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Caulobacter ClpXP Adaptor PopA's Domain Interactions in the Adaptor Hierarchy of CtrA Degradation

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Caulobacter ClpXP Adaptor PopA's Domain Interactions in the Adaptor Hierarchy of CtrA Degradation

A Thesis Presented

By

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Molecular & Cellular Biology

DEDICATION

To my family (by blood and by choice), to my friends, and to Hazel. Without their tireless love and support the following work would not have been possible.

I dedicate this thesis to my parents, Philip and Sherrie, my sister Jennifer, and my wife Grace. Thank you so much for never giving up on me even when I sometimes gave up on myself. Thank you for your encouragement, advice, support, and reproach over countless conversations.

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I would first like to acknowledge my mentor, advisor, and former professor, Dr. Peter Chien, for his unwavering ability to encourage me to improve and persistence in reminding me why I wanted to become a scientist. Thank you for seeing something in me and allowing me to join you in scientific exploration, and providing me with the space, the tools, and the skill-set necessary to continue to higher levels of academia. Thank you also for pushing me to overcome my weaknesses and delve deeper into my strengths. The lessons you have taught me as your mentee are things that have changed the way I engage with my work and pushed me to move on to the next level of serious scientific inquiry. I will always be grateful for, and indebted to, the time, energy, and insight you gave me during our time together in your office, in the lab, and over the internet.

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ABSTRACT

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The degradation and recycling of protein is a process essential for the maintenance and regulation of cellular function. More specifically, in *Caulobacter crescentus,* the ClpXP protease is responsible for driving progression through the cell cycle and protein quality control. This protease utilizes three known adaptors to selectively degrade proteins that initiate different stages of development. This thesis will elaborate on the specific binding interface on one of these adaptors, PopA, with another, RcdA, and focus in on specific residues on PopA and investigate their roles in adaptor binding and delivery of CtrA, the master regulator of *Caulobacter*. Finally, I will investigate the relationship between and necessity of these adaptors using a mutant PopA that does not require the

presence of RcdA or the other adaptor, CpdR. The remainder of this thesis will present data that arises from these projects.

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CHAPTER 1

AN OVERVIEW OF AAA+ PROTEASES, THEIR ADAPTORS, AND REGULATION

1.1 Introduction

Proteolysis is an essential process, common to all forms of life, that facilitates the healthy growth and development of cells through the degradation and recycling of protein. It is important for the cell to be able to not only recognize and remove defective protein, but also to process obsolete protein as a response to stimuli and during different developmental stages of the cell's life cycle. Proteolysis must also be highly regulated because unchecked protein degradation can lead to cell death. Eukaryotes have developed a system by which proteins are marked for degradation via polyubiquitination (Streiter, 2012), whereas bacteria utilize energy dependent AAA+ (**A**TPases **A**ssociated with diverse cellular **A**ctivities) proteases that unfold and degrade the protein. Their specificity is driven by a hierarchy of adaptor proteins, small molecule ligands and their own degradation, that regulates the exact degradation capabilities of these proteins. At the heart of this thesis is the question of how one of these adaptor proteins molecularly interacts with its surrounding adaptors, its small molecule

regulators, and its cargo, for eventual cargo delivery and degradation. In this thesis I will detail several of these proteases that use adaptors to regulate degradation target specificity, how these adaptors are regulated via proteolysis and post-translationally, and the specific characterization of the adaptor at the focus of my thesis, PopA. The research question of my thesis is driven by exploration of each of these topics.

1.2 AAA+ Protease Diversity and Anatomy

AAA+ Proteases serve a very basic function in bacterial cells and their make-up follows a generally conserved pattern. They contain two main functional constituents, the first of which being an unfoldase domain, responsible for recognizing target substrates by a short amino acid motif known as a degron (Flynn et al, 2001). After the unfoldase portion of the protease recognizes this sequence, the AAA+ unfoldase uses power derived from the hydrolysis of ATP to unwind the protein into its primary structure and feed it into the second constituent part of the protease, the proteolytic peptidase domain. (Figure 1.1) (Hanson and Whiteheart, 2005). Initial protease-substrate recognition specificity is driven by the unfoldase component. However, proteases need to be able to degrade an extremely broad range of substrates but at the same time be very

specific of their targets during different stages of the cell cycle, so additional levels of regulation are needed to prevent unwanted degradation. To achieve this specificity, proteases use proteins known as adaptors to aid in specific targeting and delivery of substrates, which select which substrates engage with the unfoldase selection component of the protease (Figure 1.2). There are several known proteases that use adaptors. Lon is a highly conserved protease that utilizes adaptors for several functions including restricting hyperflagellation in *Bacillus subtillis* via the SmiA adaptor. (Mukherjee et al., 2014, Gur et al., 2011). Another protease found prolifically in bacteria and mitochondria is the ClpXP protease (Baker and Sauer, 2011). ClpXP uses several adaptors to drive cell cycle progression through the targeted degradation of specific proteins (Mahmoud et al., 2018, Joshi et al., 2015). Other Clp proteases also utilize adaptors to drive substrate specificity. ClpAP utilizes the ClpS adaptor to target N-end rule substrates (Erbse et al, 2006), and ClpCP utilizes several adaptors, YpbH, MecA, and McsB in *Bacillus subtillis* to regulate sporulation by degrading ComK and Com S (Kirstein et al, 2007, Schlothauer et al, 2003, Persuh et al, 2002). Adaptors can also be post-translationally modified to add an additional layer of regulation to their specificity.

