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Mechanistic Insights into Diverse Protease Adaptor Functions

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Mechanistic Insights into Diverse Protease Adaptor Functions

A Dissertation Presented

by

Nathan James Kuhlmann

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

DOCTOR OF PHILOSOPHY

September 2021

Molecular and Cellular Biology

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Mechanistic Insights into Diverse Protease Adaptor Functions

A Dissertation Presented

by

Nathan James Kuhlmann

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DEDICATION

To my family, Janelle, and closest friends, for their relentless support, motivation,
and moments of relief during this process.

I dedicate this thesis to my parents, my brother Mark and Janelle. Thank you for
encouraging me to follow my dreams and pursue my Ph.D. and never giving up.
Thank you for teaching me the value of living a balanced life while also giving my
full force into my work and my career.

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all the BTP events has been something I will never forget. The BTP program has helped me get my foot in the door for starting my career in Biotechnology, and every faculty member that is a part of the BTP program has been encouraging, supportive and insightful in reaching my professional goals.

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I would like to thank all the members of the MCB program that I have had the pleasure of socializing and bonding with. Noelle, Ana, Javi, Thomas, Spencer, and all the friends I have made in MCB have made this journey worth it. I will sorely miss the MCB retreats and events that we attended and enjoyed together.

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ABSTRACT

Mechanistic Insights into Diverse Protease Adaptor Functions

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Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Peter Chien

Protein degradation is an essential cellular process that helps maintain proper homeostasis. The ClpXP protease broadly regulates bacterial development and quality control during the cell cycle. The range and order of substrates that ClpXP degrades during the cell cycle is dictated by 3 accessory proteins, which are known as adaptors. This thesis will elaborate on how dimerization tightly regulates the stability and activity of the adaptor protein at the center of this hierarchy, RcdA, and show how this affects normal cellular processes in *Caulobacter crescentus*. I will discuss the mechanism by which dimerization limits RcdA activity and how the dimerization interface contains selective regions that differentiate which substrates can engage with RcdA. Lastly, I will discuss how RcdA and the master regulator of *Caulobacter*, CtrA, interacts with the third adaptor in the hierarchy, PopA and the various surfaces on the PopA adaptor that are involved in each interaction. The remainder of the thesis will present data that arises from these projects.

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CHAPTER 1

An Overview of Adaptor-Dependent Protein Degradation and Regulation

Parts of this introduction were as written by Kuhlmann, N.J. and Chien, P., published in Current Opinions in Microbiology 2017

1.1 Overview

The controlled degradation of proteins is a necessity for cellular development and physiology. AAA+ (ATPases Associated with diverse cellular Activities) proteases dictate the lifetime of proteins after translation, offering another layer of post-translational control and recycling amino acids for metabolic networks in all organisms. While there are few proteases in every organism, they contain the capacity to completely change the balance of proteins in the cell at any given point. In most cases, proteases themselves are also tightly regulated by additional factors, be it small molecules, protein scaffolds, post-translational modifications, or even degradation by themselves or other proteases. At the center of my thesis is the question of how protein scaffolds, or adaptors regulate which substrates are chosen to be degraded at the molecular level and how degradation of adaptors affects cell physiology. I will go into detail into different proteases that use adaptors to regulate their mechanism and substrate selection, how adaptors can work in tandem to regulate substrate selection in a hierarchical manner, how adaptors themselves are regulated post-translationally, and the specific characteristics of my adaptor of focus, RcdA. Each of these topics drive the research questions of my thesis project.

1.2 Adaptor-Dependent Proteases in Nature

Proteases are, on the surface, simple enzymes. Proteases typically consist of two functional components: the unfoldase domain, which recognizes folded or unfolded substrates by a short sequence motif, called a degron. After recognition of this sequence, the AAA+ unfoldase module unravels the substrate through the power of ATP hydrolysis into its primary structure, where the substrate is fed into the proteolytic peptidase module (Figure 1.1), (Hanson and Whiteheart, 2005). The unfoldase module is therefore responsible for the initial specificity of the protease-substrate interaction. However, during specific stages of the cell-cycle and during periods of stress, additional levels of specificity are needed to prevent unwanted degradation and to prioritize certain substrates. In such cases, proteases use additional proteins called adaptors to dictate substrate selection. At the simplest level, a protease adaptor acts to select which substrates are actively engaged with the unfoldase component of a protease (Figure 1.2). Proteases that use adaptors are widespread in nature. The ClpXP protease, found in bacteria and in human mitochondria (Baker and Sauer, 2011), makes use of several adaptors to drive cell-cycle dependent protein degradation, the function of which I will go into greater detail in section 1.4. For the Clp family of proteases, there are several proteases that make use of specific adaptors to regulate substrate choice. The ClpAP protease, which shares similarities to ClpXP, uses the ClpS adaptor to prioritize N-end rule substrates (Erbse et al, 2006, Wang et al, 2007 and 2008). Similarly, in *Bacillus subtilis*, the ClpCP

protease uses several adaptors, MecA, McsB, and YpbH, to regulate the competency and sporulation of the bacterium by degrading factors such as ComK and ComS (Schlothauer et al, 2003, Persuh et al, 2002, Kirstein et al, 2007). Interestingly, the assembly of the ClpC hexameric unfoldase is mediated by interactions with MecA, suggesting that adaptors can act in other ways besides direct substrate engagement to regulate protease function (Kirstein et al, 2006). Finally, the 26S proteasome uses adaptors such as Cic1 to recruit specific proteins for proteasomal degradation (Jager et al., 2001). In each of these cases, adaptors provide an additional layer of specificity and prioritization for the protease substrate pool such that substrates of particular importance are selectively degraded. For some adaptors such as MecA, the post-translational state of the adaptor is modified by phosphorylation to control its function and this has direct consequences on the physiology and development of the bacteria. The additional layers of regulation that gate adaptor activity and stability is a central theme of my thesis work and will be further explored in Chapters 2 and 3.

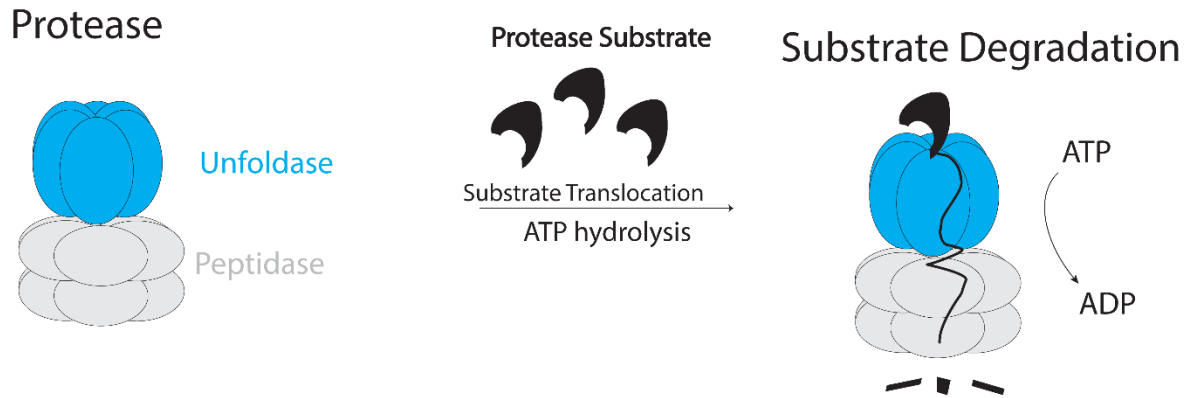


Figure 1.1: Mechanism of Protease Function.

AAA+ Proteases contain two functional modules: A hexameric unfoldase chaperone, and a peptidase chamber that contains the proteolytic active site. Through the power of ATP hydrolysis, the unfoldase portion engages substrates through a short sequence recognition motif, then unfolds the substrate into the peptidase chamber, where the protein is degraded into its amino acid components.

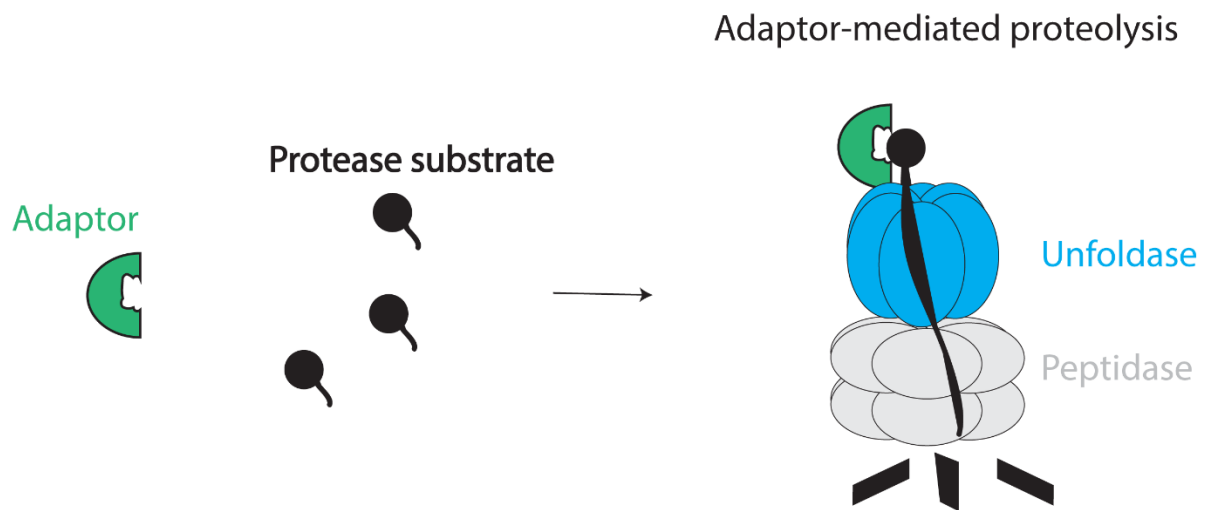


Figure 1.2: Mechanism of Protease Adaptors.

Adaptor proteins work by limiting the protease to only degrading certain targets, providing extra levels specificity to the substrate-unfoldase interaction. Many adaptors interact with the substrates to bring them in proximity of the unfoldase, where they are recognized and subsequently degraded.

1.3 Specific Adaptors Tether or Prime Proteases for Delivery of Cargo

Parts of this section were taken from Kuhlmann, Chien 2017.

Adaptors typically fall into two modes of function: that of an active primer or that of a passive scaffold. Adaptors that act as active primers prepare the protease for substrate delivery, often by directly interacting with the protease before interacting and delivering its cargo. In the case of the CpdR adaptor from *Caulobacter crescentus*, binding of this protein to the unique N-domain of the ClpX unfoldase prepares the protease for recognition of a class of substrates (Figure 1.3), (Lau et al., 2015, Iniesta et al., 2006). Here, the adaptor does not strongly interact with its cargo in the absence of the ClpX N-domain. This preparation, or priming, of the protease could arise from conformational changes in either CpdR or ClpX upon adaptor binding or could stem from a composite interaction surface that is only present in the complex. Regardless of mechanism, this type of priming function has features distinguishable from a simple scaffold. For example, high concentrations of scaffolds such as SspB inhibit substrate degradation because partial occupancy of the scaffold restricts simultaneous binding of substrate and protease. By contrast, CpdR activity resists such inhibition as the substrate binding activity is only present when the adaptor binds the protease (Lau et al., 2015).

Similar types of priming functions have been observed with other adaptor systems. Binding of the YjbH adaptor is required for Spx degradation by ClpXP in gram-positive bacteria, but YjbH does not bind ClpX well on its own. Here, YjbH

binding unveils a C-terminal degron in Spx that is in turn recognized by ClpXP (Chan et al, 2014). The RssB adaptor binds RpoS directly, but its weak binding to ClpX suggests that priming of the substrate by the adaptor is needed for protease recognition (Zhou et al., 2001, Studemann et al., 2003). Thus, it seems that the ability of adaptors to activate either protease or substrate for eventual degradation is a mechanism that has arisen in many systems.

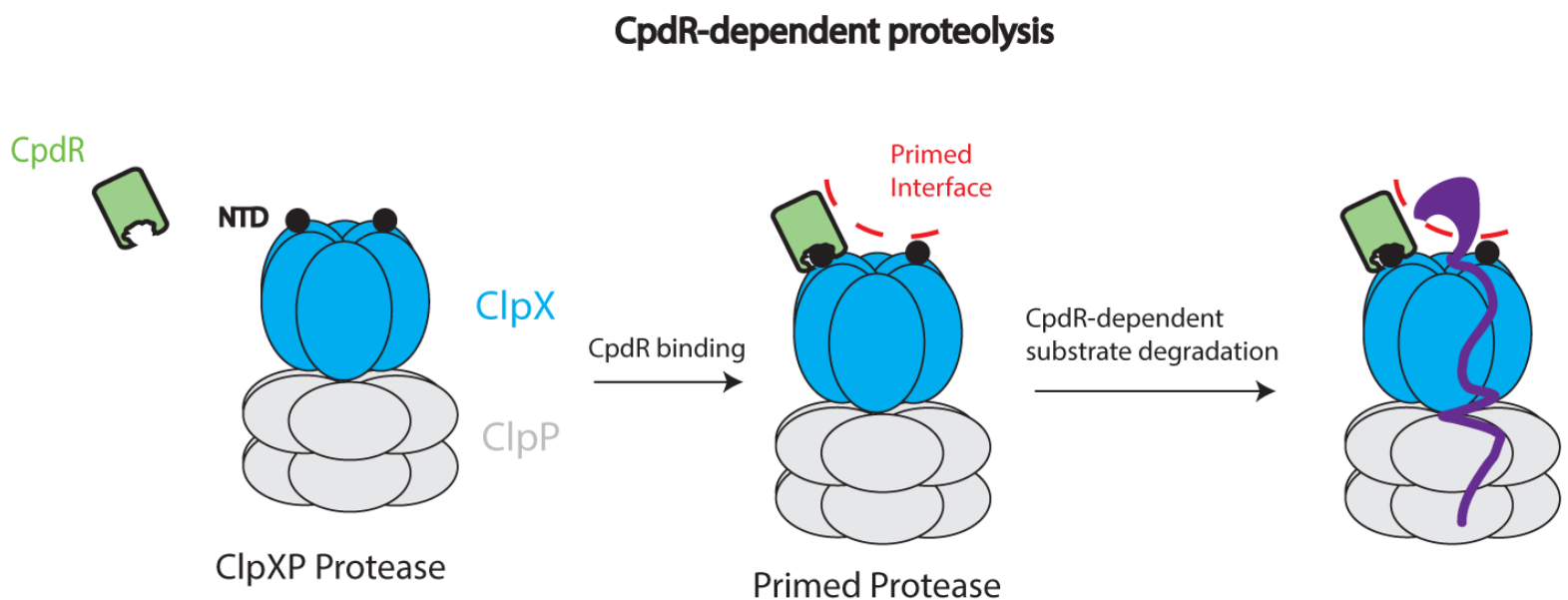


Figure 1.3: Mechanism of the Priming Adaptor CpdR.

The CpdR adaptor acts as a primer for the ClpXP protease. CpdR does not interact with its substrates on its own, rather, it directly interacts with the N-terminal domain of ClpX to prime ClpX for interactions with CpdR-dependent substrates. While the mechanism of CpdR-dependent ClpX activation is currently unknown, this mechanism might involve the formation of a new composite interface between ClpX and CpdR that recruits CpdR-dependent substrates.

The simplest mechanism for an adaptor is that of a passive scaffold. In these cases, the adaptor directly interacts with its cargo outside of the presence of a protease and increases the effective concentration of substrate that the unfoldase module encounters. In doing so, the adaptor increases the relative concentration of substrates to well past the K_M of the protease by constraining the volume of the substrate to make recognition events by the unfoldase module happen more frequently. Adaptors that follow this function are the SspB adaptor, which binds and delivers substrates tagged with the ssrA peptide appended to proteins arising from failed translation events (Levchenko et al., 2000, Gottesman et al., 1998). SspB acts as a constitutive adaptor as its levels are constant throughout the bacterial life cycle (Farrell et al., 2005). Other adaptors that follow this mechanism are ClpS (Erbse et al, 2006, Wang et al, 2007 and 2008), and the RcdA (Regulator of CtrA Degradation) adaptor in *Caulobacter crescentus* (Figure 1.4), (Joshi et al., 2015, McGrath et al, 2006), the adaptor of my research focus in my thesis. In the next subchapter, I will go into the specific functions that RcdA performs during the *Caulobacter* cell-cycle and how it coordinates its delivery functions with additional adaptors.

RcdA Adaptor Mechanism

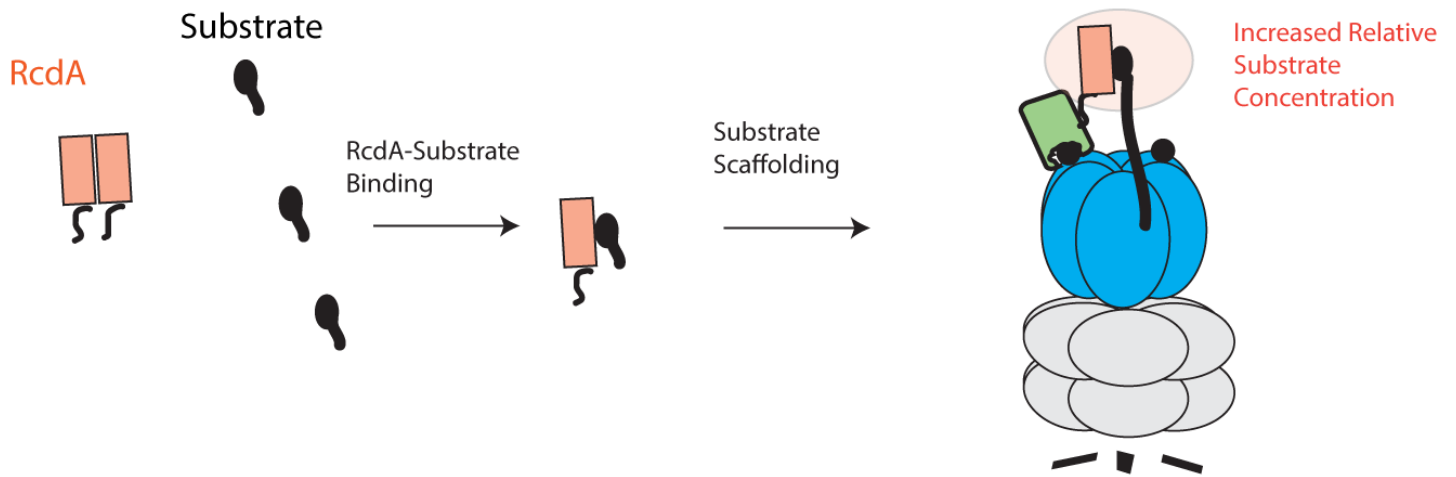


Figure 1.4: Mechanism of the Scaffolding Adaptor RcdA.

The RcdA adaptor acts as a scaffold for CpdR and ClpXP. RcdA directly binds its cargo, and upon interactions with a CpdR-primed ClpXP, can scaffold its substrates to ClpX for unfolding and subsequent degradation. RcdA's function as a scaffold increases the relative substrate concentration by constraining the volume that the pool of substrate occupies, allowing for ClpX to encounter a higher effective concentration of substrate and consequentially result in more unfolding events.

1.4 A Hierarchy of Adaptors Drive Cell-Cycle Protein Degradation in *Caulobacter crescentus*

Proteases make use of adaptors during cellular transitions and during times of stress. In *Caulobacter crescentus*, the ClpXP protease makes use of a hierarchy of adaptor proteins to dictate substrate degradation during the developmental transition from a motile swarmer developmental state to a sessile stalked developmental state. During the G1 to S transition in the *Caulobacter* life cycle, ClpXP degrades a series of key regulatory proteins that include chemoreceptors involved in cell motility (Bhat et al., 2013, Tsai et al., 2001) and several replication and transcription factors critical for promoting proper cell cycle and developmental control (Quon et al., 1996, Gora et al., 2013, Biondi et al., 2006, Gorbatyuk et al., 2005). While the ClpXP is capable of degrading important cell cycle independent substrates such as DnaX (Vass et al., 2013), ClpXP requires one or more adaptors for all substrates that are degraded in a cell-cycle dependent manner (Bhat et al., 2013, Joshi et al., 2015, Lau et al., 2015). During the *Caulobacter* cell-cycle, ClpXP makes use of 3 adaptors and oscillating levels of the small molecule cyclic-di-GMP (cdG) to dictate which substrates are degraded (Figure 1.5). During G1, the first adaptor, CpdR, binds to the N-terminal domain of ClpX, allowing the degradation of a class of substrates specific to CpdR, including the phosphodiesterase PdeA, which downregulates the levels of cdG (Lau et al., 2015). Only after CpdR is assembled on ClpXP can the second adaptor, RcdA, bind via its C-terminal tail to CpdR and deliver a class

of substrates that includes the σ^{54} transcription factor TacA, which is involved regulating genes involved in stalk biogenesis (Biondi et al., 2006, Joshi et al., 2015), and the pole-localized protein SpbR, which is implicated in inhibiting centromere translocation (Wang et al., 2019). After the assembly of CpdR and RcdA onto ClpX, the third adaptor, PopA (Duerig et al., 2009, Ozaki et al., 2014), binds the small molecule cdG and RcdA to deliver its own class of substrates, which include the replication inhibitor CtrA (Quon et al., 1996, Smith et al., 2014). This hierarchical proteolysis provides precise timings of degradation coupled to the tight regulation of cdG levels to provide several layers of regulation of this important signaling pathway in *Caulobacter*. The use of several adaptors appears to be present in Eukaryotic organelles as well, as the ClpF and ClpS1 adaptor complex is necessary for the regulated degradation of GluTR, a critical enzyme involved in tetrapyrrole synthesis in chloroplasts (Nishimura et al., 2015). The presence of several multi-adaptor systems indicates the necessity for various levels of regulation when delivering key protein targets, suggesting that other proteolytic machines may have these adaptor hierarchies in place that as of yet unknown. At the center of the *Caulobacter* adaptor hierarchy is RcdA, which coordinates binding between CpdR, PopA and the range of cargo it delivers to ClpXP. The broad focus of my thesis is understanding how RcdA and other adaptors in this pathway function mechanistically. In the following subchapters, I will go into detail into how RcdA degradation (Joshi et al., 2017) and RcdA dimerization and regulation (McGrath et al., 2006, Taylor et al., 2009, Joshi et al.,

2015) are fundamental properties that drive the major research questions of my thesis project.

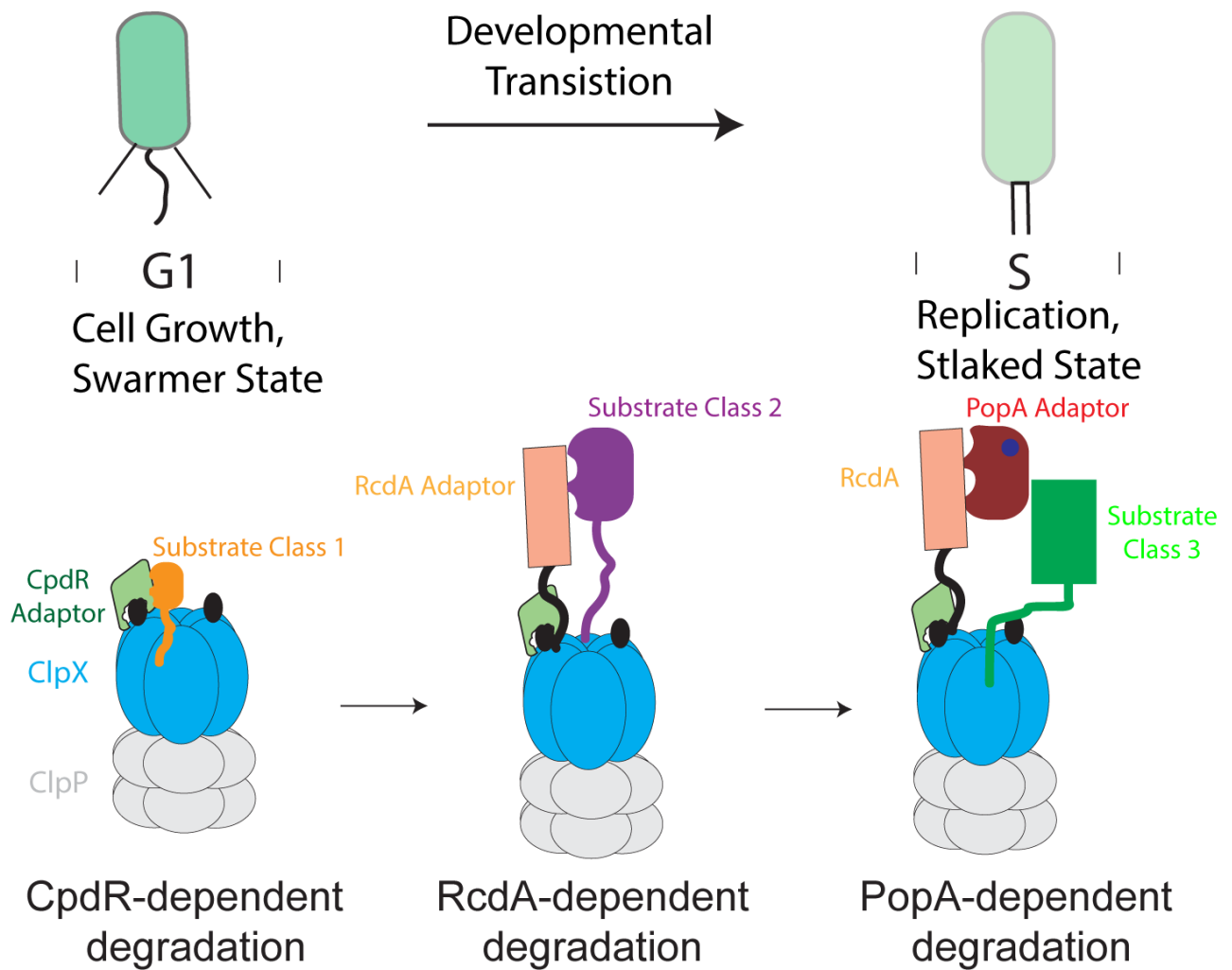


Figure 1.5: An Adaptor Hierarchy Controls Cell-Cycle Protein Degradation in *Caulobacter*

A series of adaptors controls cell-cycle degradation in *Caulobacter*. The developmental transition from a motile swarmer cell in G1 to a sessile stalked cell during the onset of S-phase and DNA replication is regulating by adaptor-dependent protein degradation. The first adaptor, CpdR, binds ClpX and delivers a class of substrates. The second adaptor, RcdA, binds to a CpdR-primed ClpX and delivers its class of substrates. Finally, the third adaptor, PopA, binds the small molecule cdG and RcdA, facilitates the degradation of CtrA, and DNA replication takes place following CtrA degradation.

1.5 Adaptors are Broadly Degraded in the Absence of Cargo

A critical conundrum exists for adaptors: what happens after they perform their delivery jobs? While not actively delivering cargo, many of these adaptors run the risk of being degraded by their respective proteases (Figure 1.6). Experimental evidence has shown that adaptors like MecA and HspQ themselves are degraded by their respective proteases, suggesting that adaptor degradation is a broadly conserved phenomenon (Puri et al., 2017, Turgay et al., 1998). In *Caulobacter*, Previous studies have shown that several ClpXP adaptors in *Caulobacter*, including RcdA, SspB and CpdR are specifically degraded by ClpXP (Joshi et al., 2017). Surprisingly, PopA does not appear to be degraded by ClpXP despite CpdR and RcdA being substrates for the protease. Interestingly, RcdA is stabilized in the presence of cargo and only appears to be degraded when there is not cargo present (Joshi et al., 2017). Nondegradable variants of RcdA substrates, such as TacADD and SpbRDD, are sufficient to protect RcdA from degradation, suggesting that the act of RcdA binding to cargo itself is responsible for this protection. The precise mechanism for this phenomenon is unknown, as it appears that only native degradation tags cause this stabilization (Joshi et al., 2017). My thesis work will provide an explanation for how cargo binding causes stabilization of RcdA and the biological consequences of RcdA degradation.

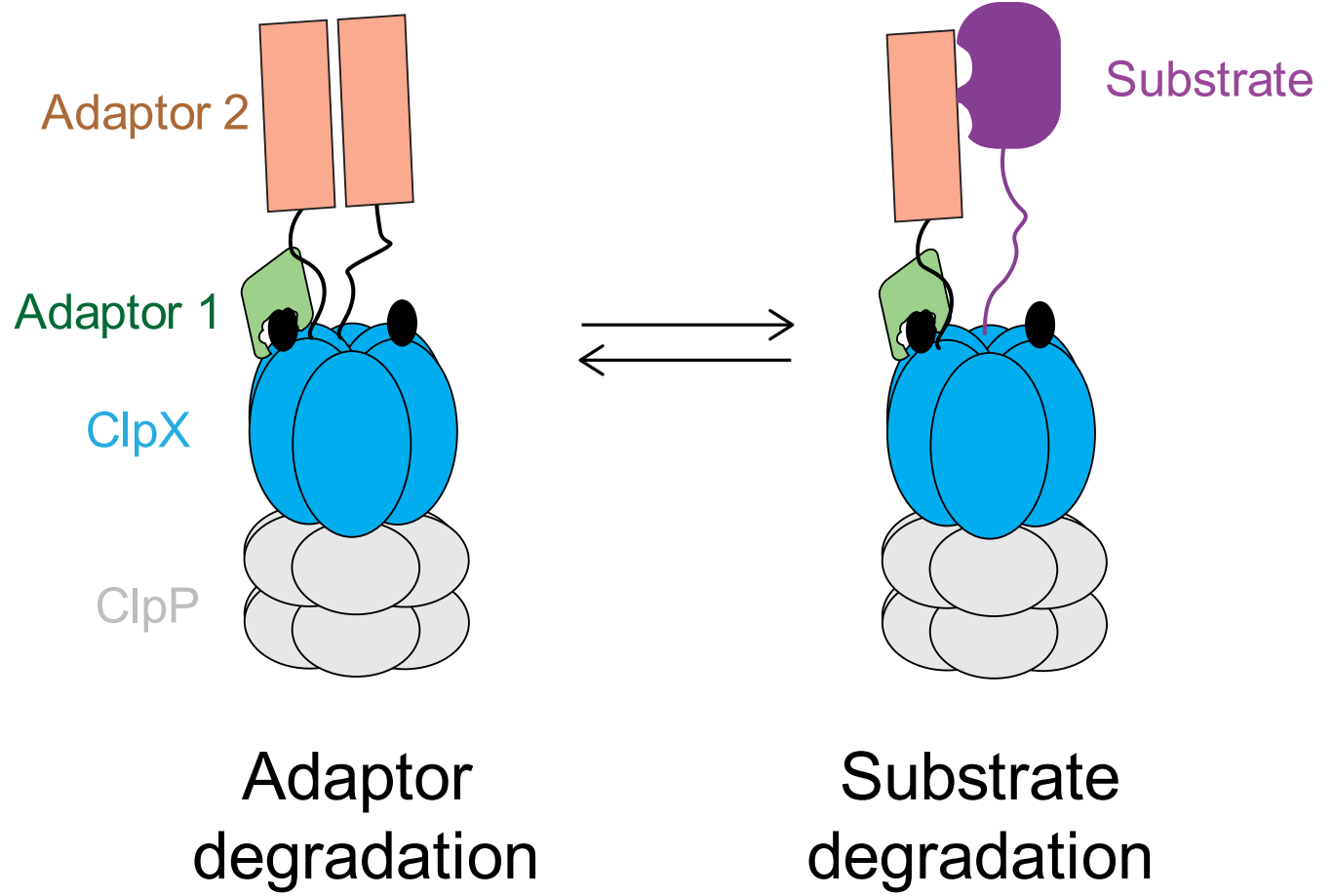


Figure 1.6: Adaptors are Degraded by their Proteases

Adaptors run the risk of degradation in the absence of cargo. In the case of the ClpXP adaptor hierarchy, adaptors are protected from degradation while delivering cargo (right cartoon). Because each adaptor physically interacts with parts of the protease, the degradation motif is near ClpX. Binding of cargo prevents degradation of each adaptor until its delivery responsibilities are completed.

1.6 RcdA Dimerization and Cargo Binding Characteristics

In this section, I will go into detail about the history of experimental characterization of RcdA to give context into the research questions I explore in my thesis work. RcdA was discovered by mining previously published genomic datasets to find gene candidates that potentially regulate CtrA function (McGrath et al., 2006). Deletion of the RcdA gene from the *Caulobacter* genome leads to stabilization of CtrA during the cell cycle, improper stalk formation and cell motility on swarm agar, and a G1 cell cycle arrest when CtrA is not being redundantly controlled by phosphorylation (McGrath et al., 2006). In 2009, RcdA was crystallized and biochemically characterized in solution as a homodimer with a disordered C-terminal tail, removal of which results in the inability to deliver CtrA (Taylor et al., 2009). The dimer interface of RcdA (Figure 1.7) is broad and covers many conserved hydrophobic and charged residues which I will explore in detail in chapter 2. Following the work from Taylor et al., detailed characterization of RcdA was performed to determine how RcdA regulates ClpXP substrate degradation during the cell cycle (Joshi et al., 2015). In this work, an affinity pulldown mass-spectrometry approach was used to identify additional cargo of RcdA, revealing the breadth of cargo that RcdA interacts with (Figure 1.8, Joshi et al., 2015). In addition to binding CpdR and PopA, the other adaptors in the hierarchy, RcdA is capable of directly binding several cargo in solution, such as SpbR and TacA, and tether those cargo to a CpdR-primed ClpXP for degradation during the cell cycle. While it is clear by biochemical data that RcdA can directly

interact with its cargo, it is unclear how RcdA can accommodate such a wide range of cargo and whether RcdA dimerization plays a role in binding.

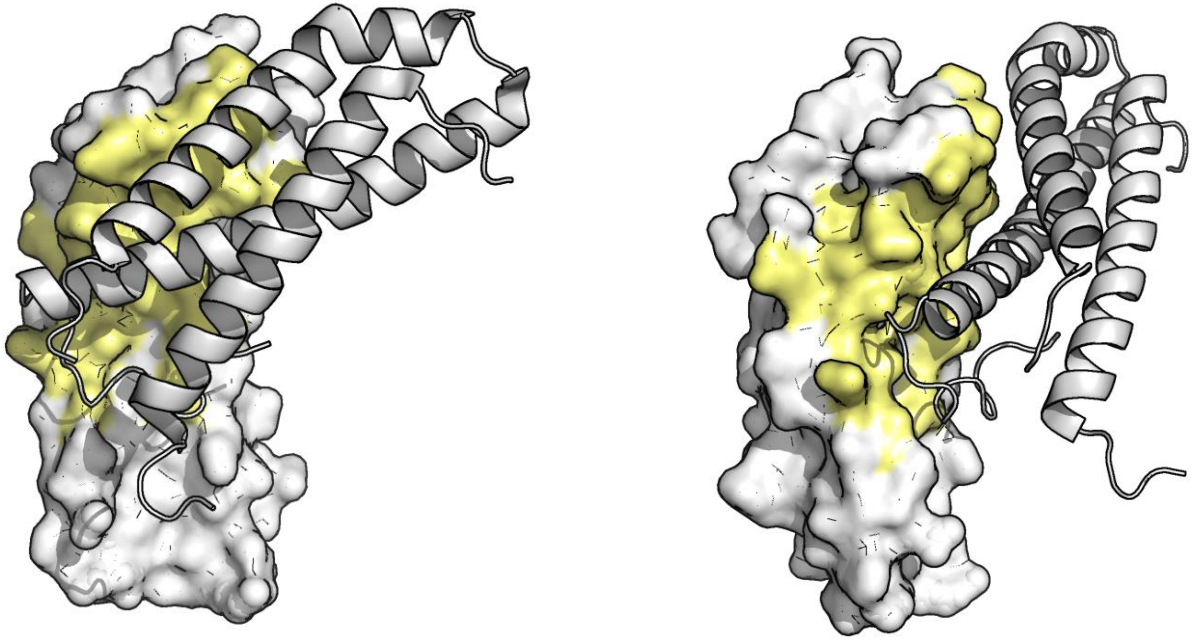


Figure 1.7: Dimerization Interface and Structure of RcdA

Two orientations of the published crystal structure of RcdA (PDB: 3ctw) with one subunit represented as a surface projection and the other subunit represented by a cartoon projection. The PDBePISA prediction of the dimer interface is highlighted in yellow.

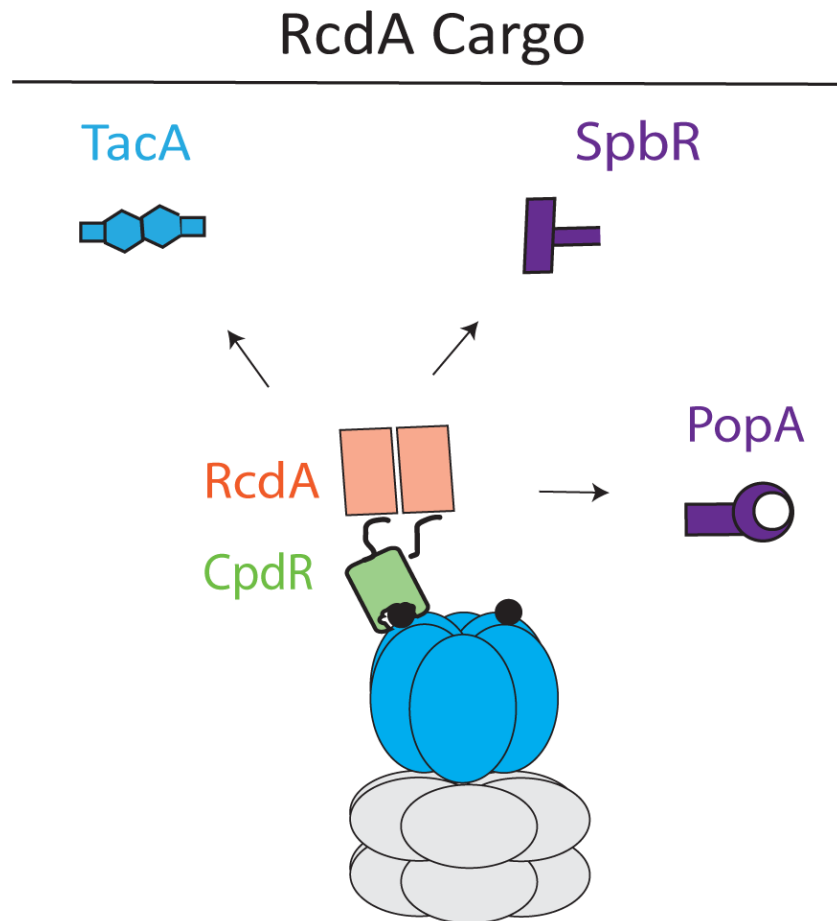


Figure 1.8: RcdA Binds a Range of Cargo

RcdA functions by directly interacting with direct substrates, such as TacA and SpbR, and interacting with other adaptors such as PopA. To deliver cargo to ClpXP, RcdA must interact with CpdR via its C-terminal tail.

1.7 Research Questions and Conclusions

The broad, overarching questions that my thesis was designed to answer involve RcdA and its mechanism. First, how does RcdA interact with such a wide range of cargo? While we know that RcdA is responsible for delivering at least 3 cargo to ClpXP for degradation (Joshi et al., 2015), none of these cargoes share any obvious sequence or fold motifs that indicate a conserved module that RcdA recognizes. How does RcdA accomplish this task while also performing its critical cell cycle responsibilities? While performing its delivery tasks, RcdA must also have a functional mechanism to protect itself from degradation when bound to cargo. How does RcdA accomplish this task? It is clear from structural and biochemical data that RcdA forms a homodimer (Taylor et al., 2009). Does dimerization play an important role in RcdA function, or is RcdA dimerization simply an artifact of crystallization? More broadly, my thesis aims to answer how RcdA coordinates binding between the third adaptor, PopA, to regulate CtrA degradation. The work presented in this thesis will highlight new mechanisms by which adaptor proteins regulate the bacterial cell cycle and explore new ways to characterize adaptor function. However, the field of adaptors and scaffolds requires new tools and screens to identify new adaptors and adaptor-dependent substrates. For identifying new adaptors, it is more challenging. RcdA was originally identified as a regulator of CtrA degradation, and through serendipity, was discovered to be an adaptor for TacA degradation. Creative genetic and biochemical screens are needed to help identify new adaptors and provide a

framework for adaptor discovery projects, as it seems clear that adaptor proteins play a critical role in regulating bacterial and organelle physiology. Advances in quantitative proteomics, combined with the ability to synchronize different cell populations of *Caulobacter*, will allow more rigorous and systematic identification of new adaptor-dependent substrates. Generation of specific allelic mutations in adaptors that differentially affect specific classes of substrates and specific phenotypes will help identify new substrate candidates that can be validated biochemically or genetically by narrowing the available substrate pool.

1.8 Thesis Structure

The second chapter of this thesis will describe my work in elucidating the mechanism behind RcdA function and its biological significance. The third chapter will describe my work in identifying the surfaces on PopA that are involved in RcdA and CtrA interactions and degradation. The fourth chapter will discuss lessons I've learned from my thesis work and provide future directions for the field of protease adaptors. In the appendix I will provide any unpublished results arising from the 2nd and 3rd chapters.

CHAPTER 2

Cargo Competition for a Dimerization Interface Restricts and Stabilizes a Protease Adaptor

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2.1 Abstract

Bacterial protein degradation is a regulated process aided by protease adaptors that alter specificity of energy dependent proteases. In *Caulobacter crescentus*, cell-cycle dependent protein degradation depends on a hierarchy of adaptors, such as the dimeric RcdA adaptor which binds multiple cargo and delivers substrates to the ClpXP protease. RcdA itself is degraded in the absence of cargo and how RcdA recognizes its targets is unknown. Here we show that RcdA dimerization and cargo binding compete for a common interface. Cargo binding separates RcdA dimers and a monomeric variant of RcdA fails to be degraded, suggesting that RcdA degradation is a result of self-delivery. Based on HDX-MS studies showing that different cargo rely on different regions of the dimerization interface, we generate RcdA variants that are selective for specific cargo and show cellular defects consistent with changes in selectivity. Finally, we show that masking of cargo binding by dimerization also limits substrate delivery to restrain overly prolific degradation. Using the same interface for dimerization and cargo binding offers an ability to limit excess protease adaptors by self-degradation, while providing capacity for binding a range of substrates.

