50%, indicating a predominantly unilamellar vesicle population. Vesicles are fully solubilized upon the addition of 400 µM Triton X-100 at 400 s. The slow fluorescence decreases are due to photo bleaching effects.

A2. Vesicle Assembly Protein Concentration Optimization

This section describes the optimization of protein conditions in order to fully maximize the vesicle surface area occupied by hexagonal arrays. We used kinase activity and stoichiometry (optimal is 6 CF: 1 CheA: 2 CheW) measured in a sedimentation binding assay to determine optimal protein concentrations for vesicle assembly (see Chapter 2 for details). Previously, Weis and coworkers utilized vesicles at a total lipid concentration of 580 µM at a 1:1 ratio of DOPC:DOGS (Shrout et al., 2003; Montefusco et al., 2007). These experiments used 30 µM CF (≈1/10 of DOGS concentration) for complete binding of CF to DOGS, and used limiting concentrations of CheA (1.2 µM),
and CheW (5 μM) so that all of CheA and CheW would be bound to CF. Koshy subsequently identified conditions with excess CheA and CheW (30 μM CF, 6 μM CheA, and 12 μM CheW) with maximal activity on 580 μM vesicles (optimization detailed in (Koshy, 2013)) so that all of the CF would be incorporated into complexes with CheA and CheW.

In addition to improving our preparation of unilamellar vesicles, we increased the total lipid concentration to 725 μM, maintaining 290 μM DOGS, in order to provide sufficient surface area for full hexagonal arrays of 30 μM CF. Thus, the optimization of new CheA and CheW concentrations was needed. To do so, we simultaneously changed CheA and CheW concentrations while keeping 30 μM CF (both CF4E and CF4Q) and 725 μM vesicle constant. CheA and CheW conditions at different ratios ranging from 6–24 μM were compared. For simplicity throughout this section, these conditions are labeled and referred to by the assembly concentration ratios of CheA to CheW (e.g. 6:12 means 6 and 12 μM CheA and CheW, respectively), with 30 μM CF in each assembly, but omitted in the label. These values are different than the measured stoichiometries of the complex determined after assembly from the pellet ratios in the sedimentation assay (e.g. 6:1:2 is 6 CF to 1 CheA to 2 CheW).

Optimal conditions for both CF4E and CF4Q assembly on 725 μM vesicles were chosen based on both the activity and complex stoichiometry. Many of the CheA and CheW concentrations tested promote similar activities, and likewise many assemblies had acceptable stoichiometries. These data were collected over the course of 2–3 days each for CF4E and CF4Q, and fresh vesicles were prepared and used no more than one day later. Replicate assembly ratios were prepared to carry over multiple days and account for
day-to-day variability. However, these replicates were reproducible, and no further correction of activities or ratios was necessary. For CF4E, 8:12 and 12:20 produced the highest activity with approximately 6:1:2 stoichiometries (Figure A.4.A and Table A.1). While 6:12 had the highest activity overall, this sample had only 2.9 µM CheA bound and the stoichiometry was 6:0.6:1.0, and thus below native levels. These conditions may be useful, however, if such stoichiometries were desired. CF4Q assemblies were typically more active than CF4E with greater CheA binding (Figure A.4.B and Table A.2). 12:24 produced the highest activity and was therefore chosen as the optimal assembly condition for CF4Q. While this assembly had an elevated complex stoichiometry of 6:1.7:2.4, we note that most CF4Q assemblies were similarly higher than the native 6:1:2.
Figure A.4. Optimization of CheA and CheW concentrations for maximal kinase activity with vesicle assembly. CF4E (A) and CF4Q (B) were each assembled at 30 µM with varying concentrations of CheA and CheW that correspond to labels (e.g. 6:12 means 6 and 12 µM CheA and CheW, respectively). Vesicle concentrations were kept constant at 725 µM total lipid (with 290 µM DOGS). Total (black bars) and specific (gray bars) kinase activities are averages of two replicates and error bars indicate ± one standard deviation. Data are also listed in Table A.1 for CF4E, and A.2 for CF4Q, with corresponding [CheA]_{bound} and pellet stoichiometries.
<table>
<thead>
<tr>
<th>CheA:CheW (µM)</th>
<th>Total Activity (s⁻¹)</th>
<th>Specific Activity (s⁻¹)</th>
<th>Bound CheA (µM)</th>
<th>Stoichiometry (CF:CheA:CheW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6:6</td>
<td>2.35 ± 0.07</td>
<td>4.87 ± 0.15</td>
<td>2.9</td>
<td>6:0.4:0.5</td>
</tr>
<tr>
<td>6:12</td>
<td>5.13 ± 0.04</td>
<td>11.40 ± 0.08</td>
<td>2.7</td>
<td>6:0.6:1.0</td>
</tr>
<tr>
<td>8:12</td>
<td>3.38 ± 0.06</td>
<td>6.48 ± 0.12</td>
<td>4.0</td>
<td>6:0.8:1.2</td>
</tr>
<tr>
<td>8:16</td>
<td>4.31 ± 0.01</td>
<td>8.61 ± 0.01</td>
<td>4.0</td>
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<tr>
<td>10:20</td>
<td>3.29 ± 0.09</td>
<td>5.58 ± 0.15</td>
<td>5.9</td>
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<tr>
<td>12:8</td>
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<td>5.19 ± 0.02</td>
<td>3.9</td>
<td>6:0.9:0.8</td>
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<td>12:12</td>
<td>2.62 ± 0.06</td>
<td>7.32 ± 0.17</td>
<td>4.3</td>
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<tr>
<td>12:16</td>
<td>3.11 ± 0.14</td>
<td>6.33 ± 0.29</td>
<td>5.9</td>
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<td>12:20^{d}</td>
<td>3.92 ± 0.10</td>
<td>9.41 ± 0.25</td>
<td>5.0</td>
<td>6:1.4:1.7</td>
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<td>12:24</td>
<td>3.29 ± 0.07</td>
<td>4.07 ± 0.09</td>
<td>9.7</td>
<td>6:1.7:2.4</td>
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<tr>
<td>16:8</td>
<td>1.42 ± 0.01</td>
<td>5.15 ± 0.02</td>
<td>4.4</td>
<td>6:1.1:1.0</td>
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<td>16:12</td>
<td>1.93 ± 0.01</td>
<td>6.67 ± 0.03</td>
<td>4.6</td>
<td>6:1.2:1.3</td>
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<tr>
<td>18:18</td>
<td>2.42 ± 0.11</td>
<td>8.52 ± 0.37</td>
<td>5.1</td>
<td>6:1.4:1.6</td>
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</table>