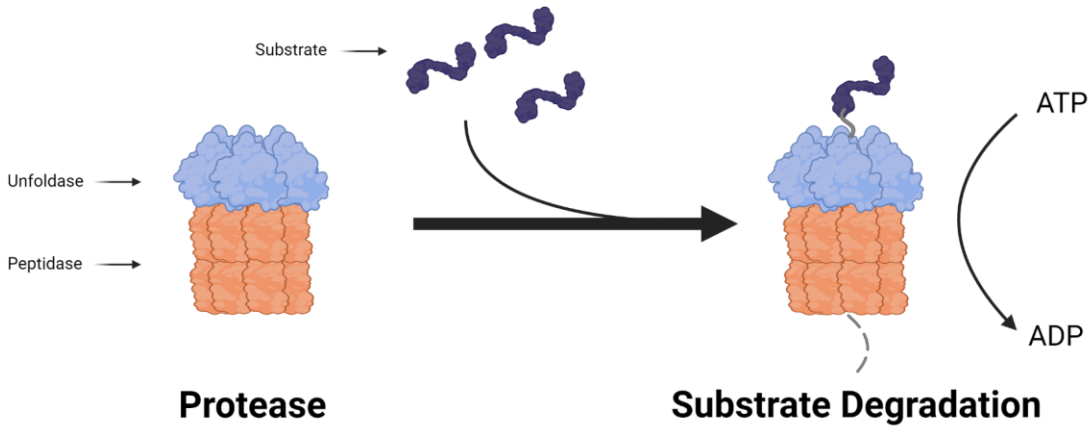

Figure 1.1: Protease Function

AAA+ Proteases consist of two main functional components: An unfoldase made up of 6 repeating subunits, and a proteolytic peptidase chamber that contains the site responsible for proteolysis activity. The unfoldase module utilizes the power derived from ATP hydrolysis to processively unwind the targeted substrate after recognizing the degron and sends it into the peptidase chamber where the protein is degraded into its amino acid subunits.

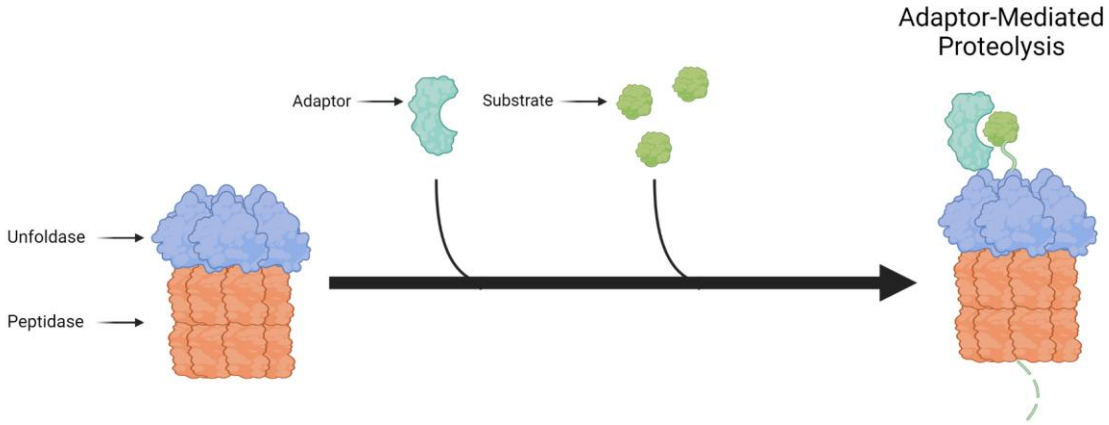

Figure 1.2: Protease Adaptors

Adaptors work by driving specificity of the protease to preferentially target certain substrates. Adaptors can bind to the protease, other adaptors, or the substrate, to bring the substrate into proximity of the unfoldase module for recognition.

1.3 Protease Specificity is Driven by Priming or Tethering via Adaptors

Adaptors can serve one or either of two functions when driving target delivery: acting as an active primer or a passive scaffold. Active primers imbue some new effect that prepares either the substrate or protease for delivery. CpdR is one such active priming adaptor in *Caulobacter crescentus* that directly interacts with the ClpX module of ClpXP. It first binds the N-terminal domain of the unfoldase, preparing the protease for substrate recognition (Figure 1.3), (Lau et al., 2015). In this case, the CpdR adaptor does not strongly bind with its target substrates in the absence of the ClpX N-terminal domain. Priming could come from allosteric changes conferred to either the adaptor or the protease upon binding or could come from an extension in surface topography of both unfoldase and substrate upon binding into a complex. This type of priming is distinct from scaffolding, as can be seen in the case of the simple scaffold SspB. In excess amounts, SspB inhibits substrate degradation because separate scaffolds binding to both substrate and ClpX prevents localization, however this inhibition is not seen with excess CpdR (Lau et al, 2015).

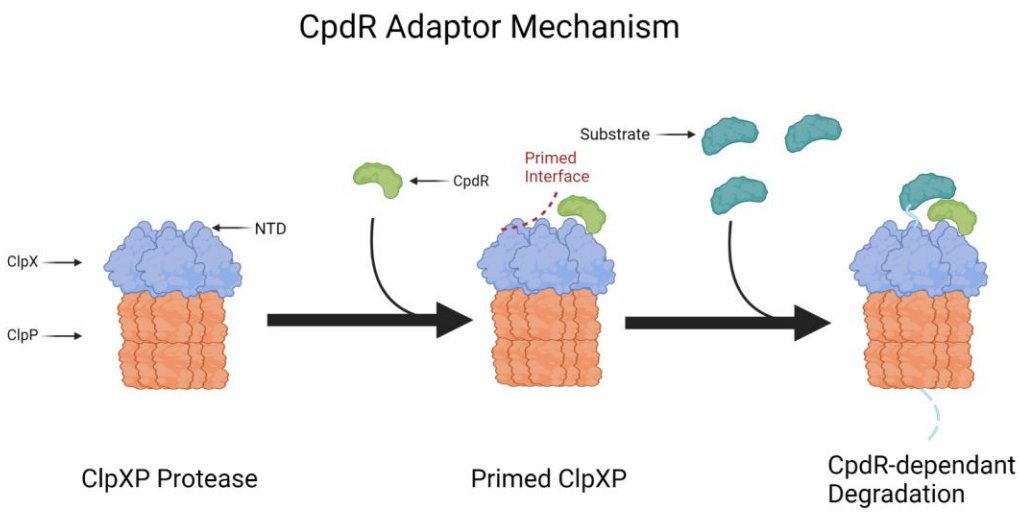

Figure 1.3: CpdR Adaptor Mechanism

CpdR is an active primer for the ClpXP protease. It has no affinity for its target substrates outside of when it is bound to the N-terminal Domain (NTD) of ClpX. Upon binding to the NTD, CpdR creates a primed interface upon which substrates can bind. The exact mechanism of this priming is unknown.