2.2 Introduction

This chapter describes our effort to elucidate how RcdA engages its cargo. Initial studies showed that RcdA is a homodimer in solution and binds directly to its cargo using several biochemical assays, including size-exclusion chromatography (SEC) and fluorescence polarization (FP) experiments (Taylor et al., 2009, Joshi et al., 2015). Initial characterization of RcdA-cargo complexes by analytical SEC confirmed that RcdA binds its cargo, resulting in a higher molecular weight species (Figure 2.1). However, it remained unclear based on the uncertainty coming from the protein standards used in analytical SEC experiments what the exact stoichiometry between RcdA and its cargo was and whether this stoichiometry was consistent for all RcdA cargo. Our expectation, based on prior work with the dimeric ClpXP adaptor SspB showing that optimal delivery is achieved when two molecules of substrate are bound to a distal site on SspB (Chien et al., 2007, McGinness et al., 2007, Bolon et al., 2004), was that RcdA would share a similar mechanism. Due to the limitations of analytical SEC, we were worried that reported masses would be inaccurate due to the dependence on internal standards that do not consider protein shape, a common pitfall in analytical SEC experiments. We decided to use sensitive biophysical techniques that take absolute measurements of mass to fully characterize the stoichiometry of RcdA-cargo complexes.

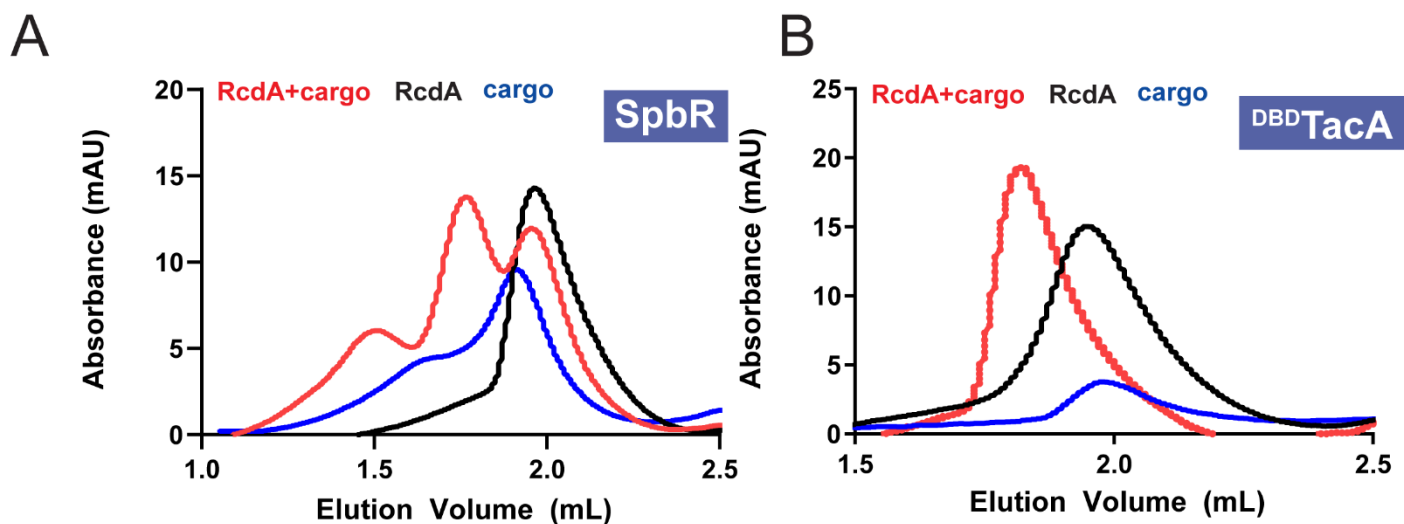


Figure 2.1: RcdA Directly Interacts with its Cargo by Analytical SEC

A) Analytical SEC of RcdA and SpbR. 25 μ M RcdA (black), 25 μ M SpbR alone (blue), or 25 μ M RcdA and SpbR together (red) were incubated for 45 minutes and ran over a 3mL analytical SEC column. **B)** Analytical SEC of RcdA and the DNA-binding domain of TacA (Joshi et al., 2015). 25 μ M RcdA (black), 25 μ M ^{DBD}TacA (blue) or 25 μ M RcdA and 25 μ M ^{DBD}TacA together were incubated and ran over a 3mL analytical SEC column.

2.3 RcdA Binds its Cargo as a Monomer

We began our studies by exploring RcdA-cargo binding using size-exclusion chromatography with multi-angle light scattering (SEC-MALS) to measure absolute molar mass. SpbR, a RcdA-dependent cargo responsible for inhibiting centromere translocation (19) is a 42 kDa monomer in solution, while as expected, the molar mass of RcdA is a dimer (36 kDa, Figure 2.2). The combination of both proteins results in a complex with a determined mass of 61 kDa (Figure 2.2). Our data is consistent with a monomer of RcdA (17 kDa) binding a monomer of SpbR (44 kDa) and inconsistent with a dimer of RcdA binding a monomer of SpbR (a predicted mass of 77 kDa) (Table 1). We confirmed this monomer:monomer stoichiometry by using analytical ultracentrifugation and found a similar mass of 60 kDa for the RcdA:SpbR complex (Figure 2.3).

We next tested other RcdA cargo. PopA, the RcdA-binding adaptor, shows an experimental mass of 41 kDa (Figure 2.2). In the presence of RcdA, the complex mass is 58 kDa consistent with a monomer of PopA (41 kDa) binding a monomer of RcdA (17 kDa) (Figure 2.2; Table 1). TacA, a transcription factor involved in stalk biogenesis, has an experimental molar mass consistent with a dimer (124 kDa). When bound to RcdA, the complex shows an apparent mass of 141 kDa, again consistent with a monomer of RcdA binding to a dimer of TacA (Figure 2.2;

A

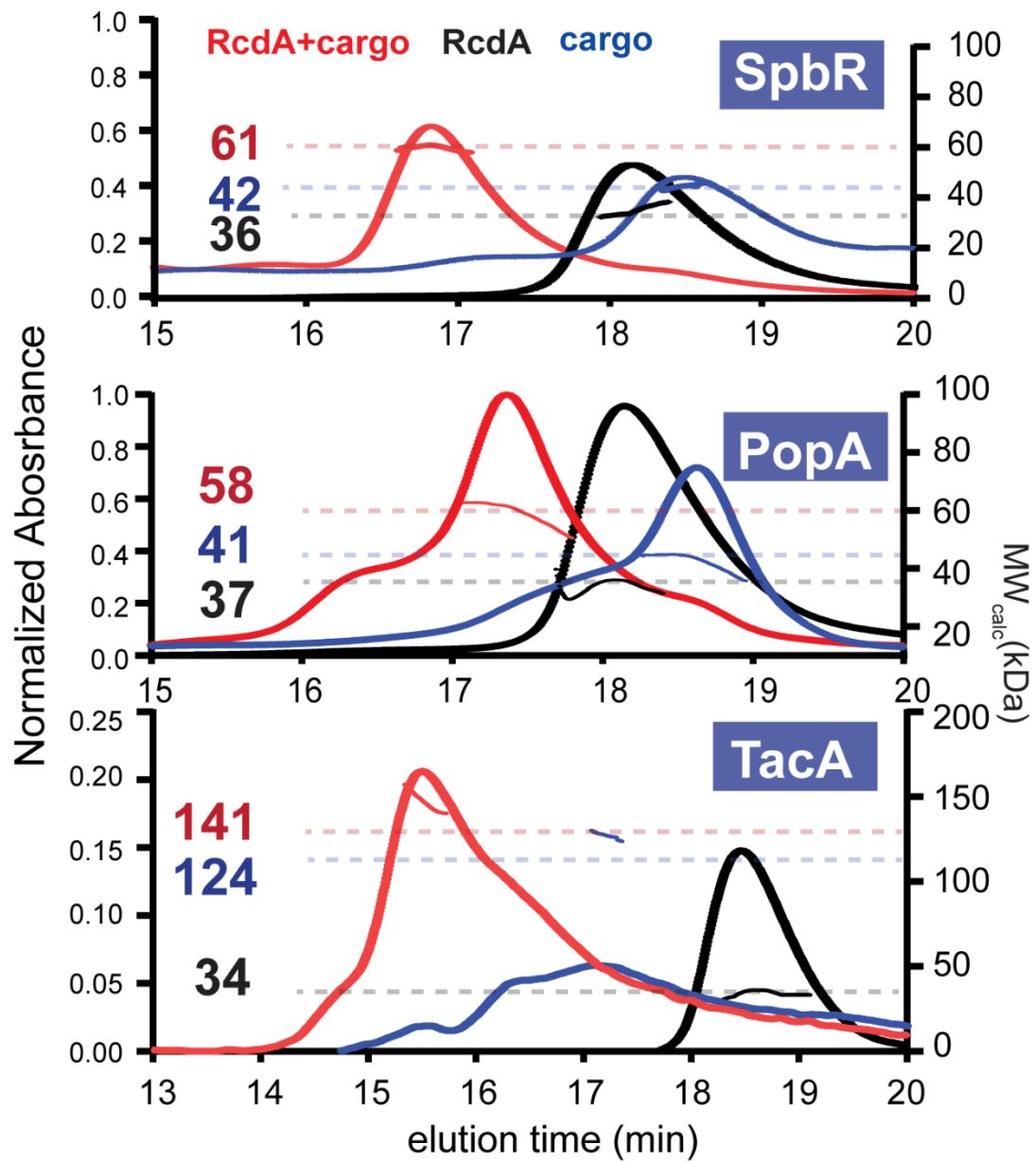


Figure 2.2: RcdA forms a Monomer when Bound to Cargo by SEC-MALS

A) SEC-MALS traces of different RcdA-cargo complexes. Representative SEC-MALS traces of the RcdA, cargo, and the RcdA-cargo complexes. Left axes show normalized absorbance traces, right axes show calculated molecular weight in kilodaltons. Dashed lines connect the scattering traces of the proteins/complexes with the calculated molecular weights as labeled. The colored lines under each peak represent the collected light scattering data as a distribution of molecular weights over the width of the chromatography peak. Numbers represent the calculated molecular weight from the scattering data. Concentrations of each component and complex are noted in the methods section.

Table 1: Cargo Binding Competes with RcdA Dimerization.

	Experimental (kDa) (± 4 kDa)	Stoichiometry	Predicted (kDa)
RcdA	36	RcdA ₂ (dimer)	37
		RcdA (monomer)	19
SpbR	42	SpbR (monomer)	43
RcdA + SpbR	61	RcdA:SpbR	62
TacA	124	TacA ₂ (dimer)	127
RcdA + TacA	141	RcdA:TacA₂	146
PopA	41	PopA (monomer)	47
RcdA + PopA	58	RcdA:PopA	66

Table of molecular weights of the different RcdA-cargo complexes. The experimental column shows the complex mass measured by SEC-MALS. The stoichiometry column gives the stoichiometry of each protein consistent with the experimental mass. The predicted column lists the EXPASY predicted mass of each stoichiometry listed. Bolded text is the experimentally measured complex masses displayed in Figure 1.

D

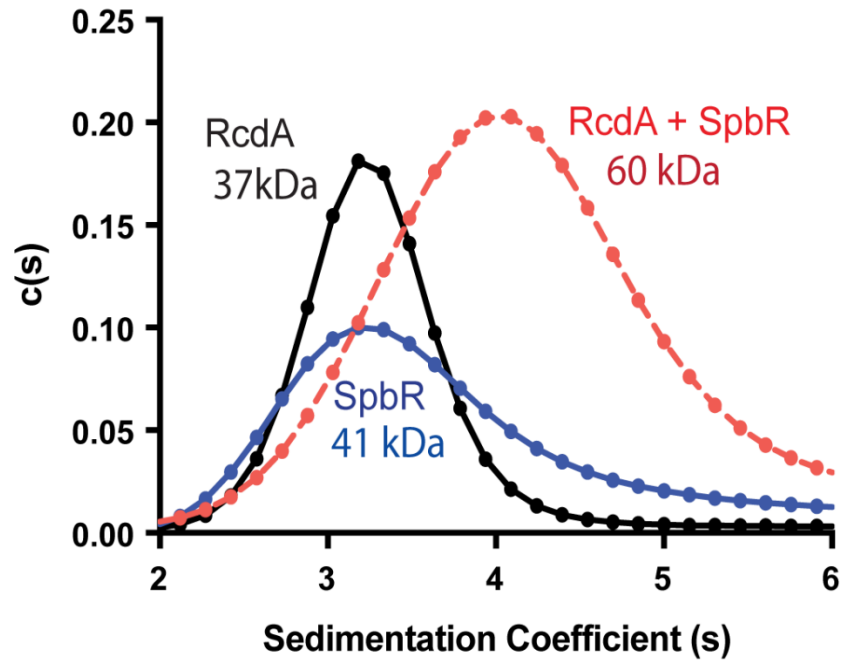


Figure 2.3: RcdA Binds SpbR as a Monomer by AUC

Sedimentation constants fitted from velocity ultracentrifugation of the RcdA-SpbR complex. Each component was present at 25 μ M, either or alone or in complex.

Table 1). Strangely, despite having more mass than RcdA, we noticed that both PopA and SpbR elute after RcdA on our SEC columns, which we attribute to non-specific interactions with the stationary phase of our column or that PopA and SpbR have different physical shapes than the RcdA dimer. We confirmed the presence of both cargo and adaptor in these complexes by SDS-PAGE (Figure 2.4).

Our observation that RcdA binds its cargo as a monomer posed an interesting thermodynamic conundrum: if RcdA dissociation occurs with a slow off-rate or with a relatively tight binding constant, how does cargo binding outcompete RcdA dimerization? We answered this question by measuring the binding affinity of RcdA to cargo and to itself using several approaches. Using isothermal calorimetry, we determined that the dimerization constant of RcdA ($K_D = 7 \mu\text{M}$, Figure 2.5) and dissociation constant between RcdA and SpbR ($K_D = 1 \mu\text{M}$, Figure 2.5) were of similar order. Measurements with a labeled RcdA reporter using fluorescence polarization also revealed similar magnitudes for dimerization ($K_D = 2.5 \mu\text{M}$) and SpbR binding ($K_D = 2.1 \mu\text{M}$), (Figure 2.6). Finally, microscale thermophoresis measurements provide additional confirmation that RcdA binds itself and SpbR with similar ranges of affinity (RcdA-SpbR complex (average $K_D = 5 \mu\text{M}$); dimerization (average $K_D = 3 \mu\text{M}$)), (Figure 2.6).

Taken together, these data suggest that RcdA binds cargo as a monomer and that binding of cargo competes with dimerization with similar binding affinities (Figure 2.7).

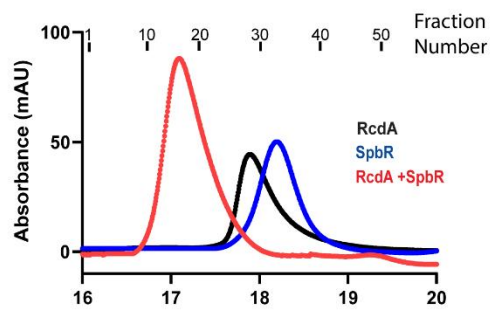
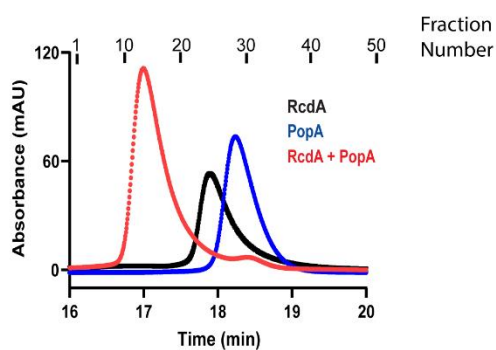
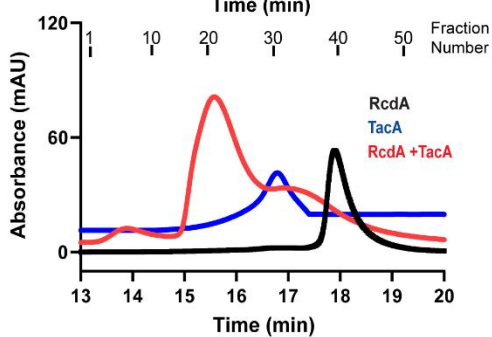
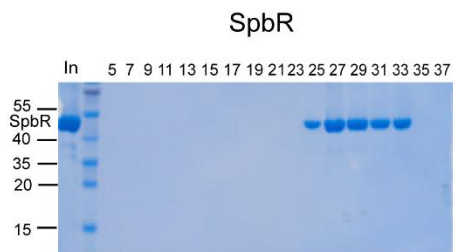
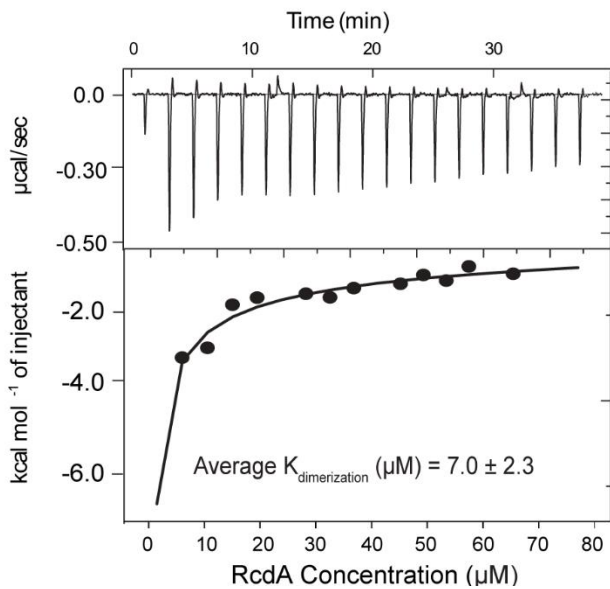


Figure 2.4: SEC Coelution of RcdA-Cargo Complexes, related to Figure 2.2

In this figure, we have replicated all of the complexes shown in Figure 2.2 and collected fractions for SDS-PAGE gel analysis. We used the chromatography setup used in the SEC-MALS experiments and collected fractions by disconnecting the light scattering detector which collects the waste and feed the output in hand fractionated tubes. We show the A_{280} signal from chromatograms of each complex and the gel profile of selected fractions for each chromatogram on the right. Fraction numbers are shown above the graph. Loaded fractions are numbered above each gel well, with the first lane showing the input sample before SEC analysis. Fractions were loaded with more volume (17uL) than the input samples (9uL) to account for dilution from the SEC column. The concentrations used were the same as the concentrations used in Figure 1 and this is noted in the methods section.

Our data showing that RcdA dimers dissociate into monomers to bind all its cargo (Figure 2.3) was surprising and opened the door for further exploration of hypotheses regarding RcdA mechanism. One apparent hypothesis that came from this observation was that the monomeric form of RcdA is responsible for its stabilization when bound to cargo (Joshi et al., 2017). Our biophysical data showing monomeric RcdA binding cargo would explain the stabilization caused by the introduction of a nondegradable form of cargo (SpbRDD, TacADD or PopA), since all three of these cargoes caused dissociation of RcdA into a monomeric species.

B



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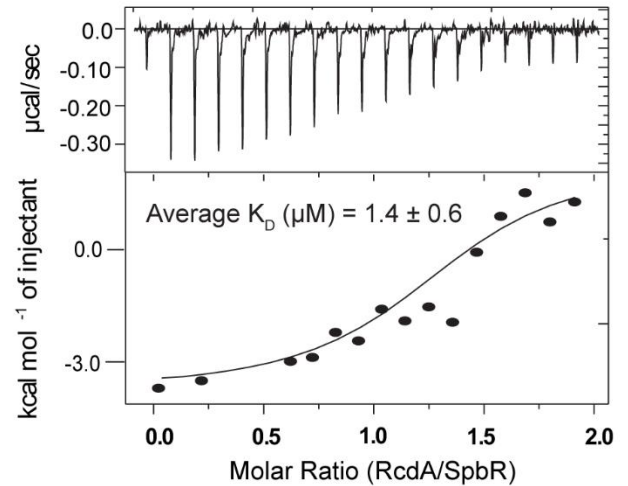
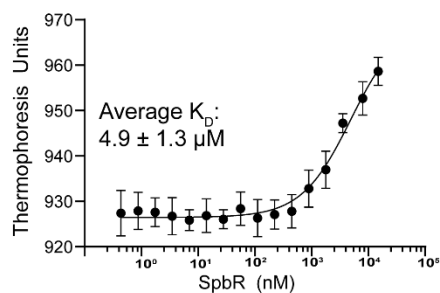


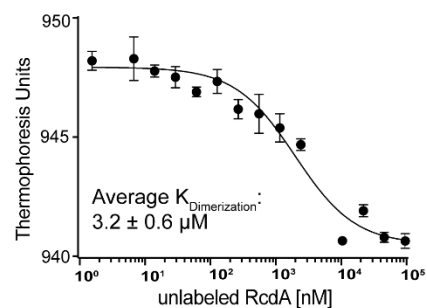
Figure 2.5: ITC analysis of RcdA Dimerization and SpbR Binding

B) Representative ITC thermogram of the heat of RcdA dissociation, heats of injection shown on top. The sample syringe was loaded with 400 μ M RcdA and titrated into a cell containing buffer. $K_{\text{dimerization}}$ values are the average of three independent replicates with error representing standard deviation. **C)** Representative ITC thermogram of the SpbR-RcdA complex formation, heats of injection shown on top.

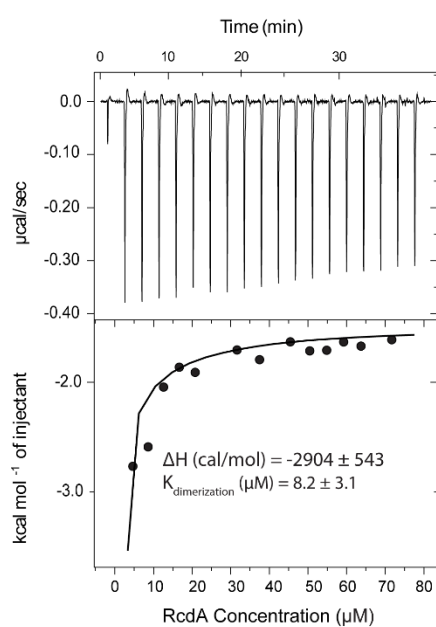
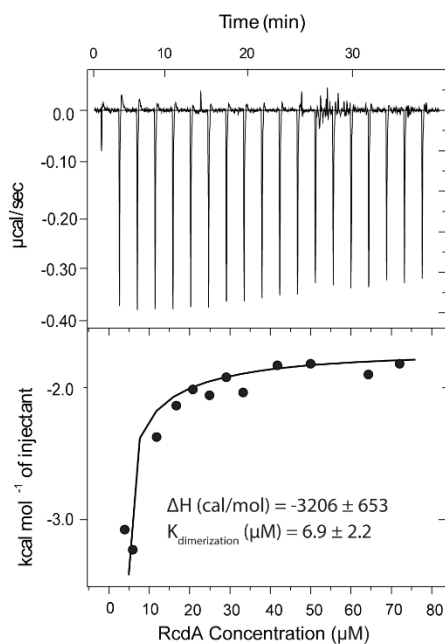
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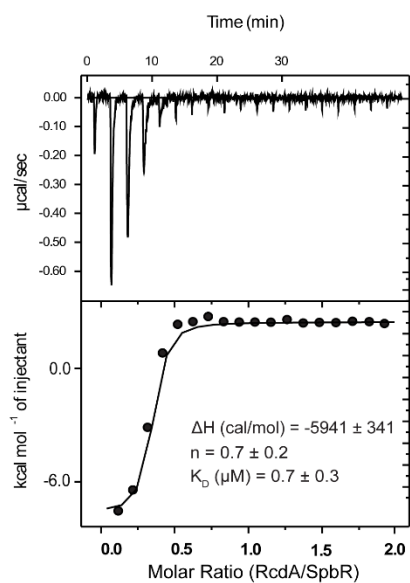
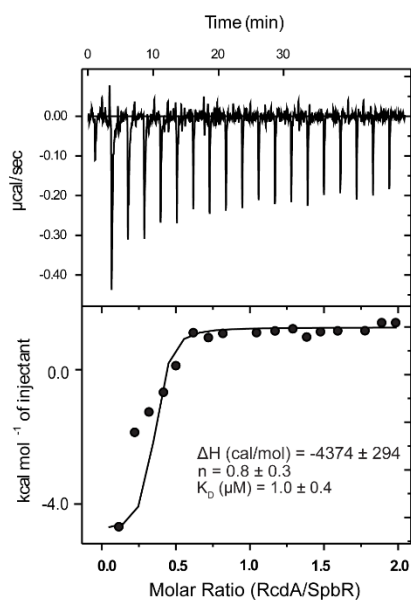


Figure 2.6: Additional biophysical measurements of SpbR-RcdA binding and RcdA dimerization, related to Figure 2.5

A) Microscale thermophoresis graph showing a titration of SpbR into labeled RcdA. A serial dilution of unlabeled SpbR starting at 30 μ M was titrated into 200 nM labeled RcdA. The average of three independent experiments and the standard deviation representing error are shown. **B)** Microscale thermophoresis graph showing a titration of RcdA into labeled RcdA. A serial dilution of unlabeled RcdA starting at 100 μ M was titrated into 200 nM labeled RcdA. The average of three independent experiments and the standard deviation representing error are shown. **C)** Additional replicates of Dimerization ITC. Experimental setup, buffers and concentration are the same as performed in Figure 1B. **D)** Additional replicates of RcdA-SpbR binding ITC. Experimental setup, buffers and concentration are the same as performed in Figure 1C.

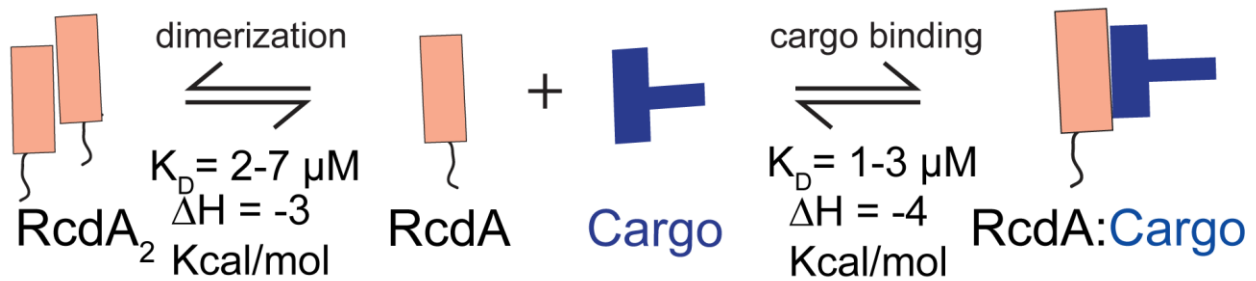


Figure 2.7: RcdA Cargo Competition Model

Cartoon illustrating the competition between dimerization and cargo binding. All RcdA concentrations are in terms of monomeric equivalents unless otherwise noted.

2.4 Heterodimerization of RcdA slows RcdA Autodegradation

Our initial hypothesis that RcdA autodegradation suppression in the presence of cargo is due to monomerization of RcdA came with two implications: that one subunit of RcdA is insufficient for RcdA degradation and that one copy of the C-terminal tail is also insufficient for degradation. We sought to devise a system where we could generate a heterodimer of RcdA that contains one subunit with a C-terminal tail and another subunit that lacks a C-terminal tail. Because the C-terminal tail of RcdA is necessary for RcdA degradation (Joshi et al., 2017) we hypothesized that we could form a heterodimer of RcdA that consist of one subunit of WT RcdA and one subunit of RcdA Δ C by mixing both species. If our hypothesis that both subunits of RcdA are needed for RcdA degradation, then our heterodimeric RcdA complex lacking one wildtype subunit should be protected from degradation. Consistent with this hypothesis, addition of excess RcdA Δ C suppressed RcdA degradation in the presence of CpdR and ClpXP (Figure 2.8). While this data is consistent with a model where monomeric RcdA is protected from degradation, we sought to test this model by generating a monomeric variant of RcdA.

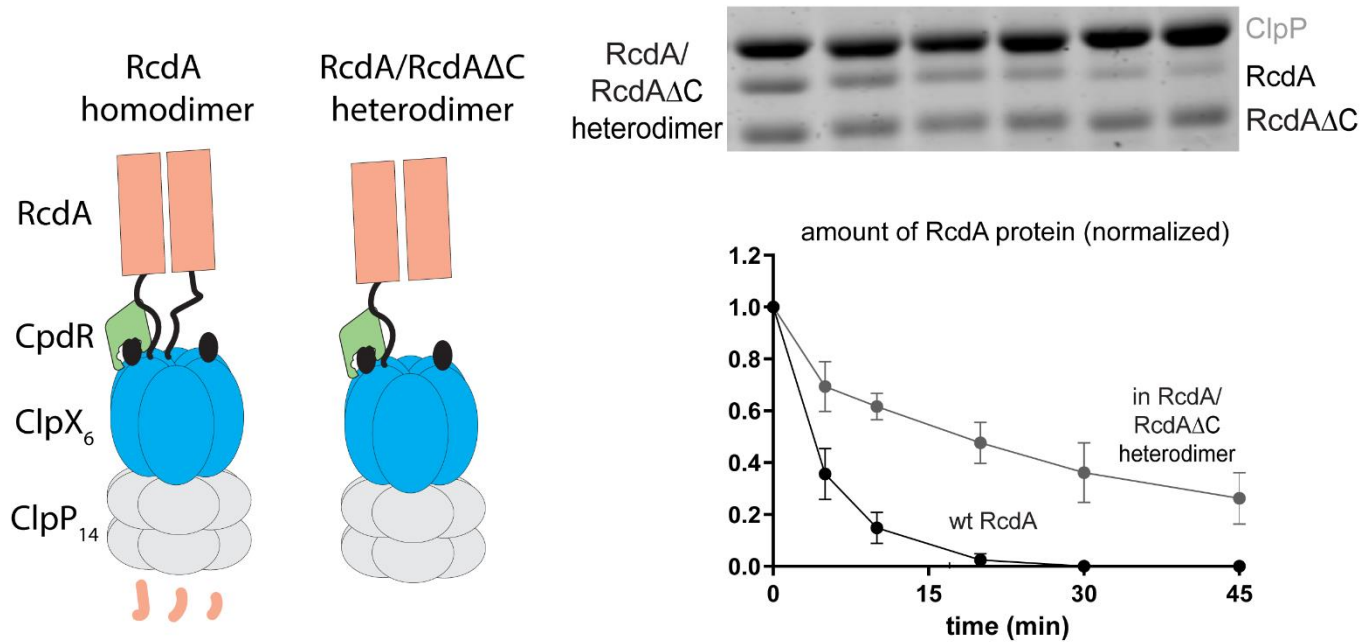


Figure 2.8: RcdA Heterodimerization Protects RcdA from Degradation

RcdA degradation at a final concentration of μM *in vitro* is inhibited in the presence of excess (10 μM) RcdAΔC. A representative gel and quantifications of triplicates are shown below. A cartoon illustrating the heterodimer complex is shown on the left.

2.5 Generation of a Monomeric Variant of RcdA

To specifically test how a monomer of RcdA affects its function, we sought to generate a monomeric variant of RcdA. During our initial attempts to co-crystallize RcdA with a TacA peptide to characterize RcdA: cargo interactions, we crystallized the apo form of RcdA in a different packing conformation than what was previously published in the original crystal structure (Crystallography completed by Madeleine Sutherland). Based on the published crystal structure of RcdA (Taylor et al., 2009, PDB: 3CTW) and our own in-house solved structure of RcdA, there were two distinct dimer interfaces that were annotated. In the published crystal structure, there were several amino acids that contain the highest percentage of buried surface area in the predicted dimer interface as predicted by the PDB PISA server for macromolecular interfaces. We identified leucine 82 (L82) as a residue that had the highest buried surface area percentage by the PDB PISA server as a candidate for mutation (Figure 2.9). In the second interface found in our in-house solved structure, we identified residues Arginine 131 and 135 (R131 and R135) as important residues in that interface (Figure 2.9). We mutated L82 to glutamic acid (L82E) and alanine (L82A) and R131 and R135 to glutamic acids (R131/5E) to disrupt each interface. We purified both proteins and ran them and performed SEC-MALS to confirm the molecular mass of each species. While the RcdA131/5E and L82A variants ran as a dimer species that coeluted with WT RcdA (Figures A.4-A.5), suggested this variant does not form a monomer, the RcdA L82E variant ran

after WT RcdA and had a mass consistent with a monomeric species of RcdA (19kDa, Figure 2.10). We confirmed that RcdA L82E is incapable of forming a dimer by using fluorescence polarization as a readout of dimerization. While wildtype RcdA was capable of increasing polarization when added to a fluorescently labeled RcdA reporter, suggesting heterodimerization, RcdA L82E did not show the same trend (Figure 2.10). Finally, we tested our hypothesis that a monomeric form of RcdA is incapable of being degraded by testing whether RcdA L82E could be degraded *in vitro* and *in vivo*. Consistent with our model, RcdA L82E was stable *in vitro* throughout the time course of a degradation assay similarly to a cargo-stabilized WT RcdA (Figure 2.11). Similarly, RcdA L82E was not turned over *in vivo* in a translational shutoff assay when expressed from the xylose promotor in a $\Delta rcdA$ strain background (Figure 2.11).

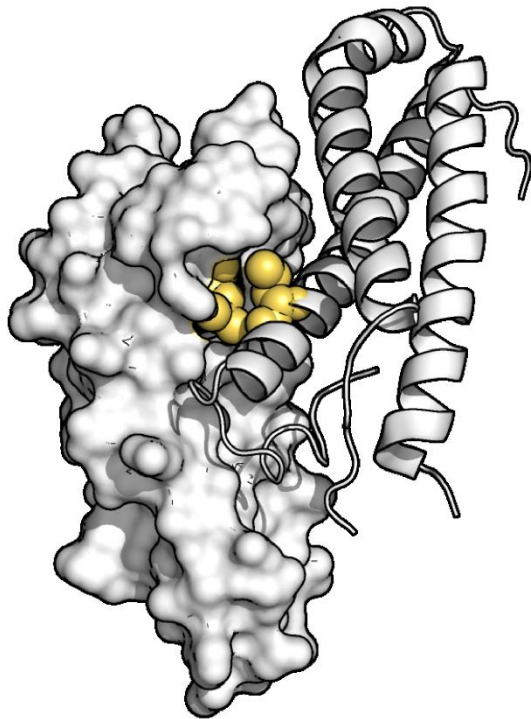
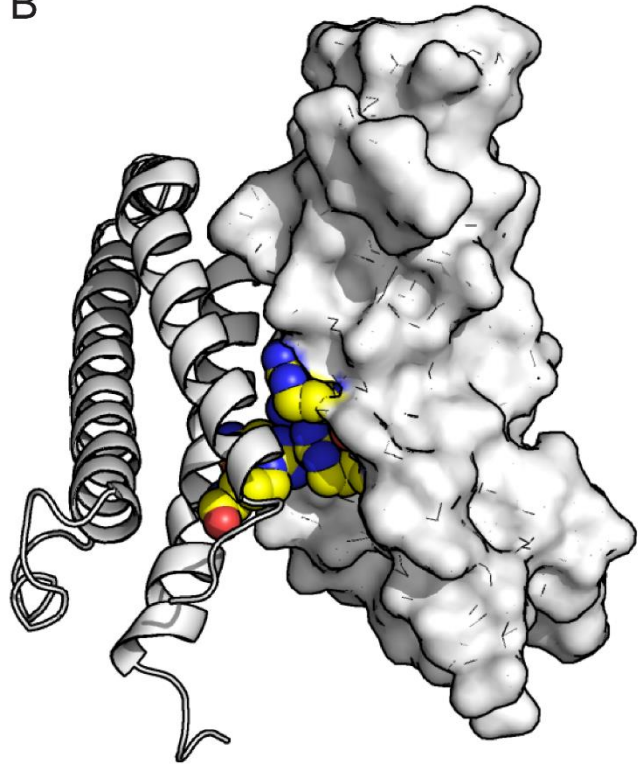
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Figure 2.9: Alternative RcdA Dimer Interfaces and Residues Important for Packing

A) Annotated dimer interface from originally published RcdA crystal structure (Taylor et al., 2009, PDB 3ctw). Leucine 82 is highlighted as yellow spheres in both subunits for visualization. **B)** Annotated dimer interface from in-house solved RcdA structure (unpublished). Arginines 131 and 135 are highlighted as residue-colored spheres in both subunits for visualization.

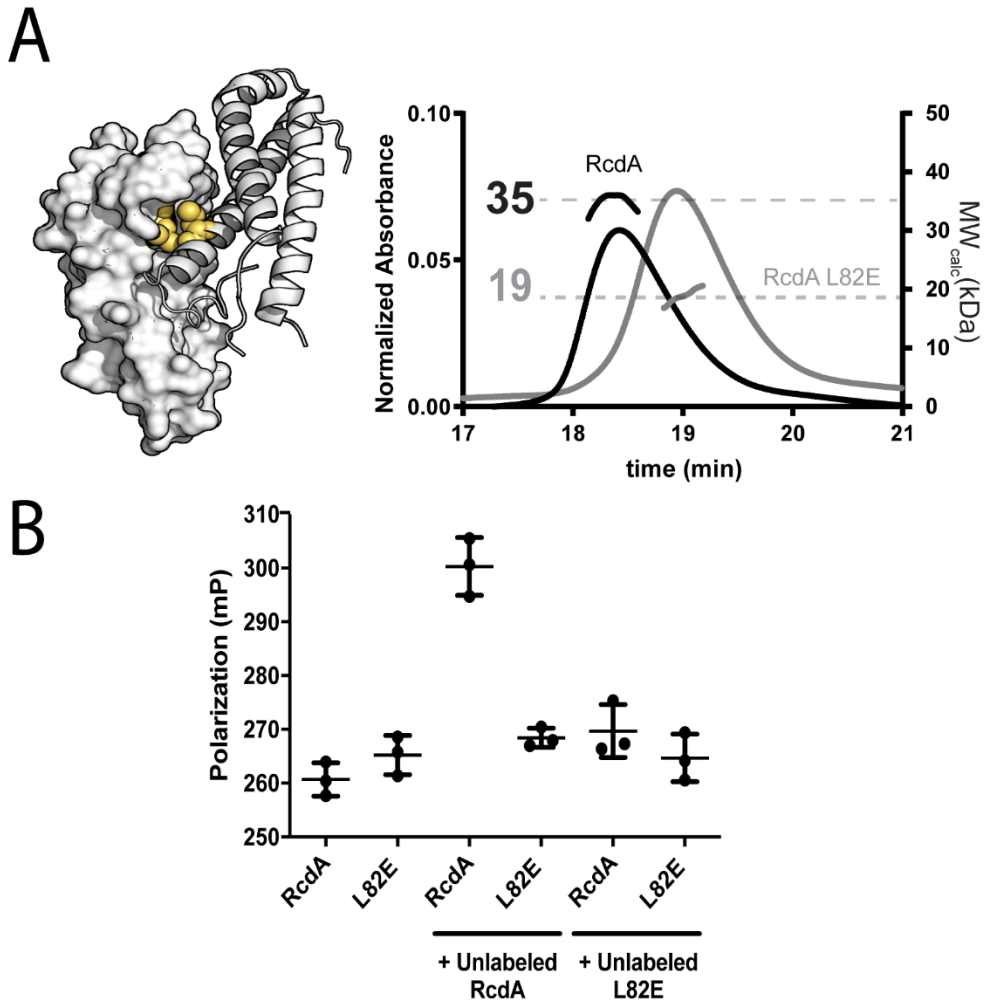


Figure 2.10: The L82E Variant Forms a Monomer by SEC-MALS and Fluorescence Polarization

A) The L82E variant forms a monomeric protein (grey trace) compared to WT RcdA (black trace) by SEC-MALS. A Pymol illustration of the L82 residue is shown on the left. **B)** Fluorescence polarization reporting on RcdA dimerization. 20 μ M unlabeled RcdA and RcdA L82E was added to each labeled reporter at 200nM.