^{a}30 µM CF4E, 725 µM vesicles (435 µM DOPC and 290 µM DOGS-NTA-Ni²⁺) and varying concentrations of CheA and CheW (listed) in each assembly.

^{b}Kinase activity per total CheA in assembly

^{c}Kinase activity per bound CheA in assembly

^{d}Optimal conditions chosen for CF4E based on high activity, CheA binding, and stoichiometry.
Table A.2. Kinase activities, CheA binding, and complex stoichiometries in CF4Q vesicle assembly optimization

<table>
<thead>
<tr>
<th>CheA:CheW (µM)</th>
<th>Total Activity (s⁻¹)</th>
<th>Specific Activity (s⁻¹)</th>
<th>Bound CheA (µM)</th>
<th>Stoichiometry (CF:CheA:CheW)</th>
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<tr>
<td>6:6</td>
<td>4.32 ± 0.25</td>
<td>7.20 ± 0.42</td>
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<td>6:0.8:0.8</td>
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<td>3.30 ± 0.37</td>
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<td>8.37 ± 0.23</td>
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<tr>
<td>12:18</td>
<td>4.31 ± 0.07</td>
<td>8.08 ± 0.14</td>
<td>6.4</td>
<td>6:1.5:2.2</td>
</tr>
<tr>
<td>12:20</td>
<td>5.38 ± 0.25</td>
<td>9.17 ± 0.43</td>
<td>7.0</td>
<td>6:1.7:1.8</td>
</tr>
<tr>
<td>12:24</td>
<td>6.54 ± 0.02</td>
<td>10.89 ± 0.03</td>
<td>7.2</td>
<td>6:1.7:2.4</td>
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<tr>
<td>14:20</td>
<td>3.87 ± 0.04</td>
<td>6.77 ± 0.07</td>
<td>8.0</td>
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</tr>
<tr>
<td>16:24</td>
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<td>7.47 ± 0.03</td>
<td>9.4</td>
<td>6:2.0:1.6</td>
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<tr>
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<td>2.32 ± 0.10</td>
<td>5.23 ± 0.03</td>
<td>8.0</td>
<td>6:1.8:1.7</td>
</tr>
<tr>
<td>18:18</td>
<td>2.53 ± 0.03</td>
<td>6.81 ± 0.07</td>
<td>6.7</td>
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<td>8.76 ± 0.13</td>
<td>6.6</td>
<td>6:1.8:2.5</td>
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<tr>
<td>24:12</td>
<td>1.92 ± 0.01</td>
<td>8.07 ± 0.04</td>
<td>5.7</td>
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<tr>
<td>24:18</td>
<td>2.00 ± 0.01</td>
<td>10.02 ± 0.01</td>
<td>4.8</td>
<td>6:1.4:1.4</td>
</tr>
</tbody>
</table>

a30 µM CF4Q, 725 µM vesicles (435 µM DOPC and 290 µM DOGS-NTA-Ni²⁺) and varying concentrations of CheA and CheW (listed) in each assembly.
bKinase activity per total CheA in assembly
cKinase activity per bound CheA in assembly
dOptimal conditions chosen for CF4Q based on high activity, CheA binding, and stoichiometry.
BIBLIOGRAPHY