Alternately, the simpler mechanism for targeted substrate specificity is passive scaffolding. In this mechanism, an adaptor simply creates a bridge between the protease and its substrate bringing them into proximity and increasing the local concentration. By doing so, the substrate surpasses the K_D of the protease for the degron of the substrate significantly, causing specific degradation of that substrate. As mentioned previously, SspB is an adapter that works as a passive scaffold which binds and delivers substrates tagged with the ssrA peptide (Levchenko et al., 2000). Another adaptor that can act as a passive scaffold is RcdA (**R**egulator of **C**trA **D**egr**a**dation), an important adaptor of ClpXP in *Caulobacter crescentus* (Figure 1.4), (Joshi et al., 2015) that is tangential to the adaptor that is the focus of my thesis, PopA. RcdA will readily dimerize at micromolar concentrations and can deliver itself for degradation as a second layer of regulation. However, this auto degradation is inhibited and RcdA levels are stabilized in the presence of the adaptor PopA, the adaptor at the focus of this thesis.

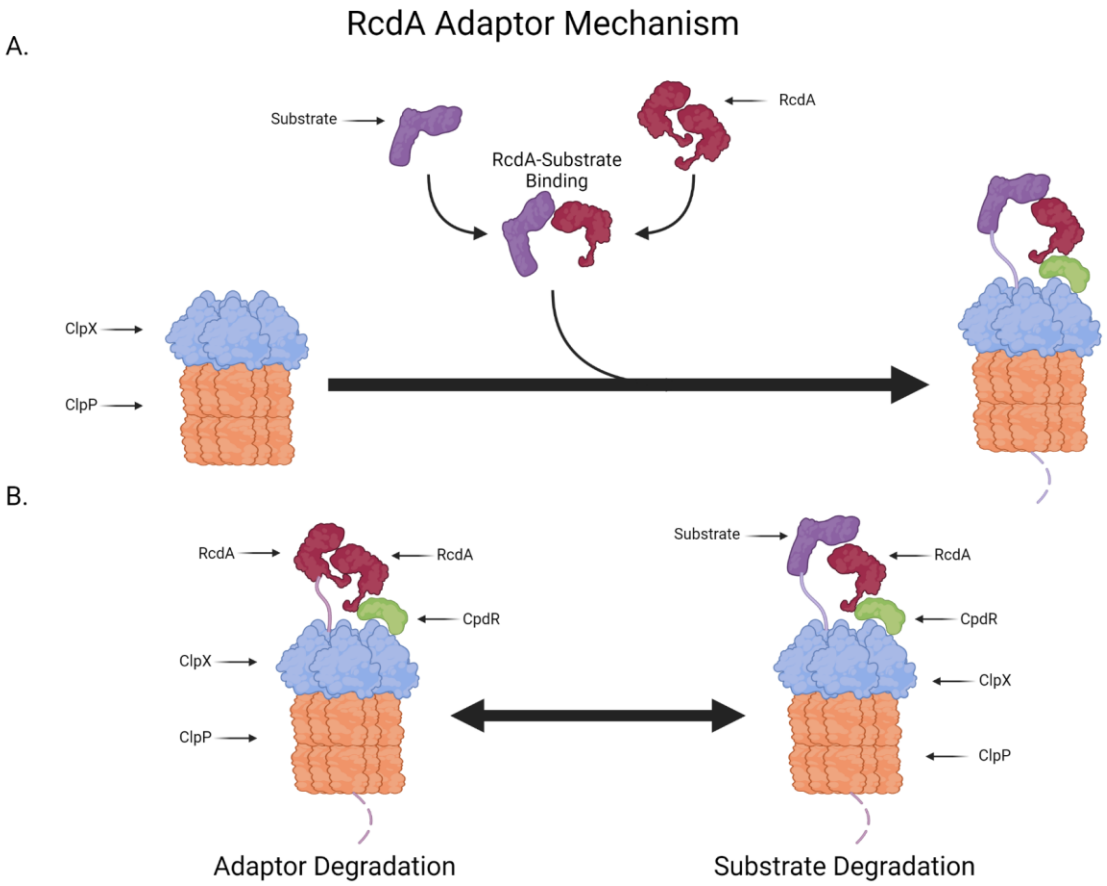

Figure 1.4: RcdA Adaptor Mechanism

A) RcdA acts as a simple scaffold for CpdR primed ClpXP. It binds its substrate and delivers it to ClpXP, increasing the local concentration and causing an increase in degradation rate. B) RcdA can autodegrade in the absence of cargo or substrate. Binding cargo or substrate prevents this autodegradation and stabilizes RcdA levels.