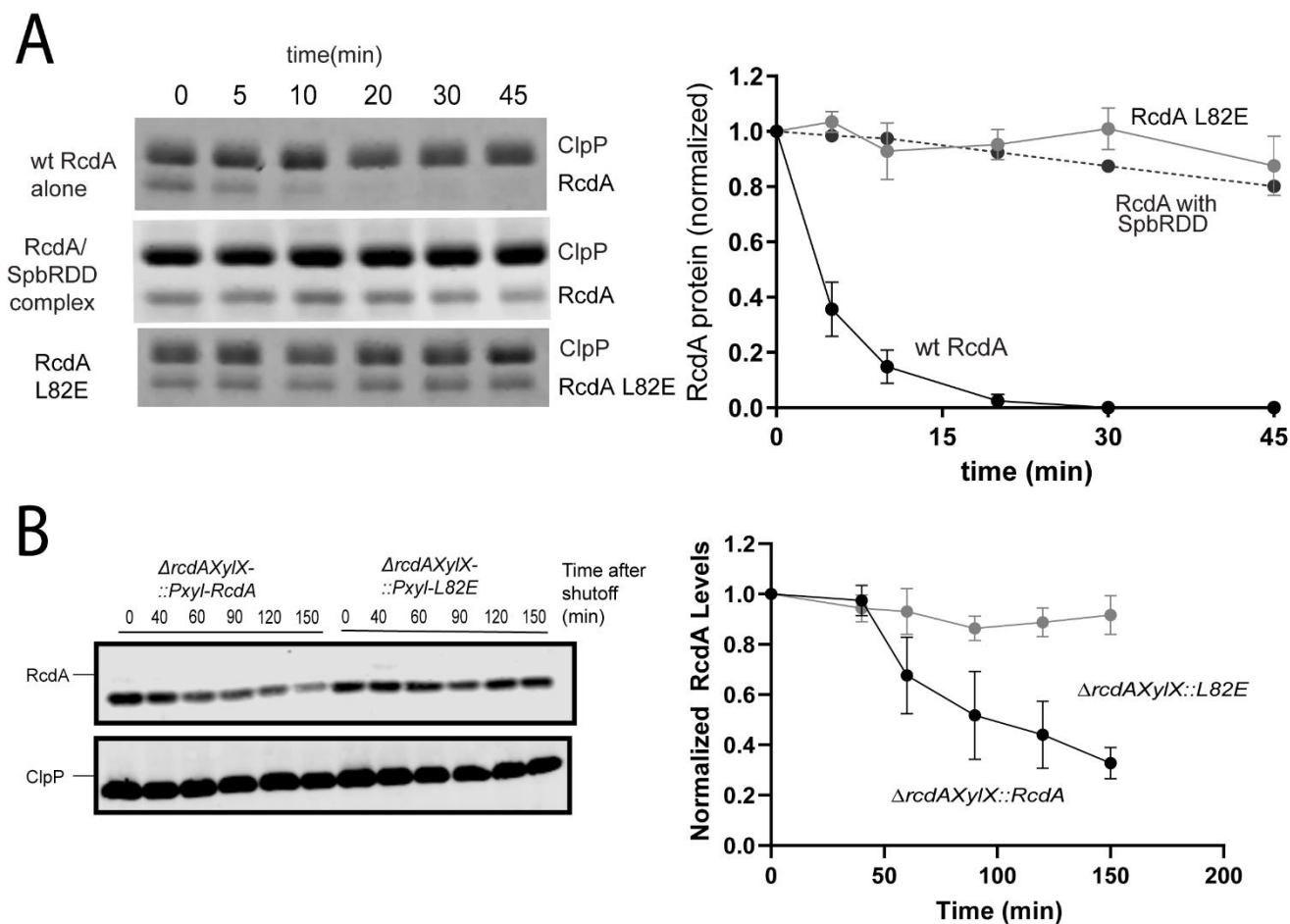


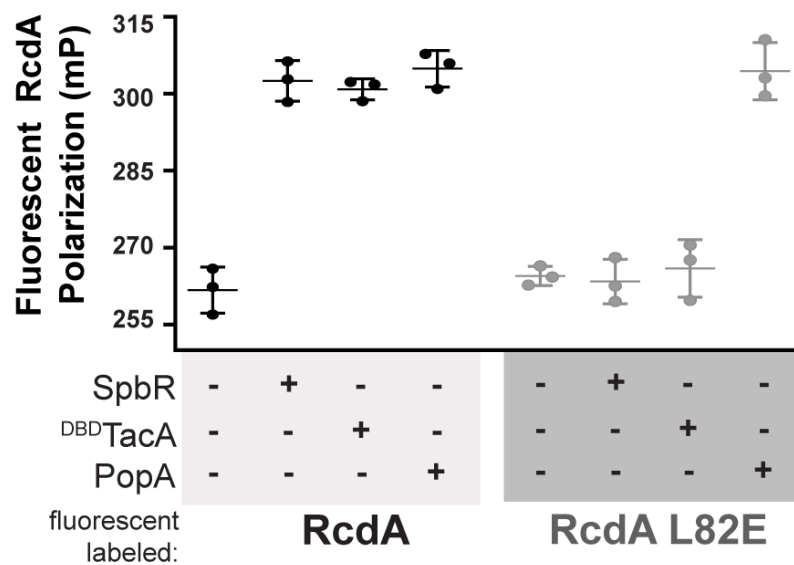
Figure 2.11: The L82E Variant is Incapable of Being Degraded

A) *In vitro* gel-based degradation of 3 μ M RcdA alone, 3 μ M RcdA with 3 μ M SpbRDD, or 3 μ M RcdA L82E alone. Quantification of three independent replicates is shown on the right, error bars represent standard deviation..**B)** Chloramphenicol shutoff assays monitoring the degradation of RcdA and RcdA L82E in cells expressing wildtype RcdA or RcdA L82E. Quantification of the average of 3 biological replicates are shown at right.

2.7 The L82E Variant is Defective in Some Cargo Binding and Delivery

Our generation of a stable monomeric variant of RcdA allowed us to test directly whether the interface used for dimerization was the same interface used for cargo binding. We next tested the ability of RcdA L82E to bind and deliver different RcdA cargo. SpbR and TacA are substrates of ClpXP that require RcdA for efficient degradation, while PopA is an adaptor that binds RcdA to expand its substrate profile (Duerig et al., 2009, Ozaki et al., 2014). We used wildtype RcdA or RcdA L82E labeled with fluorescein and fluorescence polarization as a proxy for cargo binding. Using this assay, we found that wildtype RcdA bound all three cargo (n.b., ^{DBD}TacA is the minimal domain of TacA needed for RcdA degradation) (Figure 2.12). The monomeric RcdA L82E was unable to form a complex with SpbR and ^{DBD}TacA, but surprisingly, bound PopA with affinity equivalent to wildtype RcdA (Figure 2.12). SEC-MALS and SDS-PAGE analysis of fractions confirmed these changes in cargo binding (Figure 2.13).

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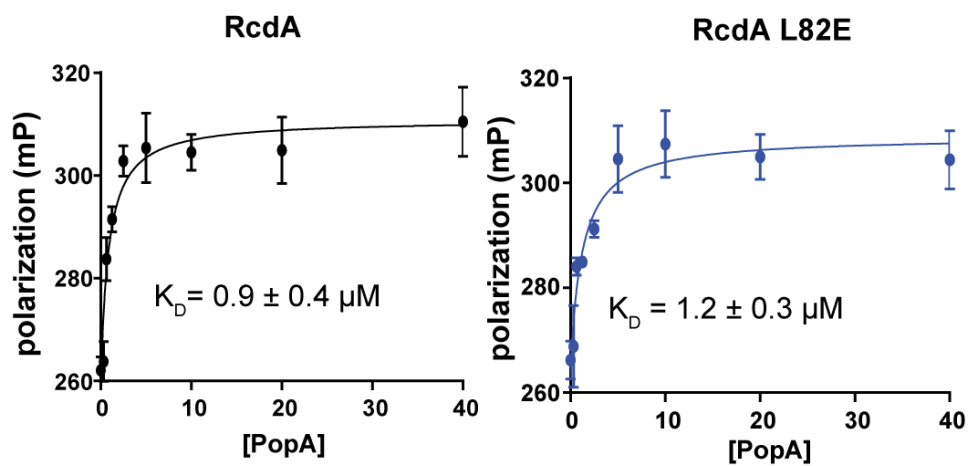
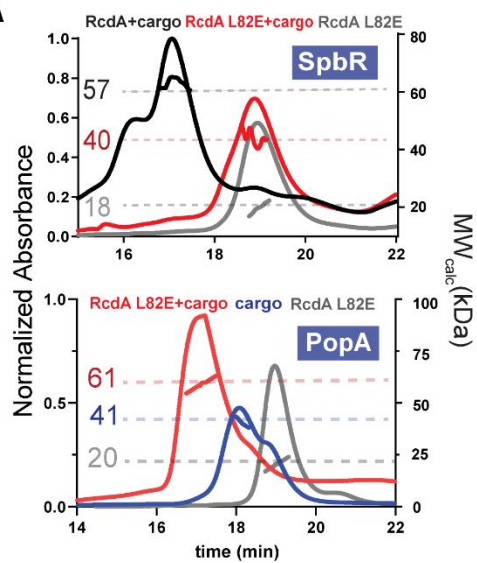


Figure 2.12: The L82E Variant is Selectively Defective in Cargo Binding by Fluorescence Polarization

A) Fluorescence polarization of labeled RcdA (black) or RcdA L82E (grey) in the presence of SpbR, ^{DBD}TacA and PopA. 100nM of each labeled reporter was incubated with 20μM cargo for 45 minutes and the polarization of labeled RcdA or RcdA L82E was measured, three independent replicates are shown with average and standard deviation. **B)** Fluorescence polarization curves of the PopA-RcdA or PopA-RcdA L82E complex. A serial dilution of unlabeled PopA starting at 40μM was titrated into 200nM labeled RcdA or 200 nM labeled RcdA L82E.

A



B

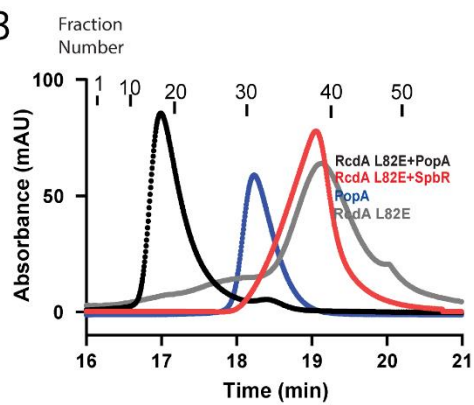
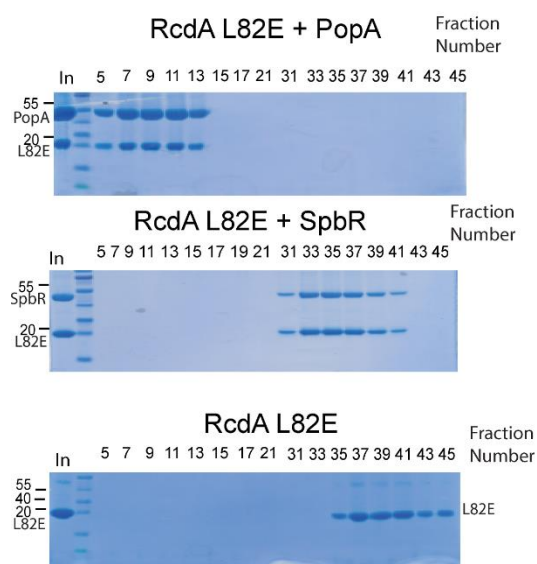


Figure 2.13: The L82E Variant is Selectively Defective in Cargo Binding by SEC-MALS and Analytical SEC

A) Representative SEC-MALS chromatograms of RcdA L82E in the presence of SpbR or PopA. Wildtype RcdA incubated with SpbR, PopA alone, and RcdA L82E alone are shown for comparison. The colored lines under each peak represent the collected light scattering data as a distribution of molecular weights over the width of the chromatography peak. Numbers represent the calculated molecular weight from the scattering data. **B)** Chromatograms and SDS-PAGE gels of collected fractions of complexes shown in Figure 2.13. Fraction numbers are shown above the graph and each gel. Loaded fractions are numbered above each gel well, with the first lane showing the input sample before SEC analysis. Concentrations used were the same as the experiments used in Figure 2.13.

Consistent with the defects in SpbR and TacA binding, the RcdA L82E variant showed less stimulation of the CpdR-ClpXP mediated degradation of these substrates *in vitro* (Figure 2.14). This deficiency was not due to a failure of RcdA L82E to bind the CpdR-ClpX complex, as fluorescence polarization assays that monitor ClpX:CpdR:RcdA ternary complex formation (Joshi et al., 2015) showed that both RcdA variants were able to bind the ClpX:CpdR complex with similar affinity (Figure 2.15)

PopA binds RcdA directly, and in a c-di-GMP dependent manner promotes degradation of CtrA (Smith et al., 2014, Ozaki et al., 2014). To test the effects on PopA-mediated substrate delivery, we used a GFP reporter fused to the minimal domains of CtrA needed for regulated degradation (GFP-CtrARD+15), (Smith et al., 2014). Unlike the case for TacA or SpbR, the RcdA L82E variant was capable of stimulating PopA-mediated CtrA degradation (Figure 2.14). These data show that disrupting the dimer interface of RcdA reduces binding to some substrates, but this monomeric variant can still bind to an activated ClpXP and deliver permissive substrates (such as CtrA). Consistent with an overlap in interfaces for cargo and dimerization, locking RcdA into a dimer by crosslinking also prevents cargo binding, while L82E treated with the same concentrations of glutaraldehyde did not affect the L82E variant's ability to bind PopA (Figure 2.16) Taken together with the observation that all cargoes can compete with RcdA dimerization, our emerging model is that different substrates rely on different regions of the RcdA dimer interface.

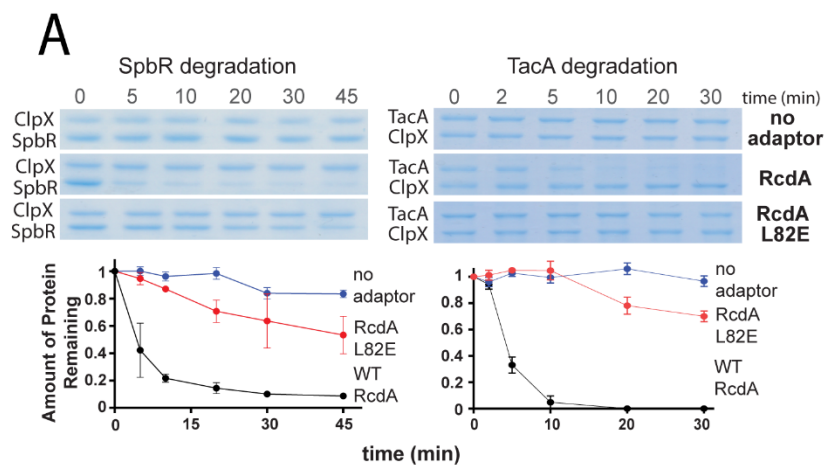
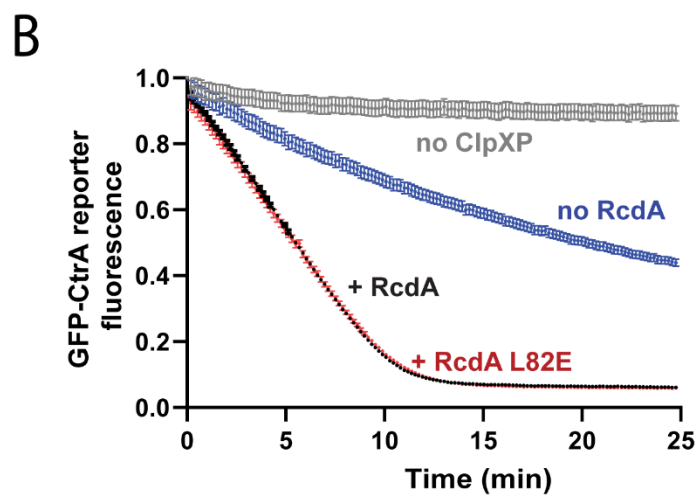
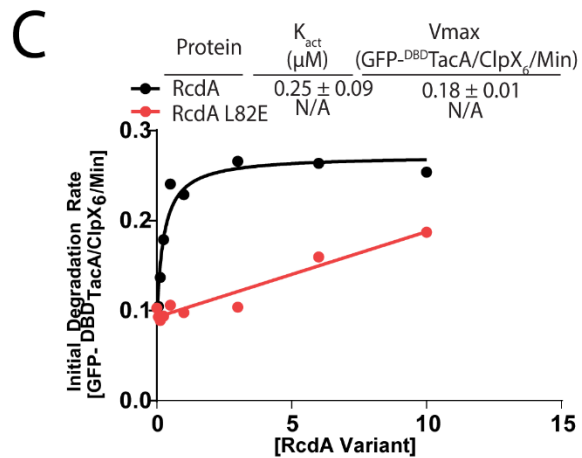
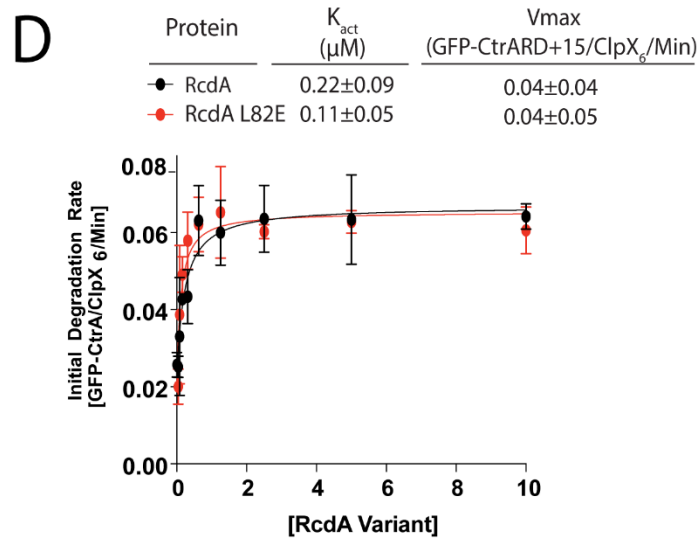
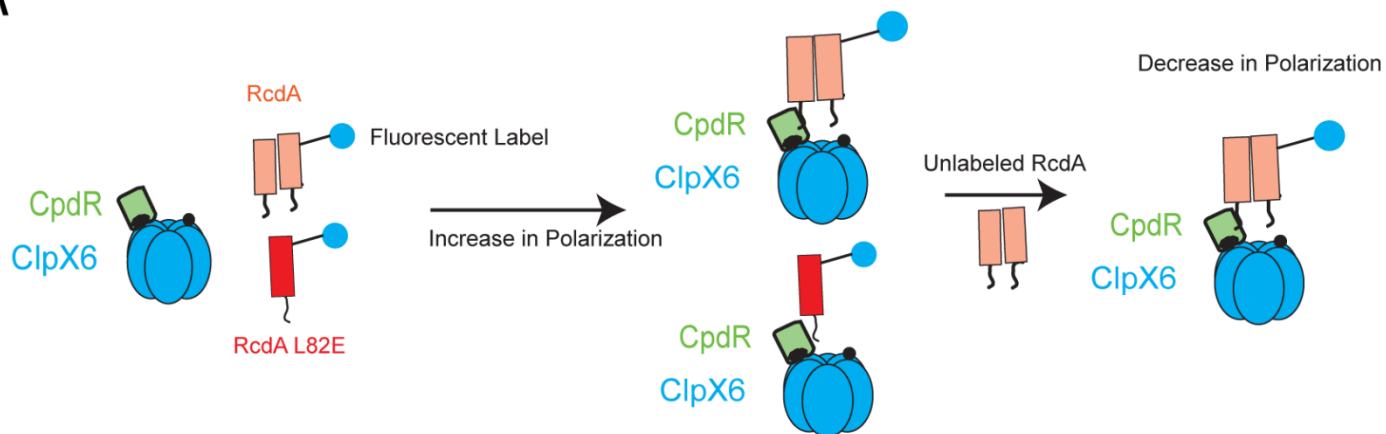


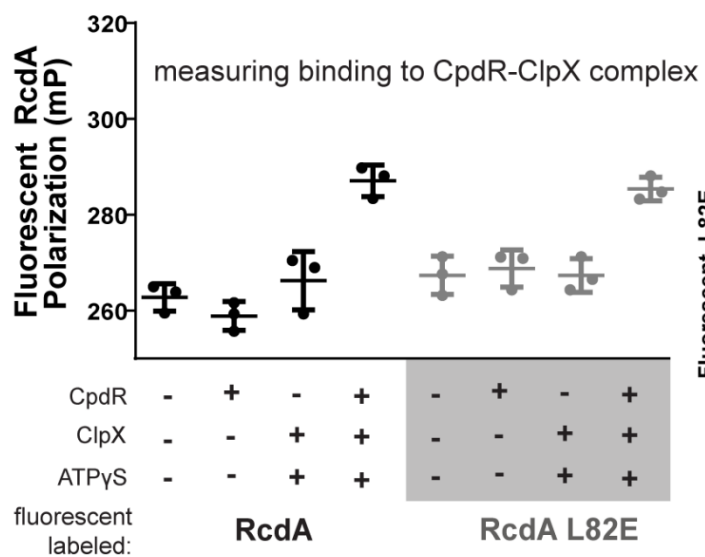
Figure 2.14: The L82E Variant is Selectively Defective in Cargo Delivery

A) *In vitro* gel-based degradation assay of SpbR or in the presence of RcdA or RcdA L82E. Full gels are shown in SI Appendix Fig. S3. Quantification of three independent replicates showing means and error bars (standard deviation). **B)** *In vitro* fluorescence degradation assay of GFP-CtrARD+15 in the presence of RcdA or RcdA L82E. Each fluorescence trace is the average of three independent replicates with error bars showing standard deviation. **C)** GFP-^{DBD}TacA reporter degradation curves with RcdA and RcdA L82E. Fits are to a modified hyperbolic equation as outlined in the methods. **D)** GFP-CtrA reporter degradation curves with RcdA and RcdA L82E. Fits are to a modified hyperbolic equation as outlined in the methods.

A



B



C

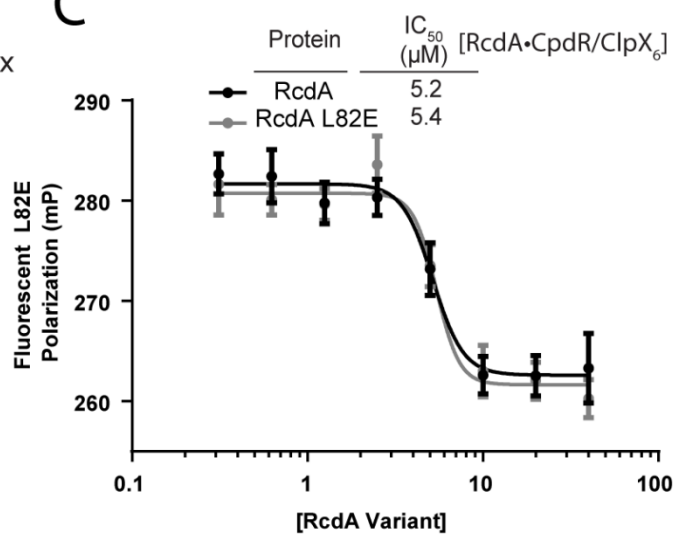
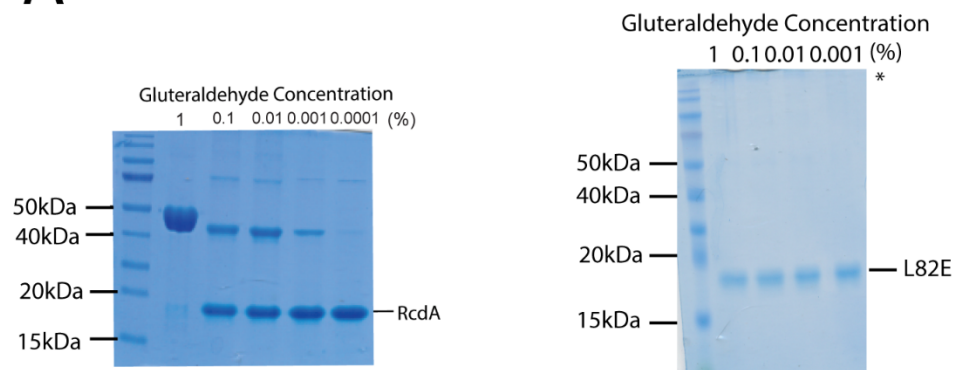


Figure 2.15: The L82E Variant Interacts with the ClpX:CpdR Complex with Same Affinity as WT RcdA

A) Illustration of polarization assay for measuring binding to CpdR-ClpX. A fluorescently labeled RcdA variant increases fluorescence polarization signal when bound to the CpdR-ClpX complex because of decreased rotation. **B)** Fluorescence polarization of 200 nM RcdA and RcdA L82E increase only with CpdR, ClpX, and ATPgS present. **C)** Competition of fluorescently labeled RcdA L82E from the CpdR-ClpX complex by unlabeled wildtype or L82E RcdA variants show they bind to the CpdR-ClpX complex equally well ($IC_{50} = 5 \mu M$). The fluorescent monomeric RcdA L82E was used to prevent formation of dimers of unlabeled and labeled RcdA, which would confound the readout.

A



B

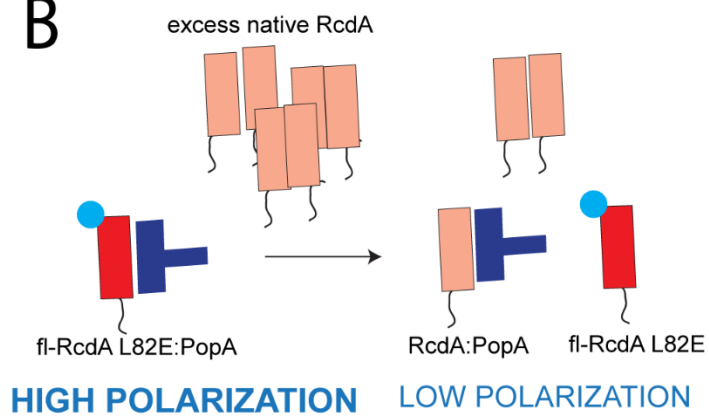
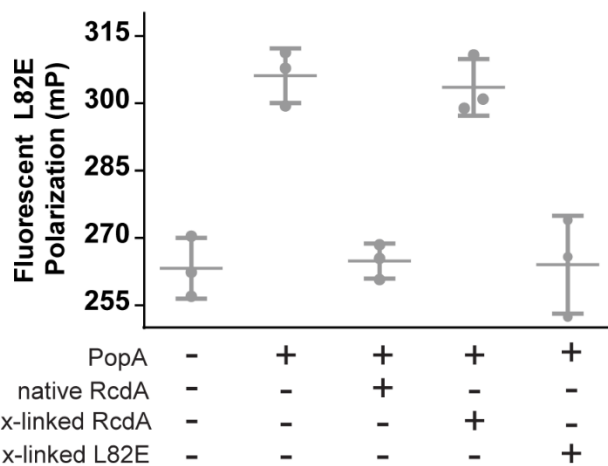


Figure 2.16: A crosslinked RcdA Dimer Cannot Interact with PopA

A) Coomassie stained SDS-PAGE gels of crosslinked 100 μ M RcdA and L82E after incubation with increasing concentrations of glutaraldehyde, demonstrating formation of a dimeric species. The asterik on the L82E crosslinking gel indicates a higher molecular weight band that did not travel through the gel matrix. **B)** (left) Illustration of assay to determine binding of RcdA to PopA using a fluorescently labeled RcdA L82E. PopA binding to fluorescently labeled RcdA L82E results in high polarization of the RcdA L82E reporter. Native RcdA will bind PopA resulting in free labeled RcdA L82E which has a low polarization signal. (right) Polarization signal from labeled RcdA L82E in the presence of the indicated components. Note that wildtype RcdA causes a decrease in polarization, while crosslinked RcdA does not, showing that locked RcdA dimers cannot bind cargo.

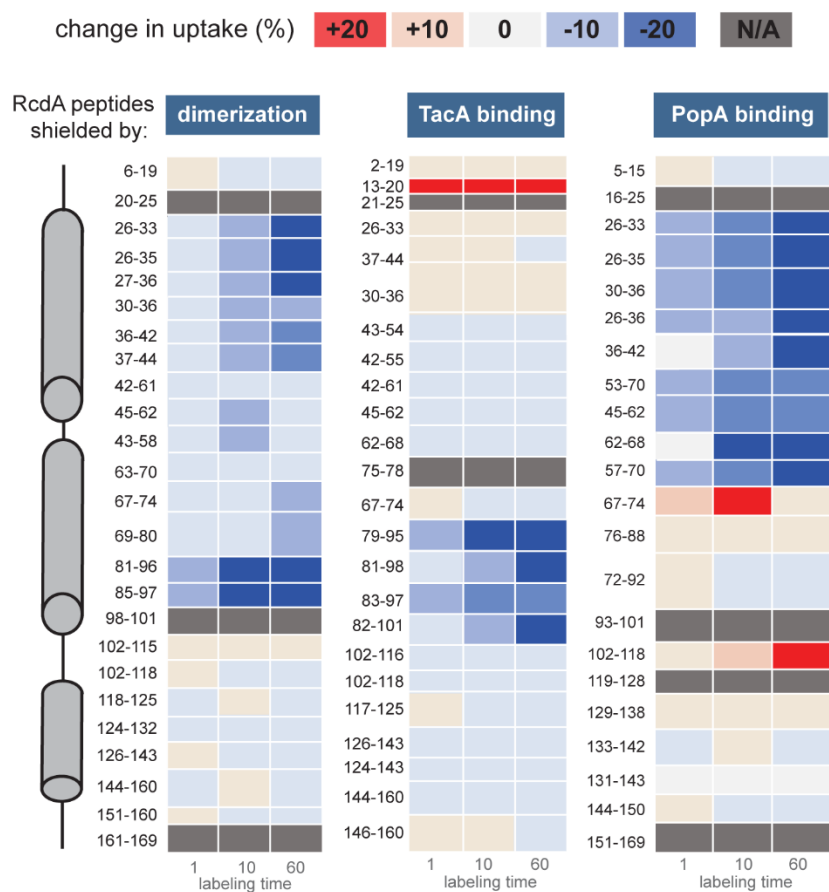
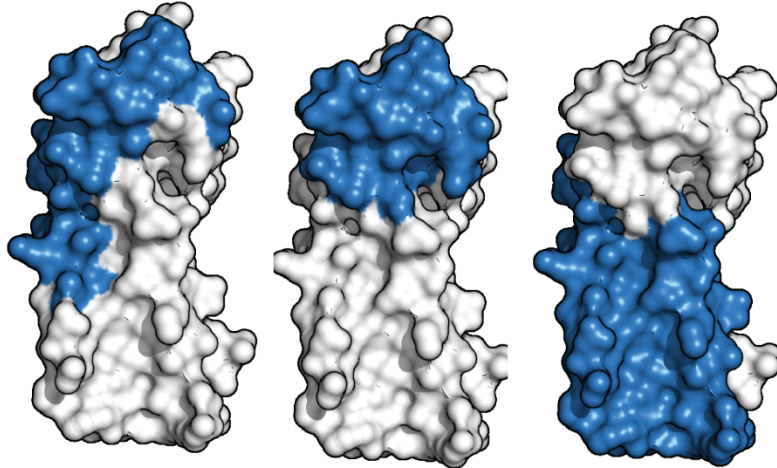
2.8 The dimer interface is protected from exchange in the presence of cargo

To determine how different cargoes interact with RcdA, we used hydrogen deuterium exchange-mass spectrometry (HDX-MS) which measures differences in deuterium uptake in the peptide backbone to determine protein-protein interaction surfaces (24-25). We first compared high and low concentrations of RcdA to map the dimer interface (Figure 2.18) and found these data to be consistent with the comparison between monomeric RcdA L82E and the dimeric wildtype RcdA that also highlights the dimerization interface (Figure 2.17A). We then measured deuterium uptake of RcdA incubated with excess ^{DBD}TacA or PopA to define potential interaction surfaces with these cargoes (Figure 2.17A). Each condition is summarized in a differential deuterium uptake plot as a heat map showing percent deuterium uptake for each peptide and regions of largest protection (>15%) mapped onto a surface rendition of a RcdA monomer (Figure 2.17A). Individual deuterium uptake plots for selected regions of largest protection are provided in Figure 2.17B and Figure 2.19.

Based on the PDBePISA server and the 3CTW crystal structure of RcdA, the most buried residues in the dimer interface lie between residues 34-45 and 71-101. We validated this in solution with our HDX-MS data which highlights residues 26-44

and 80-97 as being most protected in the wildtype dimer (Figure 2.17A). This result further confirms how the L82E mutation disrupts the dimer interface to generate a monomeric variant. Consistent with our biochemical results in Figure 3, ^{DBD}TacA also binds the region of the dimer interface containing the L82 residue with most protection at residues 79-101 supporting our observation that RcdA L82E fails to deliver TacA for degradation. Interestingly, PopA also binds the dimer interface, but principally protects residues 26-70, with the L82 containing region of RcdA showing no substantial protection. We conclude that consistent with our biochemical data, TacA and PopA both bind the RcdA homodimer interface to disrupt dimerization, but they bind at different regions of this interface.

A



B

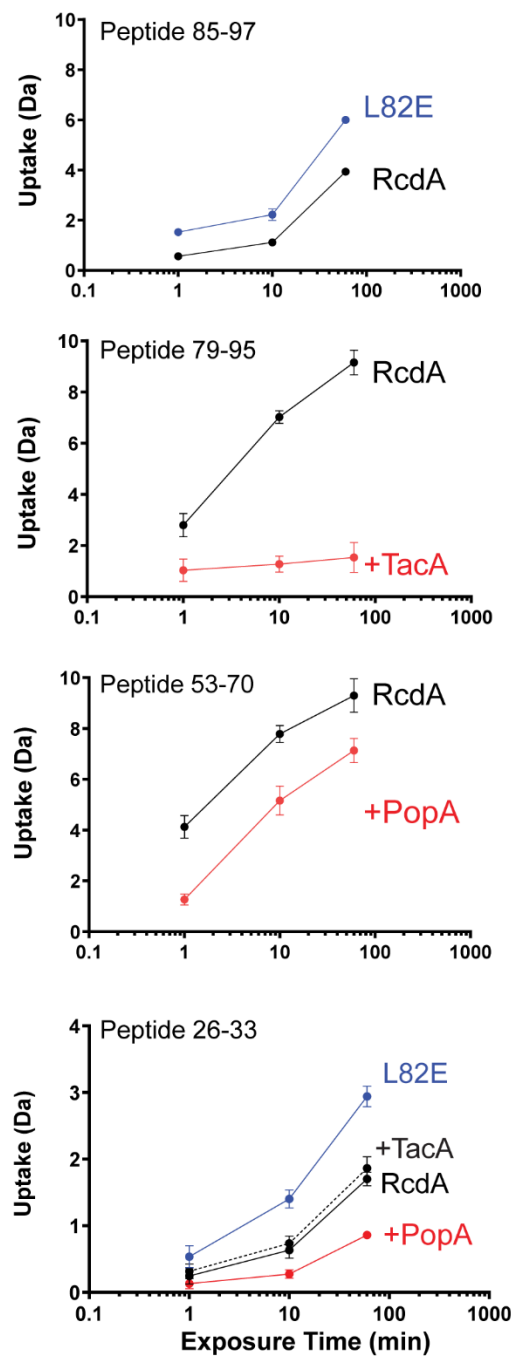


Figure 2.17: The Dimerization Interface is Protected in the Presence of Cargo

A) Differential uptake heatmap plots of 15 μ M RcdA compared with 15 μ M RcdA L82E (dimerization), 600nM RcdA incubated with 20 μ M ^{DBD}TacA (TacA), or 600nM RcdA incubated with 20 μ M PopA (PopA). Regions of 15% or greater protection are highlighted in blue on a surface rendition of the RcdA monomer structure (PDB ID: 3CTW). **B)** Deuterium uptake plots of selected peptides. Data is plotted as the mean of three replicates with the standard deviation plotted as error bars. See chapter 2 appendix for more peptides in the HDX datasets.

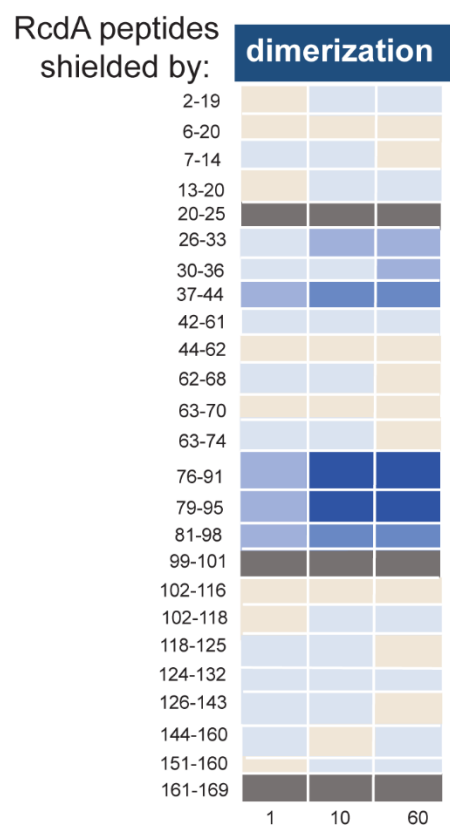


Figure 2.18: Mapping of The Solution Dimer Interface

Differential uptake heatmap plot of 20 μ M versus 600nM RcdA to illustrate dimerization interface. Given K_D determined in Figure 2.5, 20 μ M would be primarily dimer while 600 nM would be primarily monomer.

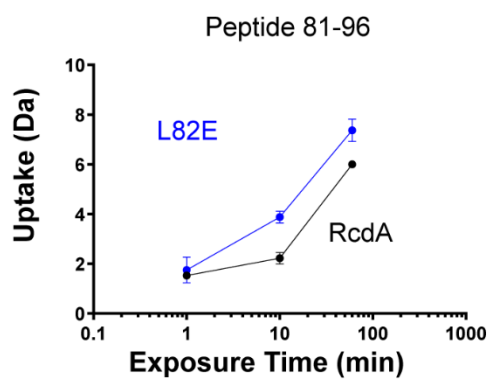
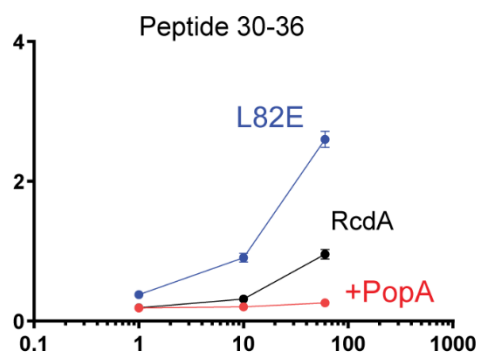
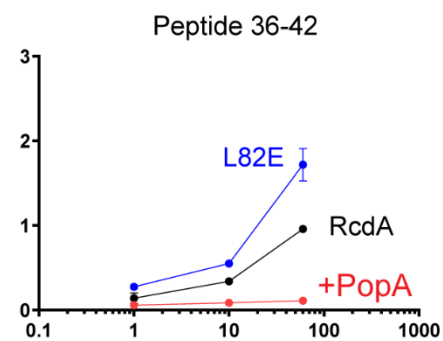
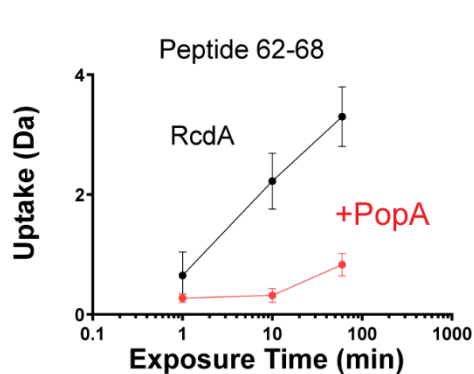
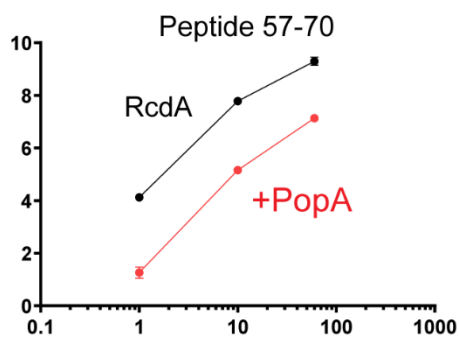
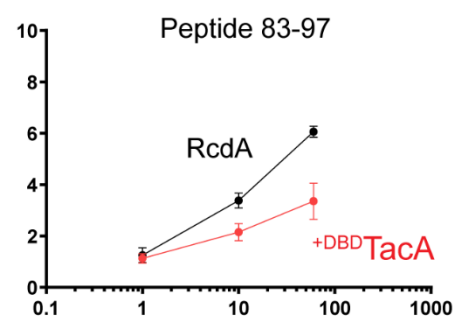
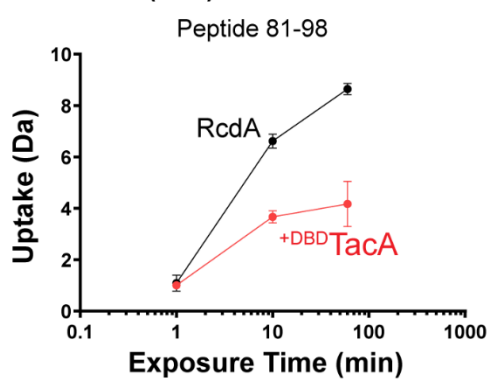
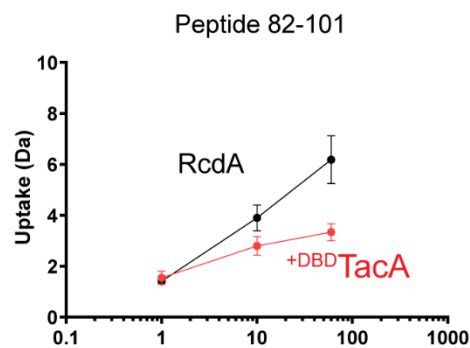


Figure 2.19: Additional Protected HDX Peptides, Related to Figure 2.17

Individual deuterium uptake plots for regions displaying >15% protection in the RcdA-L82E, RcdA-^{DBD}TacA, and RcdA-PopA HDX datasets. Quantification of three independent experimental replicates is shown with the mean and standard deviation plotted as error bars.

2.9 Mutations in the PopA interaction region highlight differences in cargo binding

Our HDX-MS data highlights two regions in RcdA as protected upon PopA binding, one of which includes a cluster of highly conserved basic residues (R49, K53, R57) (Figure 2.20). We had previously identified these residues by modeling a peptide of TacA onto the RcdA crystal structure (Appendix A.9). We mutated these residues to glutamic acid to generate a variant that we refer to as RcdA 3E for brevity (Plasmid made by Madeleine Sutherland), (Figure 2.21). Based on our biochemical studies (Figure 3) we predict that both TacA and SpbR bind similar regions of RcdA, including L82, that are distinct from the regions preferred by PopA (Figure 4). Consistent with this hypothesis, while the RcdA 3E fails to bind PopA based on SEC-MALS, it forms a complex and coelutes with SpbR with a measured mass consistent with the native complex and a dimer of mass consistent with wildtype RcdA (Figure 2.22). Given the failure to bind PopA, we were not surprised to find that RcdA 3E was unable to deliver CtrA for degradation (Figure 2.23).