1.4 Cell Cycle in Caulobacter is Driven by Degradation via an Adaptor Hierarchy

Bacterial cells must degrade specific proteins during cellular transitions or during times of stress, so it is during these times that proteases utilize adaptors to target specific substrates. *Caulobacter* undergoes a tightly regulated cell cycle where DNA replication only occurs once per cell cycle, at S-phase. This regulation occurs because of *Caulobacter*'s life cycle. Upon cellular division, *Caulobacter* creates two dimorphic daughter cells, a sessile stalked cell and a motile swarmer cell. Only the stalked cell can replicate again and so the swarmer cell must make a transition from G1 to S-phase. (Poindexter, 1981; Skerker, 2004) During this transition, ClpXP degrades key regulatory proteins involved in cell motility, replication, and transcription (Bhat et al., 2013, Gora et al, 2013, Biondi et al., 2006). To drive the cell cycle, and specifically target the DNA-binding and transcriptional regulatory protein CtrA to initiate DNA replication (Quon et al., 1996), *Caulobacter crescentus* utilizes a four-part hierarchy of three adaptors (CpdR, RcdA, and PopA) and one small ligand second messenger (Cyclic di-GMP). During G1 phase, CpdR binds the N-terminal domain of ClpX and primes it for targeting substrates including PdeA. When PdeA is degraded, Cyclic di-GMP (CdG) levels are downregulated (Lau et al. 2015). The next adaptor to bind to the

complex is RcdA. RcdA interacts with the primed CpdR/ClpX complex via it's C-terminal tail. RcdA delivers its own class of substrates including TacA which is involved in the regulation of stalk biogenesis (Joshi et al. 2015). Finally, PopA binds to RcdA (Ozaki et al., 2014, Kuhlmann, 2021) and, after also binding CdG, can deliver its own class of substrates including CtrA (Deurig et al., 2009, Smith et al., 2014), KidO (Bergé et al., 2020), and GdhZ (Beaufay et al., 2015). While the presence of the adaptor hierarchy accelerates the degradation of CtrA, ClpXP can target CtrA by itself. The main benefit of the adaptor hierarchy is seen when CtrA is bound to DNA. *In vitro*, CtrA degradation can be increased around 2x with the right concentration of adaptors and substrate. (Smith et. Al, 2014) PopA itself is an ortholog of PleD, a CdG synthesis protein, and is comprised of three domains: two N-terminal receiver domains, and a C-terminal GGDEF domain (Ozaki et al., 2014) Receiver domains are usually associated with their ability to act as phospho-switches, however the phospho-switch capability of PopA's receiver domain has been lost. Similarly, GGDEF domains are usually associated with the synthesis of CdG, as in the case with PleD, however, though PopA's GGDEF domain retains the CdG binding I-site, it has lost the catalytic GGDEF motif and can

no longer synthesize cyclic di-GMP. Previous work has implicated the most N-terminal receiver domain (REC1) in RcdA binding (Ozaki et al., 2014), however this thesis aims to test these results and dive further into specific mechanistic insights into PopA-mediated CtrA delivery.

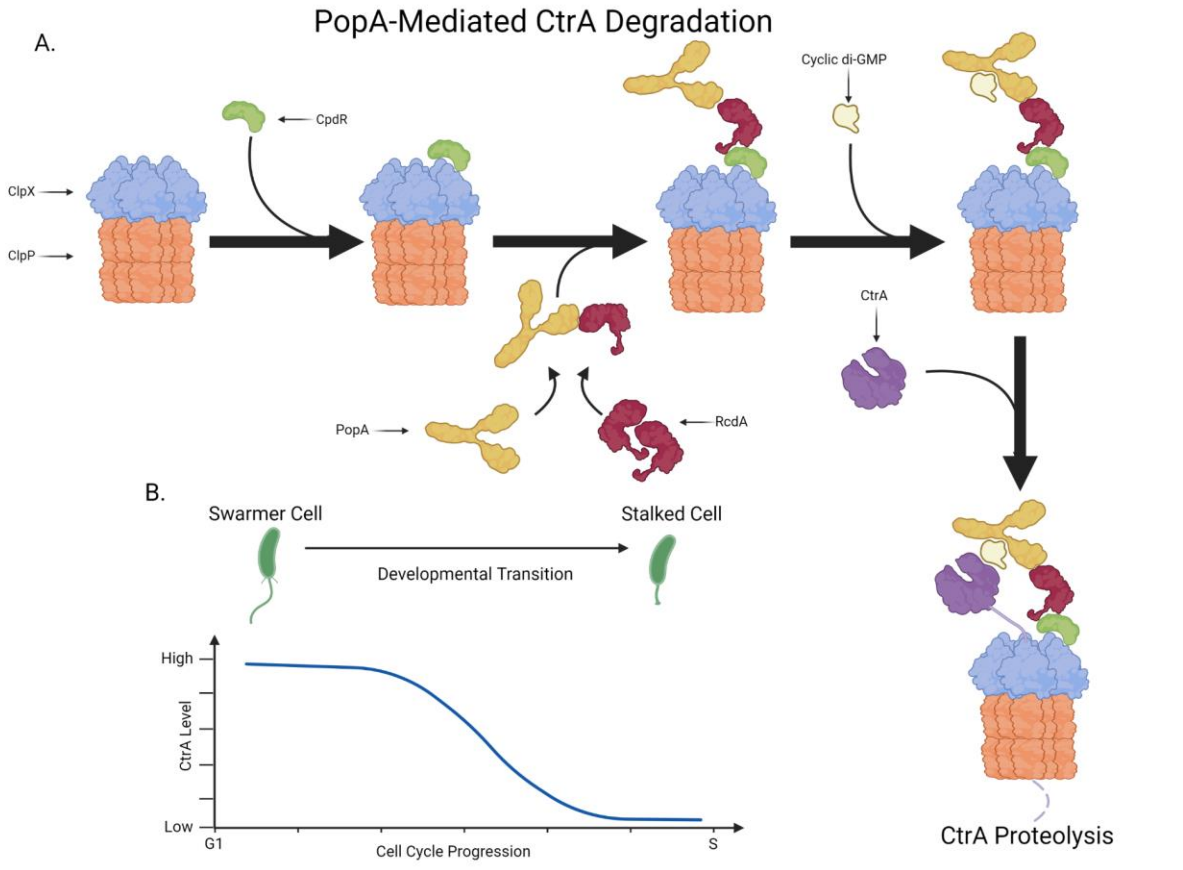

Figure 1.5: PopA-Mediated CtrA Degradation and Cell Cycle Progression

A) CtrA degradation in *Caulobacter* is regulated via a hierarchy of adaptors and small ligands. CpdR first primes ClpXP by binding to the N-terminal domain of ClpX. Then RcdA binds to CpdR via it's C-terminal tail. Then PopA binds to RcdA and, upon binding to CdG, targets and delivers CtrA. B) CtrA levels drive DNA replication and progression to S-phase in *Caulobacter*. As the G1 to S-phase transition occurs, CtrA is degraded.