Consistent with the preserved binding to SpbR and TacA, RcdA 3E was active for their degradation, although not to fully wildtype activity (Figure 2.23), which we suggest may be due to folding defects of the purified protein. Overall, these data suggest that while the homodimer interface of RcdA is generally responsible for cargo binding, PopA and SpbR/TacA rely on different aspects of this interface for effective binding.

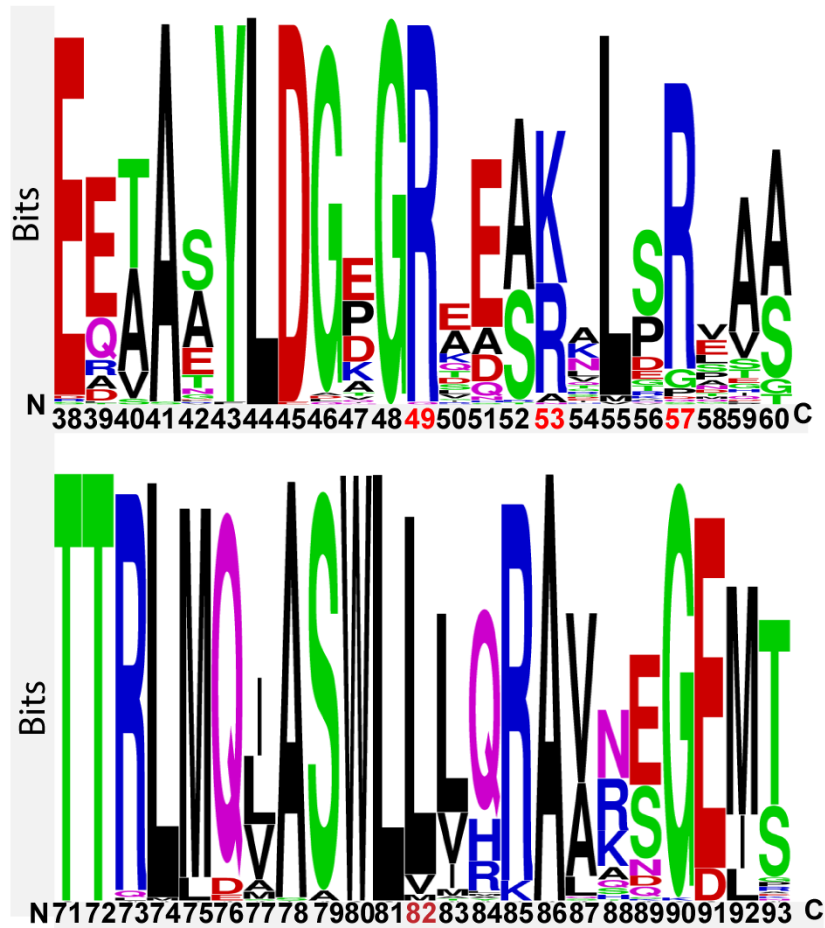


Figure 2.20: RcdA Sequence Alignment Reveals Conserved Residues

Sequence alignment of RcdA in other alpha-proteobacteria (WebLogo, Berkeley).

The upper panel highlights in red the residues (R49, K53, R57) mutated to glutamic acid to general RcdA 3E. The lower panel highlights in red the L82 residue mutated to glutamic acid to make RcdA L82E.

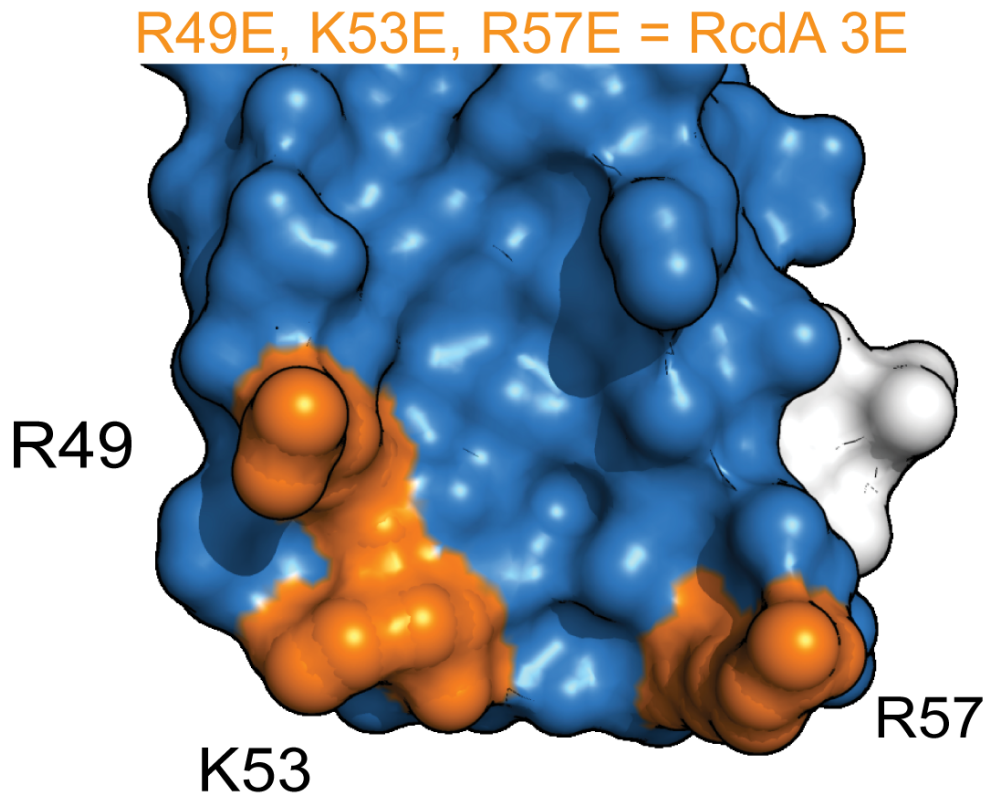
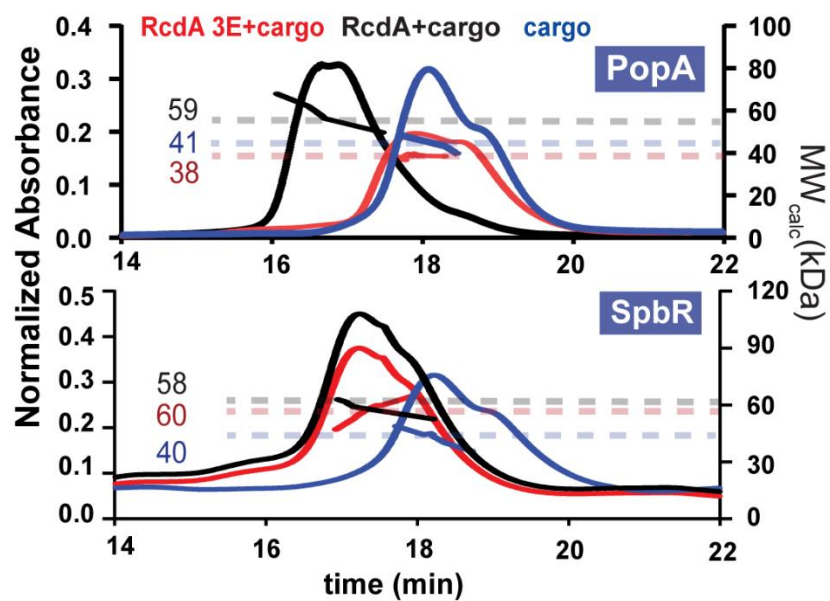


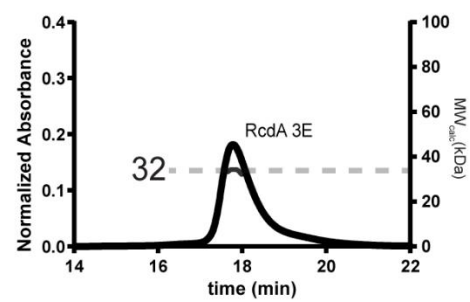
Figure 2.21: Illustration of the RcdA 3E Mutant

Illustration of region of RcdA protected upon PopA incubation (in blue). In orange are the 3 residues (R49, K53, R57) mutated to glutamic acids in RcdA 3E. These mutations were not involved in interactions in the dimer interface and we changed these amino acids solely based on charge.

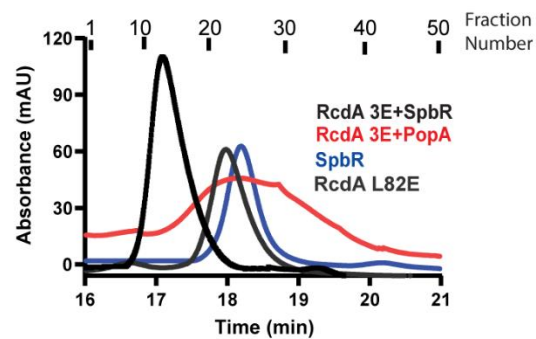
A



B



C



D

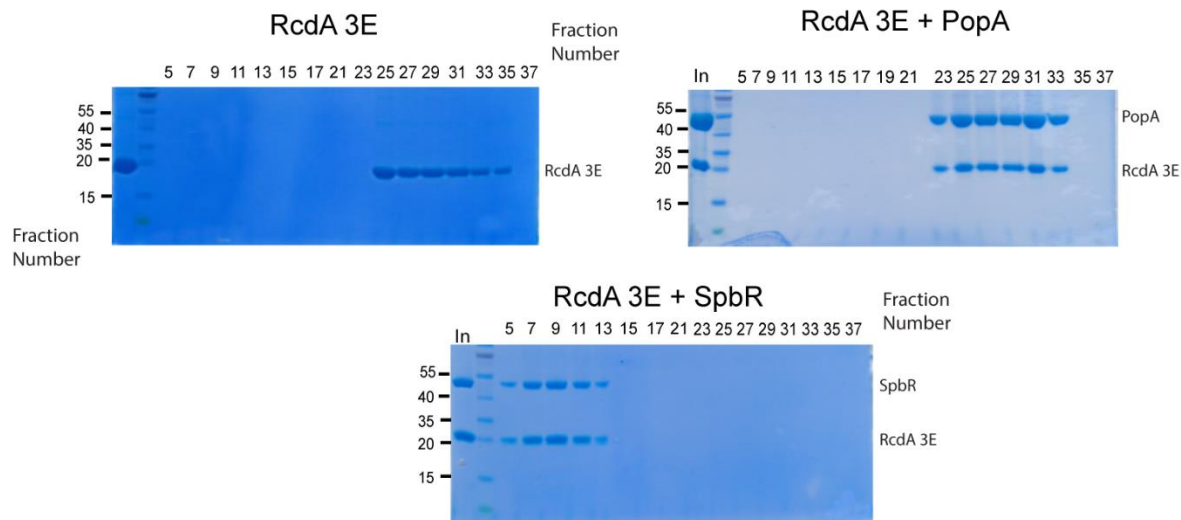


Figure 2.22: The RcdA 3E Mutant is Selective for Cargo Binding

A) Representative SEC-MALS traces for the PopA or SpbR mixtures with RcdA 3E (red). The complex of native RcdA and cargo (black) and the cargo alone (blue) is shown for comparison. The colored regions under each peak represent the collected light scattering data as a distribution of molecular weights over the width of the chromatography peak. Numbers represent the calculated molecular weight from the scattering data. **B)** SEC-MALS traces of RcdA 3E. 25 μ M RcdA 3E was injected over the column. **C)** Chromatograms of all the SEC-MALS data collected in Figure 2.21A. Hand fractionation windows are shown above the graph. The concentrations used were the same as the concentrations used in Figure 2.22A. **D)** SDS-PAGE gels of collected fractions of complexes shown in Figure 3. Loaded fractions are numbered above each gel well, with the first lane showing the input sample before SEC analysis.

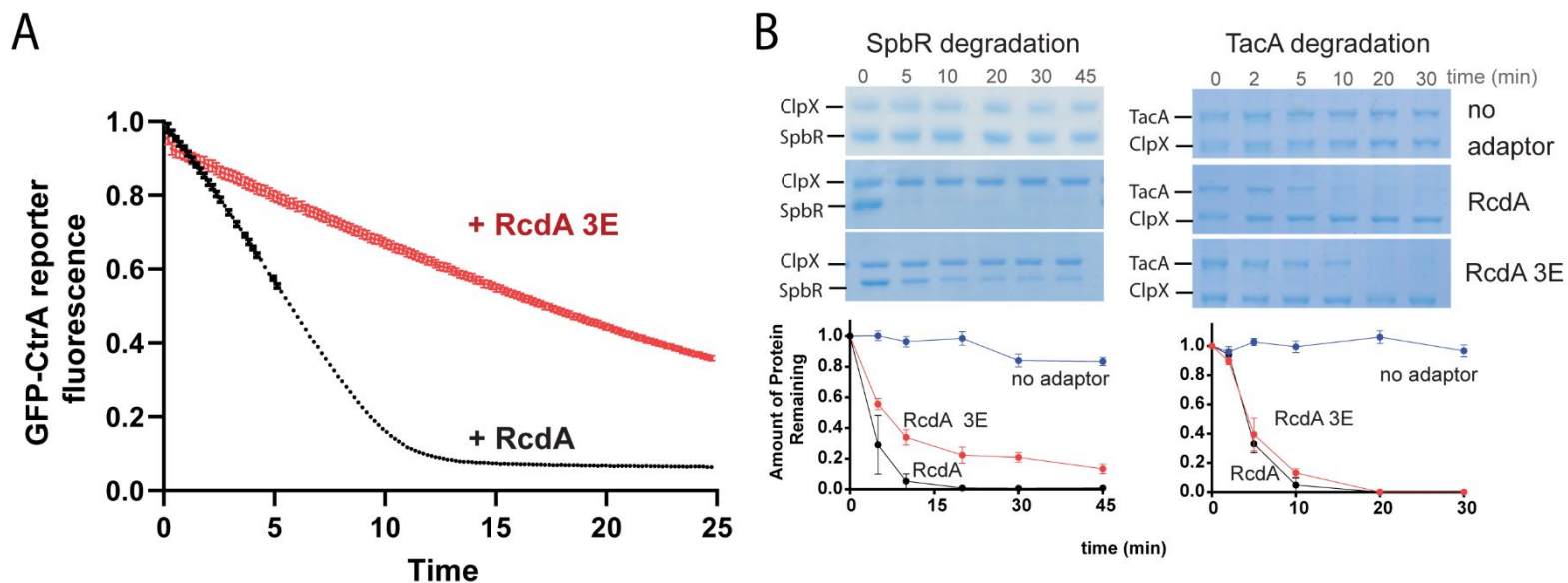


Figure 2.23: The RcdA 3E Variant is Selective for Cargo Delivery

A) *In vitro* fluorescence degradation assay of GFP-CtrA+15 in the presence of RcdA or RcdA 3E. Fluorescence traces are the average of three independent replicates with error bars showing standard deviation. The +RcdA control is the same control that was used in Figure 3C and is shown here for comparison to RcdA 3E. **B)** *In vitro* gel-based degradation assay of SpbR or TacA in the presence of RcdA or RcdA 3E. Quantification of three independent replicates is shown below.

2.10 Defects in cargo binding affect target degradation *in vivo*

We finally investigated the *in vivo* consequences of altering RcdA-cargo interactions. Previous *in vivo* work had shown that altering parts of the RcdA interface did not dramatically affect CtrA delivery during the cell cycle (Taylor et al., 2009). However, our specific mutations had not been tested for defects *in vivo*. During the cell cycle of *Caulobacter crescentus*, the transition from G1 to S phase is accompanied by a morphological change from a motile swarmer cell to a sessile stalked cell facilitated by degradation of RcdA-dependent substrates. Because of this transition, defects in cell-cycle are often accompanied by changes in morphological features. We generated strains that express RcdA, RcdA L82E or RcdA 3E at the *xylX* locus in $\Delta rcdA$ strains, which allows for titration of RcdA using the inducer xylose. $\Delta rcdA$ strains have longer stalks and reduced growth in low percentage agar, indicative of defects in cell-cycle stages and development as stalk growth (McGrath et al., 2006, Joshi et al., 2015). At low levels of inducer, RcdA variant levels driven by the *xylX* promoter were similar to that of the wildtype control (Figure 2.24), and in these conditions, stalk length was compromised in both RcdA L82E and 3E backgrounds (Figure 2.24), while growth in low percentage agar was more affected in the RcdA 3E mutant (Figure 2.25). Curiously, under high induction, where RcdA levels are well above normal, stalk length was reduced to less than wildtype for all the alleles tested (included wildtype RcdA) (Figure 2.25).

These data suggest that the different substrates stabilized by the different RcdA variants may drive defects in stalk length and agar growth separately. Consistent with this and our *in vitro* results, *in vivo* degradation assays showed that strains expressing RcdA L82E are deficient in SpbR degradation (Figure 2.26), while strains expressing RcdA 3E failed to degrade CtrA during either asynchronous growth (Figure 2.26) or during cell cycle progression (Figure 2.26).

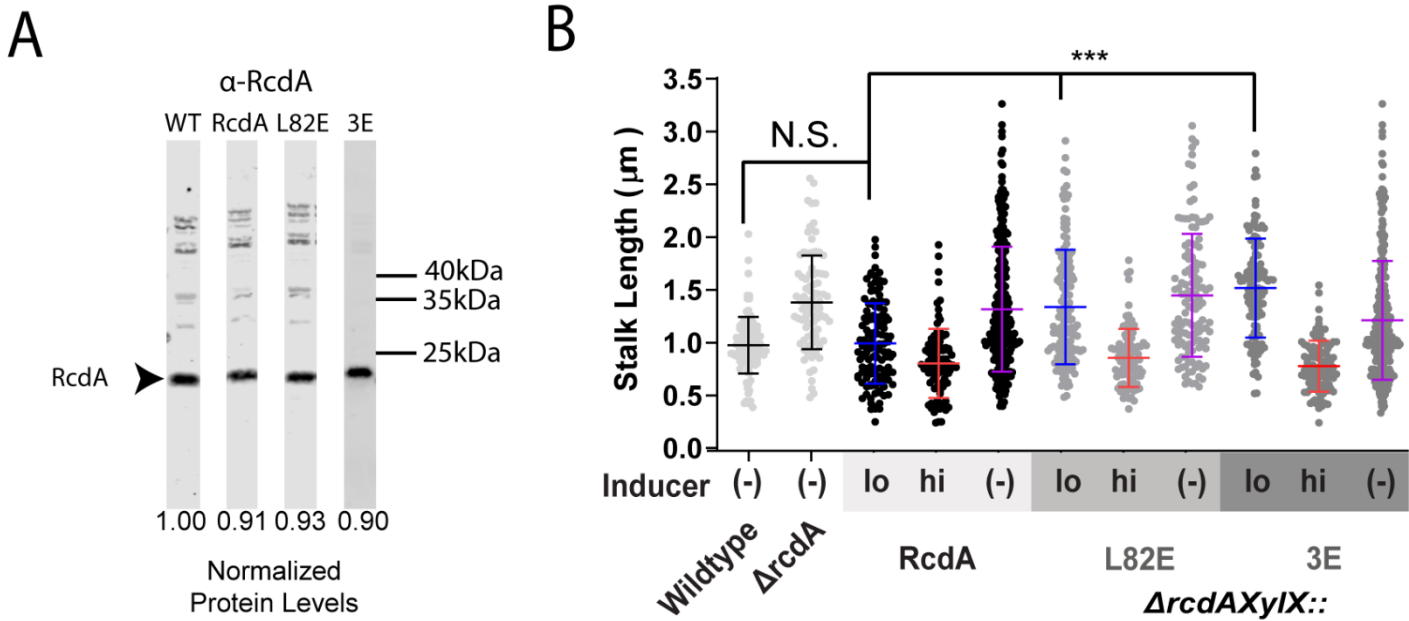
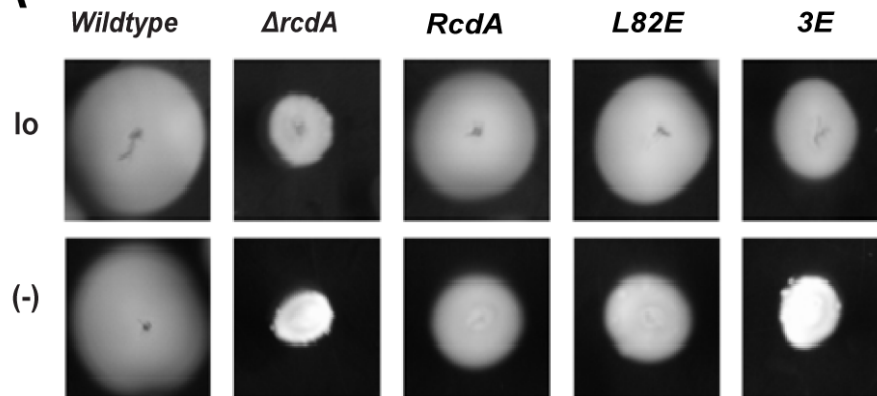


Figure 2.24: Expression of the L82E and 3E Variants causes Stalk Formation Defects

A) Western blot of steady state levels of integration strains expressing RcdA, L82E or 3E from the xylose locus at low induction (0.002% xylose). Quantifications are normalized to wildtype levels. **B)** Stalk length quantification and images of cells expressing RcdA, RcdA L82E or RcdA 3E from the xylose locus. Cells grown to exponential phase in media containing 0.2% (hi: high inducer), 0.002% xylose (lo: low inducer) or 0.2% glucose (repressed). A one-tailed, unpaired t-test with $\alpha=0.05$ was used to compare stalk lengths from indicated strains.

A



B

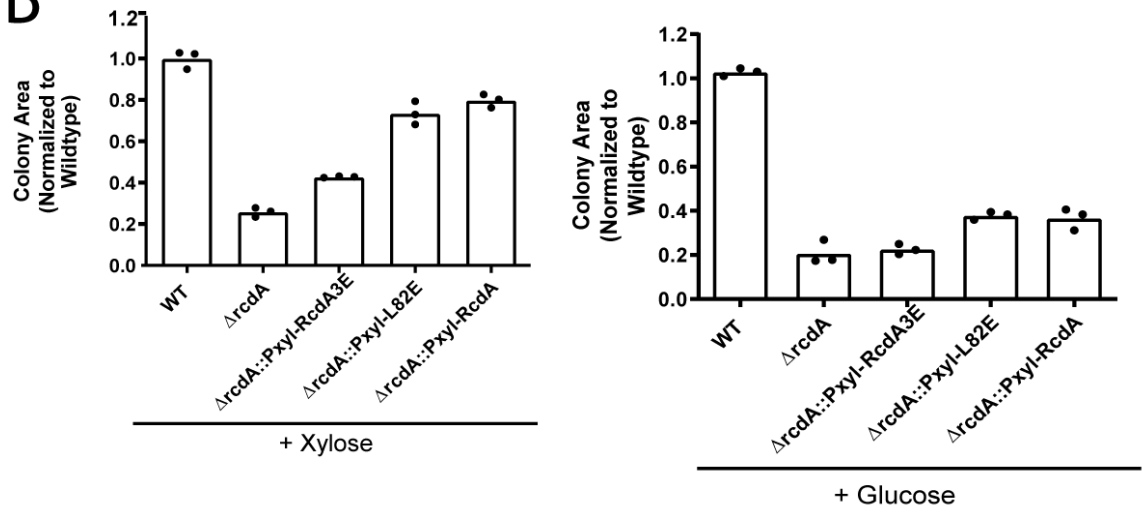


Figure 2.25: The RcdA 3E Variant has Motility Defects

A) Motility agar assay of each integration strain in PYE + low inducer (lo) or glucose repressor (-) agar plates with wildtype RcdA, RcdA(L82E) or RcdA(3E) in each spot. Images have the same absolute perimeter size. **B)** Quantifications of 3 biological replicates of the motility assays in low induction (+xylose) and no induction (+glucose) showing that cells expressing only RcdA3E are deficient in growth in low percentage agar media.

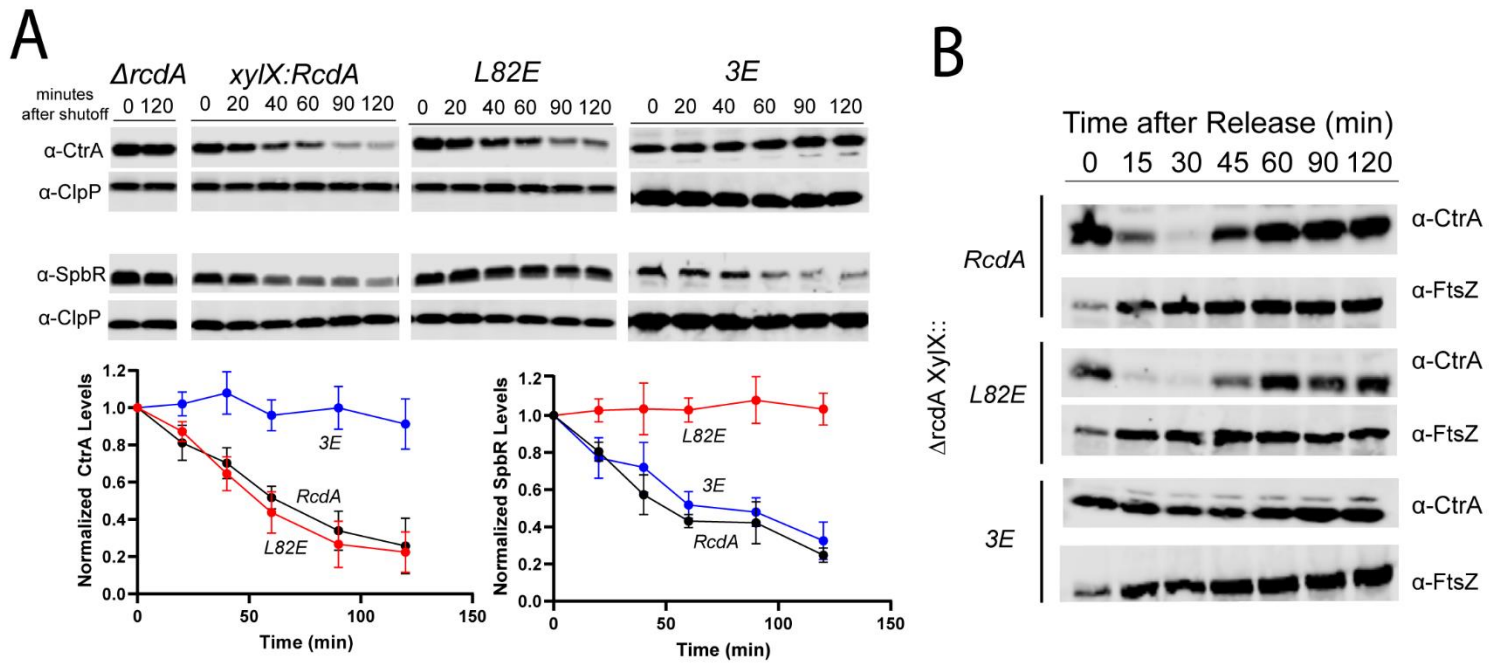


Figure 2.26: The RcdA L82E and 3E Variants are Selective for Cargo Delivery *In Vivo*

A) Chloramphenicol shutoff assays monitoring the degradation of SpbR and CtrA in cells expressing wildtype RcdA or RcdA L82E. **B)** Synchronized cell growth of each integration strain expressing wildtype RcdA, RcdA L82E or RcdA 3E, showing that CtrA oscillations are lost in cells expressing RcdA 3E. Cell samples taken at the indicated time points were probed with antibodies against CtrA and FtsZ.

2.11 Dimerization limits overly prolific RcdA activity

When we monitored cell-cycle progression, we noticed that CtrA levels were slightly lower with RcdA L82E upon minimal induction of this mutant (Figure 2.26). Interestingly, in our biochemical studies we noticed that at lower RcdA concentrations (300 nM) the rate of PopA-mediated CtrA degradation in the presence of RcdA L82E was faster than that found with wildtype RcdA (Figure 2.27). When we generated enzyme-substrate reaction curves under these conditions and fit to Michaelis-Menten kinetics, we found that RcdA L82E was 2-fold more active for PopA mediated CtrA degradation at limiting concentrations of protease and adaptor (Figure 2.27). Since the RcdA dimer interface is also used to bind cargo (such as PopA), this observation is most consistent with a model where dimerization normally limits the specific activity of RcdA for cargo delivery.

We further explored this dimerization driven limit on RcdA activity in normal physiology by constructing merodiploid strains that expressed either wildtype RcdA or RcdA L82E from the native *rcdA* promoter. We found that steady state levels of CtrA are lower and degraded faster in the RcdA L82E strain than in the wildtype strain (Figure 2.28). During synchronized growth, CtrA is lost more rapidly during the G1 to S transition, and re-accumulation of CtrA in predivisional cells is reduced (Figure 6F; SI Appendix Fig. S8G). Finally, to determine the overall fitness cost of this mutation, we used a competition assay and found that strains expressing only RcdA L82E are at a fitness disadvantage compared to wildtype (Figure 6G). Taken together with our biochemical results, these data suggest that

dimerization limits RcdA activity and bypassing this restriction results in persistent degradation of substrates such as CtrA.

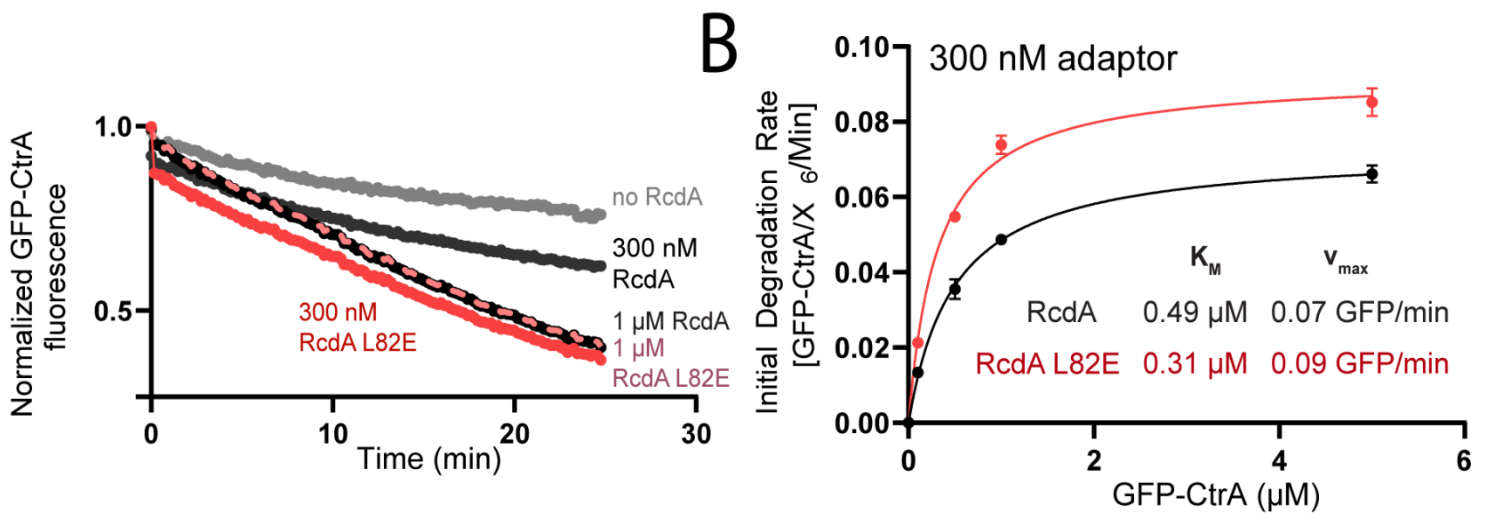


Figure 2.27: The RcdA L82E Facilitates Faster Degradation of CtrA at Lower Concentrations of RcdA

A) Normalized fluorescence degradation curves of GFP-CtrA in the presence of different concentrations of RcdA and RcdA L82E. 500nM PopA, 1 μ M cyclic di-GMP, 300nM RcdA or L82E, 0.1 μ M ClpX, and 0.2 μ M ClpP and 1 μ M of GFP-CtrA was used in this experiment. **B)** Initial rates of GFP-CtrA degradation as a function of adaptor. 500nM PopA, 1 μ M CdG, 300nM RcdA or L82E, 0.1 μ M ClpX, and 0.2 μ M ClpP and varying concentrations of GFP-CtrA reporter were used in these experiments (n=3). Fits are to the Michaelis-Menten equation.

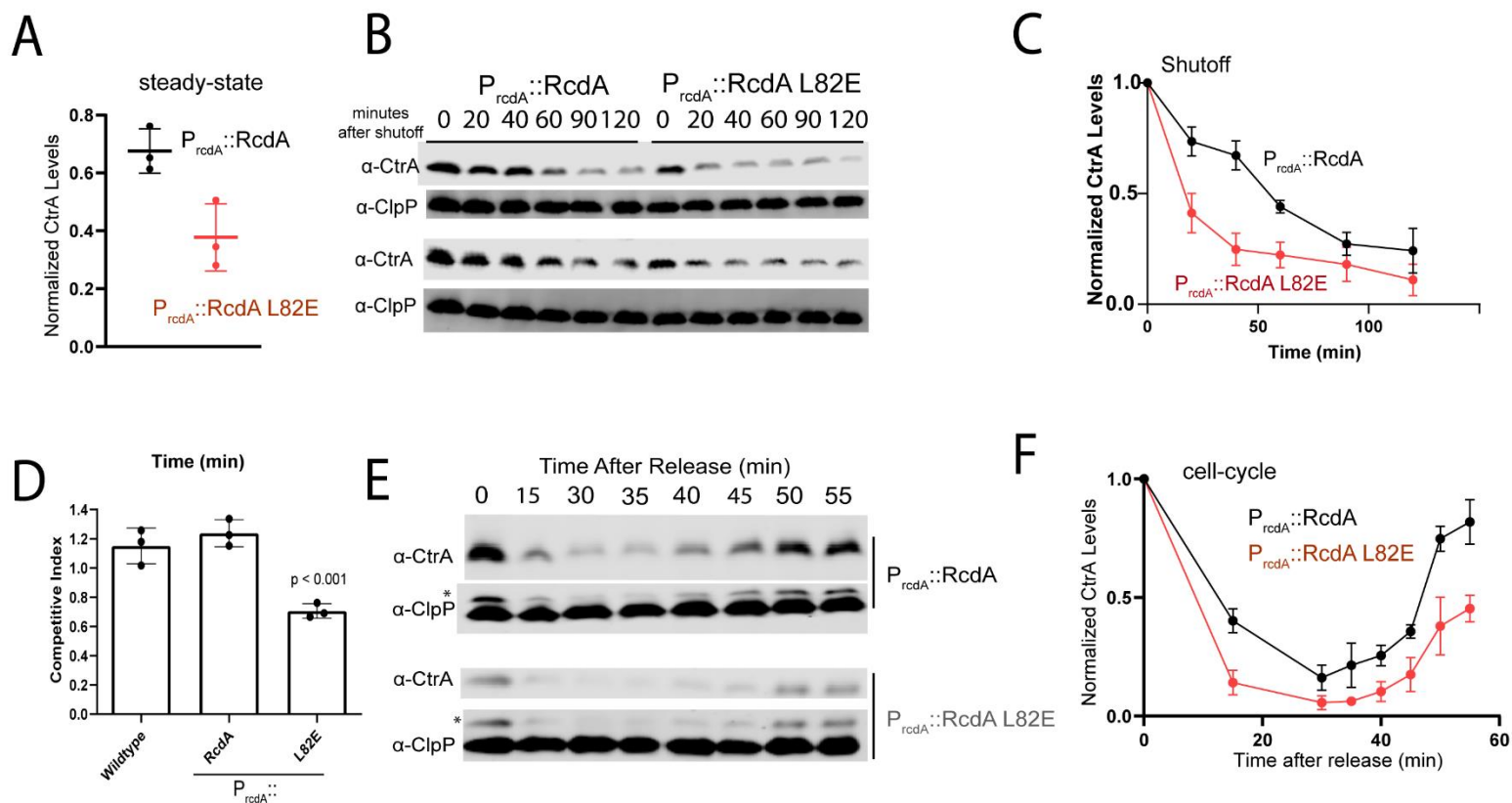


Figure 2.28: The RcdA L82E Variant Reduces Cell Fitness and Delivers CtrA Faster *In Vivo*

A) Normalized CtrA steady levels in asynchronous cultures and their degradation during shutoff. Quantifications are the averages of three biological replicates. **B)** Representative westerns of shutoff experiments for merodiploid strains expressing RcdA or RcdA L82E as the sole copy of RcdA under the control of the P_{rcdA} promoter. **C)** Quantification of replicate shutoff experiments. **D)** Fitness competition assay between wildtype and $P_{\text{rcdA}}::\text{RcdA}$ or $P_{\text{rcdA}}::\text{RcdA L82E}$. For all data except the asynchronous shutoff, which were duplicate experiments, average and standard deviation of three independent replicates is shown. **E)** Representative westerns of synchronized cell growth of merodiploid strains expressing either RcdA or RcdA L82E as the sole copy of *rcdA* under the control of the P_{rcdA} promoter. Quantification of replicate experiments are shown in Figure 6. **F)** Quantification of replicate shutoff experiments.

2.12 Discussion

Adaptor mediated degradation is critical for bacteria. Our results demonstrate a surprising feature of the cell cycle adaptor system in *Caulobacter*, where binding of a cargo to the RcdA adaptor competes with homodimerization. This competition results in stabilization of RcdA, protecting it from self-degradation by ClpXP, while providing a relatively broad binding surface for cargo binding. We note that the cellular concentration of RcdA estimated by ribosome profiling is 6 μM and SpbR, PopA and TacA are between 2-4 μM each (Aretakis et al., 2019). This implies that total cargo concentration is likely in excess of RcdA, driving the RcdA equilibrium towards the monomer form and protecting RcdA from degradation until target substrates are delivered by RcdA. Our binding data is consistent with the model that different classes of cargo can interact with specific regions of this surface, with our direct measurement by HDX-MS and our mutation data showing that we can selectively influence particular substrate binding and degradation (Figure 2.29). Interestingly, CpdR and RcdA are conserved throughout α -proteobacteria, while PopA is only present in *Caulobacter* and closely related bacteria (Brilli et al., 2010, Ozaki et al., 2014). The region mutated in our RcdA 3E variant may represent the binding interface for currently unknown adaptors that fulfill the role for PopA in other species where CtrA is degraded such as *Sinorhizobium meliloti*, (Pini et al., 2015). Recent structures show that the E3 ubiquitin ligase adaptor Skp1 buries its F-box interaction site upon dimerization (Kim et al., 2020), illustrating that masking

of cargo binding sites by self-interactions is more generally found in biological systems.

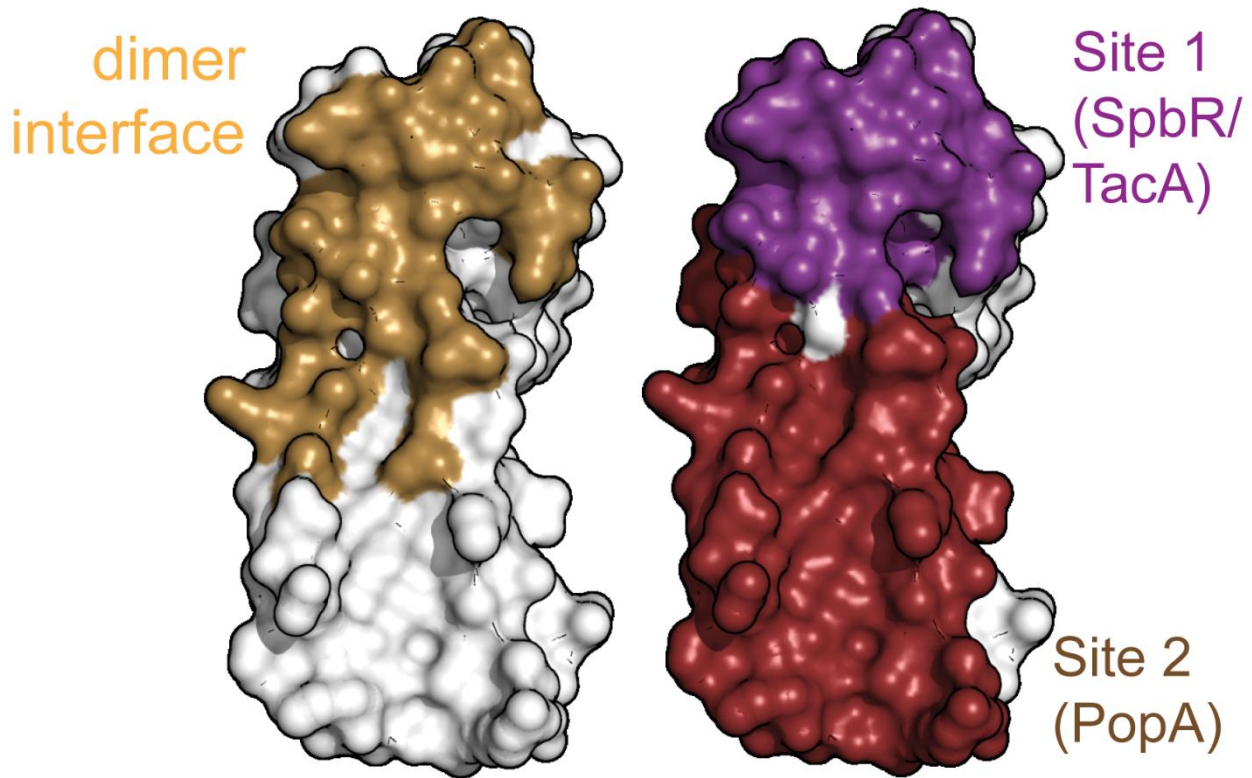
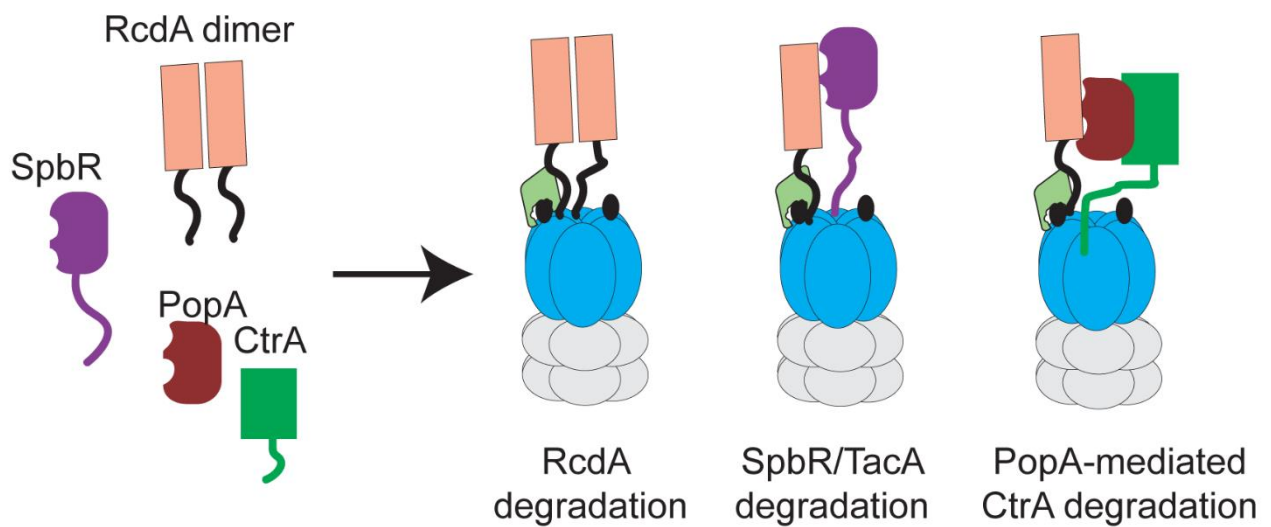


Figure 2.29: Model of RcdA binding and delivery mechanism

Highlighted structure illustrating the different interactions sites of RcdA. In gold is the dimer interface. In purple is the site involved in the SpbR and TacA interaction, which includes the L82 residue. In ruby is the site involved in the PopA interaction, which includes residues R49, K53, R57. Cartoon model of how the oligomerization state of RcdA affects its activity and self-degradation. When not bound to cargo, RcdA is dimeric and delivered to a CpdR-bound ClpXP for degradation. Cargo binding at different interaction sites by different cargo (SpbR/TacA or PopA) causes dissociation of the RcdA dimer and masks RcdA from its own degradation.

Because protease adaptors catalyze the irreversible destruction of targets, we also considered how dimerization of RcdA could regulate adaptor activity. Our results support two mechanisms for the role of dimerization in restricting adaptor activity:

First, because the RcdA homodimer is degraded robustly, overlapping the dimer interface and cargo binding site adjusts RcdA levels tuned to substrate availability. Our results show that RcdA monomers are not degraded and that binding to cargo results in loss of RcdA dimerization. Once the substrates are degraded, RcdA homodimers would form more readily and be destroyed. Similarly, RcdA would never be fully eliminated as once levels drop below the dimerization constant, the shift to the monomeric species would stabilize the adaptor.

Second, masking substrate interaction surfaces by dimerization appears to limit degradation of substrates directly as seen with accelerated PopA-mediated CtrA proteolysis in the presence of the monomeric RcdA variant (Figure 2.28). Essentially, the partner monomer of a homodimer acts as an inhibitor of cargo binding resulting in reduced degradation of substrates. Our data show that there are physiological consequences to the loss of this limitation as cells expressing only monomeric RcdA have lower levels and faster degradation of the essential transcription factor CtrA, resulting in reduced fitness. We note that other protease systems show similar constraints, for example, the N-domain of the Lon protease interacts both with itself and some substrates (30).

Not all dimeric adaptors share this competitive mechanism. For example, the SspB adaptor delivers proteins marked by the *ssrA*-tagging system to the ClpXP protease (Levchenko et al., 2000, Gottesman et al., 1998, McGinness et al., 2007). Unlike RcdA, SspB binds substrates at sites far away from the dimer interface (Chien et al., 2007) and SspB delivery is optimal when two molecules of substrates are bound per SspB dimer (McGinness et al., 2007, Bolon et al., 2004). Therefore, SspB activity would not be limited by competition from dimer formation. We reason that because *ssrA*-tagged proteins naturally arise from failed translation (Levchenko et al., 2003, Keiler et al., 2015), these targets should always be destroyed, therefore prolific SspB activity would not be toxic. By contrast, degradation of RcdA dependent substrates occurs only at a specific stage of the cell-cycle (McGrath et al., 2006, Joshi et al., 2015) and additional controls to limit this adaptor activity, such as the mechanism we describe here, would be beneficial. We speculate that other adaptors that deliver substrates in such a highly timed manner may also be subject to similar regulation.

2.13 Materials and Experimental Procedures

Protein Expression and Purification. BL21(DE3) *pLYS* or X90 cells with expression plasmids for different proteins were grown at 37°C to an OD₆₀₀ of 0.4-0.6, then induced with 0.4mM IPTG for 3-4 h. Induced cells were then centrifuged at 7,000g for 10 minutes and resuspended in buffer containing 50mM Tris pH 8.0, 300mM NaCl, 10mM imidazole, 10% glycerol, 5mM β -mercaptoethanol and 100mM PMSF and frozen at -80°C until further use. Cells were lysed using a Microfluidizer system (Microfluidics, Newton, MA). The clarified lysate was bound over a Ni-NTA column for affinity purification. H₆SUMO-tagged proteins were cleaved by Ulp1-his protease (36). Proteins were then purified using size exclusion and anion-exchange chromatography using Sephacryl 200 16/60 and MonoQ 5/50 columns. ClpX and ClpP were purified as outlined in (9). Detailed purification protocols are available upon request. For all in vitro experiments RcdA concentrations are stated in terms of monomeric equivalents unless otherwise noted.

Cloning and Molecular Biology. RcdA variants were cloned using around-the-horn site-directed mutagenesis by amplifying the desired plasmid using pET23SUMO-RcdA as a template. To generate the $\Delta rcdAXylX::Pxyl$ integration strains, the pXGFPN-1 plasmid (37) was used a template to generate a vector with the RcdA, RcdA(L82E) and RcdA(3E) coding sequences under the xylose promoter. The coding sequence of RcdA, RcdA(L82E) or RcdA(3E) was amplified with complementary overlaps to this vector and the final construct was generated

using Gibson assembly method (38). Competent *ΔrcdA Caulobacter* cells were transformed with pXGFPN-1-RcdA, RcdA(L82E) or RcdA(3E) containing plasmids and selected on 30 µg/mL spectinomycin/streptomycin plates. To generate RcdA or RcdA L82E merodiploid strains, we performed around the horn mutagenesis on the wildtype RcdA pENTR gateway cloning vector (EPC 721) using the same primers used to make the original L82E mutation. We transformed this plasmid into competent wildtype cells and selected on 50 µg/ml kanamycin containing PYE plates. We confirmed insertion of the L82E coding sequence by Sanger sequencing.

***In Vivo* Protein Stability and Synchrony Assays.** Wildtype or *Caulobacter* cells expressing different constructs from a xylose inducible promoter or from the native *rcdA* locus were grown in PYE media with appropriate antibiotic and xylose when required as outlined in the figure legends. Cells were grown to an OD₆₀₀ of ~0.4 with addition of 0.2-0.002% xylose or 0.2% glucose (when noted). Protein synthesis was blocked by addition of 30 µg/mL chloramphenicol and aliquots were taken at the timepoints indicated in the figures. For synchrony experiments, an asynchronous population of cells was grown to an OD₆₀₀ of ~0.4 in PYE. Swarmer cells were harvested and isolated using Percoll density gradient centrifugation, then released into fresh PYE media containing 0.002% xylose (when needed) for progression through the cell cycle.

Microscopy. Phase contrast microscopy was performed on glass slides layered with a 1% agarose pad. A Zeiss Scope A.1 microscope (Zeiss, Germany) equipped

with 100X (1x25 oil ∞ /0.17) objective and 60 N-C 1" 100x camera was used. Images were analyzed with BacStalk (Drescher Lab, Max Planck Institute) software. Stalk distributions were compared using a one-tailed unpaired t-test with $\alpha = 0.05$ (GraphPad Prism).

Motility Assays. Motility assays were performed as described previously (36). Briefly, 0.3% agar plates containing varying concentrations of xylose and glucose were inoculated with three independent colonies of each strain for 3 days at 30°C. Colony sizes were determined using ImageJ (NIH). Quantifications were completed using ImageJ and plotted in GraphPad Prism.

***In Vivo* Growth Competition Assay.** Overnight cultures of a strain constitutively expressing the fluorescent reporter Venus (CPC798) were mixed with Wildtype, P_{r_{cdA}}::RcdA, P_{r_{cdA}}::RcdA L82E at a 1:1 ratio. Mixed strains were then diluted 1:15000 into fresh media and allowed to outgrow for 24 hours. The initial 1:1 mixture of cells was verified by phase contrast and fluorescence microscopy. Quantification of >100 cells was performed for 3 biological replicates. All final ratios were normalized to their starting ratios prior to dilution. Statistical analysis was performed using an unpaired t-test in GraphPad Prism.

Western Blot Analysis. Aliquots withdrawn at indicated time points were spun down, resuspended in 2X SDS sample buffer, boiled at 95 °C for 10 min and then centrifuged. After centrifugation, 10 μ l of clarified supernatant was loaded onto SDS-PAGE gels. Proteins were then transferred onto a nitrocellulose membrane

at 20V for 1 hour and probed for monoclonal rabbit anti-RcdA (1:5000), monoclonal rabbit anti-ClpP (1:5000), polyclonal rabbit anti-SpbR (1:5000), rabbit anti-FtsZ (1:5000), or rabbit anti-CtrA (1:5000). Following overnight primary probing at 4°C, the membranes were washed 3 times with TBST. Proteins were then visualized using IRdye-labeled goat anti-rabbit antibody (LI-COR Biosciences) at 1:10000 dilution and an Odyssey Scanning system (LI-COR)

Fluorescence Polarization and Maleimide Labeling. Purified RcdA or RcdA mutants were labeled with Fluorescein-5-Maleimide (Thermo Scientific™). Purified protein at ~8-10 mg/mL was buffer exchanged into labeling buffer (50 mM Tris pH 7.0, 150mM NaCL, 2mM TCEP). Fluorescein-5-Maleimide was dissolved in DMSO and added to protein at a 20-fold molar excess to cysteine. Labeling reactions were completed at 4°C overnight. Free dye was removed using a PD-MidiTrap column (GE Healthcare) and Amicon Ultra-15 Centrifugal Filter Units in a buffer containing 20mM HEPES pH 8.5, 100mM KCL, 10mM MgCl₂ and 0.05% Tween. Confirmation of protein labeling was verified using a Typhoon imaging system (GE Healthcare). The labeled protein was aliquoted, and flash frozen at -80°C.

Fluorescence polarization binding experiments were performed with 100 nM labeled RcdA or RcdA L82E and varying concentrations of cargo. The binding reaction was incubated at 25°C for 1hr to reach equilibrium. Polarization measurements were read from 40uL of these mixtures using opaque black 384-well plates using a SpectraMax M5 plate reader (Molecular Devices) with excitation and emission wavelengths set at 460 and 540, respectively. Equilibrium binding

constants were calculated by fitting the polarization data using GraphPad Prism to a one site, total and nonspecific binding equation $P = P_{\text{max}}*[X]/([X] + K_d) + NS*[X] + \text{Background}$, where P_{max} is the maximum specific binding value, P is the polarization value, NS is the slope of linear nonspecific binding constrained to be greater than 0, and the background is the polarization value when $[X]$ is 0. Error bars are calculated from the standard deviation between replicates of experiments. All RcdA concentrations are in terms of monomeric equivalents unless otherwise noted.

Chemical Crosslinking. For crosslinking experiments, 100 μM RcdA or L82E was incubated with increasing concentrations of glutaraldehyde as annotated. Crosslinking was performed in 20 mM HEPES buffer, pH 7.5 with 100mM KCL and quenched after 2 hours of incubation at room temperature with 1M Tris pH 8. The resulting mix was then desalted into the same HEPES buffer used for Fluorescence Polarization experiments.

Isothermal Titration Calorimetry (ITC). ITC experiments were completed using a Malvern-autoITC200 automated system (Malvern). Measurements were taken at 25°C. The reference cell was filled with 20 mM HEPES pH 8.5, 100mM KCL and 10% glycerol. This buffer was used for all ITC experiments and for dialysis of each protein into the same buffer prior to the experiment. The sample cell was loaded with 400 μL of 40 μM SpbR and the stirring syringe was loaded with 120 μL of 400 μM RcdA. 19 injections of RcdA into SpbR were used to build the binding isotherm. Data analysis was performed with MicroCal (ORIGIN) and fitted to a single set of

identical sites equation $K_d = (\Theta)/((1-\Theta)*[X])$, where Θ is the fraction of sites occupied by ligand X and $[X]$ is the concentration of ligand X. For dimer dissociation, the sample cell was loaded with 400 μ L of the buffer described above and the stirring syringe was loaded with 120 μ L of 400 μ M RcdA. Dissociation data was fit to the dimer dissociation model in ORIGIN ($P^2 \rightarrow 2P$)

Size-Exclusion Chromatography with Multi-Angle Light Scattering. Each protein complex was allowed to bind at room temperature for 45 minutes. The complexes were then injected onto a TSKgel™ G3000 SEC column equilibrated in 20mM HEPES buffer, pH 7.0 with 100mM Potassium Chloride and 10% glycerol (Tosoh Biosciences) at room temperature. The SEC column was coupled to an 18-angle light scattering detector (DAWN HELEOS-II) and a refractive index detector (Optilab T-rEX) (Wyatt Technology). Data was collected every second and the flow rate was set to 0.5 mL/min. Data analysis was carried out using the program ASTRA (Wyatt Technology). Monomeric bovine serum albumin (BSA; Sigma) was used for calibration of the light scattering detectors and general data quality control. Measurements were taken at 25°C. The light scattering data was collected across a window containing the entire chromatographic peak for a full distribution of molecular weights, shown underneath each chromatogram. The monomeric equivalent concentrations used in the SEC-MALS experiments are as follows unless otherwise noted in the figure legends: 25 μ M RcdA, 25 μ M RcdA L82E, 25 μ M RcdA 3E, 50 μ M PopA, 50 μ M SpbR, 15 μ M His-TacA. We used 50 μ M of RcdA and

SpbR for Figure 1 and Figure 3. The concentrations we used in our coelutions studies were the same concentrations used in our SEC-MALS studies.

Microscale Thermophoresis (MST). The MST experiments were performed using a Monolith NT.115 instrument (NanoTemper). Fluorescein-5-Maleimide labeled RcdA was incubated with increasing concentrations of unlabeled RcdA in the same buffer used in the polarization experiments. The measurements were performed at 20% MST power with 40% LED Power and with 3 s laser on time and 25 s off time. The K_d values were calculated using MO Affinity Analysis software (NanoTemper) and fit to the nonlinear equation $F_{\text{norm}} = [\text{unbound} + (\text{bound} - \text{unbound}) / 2 * (\text{FluoConc} + c + K_d - \sqrt{(\text{FluoConc} + c + K_d)^2 - 4 * \text{FluoConc} * c})]$, where unbound and bound are the thermophoresis values of the unbound and bound states, FluoConc is the fixed concentration of the fluorophore, F_{norm} is the normalized fluorescence, and c is the concentration of the unlabeled protein.

Hydrogen-Deuterium Exchange Mass Spectrometry. Hydrogen-deuterium exchange was measured on a Synapt G2Si high definition mass spectrometer (Waters). Deuterium exchange and quenching steps were performed using an automated HDX robotics platform (Waters). Samples were diluted 1:16 in D_2O -containing buffer containing 0.1 mM K_2HPO_4 to final concentrations as specified in the figure legends. Deuterium exchange was allowed to take place for 0, 1, 10 and 60 minutes at 25°C, with staggered starts for each dilution reaction. After all reactions were completed, aliquots were removed and diluted 1:2 into cold quench buffer at 4°C (water with 4M Guanidine Hydrochloride at pH 2.5) and

subsequentially run over an immobilized Water ENZYMATE immobilized pepsin column (ID: 2.1 length: 30mm) at a flow rate of 0.15mL/min at high pressure (~11000 psi) for peptide digestion. Prior to HDX analysis, the quality of each sample was assessed using SDS-PAGE and size-exclusion chromatography.

Three independently prepared experimental replicates and labeling reactions were performed for each condition and averaged in the peptide uptake plots. Blank runs were run in between each analysis to avoid peptide carry-over. Continuous lock-mass correction was performed using leu-enkephalin compound. Timepoints and analysis were randomized to ensure no biasing of results and to ensure variation. Peptides were ionized and separated by electrospray ionization for analysis in MSE mode at a mass resolution of 50-2000 m/z range. Identification of peptides and analysis of the uptake plots and charge states for each peptide were completed in Protein Lynx Global Server (PLGS) and the software DynamX (Waters). Differential uptake heatmaps and uptake plots were plotted and created in Adobe Illustrator and in GraphPad Prism. Protections of greater than 15% are shown on the surface renditions of the RcdA structure (PDB 3CTW) using PyMol (Schrodinger Software)

***In vitro* Degradation Assays.** Degradation of proteins was monitored using SDS-PAGE gels as described previously (21). The concentrations of different proteins used in degradation reactions are indicated in the figure legends. Degradation of GFP-CtrARD+15 was monitored with the loss of fluorescence over time as described previously (18). The concentrations of each protein used in the reactions

were as follows unless otherwise noted: 3 μ M RcdA, 3 μ M RcdA(L82E), 3 μ M RcdA(3E), 2 μ M CpdR, 0.2 μ M ClpX₆, 0.4 μ M ClpP₁₄, 1 μ M GFP-^{DBD}TacA, 4 μ M SpbR, 4 μ M TacA, 2 μ M GFP-CtrARD+15, 5mM ATP. For GFP-CtrARD+15 experiments in Figure 6C, we used 1 μ M GFP-CtrARD+15, 500nM PopA, 1 μ M CdG, 300nM RcdA or L82E, 0.1 μ M ClpX, and 0.2 μ M ClpP. GFP-^{DBD}TacA curves were fit to a modified hyperbolic equation with the form of: $Y = ((V_{max} * [RcdA]) / (K_{act} + [RcdA])) + A$, where A is a baseline constant, or to the Michaelis-Menten equation using GraphPad Prism.

Sedimentation Velocity Analytical Ultracentrifugation. Sedimentation Velocity experiments were completed using a Beckman ProteomeLab XL-1 Analytical Ultracentrifuge (Beckman). Samples were diluted into 20 mM HEPES pH 8.5, 100mM KCl, 10% glycerol at the concentrations indicated in the figure legends. The samples were spun at 55,000 g overnight at 25°C. The ρ and v values used for data fitting were determined using SEDNTERP and the amino acid sequence for each protein. The sedimentation velocity data was directly fit to the $c(s)$ distribution method using the program SEDFIT and using the first 100 velocity scans for each condition. The resulting distributions from each experiment were then plotted in GraphPad Prism. The final concentrations used were the same concentrations used in the SEC-MALS experiments.

Bacterial Strains Used in This Study:

Organism	Name	Description	Source
<i>C. crescentus</i>	CB15N	synchronizable derivative of wild-type CB15	(Evinger and Agabian, 1977)
	CPC452	$\Delta rcdA$ (<i>hyg^R</i>)	(Mcgrath et al., 2006)
	CPC963	$\Delta rcdAXylX::Pxyl-RcdA$	This study
	CPC964	$\Delta rcdAXylX::Pxyl-L82E$	This study
	CPC965	$\Delta rcdAXylX::Pxyl-3E$	This study
<i>E. coli</i>	TOP10	Cloning strain	Invitrogen
	BL21(DE3) pLysS	recombinant protein expression	Invitrogen
	X90 T7	recombinant protein expression	From Bob Sauer
	EPC100	dh5alpha pQE70-his-ClpP	(Chien et al., 2007)
	EPC112	BL21DE3 pLysS pET23 Ulp1his protease	This study
	EPC162	BL21DE3 375 eGFP-His6-CtrARD+15	(Smith et al., 2014)
	BPC180	Top10 pXMCS-2	This study
	BPC238	BL21DE3 plysS pET23 ClpX	(Chien et al., 2007)
	EPC196	BL21DE3 plysS pET23b His6Sumo-CpdR	(Lau et al., 2015)
	EPC677	BL21DE3 plysS 375 His6-TacA	(Joshi et al., 2015)
	EPC811	BL21DE3 plysS pET23b His6Sumo-TacADBD (437-488)	(Joshi et al., 2015)
	EPC812	BL21DE3 plysS pET23b His6Sumo-SpbR	(Joshi et al., 2015)

	EPC960	BL21DE3 plysS pET23b His6Sumo- SpbRDD	(Joshi et al., 2015)
	EPC967	BL21DE3 plysS pET23b His6Sumo- RcdAΔC	(Joshi et al., 2015)
	EPC970	BL21DE3 plysS pET23b His6Sumo-RcdA	(Joshi et al., 2015)
	EPC1000	TOP10 pET23b His6Sumo-RcdA	(Joshi et al., 2015)
	EPC1037	BL21DE3 plysS pET23b His6Sumo-PopA	(Joshi et al., 2015)
	EPC1129	BL21DE3 plysS pET23b His6Sumo-RcdA 3E	This study
	EPC1517	BL21DE3 plysS pET23b His6Sumo-RcdA L82E	This study

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CHAPTER 3

Hydrogen-Deuterium Exchange Mass Spectrometry Reveals Determinants for PopA-Dependent Functions

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3.1 Abstract

Regulated protein degradation is aided by specificity factors called adaptor proteins that alter the substrate selection of their respective proteases. In *Caulobacter crescentus*, the PopA adaptor, along with two other adaptors, controls the regulated degradation of the replication inhibitor, CtrA. In doing so, PopA must make interactions with the RcdA adaptor and the small molecule, cyclic-di-GMP, to deliver CtrA to ClpXP during the cell cycle. However, how PopA coordinates interactions with RcdA and CtrA to perform its delivery function is poorly understood. Here, we provide mechanistic details into the determinants of PopA responsible for RcdA, CtrA and cdG interactions using statistically filtered Hydrogen-Deuterium Exchange Mass Spectrometry (HDX-MS) experiments. We map the surfaces involved in the binary PopA-RcdA and PopA-cdG complex and the surfaces involved in the PopA-RcdA-CtrA ternary complex and show that this ternary complex forms *in vitro*. We show that, in solution, PopA must facilitate conformational changes throughout the protein through cdG binding to facilitate its functional state while degrading CtrA. Our findings suggest that RcdA and CtrA share nonoverlapping surfaces on PopA and determine that PopA can accommodate both CtrA and RcdA interactions as a monomer. This work provides mechanistic insight into PopA-dependent delivery that will likely extend to other bacteria that use the adaptor-CtrA degradation pathway.

3.2 Introduction

Regulated protein degradation controls many key aspects of physiology and is essential for proper homeostasis. In Bacteria, AAA+ (ATPases with Associated Activities) proteases degrade misfolded and native substrates to maintain proper homeostasis and regulate cell-cycle progression (Mahmoud et al., 2018). The Clp family of proteases, which include the protease ClpXP, are essential for degrading proteins necessary for maintaining normal cell physiology in bacteria and eukaryotic organelles (Truscott et al., 2011, Olivares et al., 2018). AAA+ protease work by using two functional modules: an oligomeric unfoldase which recognizes a short degradation motif and consequentially unfolds proteins into the peptidase module, which non-specifically cleaves proteins into smaller peptide fragments (Sauer and Baker, 2011).

To properly regulate the specificity of their targets, bacterial proteases use accessory factors, or adaptors, to control the specificity of the protease (Kuhlmann et al., 2017). Adaptors can either act as scaffolds by increasing the local concentration of substrate through tethering or by priming the protease by binding to the unfoldase module and promoting recognition of substrates. In the alpha-proteobacterium *Caulobacter crescentus*, a hierarchy of adaptors controls protein degradation during cell-cycle progression and development (Joshi et al., 2015). In this proteolytic pathway, the CpdR binds to ClpX, activating it and recruiting a specific set of substrates (Iniesta et al., 2006, Lau et al., 2015). The second adaptor, RcdA, binds to CpdR by its c-terminal tail and delivers its own

set of substrates through direct interactions with its cargo (McGrath et al., 2006, Joshi et al., 2015, Kuhlmann et al., 2021). Finally, the third adaptor, PopA, interacts with RcdA and the small molecule cyclic-di-GMP (cdG) to deliver the replication inhibitor and master regulator of the *Caulobacter* cell cycle, CtrA (Quon et al., 1996, Duerig et al., 2009, Ozaki et al., 2014). PopA was discovered to be a gene duplication product of the diguanylate cyclase enzyme PleD through functional characterization of *Caulobacter crescentus* proteins involved in cdG turnover (Duerig et al., 2009). While PopA can bind cdG specifically, it lacks diguanylate cyclase (DGC) activity, suggesting that PopA evolved from PleD to serve a different function (Duerig et al., 2009). Through bioinformatic comparisons with PleD, it was found through homology modeling that PopA shares a common response regulator fold like PleD and other DGC enzymes found in other bacteria, such as WspR (De et al., 2009) and DgcB (Meek et al., 2019). The conserved response regulator structure uses input receiver domains, where phosphorylation of a conserved site shifts the response regulator from an inactive conformation to an active conformation (Gao et al., 2019). For DGC enzymes, phosphorylation and cdG binding to the GGDEF domain gate the catalytic behavior of the enzyme. For PleD, phosphorylation and cdG binding allow for dimerization of the PleD enzyme necessary for catalytic activity (Wassman et al., 2007, Chan et al., 2004). Surprisingly, despite evolving from the PleD gene, PopA is not regulated by phosphorylation (Ozaki et al., 2014) but PopA's CtrA delivery activity is gated by cdG binding (Ozaki et al., 2014).

Furthermore, PopA can interact with RcdA without its GGDEF domain, suggesting a role for its receiver domains in facilitating protein-protein interactions (Ozaki et al., 2014). It has been suggested that PopA can form dimers in the presence of cdG *in vivo* (Wang et al., 2021). While both PopA and RcdA are necessary for delivery of CtrA during the *Caulobacter* cell cycle, the mechanism by which PopA interacts with RcdA and CtrA to promote delivery remains unclear.

Here, we provide detailed characterization of the PopA-RcdA-CtrA ternary complex and the surfaces on PopA responsible for each binary interaction using hydrogen-deuterium exchange and statistical filtering of each dataset. We found that different peptides were protected across each dataset in the ternary complex versus each binary complex and provide proposed interaction surfaces arising from our HDX-MS data. We confirm that RcdA, PopA and CtrA form a ternary complex only in the presence of cdG. We show that, in solution, PopA must facilitate conformational changes throughout PopA through cdG binding to facilitate its functional state while degrading CtrA and that, surprisingly, PopA binds CtrA as a monomer only in the presence of cdG. Our findings suggest that RcdA and CtrA share nonoverlapping surfaces on PopA and that PopA binds CtrA and RcdA as a monomer.

3.3 Results

RcdA, CtrA and PopA form a Ternary Complex

We began our studies by performing interaction studies to determine if PopA can interact with RcdA and CtrA to form a ternary complex. PopA and RcdA interact directly without cdG, where a monomer of PopA binds a monomer of RcdA (Duerig et al., 2009, Smith et al., 2014, Kuhlmann et al., 2021) and it is suggested PopA can interact with CtrA only in the presence of cdG (Smith et al., 2014). We decided to perform a nickel pulldown experiment using a 6xHis tagged CtrA (His₆-CtrA) to assay for PopA-RcdA-His₆-CtrA ternary complex formation. We replicated the results from (Smith et al., 2014) showing that PopA can only be pulled down by CtrA in the presence of cdG (Figure 3.1A). In the presence of RcdA, all three proteins were present in the elution fraction, suggested binding of RcdA and His₆-CtrA to PopA simultaneously (Figure 3.1B). Interestingly, cdG does not affect RcdA's affinity for PopA, suggesting that the binding of cdG to PopA affects CtrA interactions independently of RcdA (Figure 3.2). Because PopA might form a dimer in presence of cdG, we asked whether there might be another role for cdG in facilitating PopA-CtrA interactions and whether CtrA and RcdA might share a common interaction surface on PopA.

Mapping of the RcdA-PopA Interaction Surface

We next performed Hydrogen-Deuterium Exchange Mass Spectrometry (HDX-MS), which measures changes in deuterium uptake in the peptide backbone of

proteins to map out the protein-protein interfaces of each binary complex and the PopA-CtrA-RcdA ternary complex (Konermann et al., 2011, Chalmers et al., 2011). We began by mapping out the surface on PopA masked by RcdA binding. Our initial PLGS searches yielded over 4,000 peptides, making interpretation of our data challenging. Using a statistical filtering program designed to provide more rigorous filtering of HDX-MS data (Hageman and Weis, 2019, Lau et al., 2020), we identified and narrowed our scope of peptides that showed the most protection across the time course of the HDX-MS assay. Our initial HDX data showed coverage across the entire protein, but we specifically looked for regions that showed up as the most protected after providing a p-value cutoff filter of 0.05. Peptides spanning residues 127-169, residues 267-275, and residues 296-302 showed the strongest protection on PopA in the presence of RcdA after application of statistical filtering in the Deuterios software (Figure 3.3A). Using Phyre2 fold prediction, we generated a homology model of PopA using the inactivated crystal structure of PleD as a model (Chan et al., 2004). We took the statistically filtered HDX data and mapped the protected and more exposed regions onto the modeled structure (Figure 3.3B). The protected regions formed a distinct surface on PopA that span through the last 4 amino acids of the 1st receiver domain through most of the second receiver domain. Our data is consistent with the 2nd receiver domain of PopA comprising most of the interaction surface in the PopA-RcdA interaction. Individual peptide uptake plots are provided to highlight peptides of significant difference in Figure 3.3C. To test

whether the 1st receiver domain is sufficient to bind RcdA, which has been previously suggested by BATCH, we made a 1st receiver domain truncation (1-117) that lacked the second receiver domain and tested whether this domain would be able of binding RcdA by size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS). Consistent with the second receiver domain being needed for RcdA-PopA interactions, we did not see any binding of this truncation construct by SEC-MALS (Figure 3.4). Interestingly, this construct could compete off GFP-^{DBD}TacA degradation, a direct test of RcdA-mediated degradation (A.14). To confirm our original HDX data and to ensure that the interaction surface on PopA does not change for RcdA when cdG is bound, we performed SEC-MALS with PopA incubated with RcdA and cdG and did a pairwise state comparison in Deuterios to PopA incubated with cdG. Consistent with our PopA-RcdA HDX results, we saw a large cluster of protected peptides primarily in the 2nd receiver domain, supporting a model where the PopA-RcdA interaction surface consists of the 2nd receiver domain (Figure 3.5).

Cyclic-di-GMP binding allows for PopA-CtrA interactions by Conformational Changes in PopA

We next tested to see what surfaces were protected on PopA in the presence of cdG, the small molecule necessary for CtrA delivery (Duerig et al., 2009). It has been suggested that cdG helps PopA forms dimers *in vivo* (Wang et al., 2021), however, there lacks strong evidence for PopA dimerization *in vitro*. We took into consideration that the inactive structural model of PopA might not reflect the

conformation of PopA in the presence of cdG, as the active conformation of PleD when it dimerizes in the presence of cdG is different than the inactive conformation. In the presence of cdG, we saw much of the PopA protein become protected from deuterium uptake over the course of the assay (Figure 3.6A). We looked for specific interfaces that could function as the dimer interface between two copies of the modeled PopA structure based on the inactive and active crystal structures of PleD (Wassman et al., 2007, Chan et al., 2004).

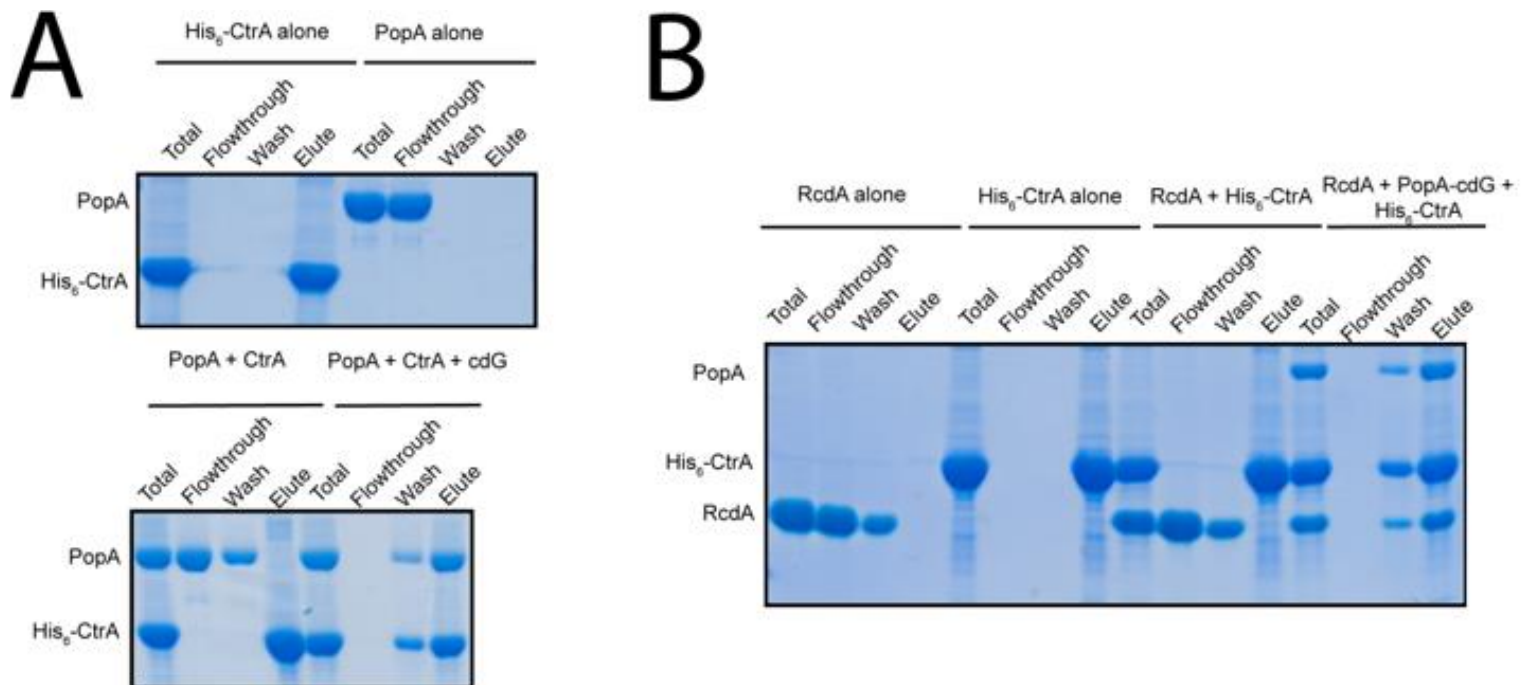


Figure 3.1: His₆-CtrA Nickel Pulldown Assay

A) SDS-Page gels of Nickel Pulldown assay of His₆-CtrA alone, PopA alone and PopA + CtrA in the presence and absence of cdG. 10μM PopA or His₆-CtrA were incubated with 50μL of pre-equilibrated Ni-NTA resin. The resin was washed and eluted as described in the methods. **B)** SDS-Page gels of Nickel Pulldown assay of His₆-CtrA alone, RcdA alone and His₆-CtrA + RcdA, and His₆-CtrA + RcdA and PopA in the presence of cdG. 10μM PopA, His₆-CtrA or RcdA were incubated with 50μL of pre-equilibrated Ni-NTA resin. The resin was washed and eluted as described in the methods section.

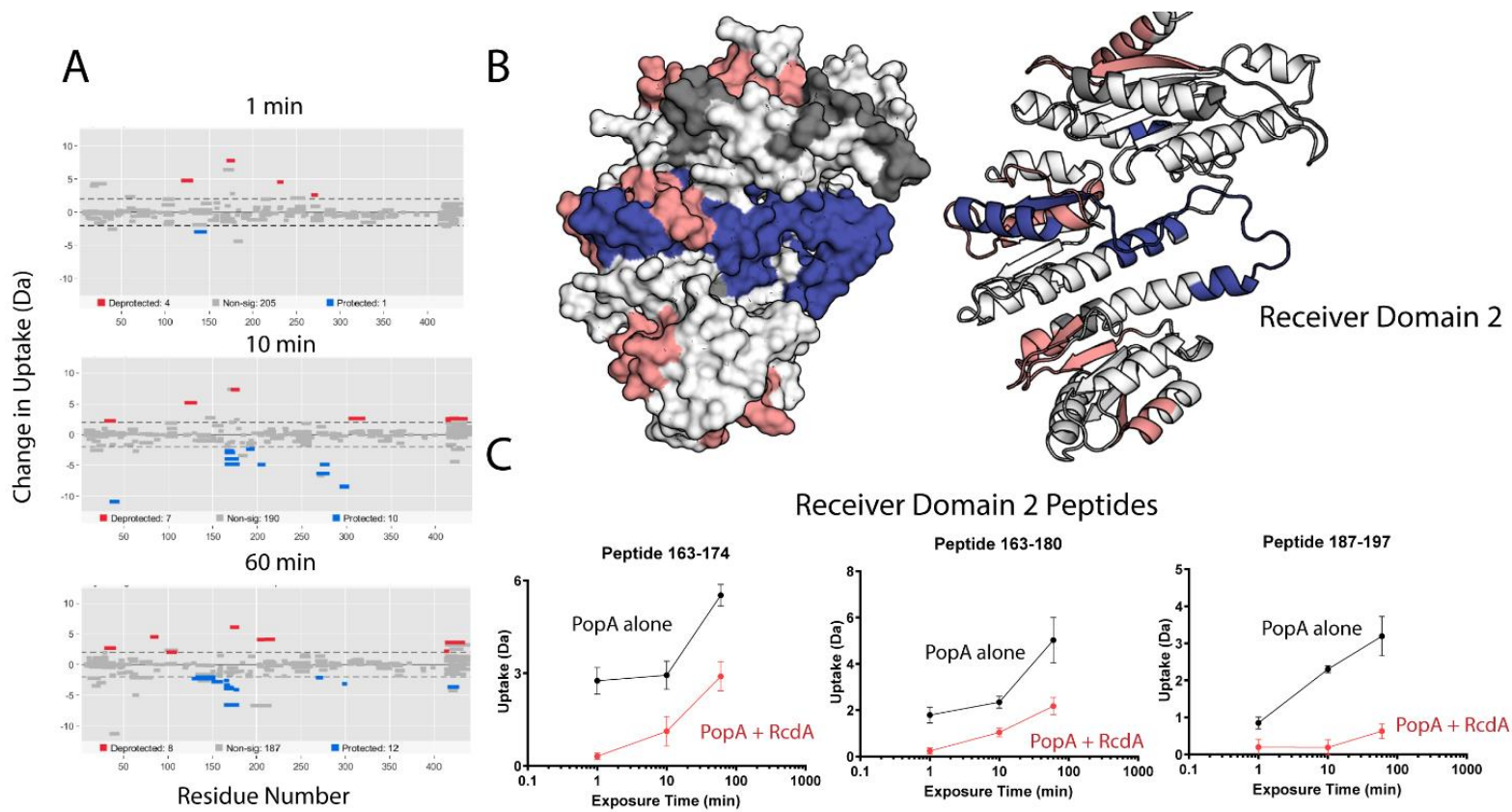


Figure 3.2: RcdA Interacts with the Second Receiver Domain of PopA by HDX-MS

A) Hybrid statistical Woods plot of filtered HDX peptides. Peptides from the each timepoint are highlighted, with peptides showing lower statistical HDX change in blue, and higher statistical HDX change in red. Insignificantly changing peptides are in grey. Each domain of PopA is annotated under the residue number. A significance filter of $\alpha = 0.05$ was used. **B)** Mapped HDX changes from the Woods plot onto a SWISS-MODEL modeled PopA structure based on the inactivated crystal structure of PleD (PDB code: 1W25) for the 60 minute timepoint. Highlighted surfaces, peptides and regions on PopA are shown in each depiction **C)** Selected peptides from the second receiver domain that are highly protected upon RcdA binding from the Woods plot.