1.5 Research Questions and Conclusions

The main questions this thesis was meant to answer are whether REC1 of PopA is indeed the binding interface between PopA and RcdA, and whether RcdA is acting as a passive scaffold in CtrA delivery or an active primer. While there is evidence that REC1 of PopA is involved in RcdA binding, preliminary data seemed to imply that Receiver Domain 2 (REC2) of PopA could be the binding surface (Nate Kuhlmann Thesis, UMass Amherst, 2021). If it is REC2 of PopA that is the interface for binding to RcdA, which residues are important for this interaction? Are all residues implicated in delivery of CtrA also responsible for binding to RcdA? The work presented in this thesis will highlight new mechanistic insights into CtrA delivery to ClpXP in a PopA-mediated manner.

1.6 Thesis Structure

The second chapter of this thesis will describe my work in elucidating the specific domain and residues responsible for PopA binding to RcdA and for CtrA delivery to the ClpXP protease. The third chapter will describe my work identifying if PopA is acting as a passive scaffold or an active primer. The fourth

chapter will discuss future directions to continue my work, as well as other work conducted during my master's degree that does not neatly fit into the story of my thesis, as well as lessons I've learned during my degree.

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CHAPTER 2

DOMAIN AND RESIDUES OF POPA RESPONSIBLE FOR RCDA BINDING AND CTRA DELIVERY

2.1 Abstract

The regulation of proteolysis in bacteria is accomplished, in part, by various adaptors that drive target specificity of the AAA+ proteases. In *Caulobacter crescentus*, the master cell-cycle regulator, CtrA, is degraded by ClpXP, and its specific targeting and delivery is driven by a hierarchy of adaptors. First, CpdR binds to the N-terminal domain of ClpX and primes ClpXP for RcdA binding. Next, when RcdA binds, PopA binds to RcdA. Finally, after the small ligand CdG binds to PopA, CtrA can finally be targeted and delivered to the protease. In previous work, the REC1 domain of PopA has been implicated in the binding surface location between PopA and RcdA. Here we show that it is the REC2 domain that is the location of the PopA/RcdA binding interface using computational predictions, HDX-MS, fluorescence polarization anisotropy binding assays, and in vitro degradation assays. We then create mutants where four residues on the REC2 domain are mutated and show that both mutants show an inhibition for binding to RcdA. Upon investigating one of the mutants further, we also confirm

that the mutant is deficient in its ability to deliver CtrA. Finally, we create single-residue PopA mutants using the same residues chosen before and show that some cause a binding deficiency while others do not. One of these mutants, while having no binding deficiency, fails to deliver CtrA, giving credence to the idea that RcdA may be imbuing an allosteric affect to PopA and PopA is acting as an active priming adaptor for CtrA delivery.

2.2 Introduction

This chapter describes our effort to elucidate the domain and then the residues of PopA necessary for RcdA binding and CtrA delivery. Previous work utilizing BacTH (**Bac**terial **T**wo **H**ybrid) assays has shown that the REC1 domain of PopA is the domain where the binding interface between PopA and RcdA is located (Ozaki et al., 2014). However, preliminary HDX-MS data (Nate Kuhlmann Thesis, UMass Amherst, 2021) has implicated the possibility that this binding surface exists on the REC2 domain. After a full analysis of the HDX-MS data, as well as analysis with Alphafold 2.0 Multimer (Figure 2.1) (Drake et al., 2022), there was agreement between those two methods, both pointing to REC2 of PopA binding RcdA. However, because the data was circumstantial, more direct assays were required to show REC2 binding to RcdA.