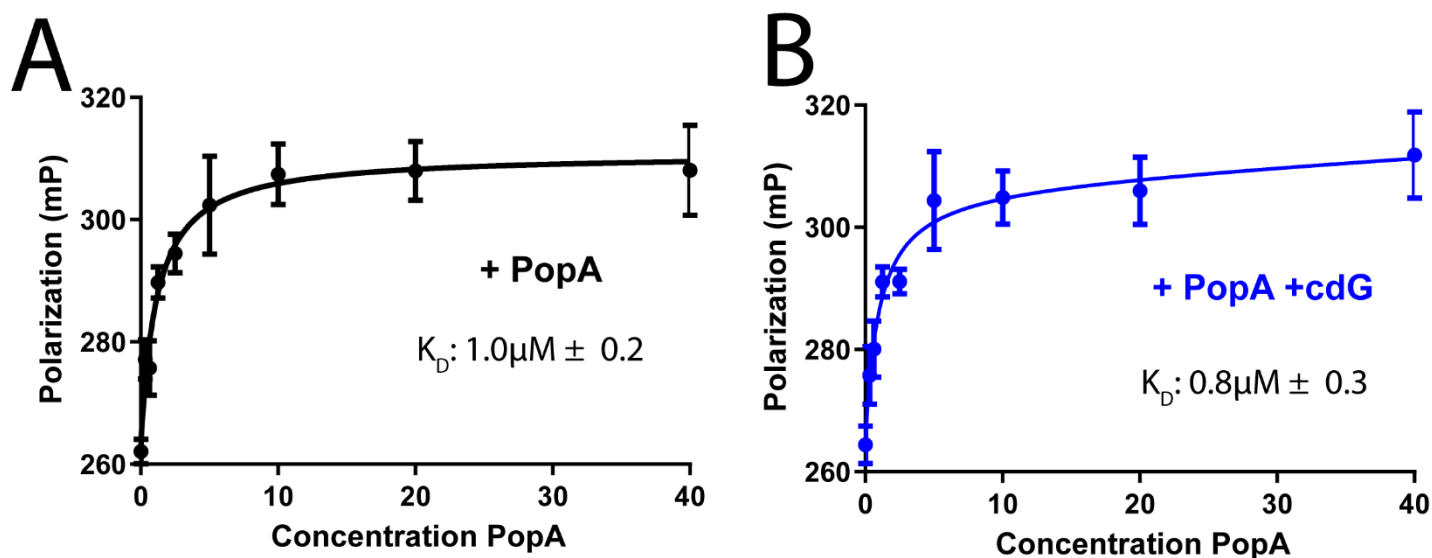


Figure 3.3: RcdA binds PopA and PopA-cdG with Similar Affinity

A) Fluorescence polarization binding curve of a fluorescently labeled RcdA with increasing concentrations of PopA. 100nM RcdA was incubated with up to 40uM PopA. Polarization data was fit to a single site binding model as described in the methods. **B)** Fluorescence polarization binding curve of a fluorescently labeled RcdA with increasing concentrations of PopA. 100nM RcdA was incubated with up to 40uM PopA and 100uM cdG.

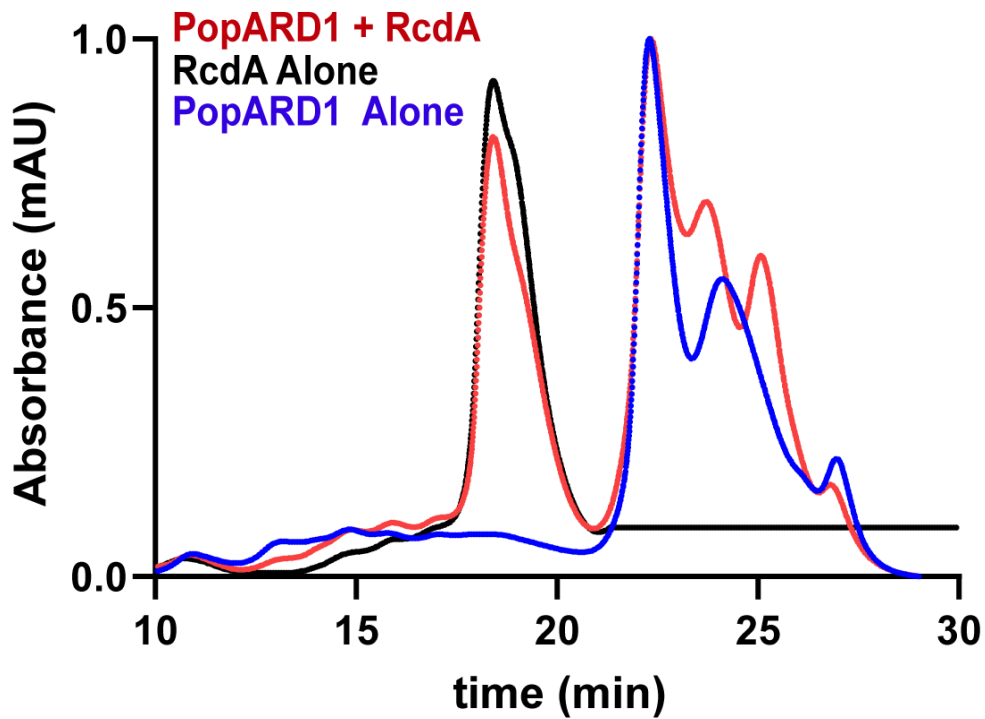


Figure 3.4: The First Receiver Domain is Insufficient to bind RcdA by SEC-MALS

SEC-MALS analysis of PopARD-1 (1-117) incubated with RcdA by SEC-MALS.

The first receiver domain is incapable of forming a higher-order complex with RcdA, as evidenced by the lack of a peak shift in the chromatogram. Surprisingly,

The PopARD1 could compete off GFP-TacA Degradation (Appendix A.14)

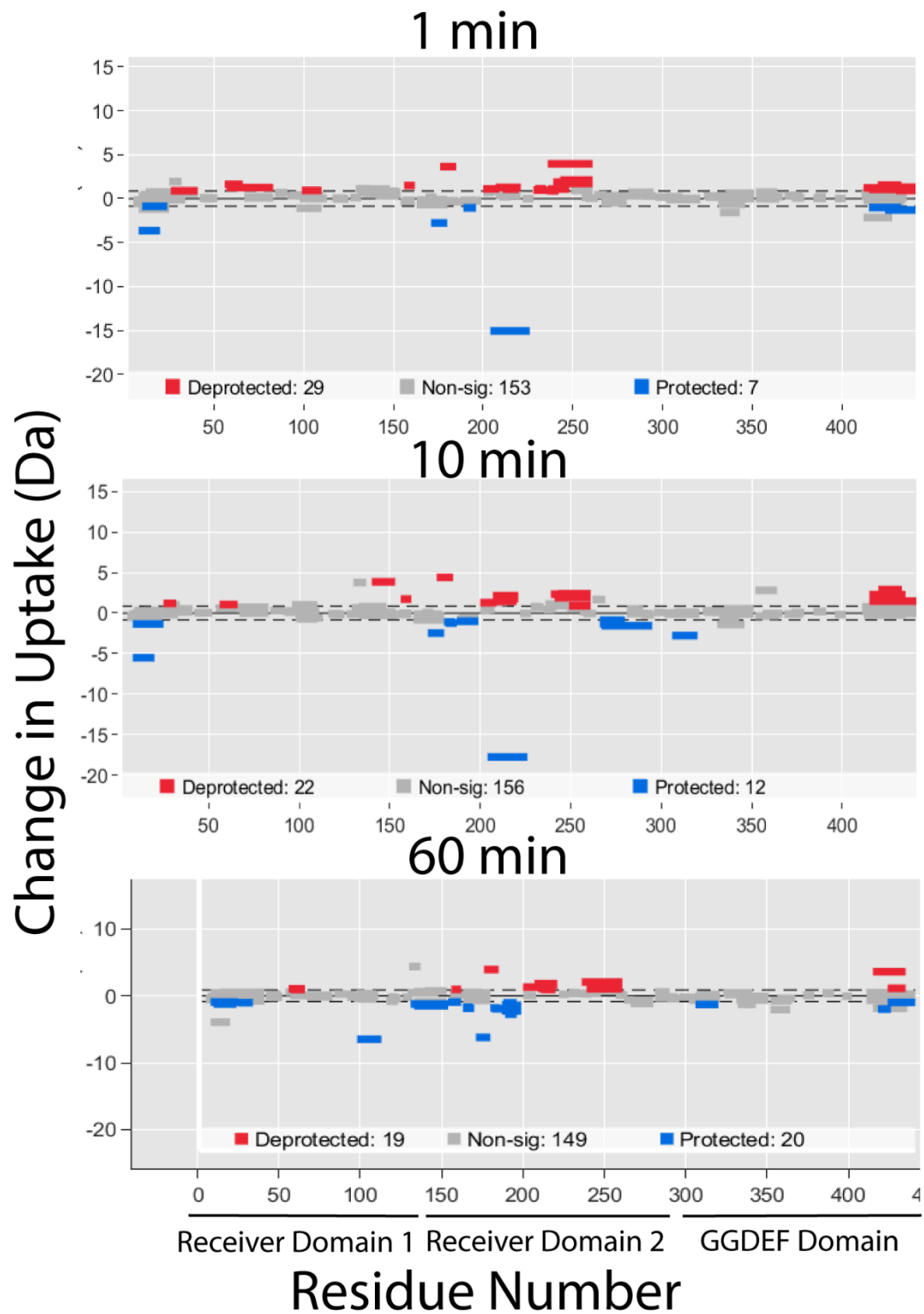


Figure 3.5: RcdA Interacts with the Second Receiver Domain of PopA when cdG is present

Hybrid statistical Woods's plot of filtered HDX peptides for PopA-RcdA-cdG compared to PopA-cdG. Peptides from the 1 minute, 10 minute and 60 minute timepoints are highlighted, with peptides showing lower statistical HDX change in blue, and higher statistical HDX change in red.

Nonsignificant changing peptides are in grey. A significance filter of $\alpha = 0.05$ was used. This experiment shows that the PopA-RcdA interaction surface does not dramatically change when PopA is bound to cdG.

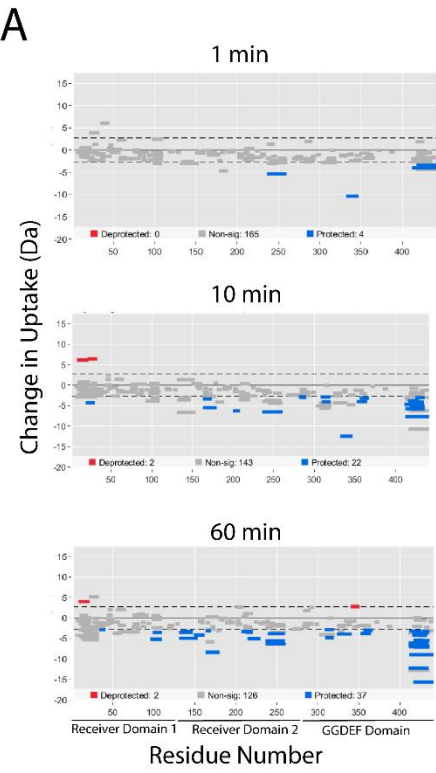
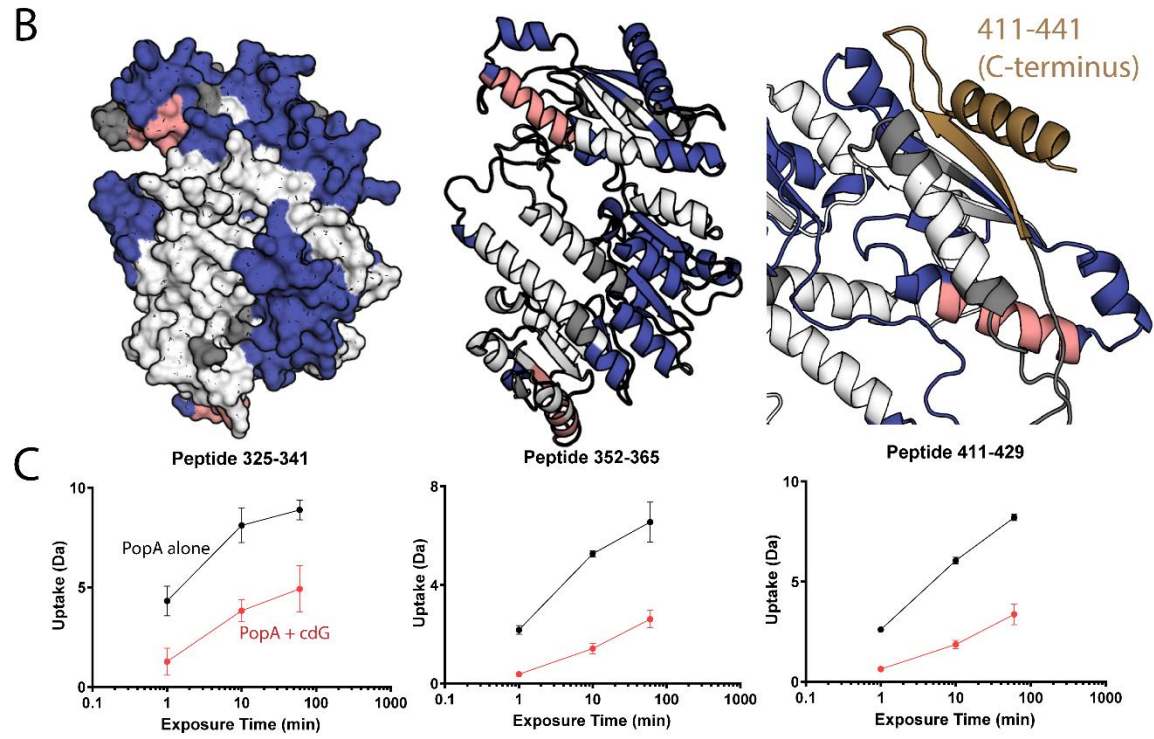


Figure 3.6: CdG Binding Causes Conformational Changes in PopA

A) Hybrid statistical Woods plot of filtered HDX peptides. Peptides from the 1 minute, 10 minute and 60 minute timepoints are highlighted, with peptides showing lower statistical HDX change in blue, and higher statistical HDX change in red. Nonsignificant changing peptides are in grey. **B)** Mapped HDX changes from the Woods plot onto a SWISS-MODEL modeled PopA structure based on the inactivated crystal structure of PleD (PDB code: 1W25) for the 60 minute timepoint. Highlighted surfaces, peptides and residues in each patch are shown in each graphical representation. The C-terminal patch that is highlight in the PopA-cdG HDX data is highlighted in gold. **C)** Selected peptides that are highly protected upon cdG binding from the Woods plot.

We first checked to see if residues in the primary I site of the GGDEF domain were protected in our dataset, as mutations in this site disrupt cdG binding and this was a good indication that PopA and cdG were binding in our experiments. Consistent with this data, we saw protection from deuterium in the primary I-site spanning over R357, the residue shown to be necessary for cdG binding (Figure 3.2A, Duerig et al., 2009). Even with the most stringent statistical cutoffs ($p = 0.001$) selective peptides in the I-site were protected from deuterium exchange. Next, we looked for any interfaces that might potentially form a dimer in the active modeled structure. Work from (Ozaki et al., 2014) identified the dimer interface forming between the rec1 and rec2 domains of PopA in this model, which was primarily formed by a salt bridge comprised of residues R118, E125 and E129. However, mutations in these residues did not affect CtrA delivery, which is dependent on PopA-cdG interactions (Ozaki et al., 2014). This suggests that the rec1-rec2 dimer interface model based on the PleD crystal structure does not contain any potential dimer interface of PopA. Consistent with this, these residues were not protected in any timepoint throughout the HDX-MS experiment (Figure 3.6A). There did not seem to be any interfaces formed in both the inactive and active state modeled structure of PopA that could lend itself to a dimer interface. At the concentrations used in our HDX experiments (5uM PopA and 40uM cdG), we wanted to see if our HDX data could be explained by changes in the PopA structure rather than dimerization. We tested to see if PopA could form dimers by analytical ultracentrifugation at concentrations of PopA and

cdG that fully stimulate CtrA degradation, which we found to be at its maximal degradation rate at 10uM of cdG (Figure 3.7)

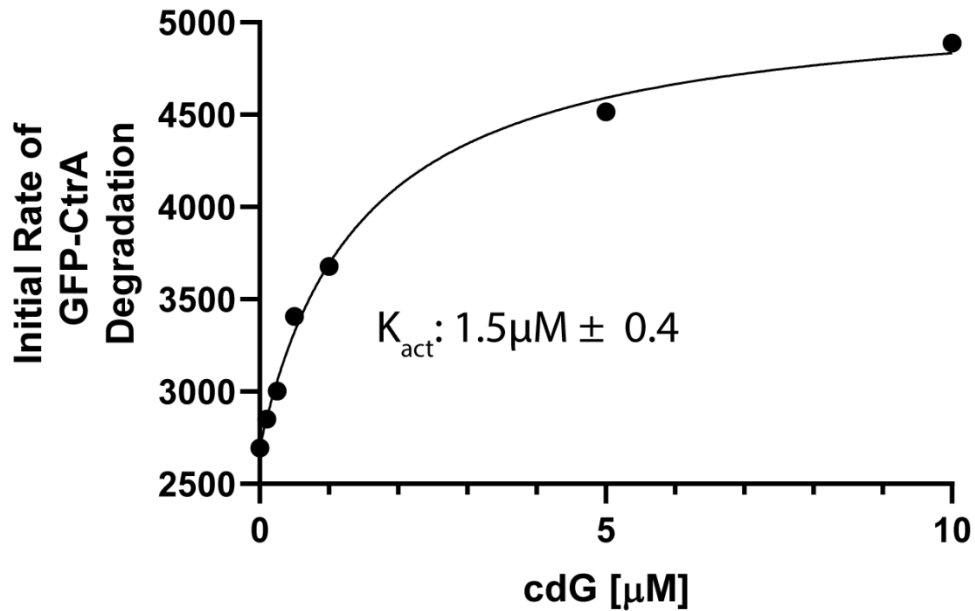


Figure 3.7: Activation curve of GFP-CtrA Degradation by cdG

$K_{activation}$ curve of GFP-CtrA degradation in the presence of varying concentrations of cdG and 5uM PopA, 3uM RcdA, 3uM CpdR, 0.2uM ClpX, 0.4uM ClpP and 1X ATP Regeneration mix. Data was fit to a modified version of the Michaelis-Menten equation to account for background degradation as noted in the methods.

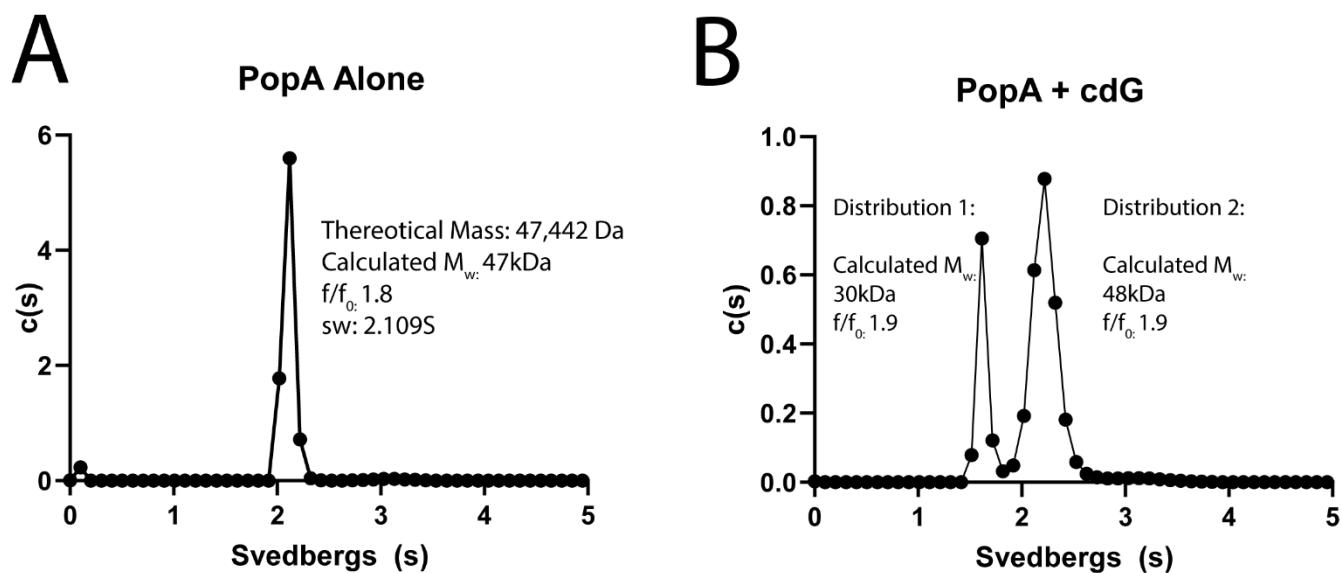


Figure 3.8: Sedimentation Velocity-AUC of PopA in the Presence of cdG

A) Sedimentation-Velocity AUC Distribution of 25 μ M PopA. The distribution statistics and fitted parameters are listed next to the graph. **B)** Sedimentation-Velocity AUC Distribution of 5 μ M PopA in the presence of 40 μ M cdG. The distribution statistics and fitted parameters are listed next to the graph.

Consistent with this, we did not see dimerization of PopA at fully stimulatory concentrations by AUC and no evidence of dimerization at high concentrations of PopA alone without cdG present (Figure 3.8). Surprisingly, the frictional ratio of PopA was 1.9, indicated an elongated conformation like that of an antibody. This ratio did not change upon cdG addition (Figure 3.8B). Therefore, the deuterium protection and CtrA binding in the presence of cdG is more likely explained by conformational changes in PopA upon cdG binding to promote ctrA binding. We wanted to see if CtrA affected the dimerization state of PopA upon binding, which we could not do by AUC due to limitations in the absorbance optics of the instrument. To do this, we used SEC-MALS to assess the oligomerization state of PopA upon CtrA binding. We ran both CtrA, PopA and RcdA on SEC-MALS with buffer that contained cdG. CtrA ran as a 27kDa monomer by SEC-MALS, while PopA ran as a 44kDa monomer. As predicted by previous experiments, the RcdA + PopA complex ran as a 59kDa complex (Figure 3.9A). We next ran PopA and CtrA together and found the complex to be approximately 70kDa, close to a predicted mass of a monomer of PopA bound to a monomer of CtrA (Figure 3.9B). We next tested to see if we could see the ternary complex being formed when we added RcdA to a PopA-CtrA reaction. The chromatography peak that formed did not scatter light and ran as a smear (Figure 3.9B), making it challenging to determine the stoichiometry of the ternary complex. Combined with the fact that the $K_{\text{activation}}$ constant for cdG-stimulated CtrA delivery is $\sim 1.5\mu\text{M}$ and PopA-CtrA form a 1:1 complex, we reason that cdG causes changes in the

PopA structure upon binding to accommodate CtrA interactions. We analyzed our HDX further to see if any specific domains or regions on PopA particularly changed upon cdG binding. The region with the most protected peptides was centered around residues 410-441, the end of the C-terminus of PopA (Figure 3.5B). One possible model is that proper orientation of the C-terminus of PopA is required for CtrA binding, explaining why this region is highly protected upon cdG binding. Taken together, our data favors a model where PopA dimerization by cdG is not necessary for CtrA delivery but rather cdG binding is required for priming PopA for CtrA interactions by conformational changes that provide a more compact structure or surface that promotes CtrA binding.

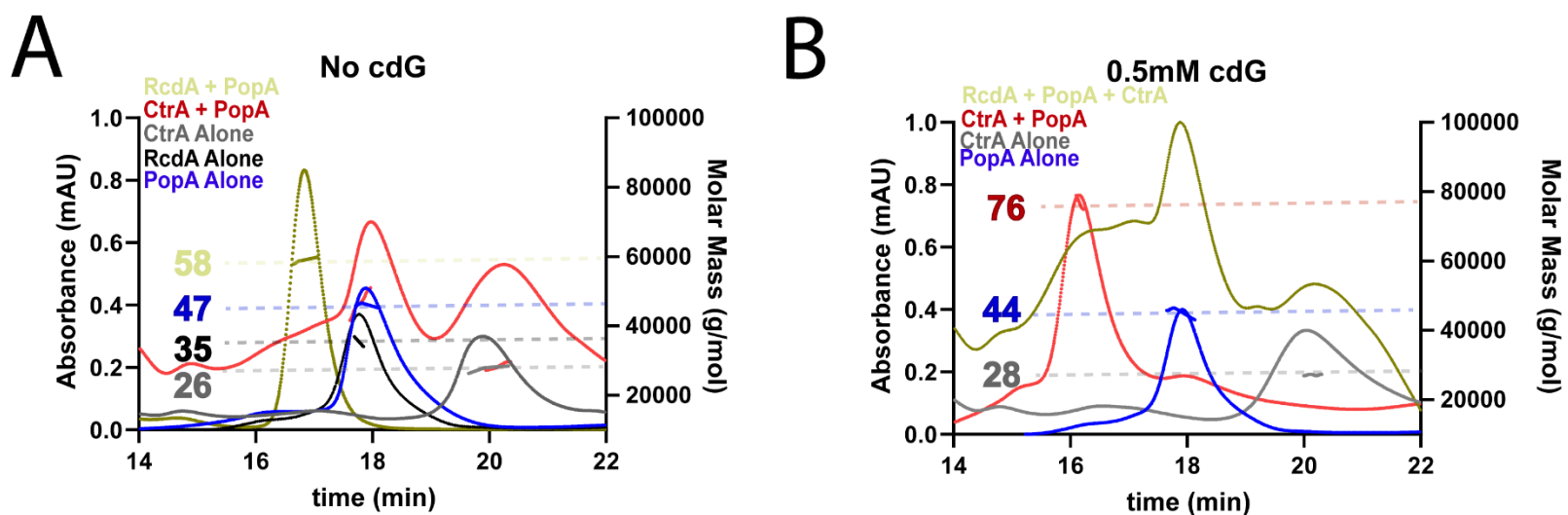


Figure 3.9: PopA and CtrA form a Monomer:Monomer Stoichiometry by SEC-MALS

A) SEC-MALS traces of RcdA, PopA, CtrA or different pairwise combinations of each protein in buffer not containing cdG. Each protein was run at 50uM alone or together. The dotted lines indicate the weight-averaged mass from the light scattering detector of each protein or protein complex. **B)** SEC-MALS traces of RcdA, PopA, CtrA or different pairwise combinations of each protein in buffer containing 0.5mM cdG that was freshly prepared. Each protein was run at 50uM alone or together. The dotted lines indicate the weight-averaged mass from the light scattering detector of each protein or protein complex.

CtrA interacts at a Nonoverlapping Surface on PopA

We concluded our HDX studies by comparing the PopA-cdG bound complex to a reaction consisting of PopA, cdG and RcdA and another reaction containing PopA, cdG, RcdA and CtrA. Our reasoning for this was that we could only compare the interactions of CtrA in the context of cdG and RcdA, as both are necessary for CtrA delivery and RcdA also makes direct interactions with PopA during this delivery process. By performing these comparisons in the same HDX experimental run, we can compare the peptide uptake and see which regions are protected in the presence of CtrA specifically after subtracting the contributions from cdG and RcdA. Most of the surface that was identified in the PopA-RcdA dataset did not change upon addition of CtrA, suggested that no further protection of this region occurs in the presence of CtrA as was seen for the PopARcdAcdG dataset (Figure 3.10A). However, there was some residues and patches that were shared with the RcdA dataset, such as regions 155-162 and 266-281 (Figure 3.3A, 3.8A). Interestingly, there was a surface that was primarily unique to the CtrA dataset, spanning from residues 255-295 and 323-341 (Figure 3.8A,B). We have shown peptides in the region showing strong protection through peptide uptake plots (Figure 3.8C). This region consists of the loop connecting the second receiver domain and the GGDEF domain and forms a surface ideal for binding (Figure 3.8B). This surface shares some overlap with the surface proposed in the RcdA dataset but does not grossly overlap with the RcdA surface, suggesting that CtrA and RcdA can both interact with PopA

simultaneously and provides supporting evidence for ternary complex formation and different surfaces being involved in the RcdA and CtrA interactions with PopA. Taken together, the peptides protected in the PopARcdAcdGctrA dataset are widely unique to the addition of CtrA compared to the PopARcdAcdG dataset. Considering both RcdA and CtrA appear to interact with a monomer of PopA and can form a ternary complex, it is logical for CtrA and RcdA to use nonoverlapping interfaces on PopA to facilitate ternary complex formation. We designed a fluorescence polarization-based competition assay to see if CtrA can compete off RcdA-PopA interactions using a constitutively monomeric variant of RcdA (RcdA L82E) labeled with Fluorescein-5-Maleimide (Kuhlmann et al., 2021). Upon incubation of L82E with PopA, we see a characteristic increase in polarization (Figure 3.11A). Adding an excess of unlabeled RcdA competes off L82E-PopA interactions by formation of the RcdA-PopA complex, causing the polarization to return to unbound levels. We tested for competition of L82E-PopA in the presence of cdG and CtrA and found that CtrA could not compete off L82E-PopA interactions by formation of the PopA-cdG-CtrA complex (Figure 3.11A). This data suggests that CtrA binding does not overlap with RcdA-PopA interactions when it is bound to monomers of PopA.

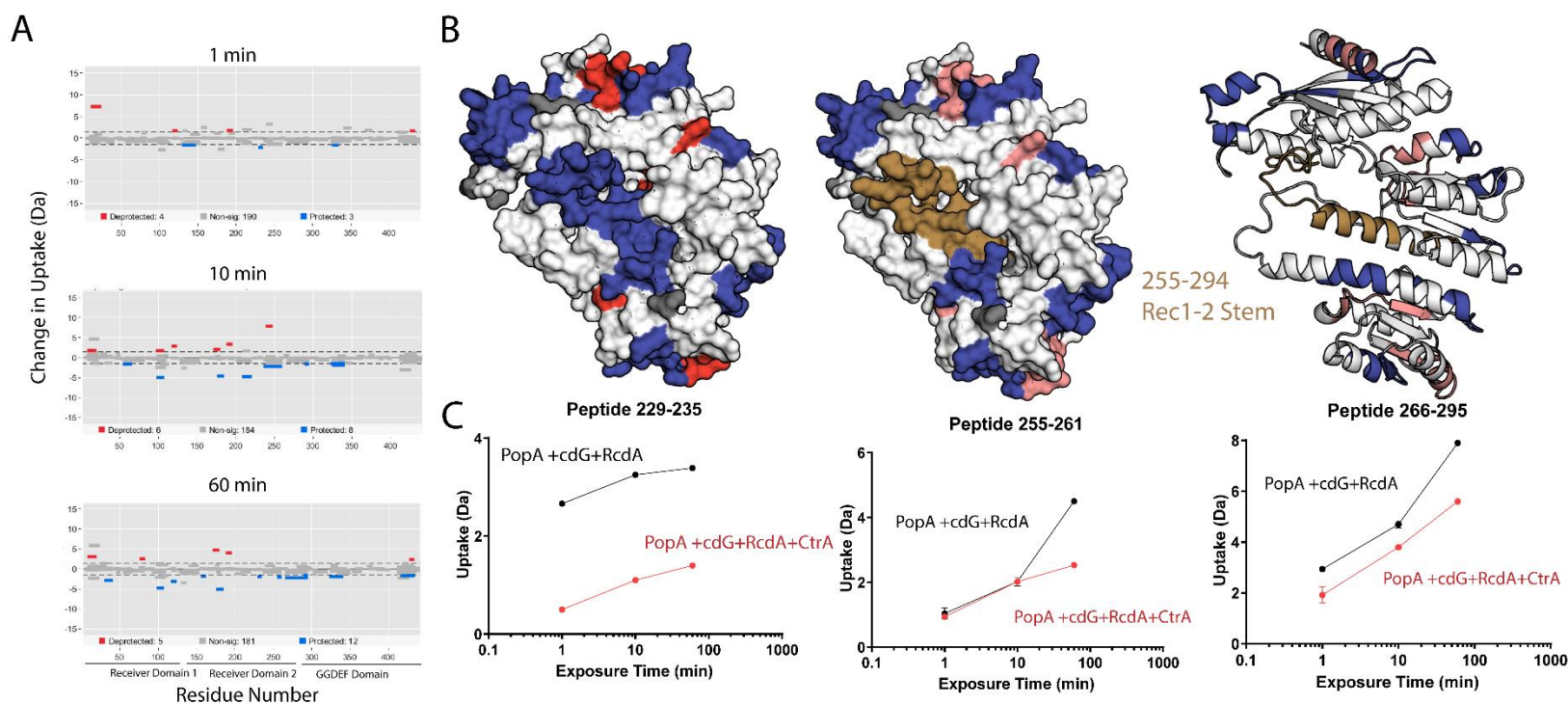


Figure 3.10: HDX Data from PopA-RcdA-CtrA Ternary Complex

B) Hybrid statistical Woods plot of filtered HDX peptides. Peptides from each timepoint are shown, with peptides showing lower statistical HDX change in blue, and higher statistical HDX change in red. Nonsignificant changing peptides are in grey. This data is the PopA-RcdA-CtrA-cdG reaction subtracted from the PopA-RcdA-cdG reaction.

B) Mapped HDX changes from the Woods plot onto a SWISS-MODEL modeled PopA structure based on the inactivated crystal structure of PleD (PDB code: 1w25). Highlighted in gold is the Rec1-2 stem that could form a potential surface for CtrA binding. **C)** Selected peptides that are highly protected upon CtrA binding from the Woods plot.

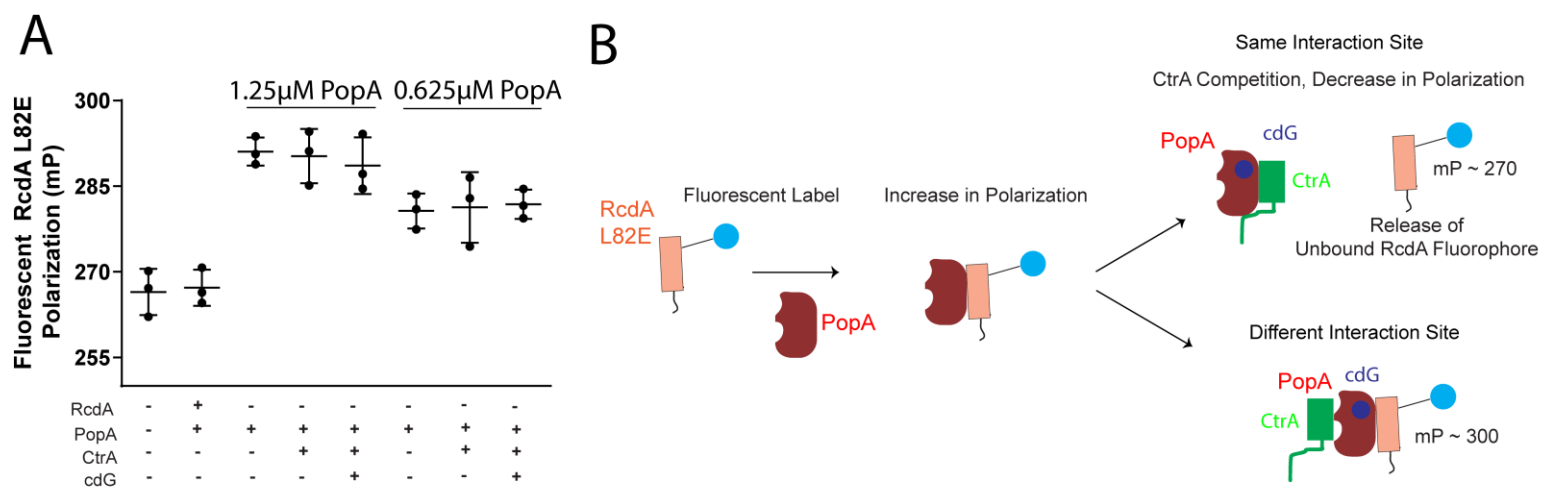


Figure 3.11: Fluorescence Polarization CtrA Competition Assay

A) Fluorescence polarization CtrA competition experiment. Fluorescently labeled RcdA L82E was incubated with 20uM PopA, RcdA, cdG or CtrA or in varying combinations. The polarization values were then read. **B)** Cartoon illustrated the workflow of the fluorescence polarization experiment with the different experimental outcomes. Competition of CtrA leads to a release of the unbound RcdA fluorophore, causing the polarization to return to unbound levels. Interaction of CtrA at a different interaction site will not cause the polarization to return to unbound levels.

Taken together, our data suggests a model where the delivery of CtrA is regulated by the formation of a ternary complex of RcdA, cdG, PopA and CtrA (Figure 3.12). The interaction between PopA and CtrA is gated by cdG binding, which initiates conformational changes that allow PopA to accommodate CtrA binding to monomers of PopA. RcdA also binds monomers of PopA, primarily through interactions with the second receiver domain of PopA. We propose that CtrA and RcdA interact on nonoverlapping surfaces as shown by HDX and fluorescence polarization studies and formation of this complex is necessary for CtrA delivery.

Different, Nonoverlapping Interfaces

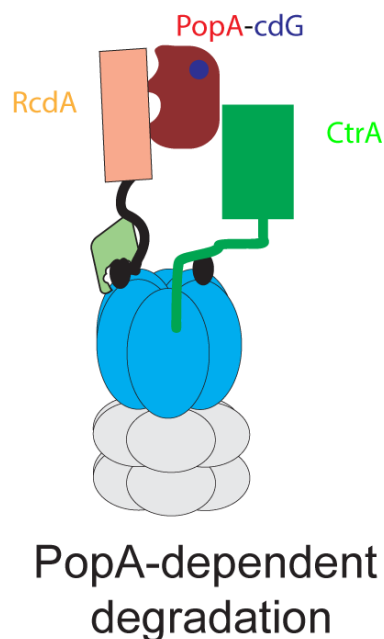


Figure 3.12: Model of PopA-CtrA-RcdA Complex and Delivery Mechanism

As a monomer, PopA is capable of binding RcdA, cyclic-di-GMP, and CtrA simultaneously. RcdA-PopA interactions primarily take place at the 2nd receiver domain, while CtrA interacts with PopA on a different interface. Proper CtrA delivery is dependent on cdG binding to PopA, which induces conformational changes that allow for CtrA binding. The formation of this ternary complex is needed for proper CtrA degradation by the ClpXP protease.

3.4 Discussion

Adaptor mediated degradation is critical for bacteria for coordinating stress responses and cellular transitions. Because of CtrA critical importance in regulating the bacterial cell cycle, understanding the mechanisms of binding and complex formation will advance our understanding of CtrA's role in alphaproteobacteria, such as the pathogen *Brucella abortus* (Francis et al., 2017). Our HDX and biochemical studies reveal how PopA accommodates RcdA and CtrA interactions and how cdG gates PopA-CtrA interactions. RcdA and CtrA do not appear to interact on similar interfaces on the PopA structure.

PopA is a unique protease adaptor in *Caulobacter* in that it is not degraded like other adaptors such as CpdR, RcdA and SspB (Joshi et al., 2017), is regulated by the binding of a small molecule to gate its CtrA delivery function and is limited to only a small subset of alphaproteobacteria closed related to *Caulobacter* (Brilli et al., 2010). Interestingly, CpdR, RcdA and CtrA are present in *Brucella abortus*, while PopA is not present (Francis et al., 2017). How *Brucella abortus* regulates CtrA post-translationally through degradation remains an outstanding question

and may be controlled by an adaptor like PopA that fulfills its function through RcdA interactions. In that case, the fold of PopA, which contains receiver domains and a cdG-binding site, contains specific elements that allow for protein-protein interactions through the receiver domains and regulation of activity by cdG binding. The properties, fold, and functional characteristics of PopA serve as a model for how unknown adaptors may work in *Brucella abortus* and other bacteria that contain the cpdR-RcdA-ctrA pathway. PopA's evolution from PleD to fulfill the specific role of delivering cargo in an adaptor-dependent manner is remarkable. The conversion of PleD, a cdG produced enzyme, to an adaptor that has no catalytic activity towards cdG highlights the importance of having PopA as an adaptor that adopts the general fold of a Diguanylate Cyclase yet lacks DGC activity. Why does PopA need to be able to bind cdG to fulfill its function? Published literature about DGC's show that cdG binding shifts the conformation from an inactive state to an active state and this promotes dimerization of the enzyme. However, for PopA, this dimerization is very weak and it does not need to dimerize to promote CtrA binding and delivery. Could there be a role for PopA dimerization in delivering other PopA-dependent cargo? Recent literature suggests that there could be a link between PopA dimerization and the protein SmrF (Wang et al., 2021). One potential model is that PopA adopts both monomeric and dimeric states to accommodate the delivery of different cargo, dependent on the local cdG concentration. More work needs to be done to clarify

the link between cdG and PopA dimerization and how cdG tightly regulates PopA-dependent activity, regardless of the oligomerization state it is in.

3.13 Materials and Experimental Procedures

Protein Expression and Purification. BL21(DE3) *pLYS* or X90 cells with expression plasmids for different proteins were grown at 37°C to an OD₆₀₀ of 0.4-0.6, then induced with 0.4mM IPTG for 3-4 h. Induced cells were then centrifuged at 7,000g for 10 minutes and resuspended in buffer containing 50mM Tris pH 8.0, 300mM NaCl, 10mM imidazole, 10% glycerol, 5mM β-mercaptoethanol and 100mM PMSF and frozen at -80°C until further use. Cells were lysed using a Microfluidizer system (Microfluidics, Newton, MA). The clarified lysate was bound over a Ni-NTA column for affinity purification. H₆SUMO-tagged proteins were cleaved by Ulp1-his protease (36). Proteins were then purified using size exclusion and anion-exchange chromatography using Sephacryl 200 16/60 and MonoQ 5/50 columns. ClpX and ClpP were purified as outlined in (9). Detailed purification protocols are available upon request.

Cloning and Molecular Biology. PopA variants and truncations were cloned using around-the-horn cloning by amplifying the appropriate domains using pET23SUMO-PopA as a template.

Fluorescence Polarization and Maleimide Labeling. Purified RcdA or RcdA mutants were labeled with Fluorescein-5-Maleimide (Thermo Scientific™). Purified protein at ~8-10 mg/mL was buffer exchanged into labeling buffer (50 mM Tris pH

7.0, 150mM NaCL, 2mM TCEP). Fluorescein-5-Maleimide was dissolved in DMSO and added to protein at a 20-fold molar excess to cysteine. Labeling reactions were completed at 4°C overnight. Free dye was removed using a PD-MidiTrap column (GE Healthcare) and Amicon Ultra-15 Centrifugal Filter Units in a buffer containing 20mM HEPES pH 8.5, 100mM KCL, 10mM MgCl₂ and 0.05% Tween. Confirmation of protein labeling was verified using a Typhoon imaging system (GE Healthcare). The labeled protein was aliquoted, and flash frozen at -80°C.