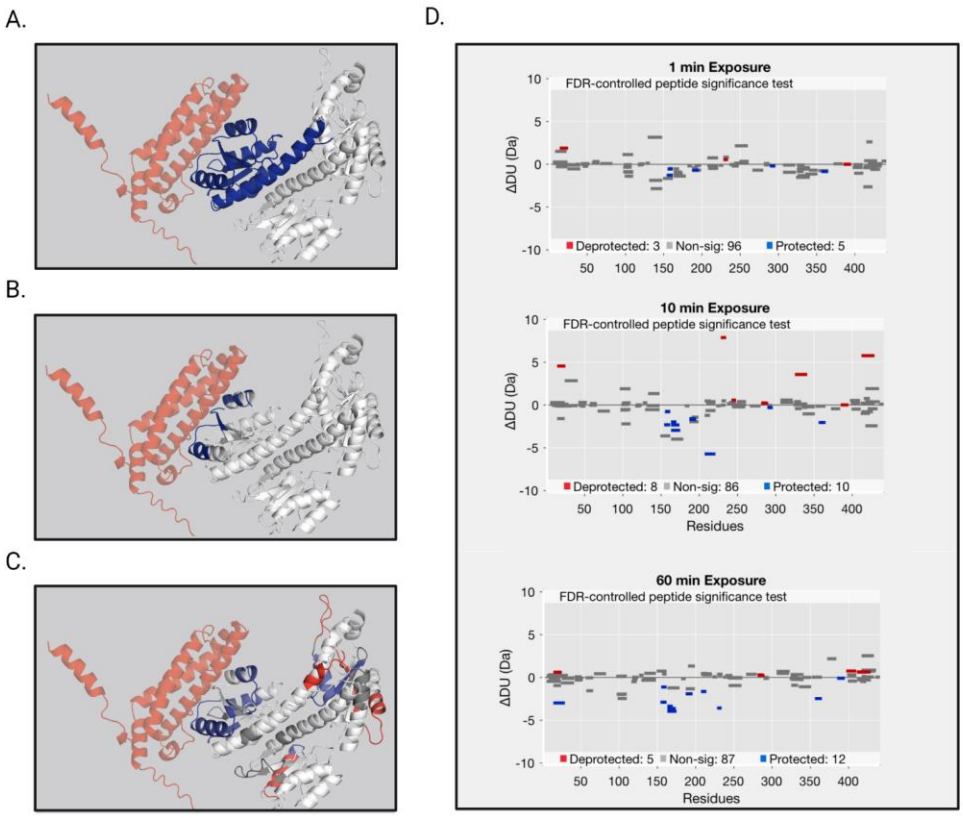


Figure 2.1: Structural Prediction and HDX Analysis of RcdA/PopA Complex

A) Alphafold 2.0 Multimer structural/binding prediction of RcdA (Translucent Red) and PopA (White/Blue). The REC2 domain of PopA is highlighted in blue while the rest of the protein is white. B) Alphafold 2.0 Multimer structural/binding prediction of RcdA (Translucent Red) and PopA (White/Blue). The region of PopA that is within 5 Angstroms of RcdA is highlighted in blue while the rest of the protein is white. C) Alphafold 2.0 Multimer structural/binding prediction of RcdA (Translucent Red) and PopA (White/Blue/Red/Grey). The blue regions of PopA are those showing more protection during HDX when RcdA is present after 60

minutes, the red regions less protection with RcdA present, the grey regions the same protection with or without RcdA, and the white regions no protection. D) Heat map showing relative protection of regions of PopA after 1, 10, and 60 minutes of HDX exposure. Blue regions have more protection with RcdA present, red regions have less protection with RcdA present, grey regions have similar protection with and without RcdA, and white regions have no protection.

2.3 REC2 Domain is Sufficient to Bind RcdA

We began our studies by cloning a truncated form of PopA that included only the REC2 domain (15.9kDa) (Ozaki et al., 2014) and using this construct to test binding compared to WT PopA (47kDa) using fluorescence polarization anisotropy (Figure 2.2). This showed apparent binding of the REC2 domain to fluorescently labeled RcdA L82E (F. L82E) (an RcdA mutant that lacks the ability to dimerize) with a 3.8-fold reduction in maximum polarization change (33mP for WT PopA to 8.5mP for REC2) corresponding to the ~3-fold decrease in size of the REC2 domain to full length PopA, and similar Kd (Kd=3.9 for WT PopA and Kd=2.1 for REC2).

We next tested the REC2 domain's ability to function as a replacement for WT PopA in the full adaptor complex for ClpXP degradation of CtrA using a GFP tagged CtrA reporter. (Figure 2.3) CtrA reporter levels were monitored for 10 minutes, and the maximum rate of degradation was analyzed for the different conditions. As expected, degradation rate of the GFP-CtrA reporter was about 2x the rate with the full WT Adaptor complex than without. Degradation rate with REC2 in replacement of WT PopA in the full adaptor complex didn't show a significant change from degradation without the full adaptor complex, indicating that REC2 wasn't functioning like WT PopA.

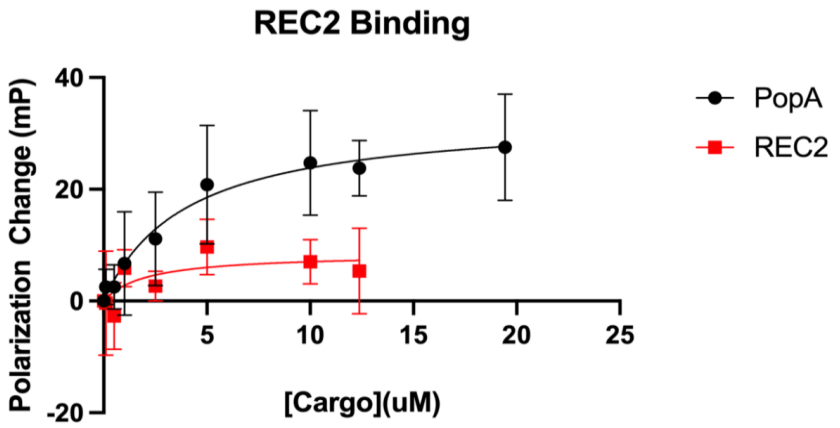
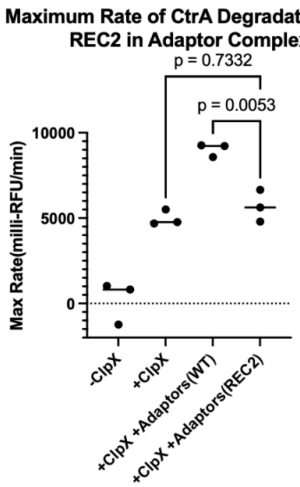


Figure 2.2: REC2 Binding

Fluorescence polarization anisotropy showing REC2 binding to RcdA L82E. REC2 was titrated at increasing concentrations to show increasing polarization value, indicating binding to the fluorescently tagged RcdA L82E.

Knowing that REC2 was not a functional PopA replacement, REC2 binding to RcdA was verified in degradation assays where REC2 was used to compete off WT PopA in the full Adaptor complex (Figure 2.4). With REC2 in excess (4.25uM), PopA is successfully competed off and the GFP-CtrA reporter's degradation rate returns to a rate not significantly different from the rate of degradation without the full adaptor complex (Figure 2.4, A.). Notably, including excess REC2 without the adaptor complex did not inhibit reporter degradation, indicating the REC2 is inhibiting degradation via acting on the adaptor complex. Titrating in REC2 showed the expected result if REC2 was indeed binding with RcdA and competing off WT PopA (Figure 2.4, B.)