Fluorescence polarization binding experiments were performed with 100 nM labeled RcdA or RcdA L82E and varying concentrations of PopA, CtrA or cdG. The binding reaction was incubated at 25°C for 1hr to reach equilibrium. Polarization measurements were read from 40uL of these mixtures using opaque black 384-well plates using a SpectraMax M5 plate reader (Molecular Devices) with excitation and emission wavelengths set at 460 and 540, respectively. Equilibrium binding constants were calculated by fitting the polarization data using GraphPad Prism to a one site, total and nonspecific binding equation $P = P_{max} * [X] / ([X] + K_d)$ where P_{max} is the maximum specific binding value, P is the polarization value, NS is the slope of linear nonspecific binding constrained to be greater than 0, and the background is the polarization value when $[X]$ is 0. The NS term usually fits to 0 and is not included in the final fit. Error bars are calculated from the standard deviation between replicates of experiments. All RcdA concentrations are in terms of monomeric equivalents unless otherwise noted.

Size-Exclusion Chromatography with Multi-Angle Light Scattering. Each protein complex was allowed to bind at room temperature for 45 minutes. The complexes were then injected onto a TSKgel™ G3000 SEC column equilibrated in 20mM HEPES buffer, pH 7.0 with 100mM KCL and 10% glycerol (Tosoh Biosciences) at room temperature. The SEC column was coupled to an 18-angle light scattering detector (DAWN HELEOS-II) and a refractive index detector (Optilab T-rEX) (Wyatt Technology). Data was collected every second and the flow rate was set to 0.5 mL/min. Data analysis was carried out using the program ASTRA (Wyatt Technology). Monomeric bovine serum albumin (BSA; Sigma) was used for calibration of the light scattering detectors and general data quality control. Measurements were taken at 25°C. The light scattering data was collected across a window containing the entire chromatographic peak for a full distribution of molecular weights, shown underneath each chromatogram.

Hydrogen-Deuterium Exchange Mass Spectrometry. Hydrogen-deuterium exchange was measured on a Synapt G2Si high definition mass spectrometer (Waters). Deuterium exchange and quenching steps were performed using an automated HDX robotics platform (Waters). Samples were diluted 1:16 in D₂O-containing buffer containing 0.1 mM K₂HPO₄ to final concentrations as specified in the figure legends. Deuterium exchange was allowed to take place for 0, 1, 10 and 60 minutes at 25°C, with staggered starts for each dilution reaction. After all reactions were completed, aliquots were removed and diluted 1:2 into cold quench buffer at 4°C (water with 4M Guanidine Hydrochloride at pH 2.5) and

subsequentially run over an immobilized Water ENZYMA^{TE} immobilized pepsin column (ID: 2.1 length: 30mm) at a flow rate of 0.15mL/min at high pressure (~11000 psi) for peptide digestion. Prior to HDX analysis, the quality of each sample was assessed using SDS-PAGE and size-exclusion chromatography. The output peptides were filtered using DynamX using the following filtering parameters: minimum intensity of 1000, minimum and maximum peptide sequence length of 5 and 30, respectively, minimum MS/MS products of 2, minimum products per amino acid of 0.25 (this can be further adjusted to prune peptides), minimum score of 5, and a maximum MH⁺ error threshold of 15 p.p.m. After analysis in DynamX, the data was exported as a cluster file for further analysis using Deuterios. For statistical analysis, filtering of peptide uptake plot data was performed in Deuterios (Hageman and Weis, 2019, Lau et al., 2020) using a Hybrid statistical model for the woods plot with $\alpha = 0.02$ for PopA-cdG HDX and $\alpha = 0.05$ for all other experimental datasets. Mapping of Deuterios filtered data was performed using a pymol script exported from the Deuterios woods plot and mapped onto a modeled structure of PopA based on the inactive structure of PlcD (Two independently prepared experimental replicates and labeling reactions were performed for each condition and averaged in the peptide uptake plots. Blank runs were run in between each analysis to avoid peptide carry-over. Continuous lock-mass correction was performed using leu-enkephalin compound. Timepoints and analysis were randomized to ensure no biasing of results and to ensure variation. Peptides were ionized and separated by electrospray ionization for analysis in

MSE mode at a mass resolution of 50-2000 m/z range. Identification of peptides and analysis of the uptake plots and charge states for each peptide were completed in Protein Lynx Global Server (PLGS) and the software DynamX (Waters). Uptake plots were plotted and created in Adobe Illustrator and in GraphPad Prism.

***In vitro* Degradation Assays.** Degradation of GFP-CtrARD+15 was monitored with the loss of fluorescence over time as described previously (Smith et al., 2014). The concentrations of each protein used in the reactions were as follows unless otherwise noted: 3 μ M RcdA, 3 μ M RcdA(L82E), 2 μ M CpdR, 0.2 μ M ClpX₆, 0.4 μ M ClpP₁₄, 2 μ M GFP-CtrARD+15, 5mM ATP. GFP-CtrARD+15 with cdG curves were fit to a modified hyperbolic equation with the form of: $Y = ((V_{max} * [RcdA]) / (K_{act} + [RcdA])) + A$, where A is a baseline constant, or to the Michaelis-Menten equation using GraphPad Prism.

Sedimentation Velocity Analytical Ultracentrifugation. Sedimentation Velocity experiments were completed using a Beckman ProteomeLab XL-1 Analytical Ultracentrifuge (Beckman). Samples were diluted into 20 mM HEPES pH 8.5, 100mM KCl, 10% glycerol at the concentrations indicated in the figure legends. The samples were spun at 55,000 g overnight at 25°C. The p and v values used for data fitting were determined using SEDNTERP and the amino acid sequence for each protein. The sedimentation velocity data was directly fit to the $c(s)$ distribution method using the program SEDFIT and using the first 100 velocity

scans for each condition. The resulting distributions from each experiment were then plotted in GraphPad Prism.

Ni-NTA Nickel Pulldown Assays. Nickel pulldown assays were performed using a Ni-NTA resin (Thermo Scientific). 50 μ L of Ni-NTA resin was washed and equilibrated with 200 μ L wash buffer (20mM Hepes pH 8.5, 100mM KCL, 10% glycerol, 30mM Imidazole). Each protein or protein mixture was combined at 200 μ L final volume and added to resin to incubate at 4°C for 2 hours. The Ni-NTA resin was spun down at 700xg for 2 minutes and the flowthrough samples were collected. 200 μ L of wash buffer was then added and a 700xg spin was performed again with a wash sample taken after the spin. Finally, 200 μ L of elution buffer (20mM Hepes pH 8.5, 100mM KCL, 10% glycerol, 300mM Imidazole) was added to the resin and spun down with the collection of an elution sample taken after the spin. Each sample was then run on SDS-PAGE gels for analysis.

Bacterial Strains Used in This Study:

Organism	Name	Description	Source
<i>E. coli</i>	TOP10	Cloning strain	Invitrogen
	BL21(DE3) pLysS	recombinant protein expression	Invitrogen
	X90 T7	recombinant protein expression	From Bob Sauer
	EPC100	dh5alpha pQE70- his-ClpP	(Chien et al., 2007)
	EPC112	BL21DE3 pLysS pET23 Ulp1his protease	This study
	EPC162	BL21DE3 375 eGFP-His6- CtrARD+15	(Smith et al., 2014)
	BPC238	BL21DE3 plysS pET23 ClpX	(Chien et al., 2007)
	EPC196	BL21DE3 plysS pET23b His6Sumo-CpdR	(Lau et al., 2015)
	EPC970	BL21DE3 plysS pET23b His6Sumo-RcdA	(Joshi et al., 2015)
	EPC1000	TOP10 pET23b His6Sumo-RcdA	(Joshi et al., 2015)
	EPC1037	BL21DE3 plysS pET23b His6Sumo-PopA	(Joshi et al., 2015)
	EPC1517	BL21DE3 plysS pET23b His6Sumo-RcdA L82E	Kuhlmann et al., 2021
	EPC520	Pet28-His ₆ CtrA	This Study

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Chapter 4

Future Perspectives and Impact of Thesis on the Adaptor Field

4.1 Overview

In this chapter I will explore future directions that I think are worth exploring for the field of protease adaptors. The fundamental, mechanistic work on RcdA and PopA function that my thesis is focused on will allow for further dissection on the details of adaptor-mediated proteolysis in *Caulobacter* and other bacteria. The mechanistic work that my thesis is focused on will provide tools to solve further research questions about the ClpXP adaptor hierarchy. I will discuss the lessons and perspectives about adaptors and adaptor-mediated protein degradation that I have gathered throughout my Ph.D. and its impact to the adaptor field. Finally, I will discuss future avenues of research that are of particular interest to me and will further our basic understanding of adaptor-dependent proteolytic pathways.

4.2 Impact of Thesis Work on Protease Adaptor Field

My thesis built on the work and the fundamental observation that adaptors can work synergistically to provide additional layers of regulation to protein degradation during critical cellular stages of growth and development (Joshi et al., 2015). Furthermore, my thesis work expanded on the initial observation that when an adaptor protein is not actively performing its delivery function, it is actively degraded by the protease (Joshi et al., 2017). However, these observations did not fully explain how each of the adaptors serve their regulatory role and why adaptor degradation matters *in vivo*. The composite work of my mechanistic studies of RcdA and PopA demonstrates that in addition to the basic function of an adaptor choosing specific substrates by acting as a scaffold, cell-cycle specific adaptors such as RcdA and PopA possess additional layers of regulation that are needed to tune the activity levels of each adaptor during the cell-cycle, and in the case of RcdA, this additional layer of regulation also controls its proteolytic stability. RcdA's regulation of activity and degradation is linked through dimerization by acting as an inhibitory role against prolific RcdA activity. The work presented in Chapter 2 was the first demonstration of adaptor dimerization having a direct consequence on its stability and this stabilization having a physiological consequence, as adaptor degradation has been observed in other bacteria (Puri et al., 2017, Turgay et al., 1998) but not elaborated upon. This piece of information can perhaps lend itself to future studies of adaptor stability and its contribution to cell viability in other organisms by looking for

determinants of adaptor degradation, modifying those determinants, and looking for changes in cell viability or physiology. Perhaps even more fascinating concerning adaptor degradation in *Caulobacter crescentus* is that PopA does not get degraded, while CpdR and RcdA are degraded robustly (Joshi et al., 2017). What properties about PopA prevent its self-degradation that CpdR and RcdA do not have, and why is PopA not found in every organism that contains CtrA, such as *Brucella abortus*? A possible explanation is that the constitutive presence of PopA is necessary for proper timing of the delivery of CtrA during the cell-cycle, while for CpdR and RcdA and their respective substrates this is not a requirement. Further studies elaborating on the specifics of CpdR degradation and consequentially their effects on cell viability and substrate delivery would help clarify the overall significance of adaptor degradation not only in *Caulobacter*, but in all organisms that use adaptors as protease regulators. The specific studies needed for RcdA characterization might not generalize to other adaptors and for me, personally, this is an aspect of adaptors that should be explored further in the field to really understand the significance of adaptors in maintaining cell physiology. These types of studies would lend themselves well in organisms such as *Caulobacter* that have distinct developmental transitions or stress responses that are aided by adaptor-mediated protein degradation. The finding that RcdA reserves different aspects of its dimerization interface for different cargo provides further understanding for how an adaptor that has a pool of cargo to deliver can accommodate different classes of substrates. By sharing

a common interface, specificity is achieved by tailoring different aspects of the interface to different classes of cargo. The RcdA pulldown data (Joshi et al., 2015) suggested a large range of otherwise uncharacterized RcdA interacting partners. It is possible that RcdA represents a general adaptor specifically evolved to fulfill the role of delivering dozens of substrates during the cell-cycle that have no shared similarities, functions, or structures. In this case, the experimental approaches outlined in Chapter 2 would help identify the overlap between different classes of cargo: some cargo might interact at the SpbR/TacA site, while others might interact at the PopA site, including as-of-yet to be discovered adaptor proteins. Using mutants such as the RcdA L82E or RcdA 3E could be used to filter out false positives. For organisms that undergo specific developmental transitions that are accompanied by coordinated protein degradation by adaptors, having a general adaptor that can degrade several classes of cargo such as RcdA allows the cell to preserve energy by not requiring many different adaptors to be synthesized. Other bacteria that undergo morphological differentiation that is dependent on protein degradation might make use of an adaptor that contains a large interface that is tailored to accommodate different varieties of cargo. In contrast, bacteria that do not undergo distinct morphological transitions during their life cycle, such as *E. coli*, use adaptors such as SspB and RssB have a limited range of cargo in comparison to RcdA. The use of a smaller, more specific interaction site fits the purpose of those adaptors more appropriately.

PopA represents a different adaptor than RcdA in that its delivery activity is regulated by a small molecule, rather than oligomerization. It was hinted that PopA dimerization might be important *in vivo* (Wang et al., 2021). However, the data presented in Chapter 3 suggests that binding of cdG to PopA allows for conformational changes in PopA to accommodate CtrA binding and delivery independent of PopA dimerization, as concentrations of PopA and cdG that promote full delivery and binding of CtrA do not induce dimerization. Binding of cdG accomplishes the same purpose of the dimer interface of RcdA: to gate CtrA delivery activity of PopA only when it is necessary. For the adaptors in the ClpXP adaptor hierarchy, an additional layer of regulation for each adaptor is present, whether it is phosphorylation of CpdR, dimerization of RcdA, or cdG binding to PopA. Each adaptor in the hierarchy is mechanistically and structurally diverse from each other, yet a need exists for gated activity because of the role each adaptor plays in the *Caulobacter* cell cycle. One interesting fact is that PopA does not exist in other bacteria and is only present in closely related alphaproteobacteria to *Caulobacter* (Brilli et al., 2010). However, alphaproteobacteria such as *Sinorhizobium meliloti* and *Brucella abortus* contain the CtrA gene network (including CpdR and RcdA) yet do not possess PopA. In the case of *Sinorhizobium meliloti*, the proteolysis of CtrA is essential for proper cell morphology and this proteolysis is dependent on CpdR and RcdA and perhaps an unknown adaptor that fulfills the role of PopA (Pini, et al 2015). My basic studies on how PopA is regulated and how PopA interacts with RcdA and

CtrA will hopefully develop a template for how an unknown adaptor that is necessary for CtrA degradation might function in other organisms. This would potentially lend itself well to a screen for RcdA and CtrA binders, as the interaction surfaces and specific folds PopA adopts might be similarly evolved in a different protein to serve the same function for CtrA degradation.

My thesis provides insight into how adaptor activities can be gated by additional layers of regulation, depending on the need for the adaptor in the cell. My specific studies of PopA and RcdA identify key characteristics of each adaptor that are critical to its function and provide a framework for how the basic interactions and determinants between RcdA and PopA might function in other organisms. The future of the adaptor field will likely be influenced by the development of genetic and proteomic screens to reliably identify new adaptor-substrate pairs that regulate critical stress responses and developmental transitions in pathogenic bacteria to identify which adaptors could be targeted as avenues for antibiotic drug discovery. The remainder of this chapter will discuss future avenues of adaptor research that are of particular interest to me.

4.3 CryoEM characterization of the ClpXP Adaptor Hierarchy

Recent Cryo-EM structures of the ClpXP machinery have uncovered critical information regarding the fundamental mechanism of ClpXP activation and substrate interactions (Gatsogiannis et al., 2019, Fei et al., 2020, Bell et al., 2020). However, these CryoEM studies were limited to variants of ClpX lacking the N-terminal domain, the part of ClpX that is necessary for CpdR interactions and adaptor-mediated protein degradation (Lau et al., 2015). Therefore, mechanistic insight into how the adaptor hierarchy structural forms and performs its function is sorely lacking as of now. During my Ph.D., I was part of a 3-year collaboration with Gabe Lander's lab to solve CryoEM structures of the adaptor hierarchy. Unfortunately, we have not been able to solve the CryoEM structure of any of portion of the adaptor hierarchy that involves ClpX with its N-terminal domain intact and in the presence of other adaptors. While we made progress in forming stable adaptor-protease complexes suitable for CryoEM analysis, more work is necessary to produce enough of these ternary complexes for CryoEM. Even a CryoEM structure of native ClpXP bound to CpdR would inform a great deal on the composite interface that is hypothesized to be necessary for CpdR-mediated degradation (Lau et al., 2015). Furthermore, a native CryoEM structure of ClpXP with adaptors bound will help explain how any potential avidity effects help aid in degradation. For the case of RcdA, my thesis work and the previous work on RcdA completed by Dr. Kamal Joshi describe the mechanism of RcdA

degradation in detail, but do not fully explain why all these adaptors are necessary for recruiting their substrates for ClpX recognition and degradation. An atomic snapshot of ClpXP with CpdR, RcdA and their respective cargo would elucidate how the adaptor hierarchy accomplishes this handoff and the interactions that facilitate this sequence of events. The primary challenge is obtaining stable, highly concentrated Adaptor-bound complexes needed for CryoEM. Chemical crosslinking and using a small SEC column that prevents dilution, along with more optimized purification protocols for ClpX to increase yield, are necessary to move this project forward. The concentration of is the limiting factor in getting high quality samples, as ClpP, CpdR, and RcdA are all very soluble at high concentrations, while concentration of ClpX results in drastic loss of protein in a spin concentrator. Using different expression systems and purification schemes to increase ClpX yield would speed up preparing highly soluble, concentrated samples necessary for CryoEM. The value of having such a CryoEM structure would answer questions about how the delivery mechanism of an adaptor-mediated substrate, such as PdeA, TacA or SpbR, is determined by the collective interactions between adaptors and protease.

4.4 Structural characterization of RcdA-Cargo Interactions

My HDX-MS studies highlighted two surfaces that, by mutagenesis, were mutually exclusive for direct RcdA cargo and other adaptors like PopA. However, due to the limited resolution of HDX, we cannot assign atomic-level information on the RcdA-cargo interaction surfaces. To fully understand how cargo binding

causes RcdA to dissociate into monomers, a crystal structure of RcdA bound to cargo is needed. In the past, we have tried to crystallize RcdA bound to SpbR and TacA as a co-crystal complex, but that came with its own issues. The RcdA homodimer appears to crystallize more readily than the RcdA-cargo complex, and the binding affinities of dimerization and cargo binding are similar, making crystallization of native RcdA-cargo complexes challenging. Because the L82E variant does not dimerize and can bind PopA with affinities like native RcdA, a viable approach would be to solve a structure of L82E bound to PopA or bound to the receiver domains responsible for interacting with RcdA. Furthermore, NMR structures of the L82E variant bound to the receiver domains of PopA might be a viable approach for mapping RcdA-cargo interactions at the atomic level.

4.5 Mechanistic Characterization of CpdR Function

CpdR is responsible for the regulated degradation of all known cell-cycle dependent ClpXP substrates (Lau et al., 2015). The biological consequences of mis regulation of CpdR function are distinct, causing motility defects and loss of ClpX localization to the cell poles (Lau et al., 2015). While it seems clear that CpdR must prime ClpX for degradation of its substrates, mechanistic characterization of these interactions and how this specificity is achieved remains unclear. Several remaining questions and obvious experiments could possibly shed insight into this question. It seems clear that the interaction of CpdR and ClpX is necessary for degrading CpdR-dependent substrates. How does this interaction happen? HDX-MS studies of the CpdR bound to ClpX and substrate,

using purified proteins or a linked complex with native ClpX or just the NTD, would help explain this observation. Furthermore, NMR studies of CpdR bound to the NTD would help identify the interface that CpdR binds to. Mutagenesis of residues that are identified in these datasets would help confirm these interfaces. Finally, limited proteolysis of a linked CpdR-^{NTD}ClpX or even a linked RcdA-CpdR-^{NTD}ClpX complex could also be an approach to identify regions that are more resistant to proteolysis, suggesting protein-protein interaction surfaces. ITC or Fluorescence Polarization studies of CpdR-^{NTD}ClpX would help aid in these studies to determine binding affinities and stoichiometries. CpdR could also be negatively regulated by dimerization through an active, unphosphorylated conformation. In this model, phosphorylation of CpdR leads to dimerization and inhibition of CpdR activity. Interestingly, The D9G mutant appears to form less higher-molecular weight aggregates during SEC purification (Christine Battle, unpublished data), suggesting that this variant may have differences in oligomerization. SEC-MALS or AUC using the D9G variant, a nonphosphorylatable CpdR variant (CpdR D51A) or using native CpdR in the presence of the phosphorelay system might help elucidate this mechanism of activation by dimerization.

4.6 Identification of New Adaptor-Dependent Substrates and Pathways

Discovery of new adaptors and adaptor-substrates pairs is a circular problem. We identify adaptor function by showing enhanced degradation of a substrate, but that approach only works if we know the substrate beforehand. In the case of

TacA and PdeA, prior knowledge from a ClpP trapping experiment identified these proteins as ClpXP substrates prior to our labs discoveries as CpdR and RcdA as their respective adaptors (Bhat et al., 2013). Biochemical reconstitution of these adaptor-substrate pairs confirmed the enhancement of PdeA and TacA degradation (Lau et al., 2015, Joshi et al., 2015). However, more systematic approaches for identifying new adaptor-substrate pairs are needed. Our labs recent optimization of systems biology approaches such as Transposon Sequencing (Tn-seq) and Tandem Mass Tag Proteomics (TMT) will provide a roadmap to developing methods for identifying new adaptor-substrate pairs and adaptor-dependent pathways. For example, particular adaptor variants that change adaptor function (such as RcdA L82E, RcdA 3E, CpdR H106A, CpdR R69A, PopA R357G) all contain specific changes in their activity that result in specific changes in substrate delivery and morphology. Along with adaptor deficient strains ($\Delta cpdR$, $\Delta rcdA$, $\Delta popA$), making strains containing these mutations would allow for optimal controls for identifying changes in our adaptor deficient strains, as we know that RcdA L82E and RcdA 3E contain substrate binding deficiencies, and PopA R357G cannot deliver CtrA during the cell cycle due to loss of cdG binding (Duerig et al., 2009). For example, to identify a potential new RcdA binding partner, deletion of RcdA show result in an increase in protein abundance of a particular candidate by TMT. If the RcdA L82E showed no change in that substrate's abundance, but the RcdA 3E showed a relative increase in that protein's abundance, then it could be concluded that changes in

the abundance of that substrate are due to RcdA binding at the surface implicated in PopA binding and it would make it much easier to identify highly specific RcdA binders. The same approach could be used for CpdR and PopA to identify new substrates by TMT. Identifying new adaptor-dependent pathways could follow a similar roadmap: adaptor deficient strains will likely see changes in the insertion profile of many substrates. If that insertion profile is differentially affected in one RcdA variant versus another, then it could be concluded that RcdA is involved in this pathway. If it is found that upon purification of candidates that show a change in insertions are not enhanced in their degradation upon addition of adaptor, then a potential novel role for an adaptor in that pathway independent of enhancing proteolysis could be hypothesized. The use of adaptor variants could potentially help weed out potential false positives amid the large quantity of TMT and Tn-seq data, allowing for the pruning of a few promising candidates.

APPENDIX

Uncropped Gels and Blots used for Figures in Chapter 2

Figure A.1: Uncropped Degradation Gels, Related to Figure 2.11

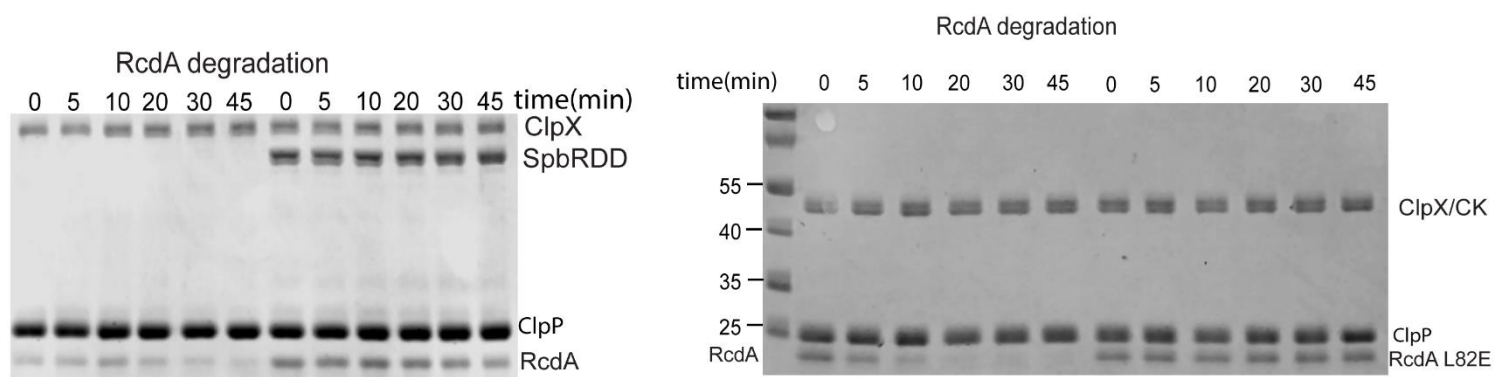


Figure A.2: Uncropped Degradation Gels, Related to Figure 2.14, Figure 2.23

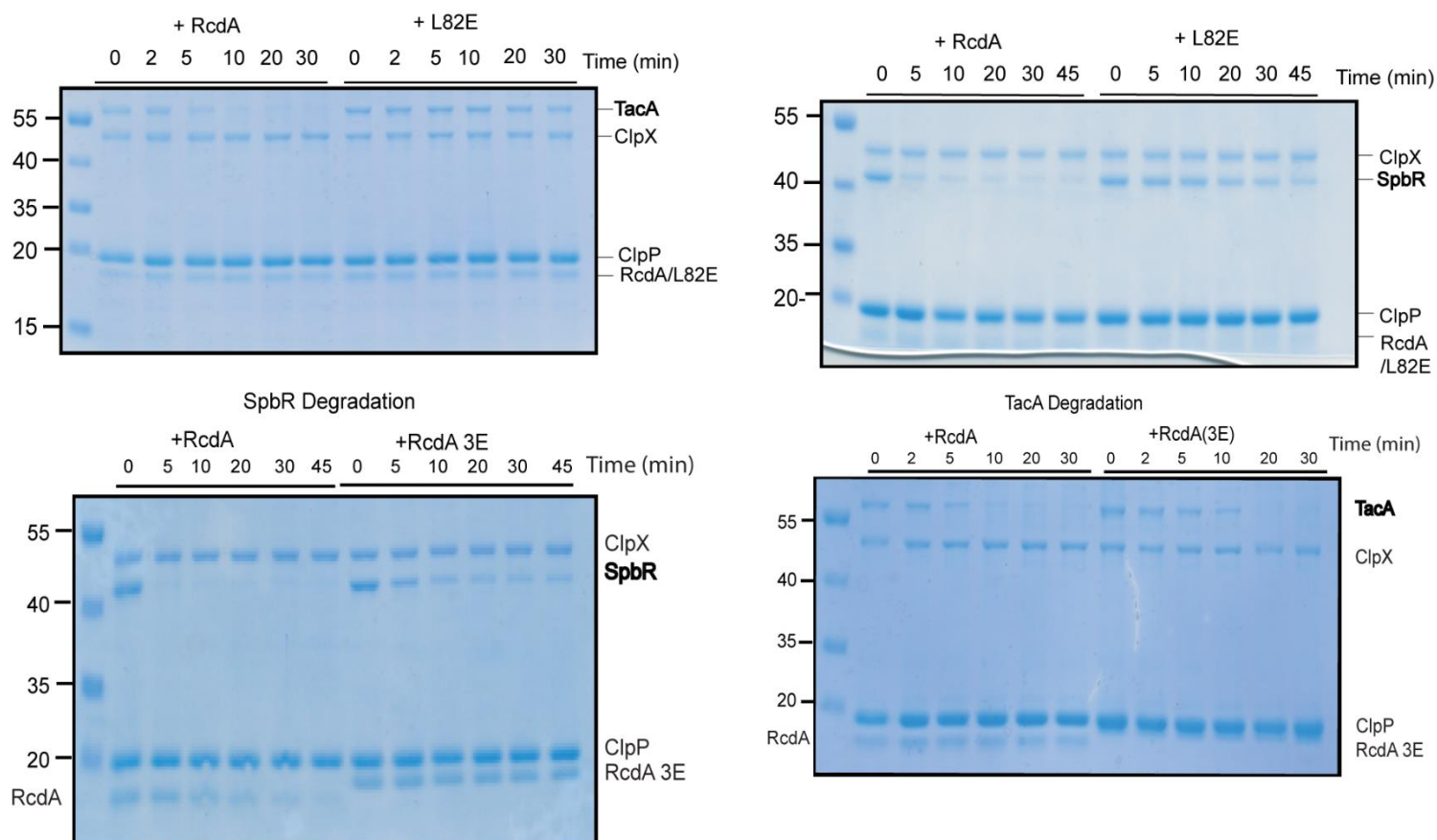
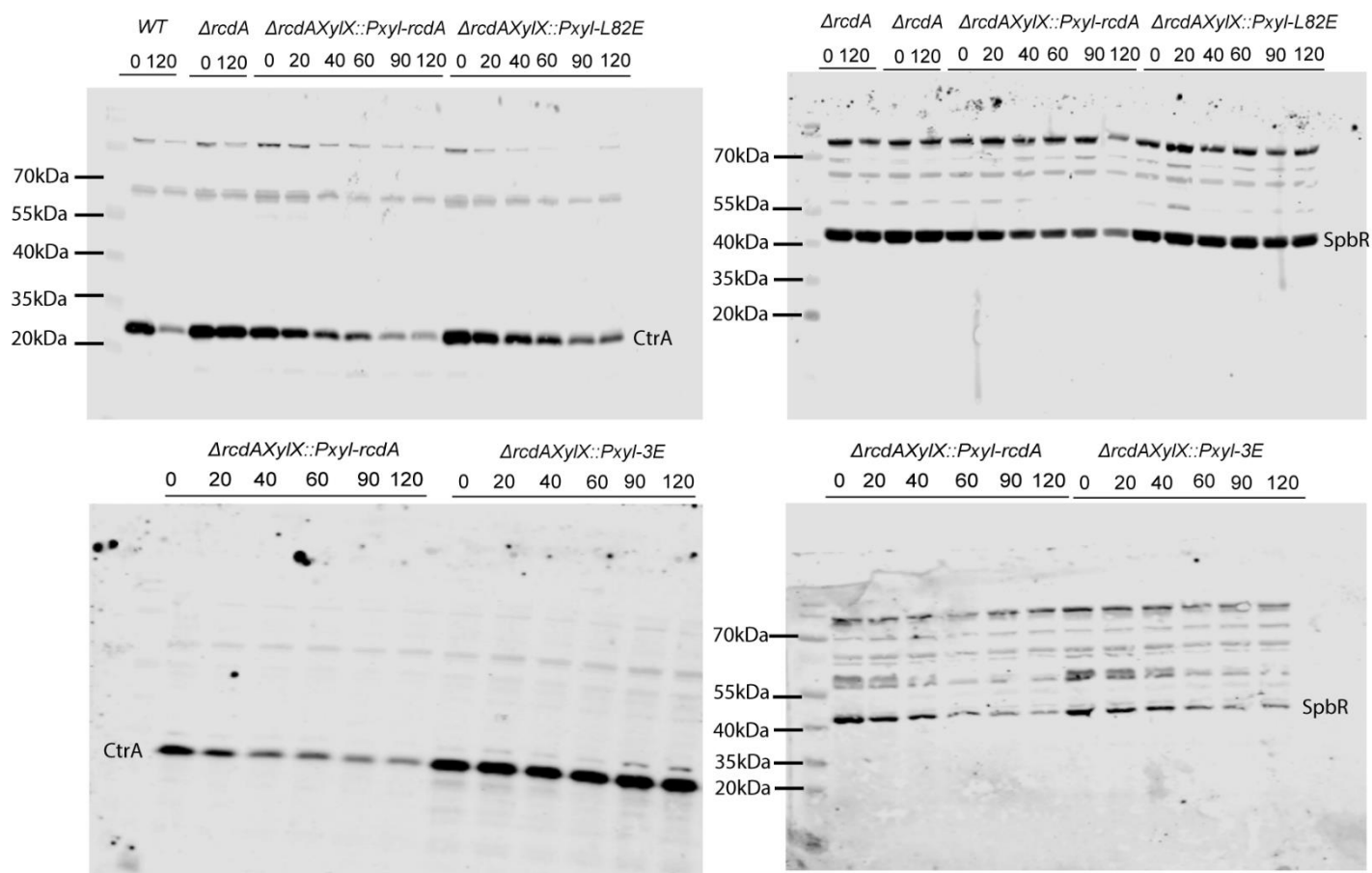


Figure A.3: Uncropped Western Blots, Related to Figure 2.26



Additional Biochemical Data

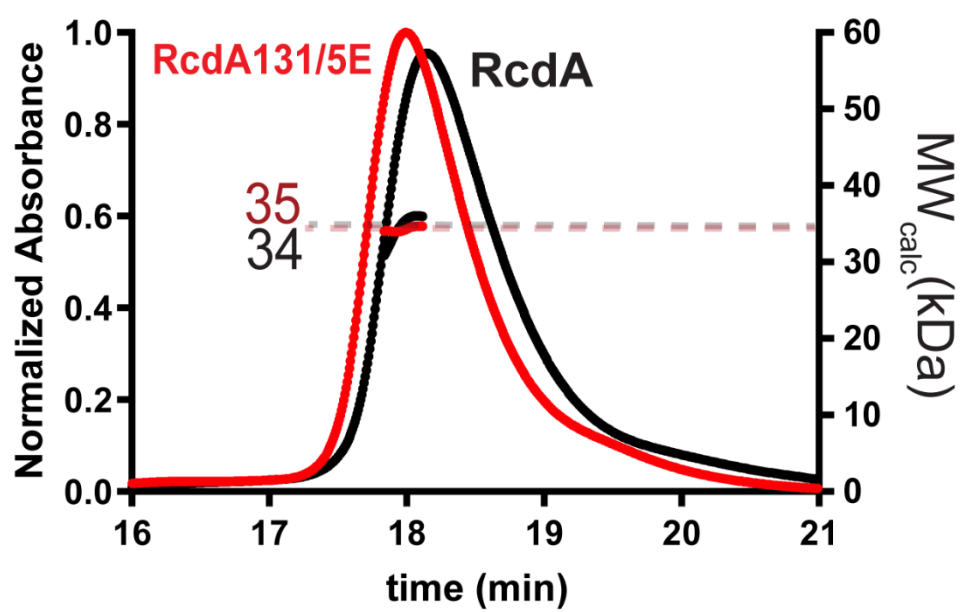


Figure A.4: The RcdA R131/5E Variant is Dimeric

SEC-MALS analysis of the R131/5E variant compared to WT RcdA. Both proteins were run over the column at 25uM. Dotted lines indicated collected MALS data and calculated mass. The R131/5E mutant was designed to disrupt the crystallographic dimer interface of our in-house solved crystal structure of RcdA, but that interface was shown to be the incorrect interface. The dimer interface described in (Taylor et al., 2009) is the correct dimer interface of RcdA.

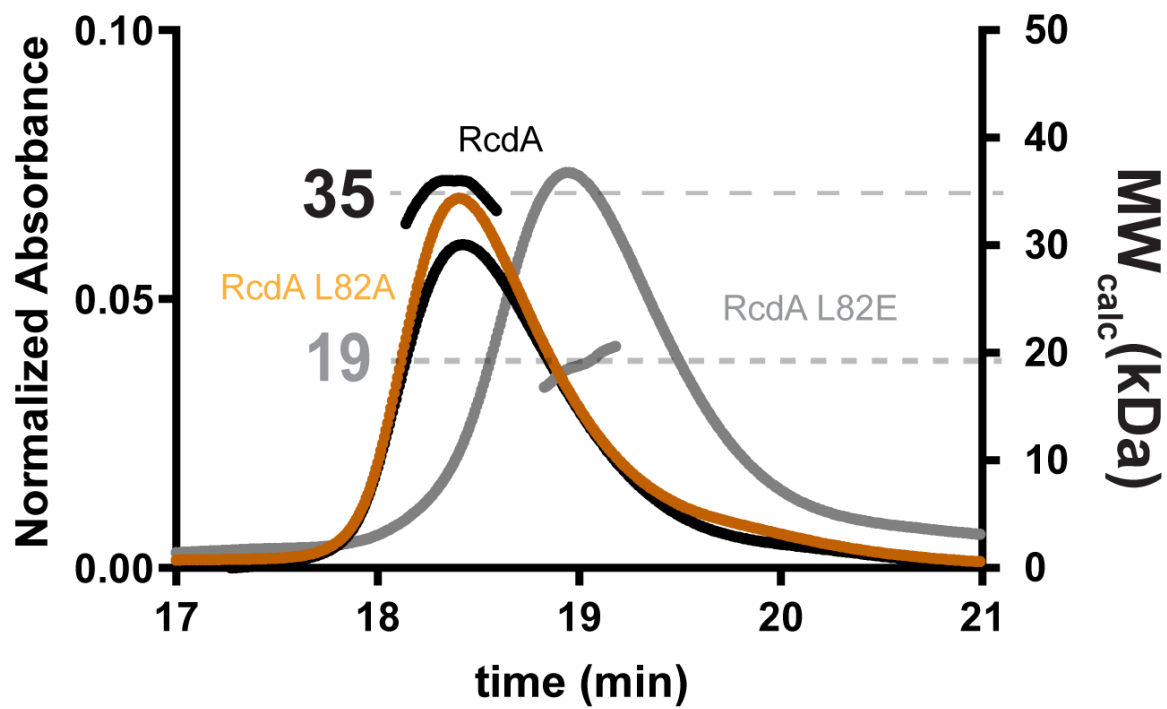


Figure A.5: The RcdA L82A Variant is Dimeric

SEC-MALS analysis of the L82A variant compared to WT RcdA and RcdA L82E (RcdA and L82E SEC-MALS Data is from Figure 2.10). Dotted lines indicated collected MALS data and calculated mass. The L82A variant did not scatter light in this experiment, but coeluted perfectly with WT RcdA, suggesting dimerization. The L82A mutant was designed to test whether we could form an obligate monomer of RcdA that did not affect cargo binding or delivery. This data tells us that the presence of L82 is not necessary for dimerization, as mutation to another hydrophobic residue of different atomic geometry does not disrupt dimerization.

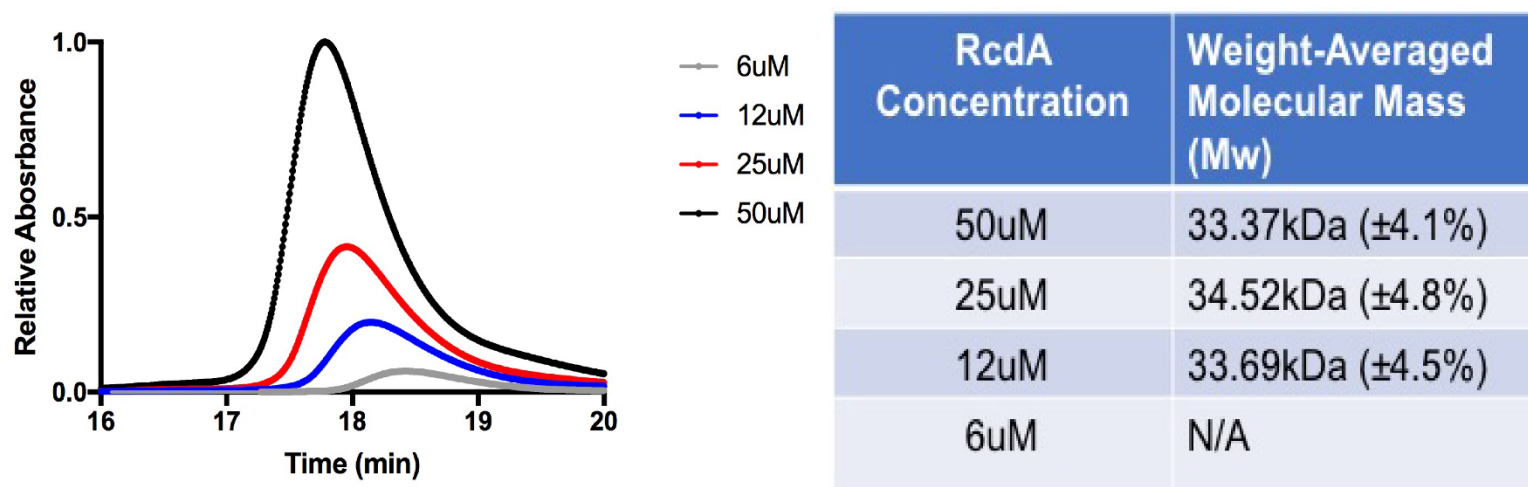


Figure A.6: RcdA Monomerization is Not Detectable by SEC-MALS

Serial Dilutions of RcdA were ran over the SEC-MALS column. At the concentrations detectable by absorbance, RcdA monomerization was not able to be determined by SEC-MALS. This describes a key limitation to SEC-MALS: the light scattering signal is proportional to the concentration of your injected protein, it becomes challenging for proteins of smaller mass to design SEC-MALS experiments at lower concentrations.