A.



B.

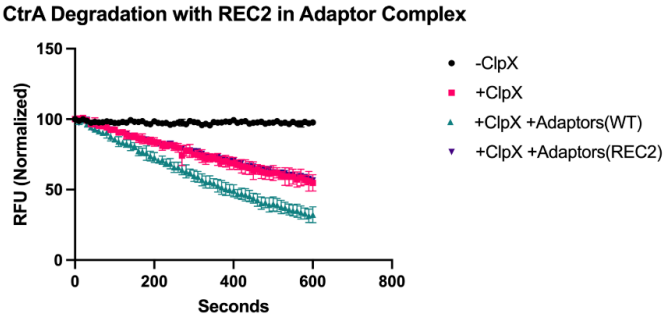


Figure 2.3: CtrA Degradation with REC2 in Adaptor Complex

A) Maximum rate of CtrA reporter degradation without the addition of ClpX; in the presence of ClpX; in the presence of ClpX and the full WT adaptor complex; and the full adaptor complex with REC2 in place of WT PopA. B) Normalized CtrA reporter degradation over 10 minutes without the addition of ClpX; in the presence of ClpX; in the presence of ClpX and the full WT adaptor complex; and the full adaptor complex with REC2 in place of WT PopA.

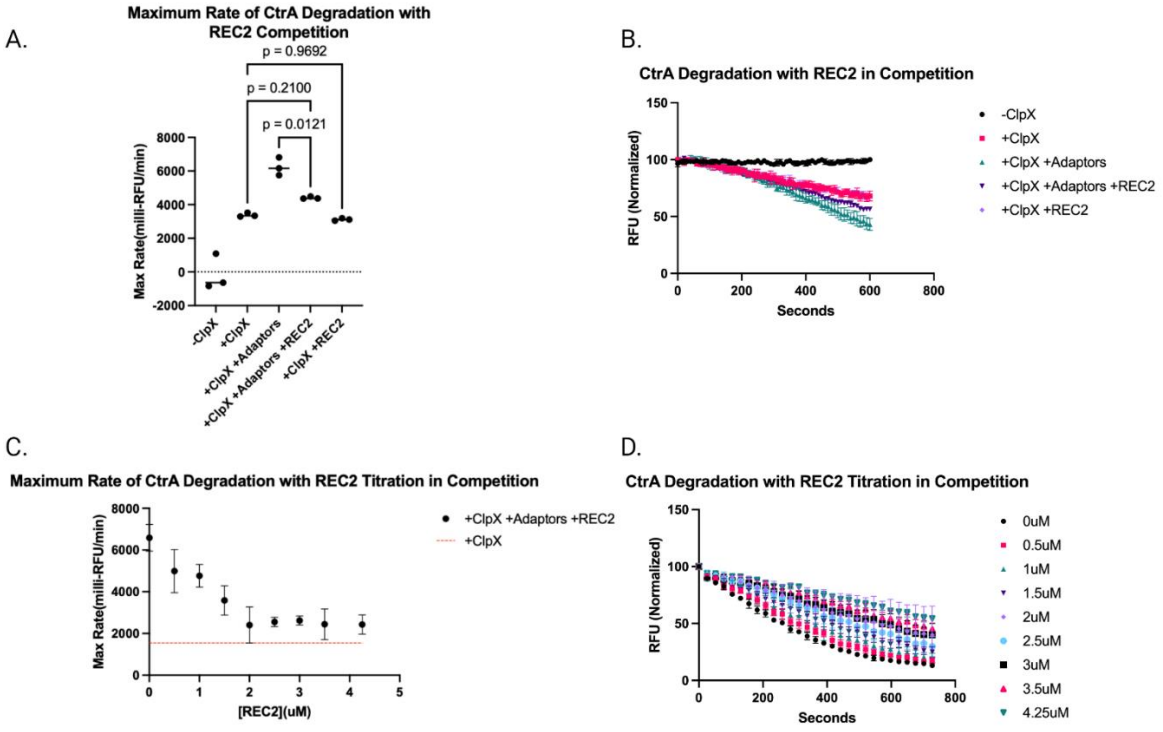


Figure 2.4: CtrA Degradation with REC2 Competing with PopA

A) Maximum rate of GFP-CtrA reporter degradation without ClpX; with ClpX; with ClpX and the full WT Adaptor complex; with ClpX, the full WT adaptor complex and excess REC2 (4.25uM); and with ClpX and excess REC2 (4.25uM). B) Normalized GFP-CtrA reporter degradation over 10 minutes without ClpX, with ClpX, with ClpX and the full WT Adaptor complex, with ClpX, the full WT adaptor complex and excess REC2 (4.25uM), and with ClpX and excess REC2 (4.25uM). C) Maximum rate of GFP-CtrA reporter degradation with increasing concentrations

of REC2 competing with the full WT adaptor complex. D) Normalized GFP-CtrA reporter degradation over 12 minutes with increasing concentrations of REC2 competing with the full WT adaptor complex.

2.4 PopA Four-Mutants Fail to Bind RcdA and Deliver CtrA

Upon confirmation that, indeed, the REC2 domain of PopA is where the binding interface between PopA and RcdA is located, the Alphafold 2.0 Multimer binding predictions were further probed to elucidate which residues might possibly be involved in RcdA binding and CtrA delivery. Using the computational predictions, four residues of PopA were chosen for their proximity to protected regions based on the HDX-MS data, and their predicted interactions from the computational model. PopA residue F180 was predicted to interact hydrophobically with RcdA residues L74 and M75, PopA residue T184 was predicted to interact with RcdA residue D45 via a polar-charge noncovalent-interaction, PopA residue D187 was predicted to interact with RcdA residue R49 via the formation of a salt-bridge, and PopA residue Y188 was predicted to interact hydrophobically with RcdA residue M68 (Figure 2.5). Interestingly, R49 of RcdA has already been implicated in PopA binding and CtrA delivery with the creation of the RcdA3E mutant where R49, K51, and R53 are all mutated to Glutamic Acid. (Kuhlmann et al., 2021).