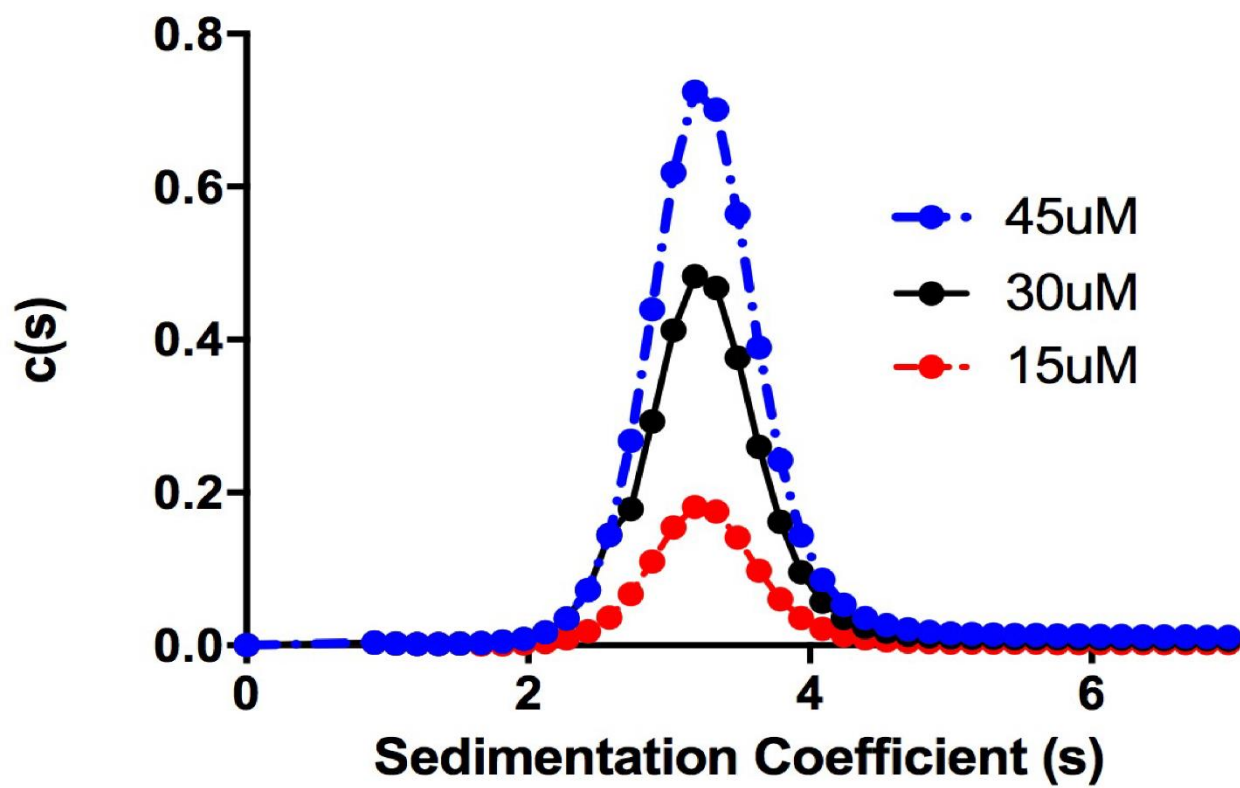
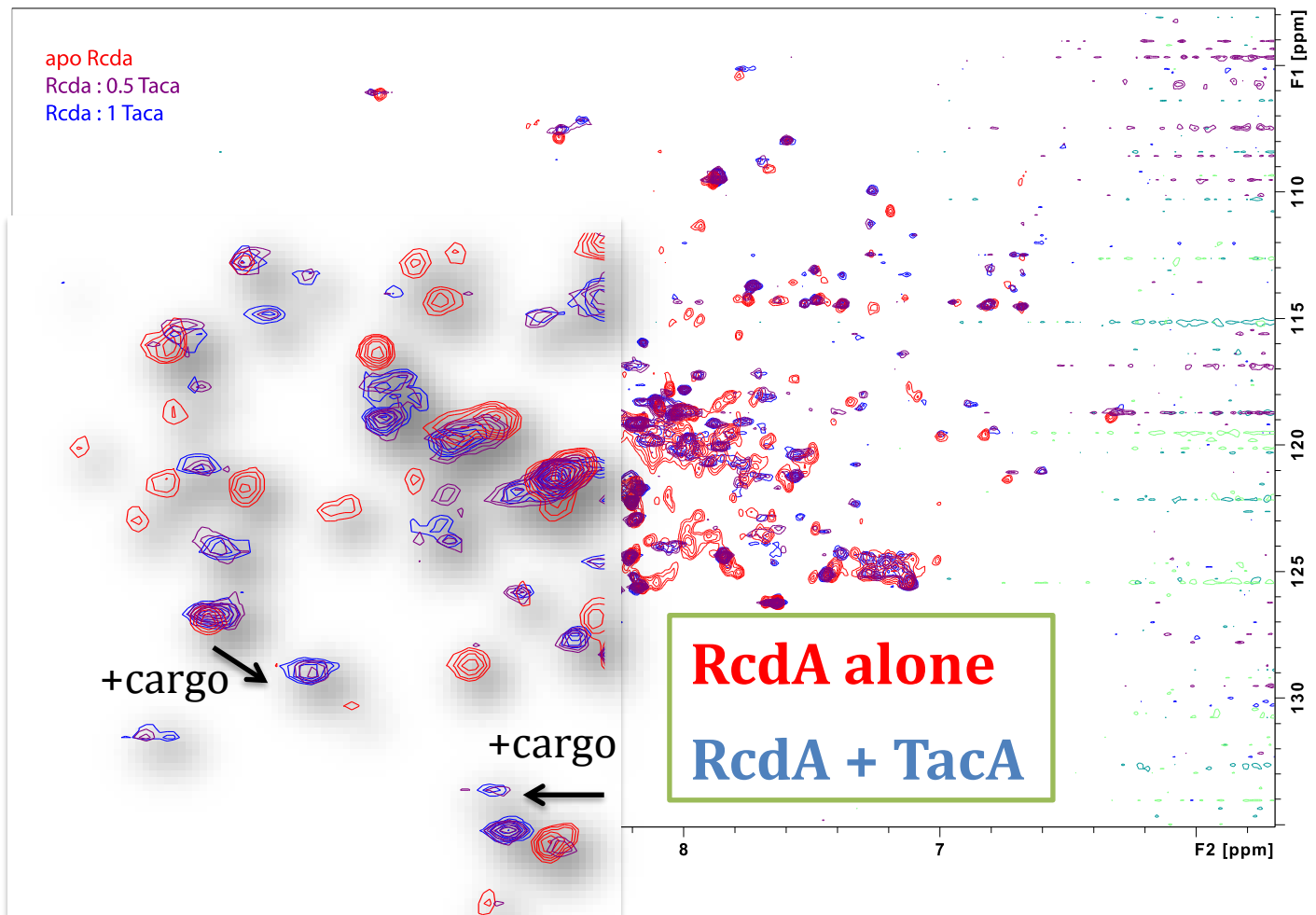


Figure A.7: RcdA Monomerization is Not Detectable by SV-AUC

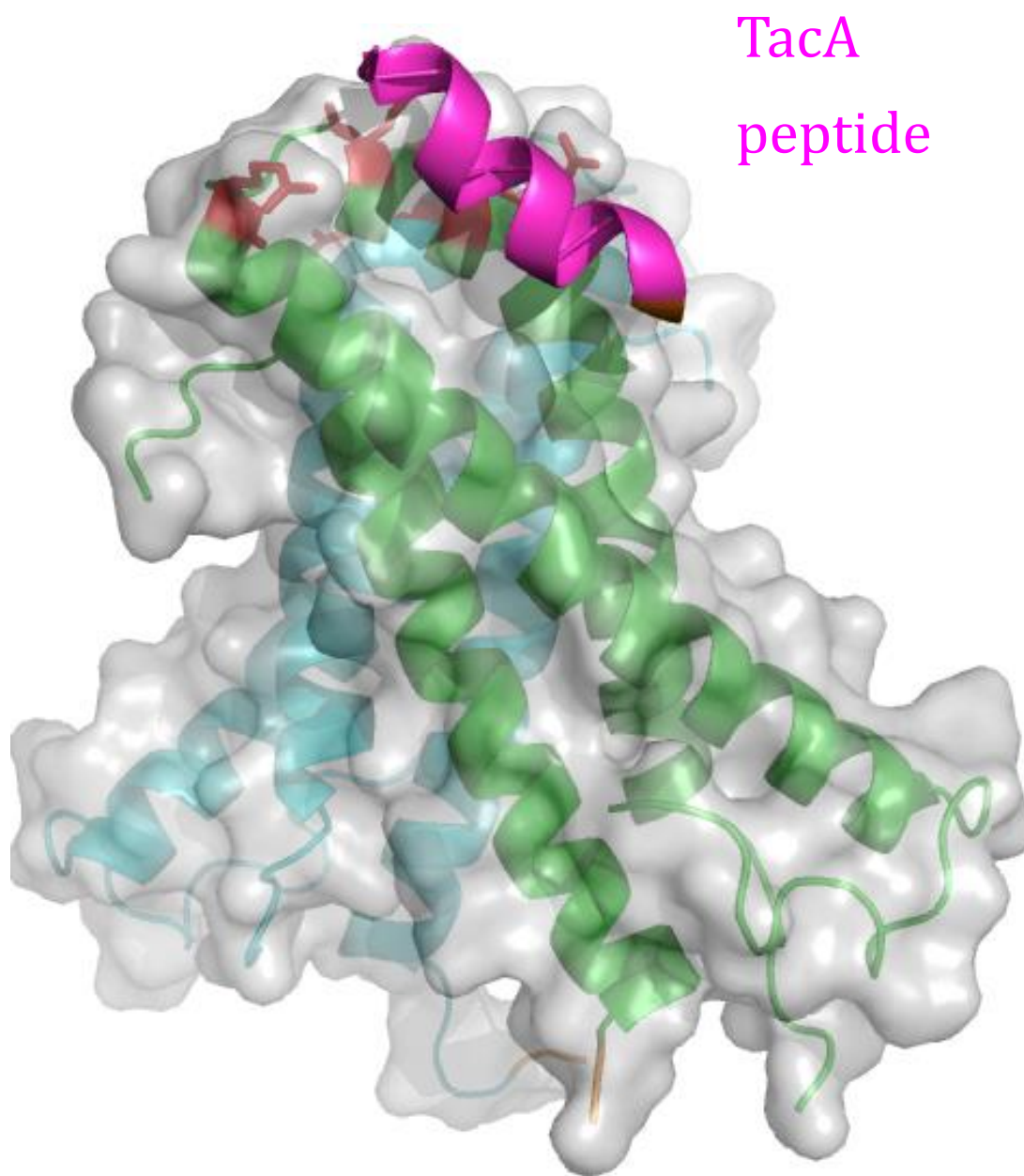
Different concentrations of RcdA were ran on the analytical centrifuge and analyzed by sedimentation velocity. At the concentrations detectable by absorbance, an RcdA monomer-dimer equilibrium was not observed in the $c(s)$ distributions provided by SV-AUC. Since we could not detect RcdA monomerization by absorbance detection, we decided to use a fluorescent reporter (Fluorecin-5-maleimide RcdA) and isothermal titration calorimetry to monitor RcdA dimerization more sensitively.



HSQC; Triple labeled RcdA + unlabeled TacA

Figure A.8: HSQC NMR Spectrum of RcdA-^{DBD}TacA interaction

Early in my graduate career, we collaborated with the Strieter lab to perform protein NMR studies (Lin Hui, Eric Strieter) to see if we could possibly elucidate the binding site of RcdA to ^{DBD}TacA. While we did see changes in the peak assignments when ^{DBD}TacA was added (inset), we were unable to determine the final binding site. Repeating this experiment with the RcdA L82E variant with PopA truncations (receiver1-2) would likely yield cleaner results.



TacA
peptide

Figure A.9: Schrodinger-Modeling TacA Peptide on RcdA Crystal Structure

The original identification of the RcdA 3E residues was through Schrodinger modeling (collaboration with Sergey Savinov) of a TacA peptide on our in-house solved structure of RcdA, which revealed a “pocket” that was proposed as important for ligand binding. In purple is the modeling TacA peptide, while in red sticks are the residues of the RcdA 3E mutant: R49, K53, R57. These residues were mutated to glutamic acids by Ashley Kosowicz, a previous undergraduate in the lab, to make the original RcdA 3E expression plasmid.

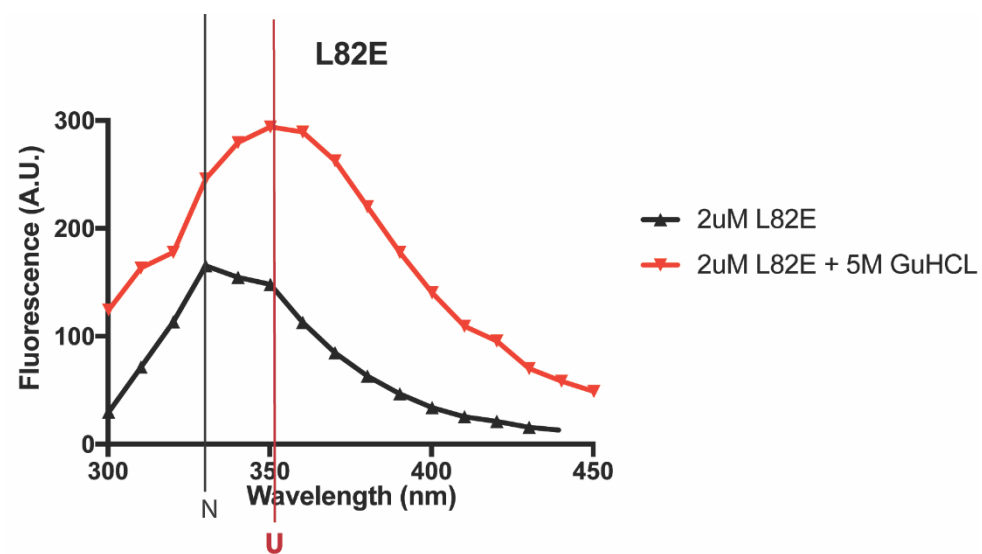
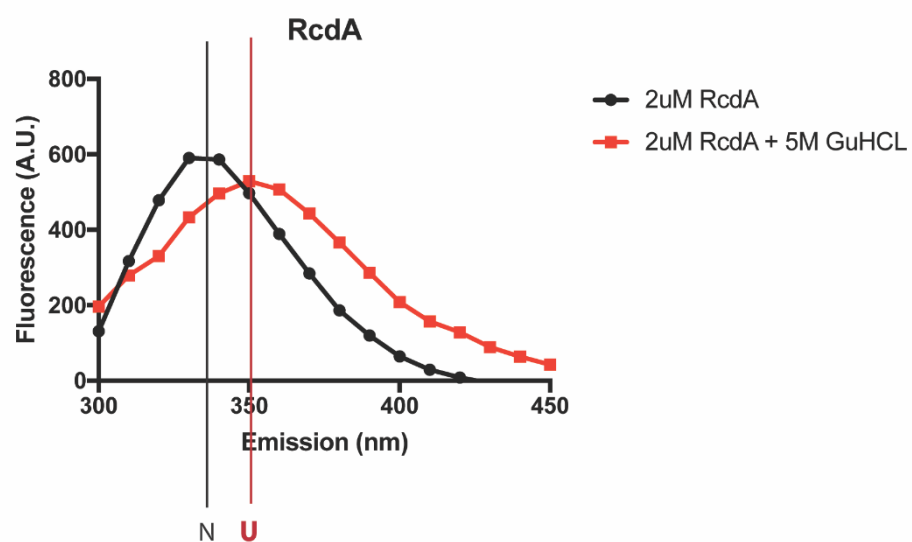


Figure A.10: Intrinsic Tryptophan Fluorescence Scans of RcdA and RcdA L82E

Intrinsic Tryptophan Fluorescence Profiles of RcdA and RcdA L82E in the absence or presence of denaturant (5M Guanidine Hydrochloride). In the presence of GuHCL, both RcdA and RcdA L82E shift in $\lambda_{\max}$ (330nm to 350nm), indicating the unfolding of the protein. Interestingly, upon unfolding, the fluorescence of RcdA L82E increases relative to the native state, while the fluorescence of RcdA decreases relative to the native state. The intrinsic tryptophan fluorescence per monomeric concentration unit of RcdA L82E is lower than that of RcdA at equal monomeric concentrations of protein.

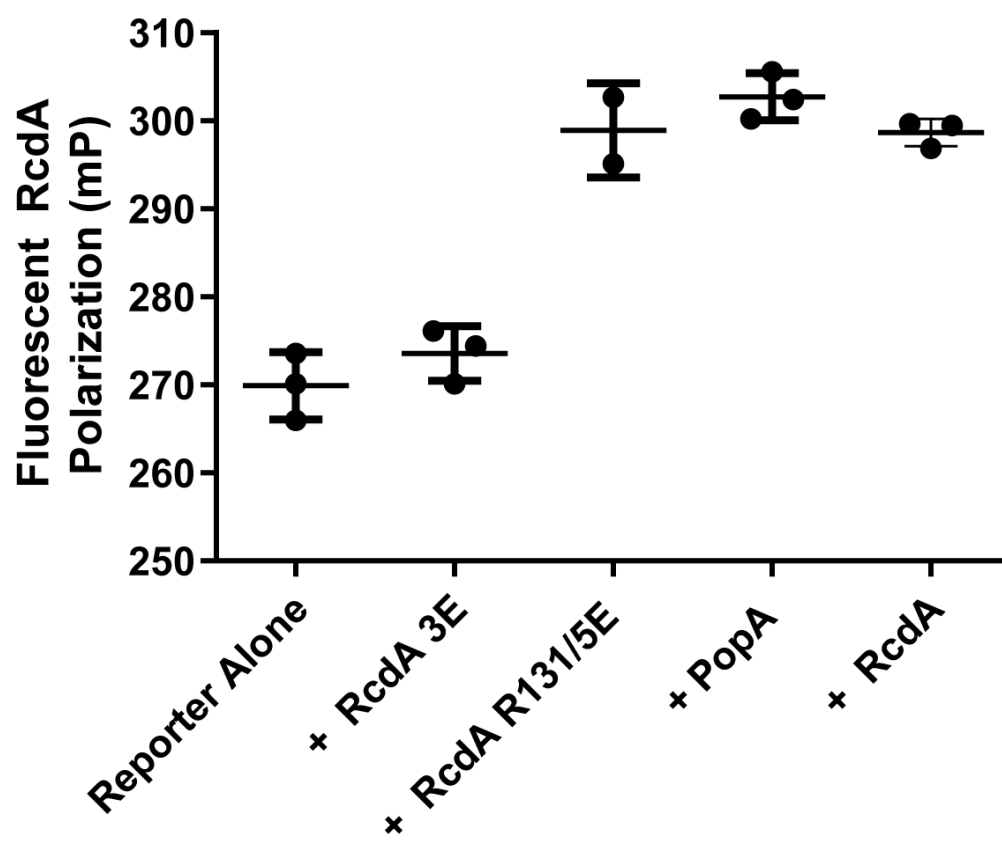


**Figure A.11: The RcdA 3E Variant does not form a Heterodimer by
Fluorescence Polarization**

Fluorescence polarization looking at heterodimerization of fluorescently-labeled RcdA with unlabeled RcdA variants. Assays were performed exactly as described earlier, with 20uM of unlabeled RcdA 3E, RcdA 131/5E, PopA and RcdA added to 100nM labeled RcdA. The only variant to not show an increase in polarization was the RcdA 3E variant, which did not show a characteristic increase in polarization when added. Unlabeled PopA and RcdA were used as positive controls in this assay.

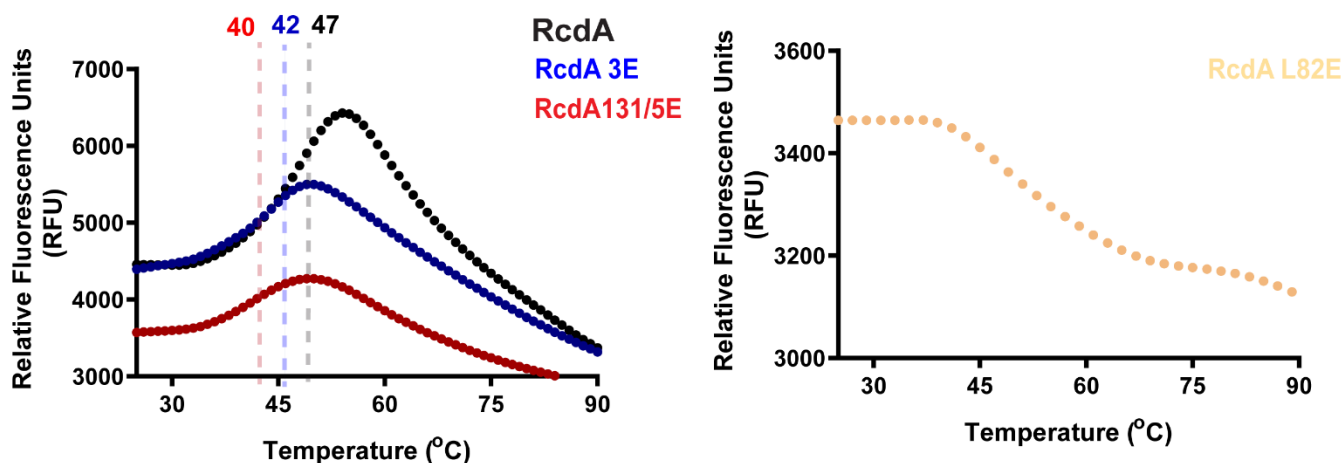


Figure A.12: Differential Scanning Fluorimetry Melt Curves of RcdA

Variants

DSF melt curves of various RcdA variants at 10uM final concentration. Melt curves were generated as described in appendix methods. Fitted T_m values are listed above the dotted line for each variant. The RcdA 3E and RcdA R131/5E variants are destabilized relative to WT RcdA as observed by their lower T_m values. Interestingly, the RcdA L82E monomer did not produce a melt curve or show a steady increase in fluorescence as the protein was heated, indicating that the Sypro orange fluorescent dye was incompatible or could not bind due to the RcdA L82E mutation.

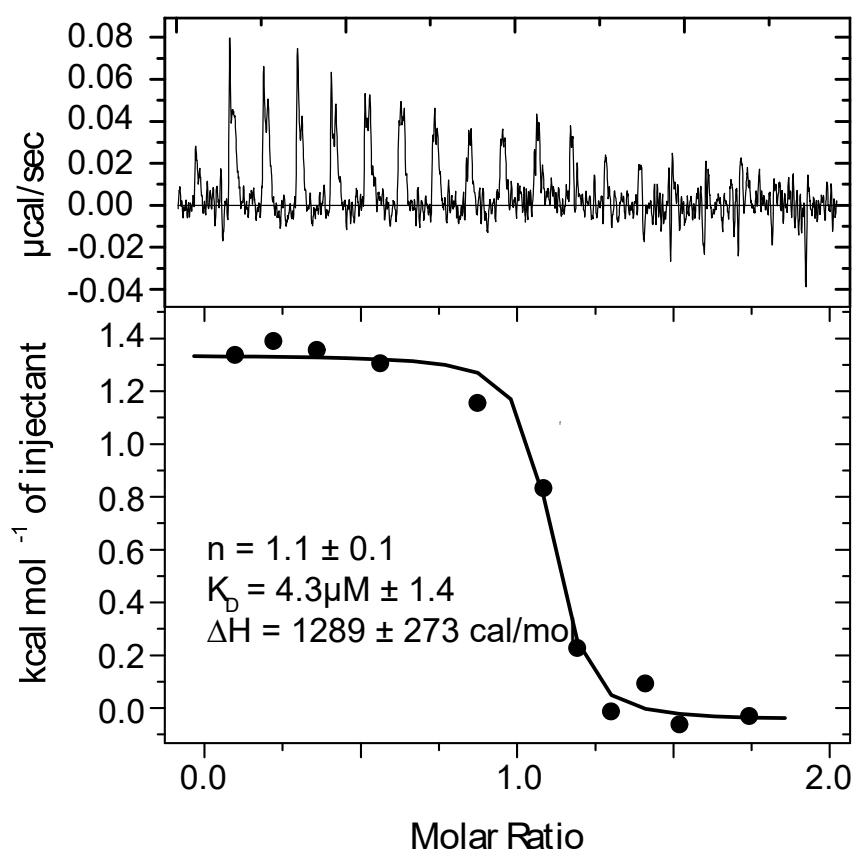


Figure A.13: ITC Thermogram of L82E-PopA Interaction

ITC Thermogram of 400 μM L82E titrated into a pool of 40 μM PopA. Both proteins were purified into 20mM Hepes, pH 8.5, 100mM KCL over an S200 size-exclusion column prior to injection. Data was fit to a single set of sites binding model.

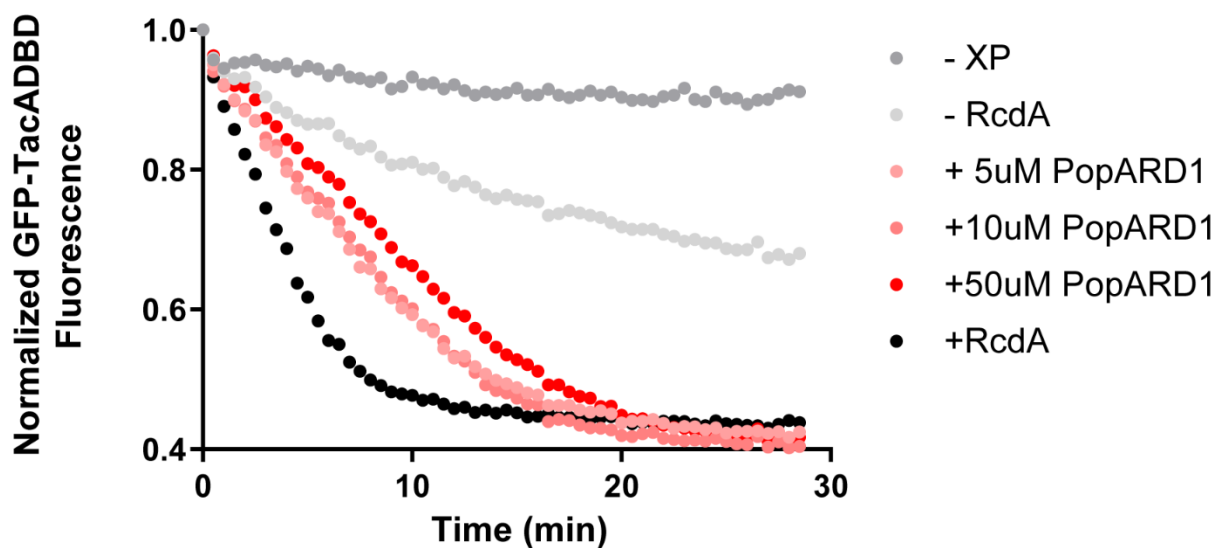


Figure A.14: GFP-^{DBD}TacA Assays in the Presence of PopA-RD1 truncation (aa 1-117)

GFP-^{DBD}TacA fluorescence degradation assay in the presence of different concentrations of PopA-RD1. 1 μ M GFP-^{DBD}TacA 1 μ M RcdA, 4 μ M CpdR, 0.2 μ M ClpX, 0.4 μ M ClpP, and varying concentrations of PopA-RD1 were used in this assay.

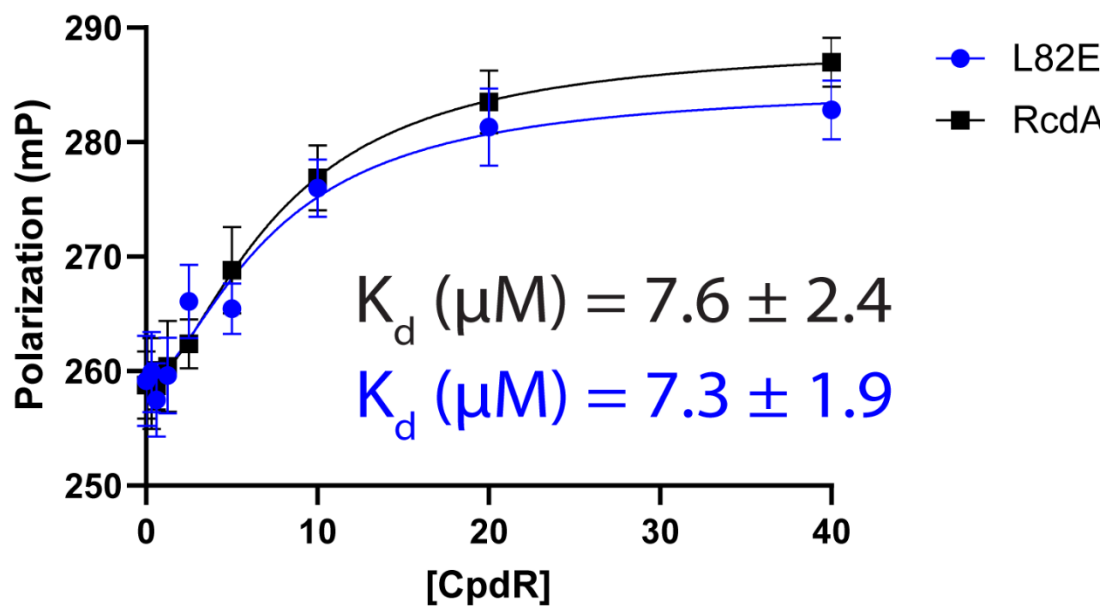


Figure A.15: CpdR-ClpX Binding Curves with L82E and RcdA Fluorescent Reporters

ClpX-CpdR interactions using a fluorescent RcdA or RcdA L82E Reporter. 1μM ClpX, 1mM ATPyS, 100nM FL-L82E or RcdA and varying concentrations were used. A single site binding model was used to fit the data in prism as outlined in the methods of Chapter 2.

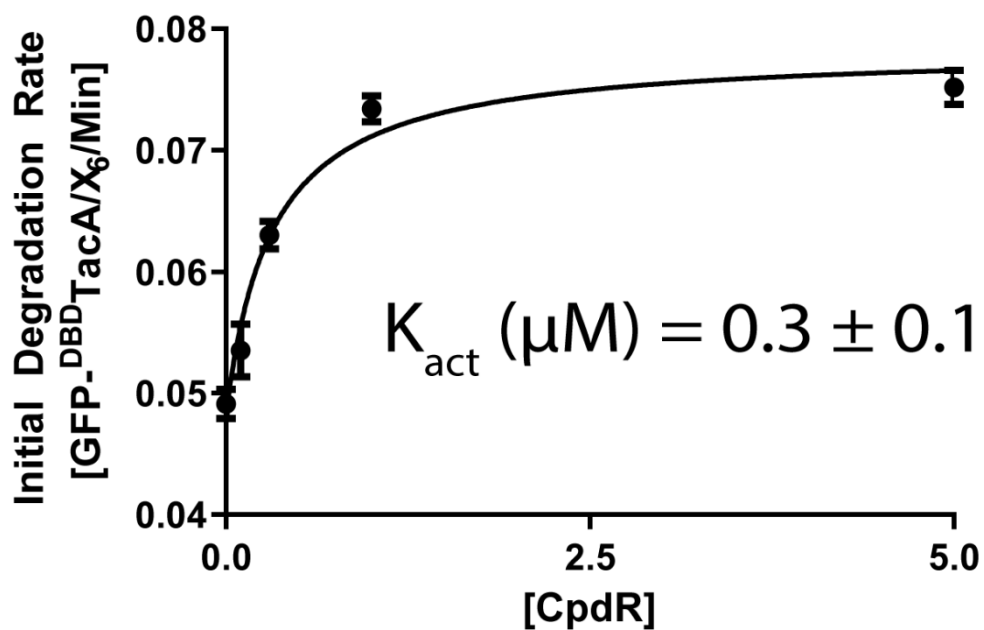


Figure A.16: CpdR $K_{\text{activation}}$ Curve with GFP-^{DBD}TacA

GFP-TacADBBD fluorescence degradation assay in the presence of different concentrations of CpdR. 1μM GFP-^{DBD}TacA, 1μM RcdA, 0.2μM ClpX, 0.4μM ClpP, and varying concentrations of CpdR were used in this assay. Data was fit to a modified form of the Michaelis-Menten equation as noted in the methods of Chapters 2 and 3.

Additional *In Vivo* Data

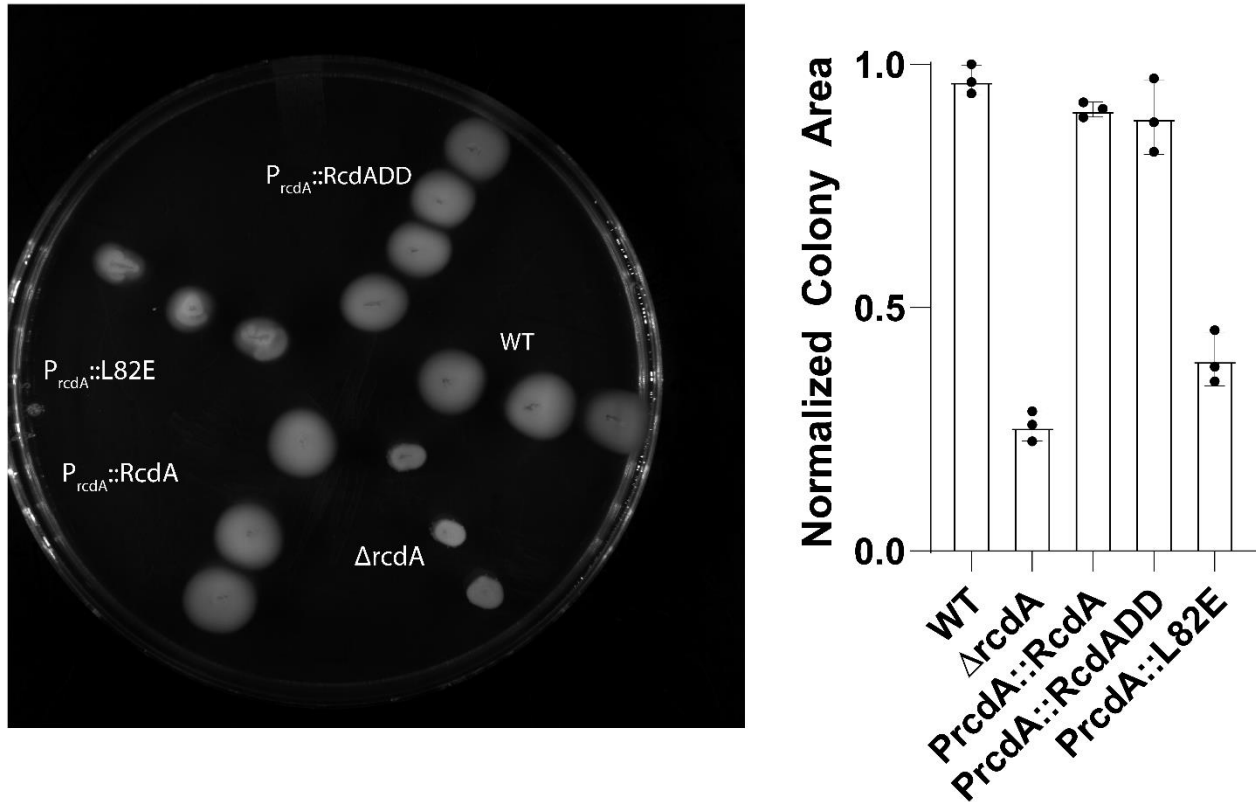


Figure A.17: Agar Motility Assay of $P_{rcdA}::L82E$ and $P_{rcdA}::RcdADD$, related to data presented in Figure 2.28

While generating the $P_{rcdA}::L82E$ strain, we looked for phenotypes that might be used to screen for positive mutants. Since we know that $\Delta rcdA$ strains have a motility defect, we used motility as a readout of positive strain construction. Interestingly, the $P_{rcdA}::L82E$ has a defect in motility while the $P_{rcdA}::RcdA$ and $P_{rcdA}::RcdADD$ strains do not have motility defects, suggesting that the motility phenotype is likely linked to deficiencies in cargo delivery due to the L82E mutation, not RcdA autodegradation.

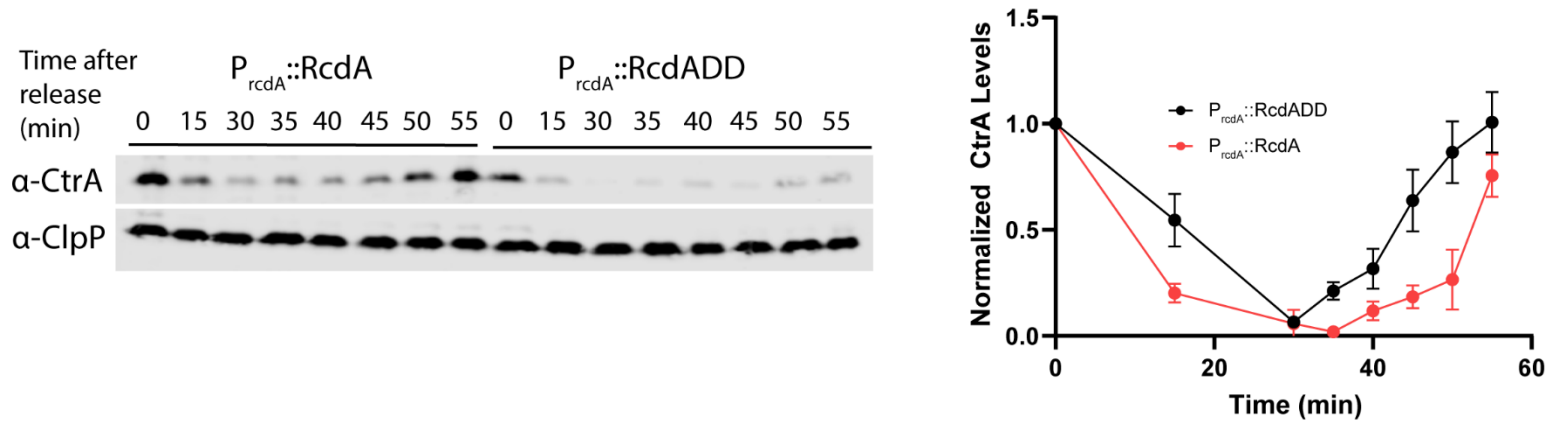


Figure A.18: CtrA Synchrony assay of $P_{rcdA}::RcdADD$

Before we generated the $P_{rcdA}::L82E$ strain, we hypothesized that RcdADD might deliver substrates faster *in vivo* because of its prolonged time on the CpdR-ClpX complex due to its inability to be degraded. The $P_{rcdA}::RcdADD$ strain was capable of delivering CtrA to ClpXP more quickly throughout the cell cycle. Shown on the left is a representative western blot of this timecourse and on the right is the quantification of this western blot, normalized to the ClpP control band.

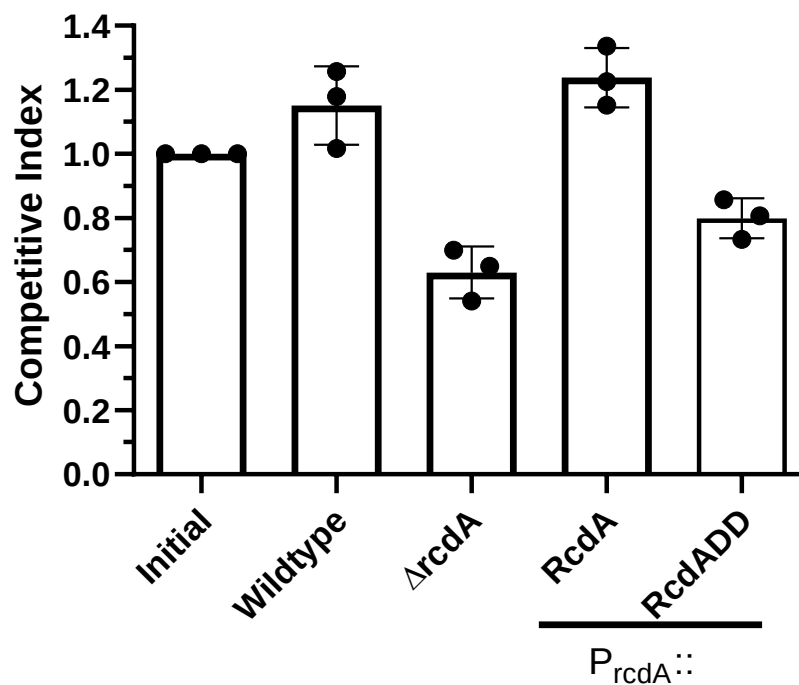


Figure A.19: $P_{rcdA}::RcdADD$ Competition Fitness Assay

Fitness competition assay between wildtype and $P_{rcdA}::RcdA$ or $P_{rcdA}::RcdADD$.

Like Figure 2.28D, the wildtype and $\Delta rcdA$ data are the same controls used.

Average and standard deviation of three independent replicates is shown.

Competition assays were completed as noted in Chapter 2 methods section.

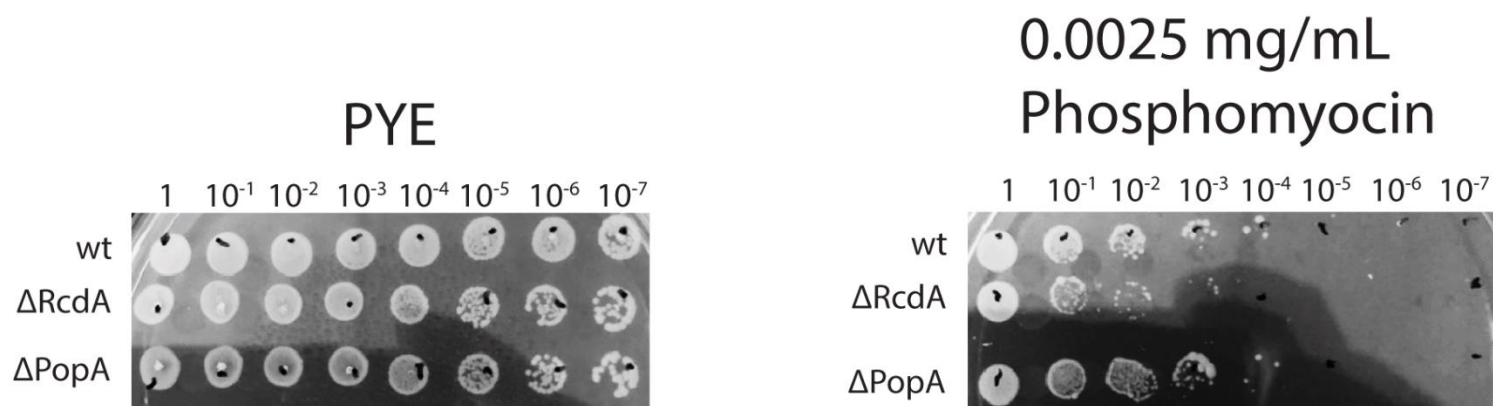


Figure A.20: Phosphomycin Spot Assay of Adaptor Deficient Strains

Upon characterization of RcdA-dependent phenotypes, we found that addition of the cell wall stressor that targets MurA, Phosphomycin, caused viability defects on an agar plate. Shown is a spot assay of *ΔrcdA* and *ΔpopA* strains on PYE and on PYE + 0.0025mg/mL of Phosphomycin.

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