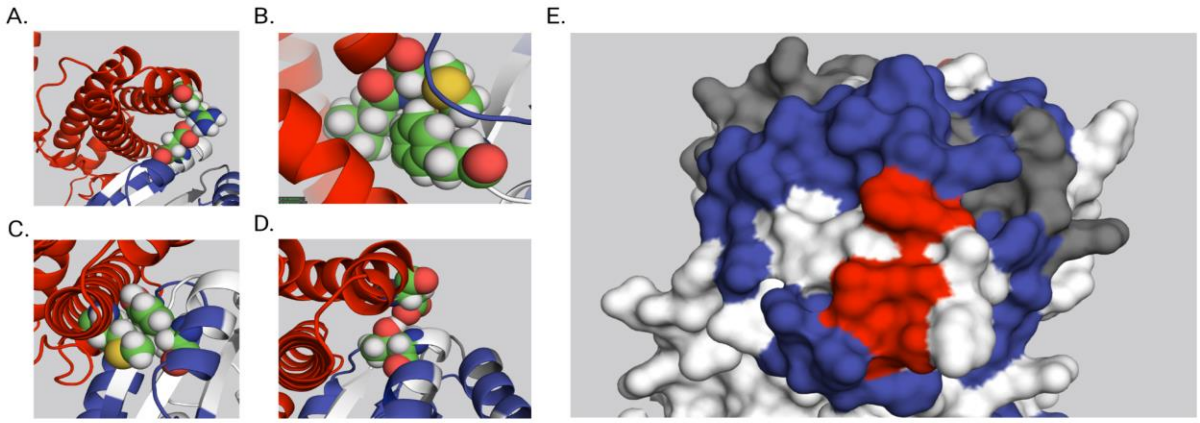


Figure 2.5: Residue Interaction Predictions

A) Alphafold 2.0 Multimer structural prediction showing D187 of PopA forming a salt-bridge with R49 of RcdA. B) Alphafold 2.0 Multimer structural prediction showing F180 of PopA forming a hydrophobically interacting with with L74 and M75 of RcdA. C) Alphafold 2.0 Multimer structural prediction showing Y188 of PopA forming a hydrophobic interaction with M68 of RcdA. D) Alphafold 2.0 Multimer structural prediction showing T184 of PopA forming a polar-charge interaction with D45 of RcdA. E) Alphafold 2.0 structural prediction of Receiver

Domain 2 of PopA with HDX coverage in blue and F180, T184, D187, and Y188 in red.

Using these predictions, two separate mutants were created where all four of these residues were mutated. The first, PopA4A, is a mutant PopA with F180A, T184A, D187A, and Y188A mutations. The second was a separate four-mutant that we called the PopA Combo Mutant with F180S, T184D, D187K, and Y188S mutations. Binding of both mutants was tested using fluorescence polarization anisotropy assays (Figure 2.6). In the case of both mutants, binding was totally inhibited compared to WT PopA with no measurable change in polarization at any concentration of either mutant.

We next focused on the PopA4A mutant and tested whether it could act as a functional replacement for WT PopA in the full adaptor complex for CtrA degradation (Figure 2.7). When compared to degradation with and without adaptors, GFP-CtrA reporter degradation in the presence of the adaptor complex with PopA4A replacing WT PopA saw degradation that was not significantly different from degradation without the full WT adaptor complex, indicating that PopA4A cannot functionally replace WT PopA and agreeing with our observation that PopA4A does not bind RcdA.

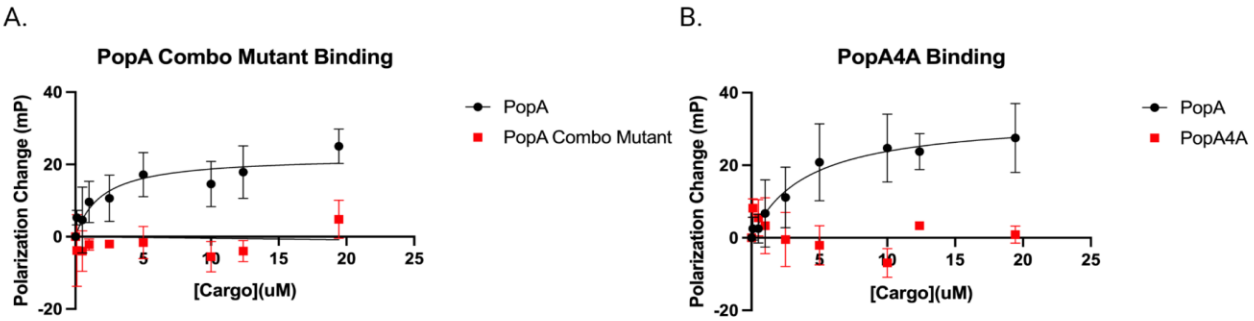


Figure 2.6: PopA Four-Mutant Binding

A) Fluorescence polarization anisotropy showing PopA Combo Mutant binding to RcdA L82E. PopA Combo Mutant was titrated at increasing concentrations to show no increasing polarization value, indicating no binding to the fluorescently tagged RcdA L82E. B) Fluorescence polarization anisotropy showing PopA4A binding to RcdA L82E. PopA4A was titrated at increasing concentrations to show no increasing polarization value, indicating no binding to the fluorescently tagged RcdA L82E.

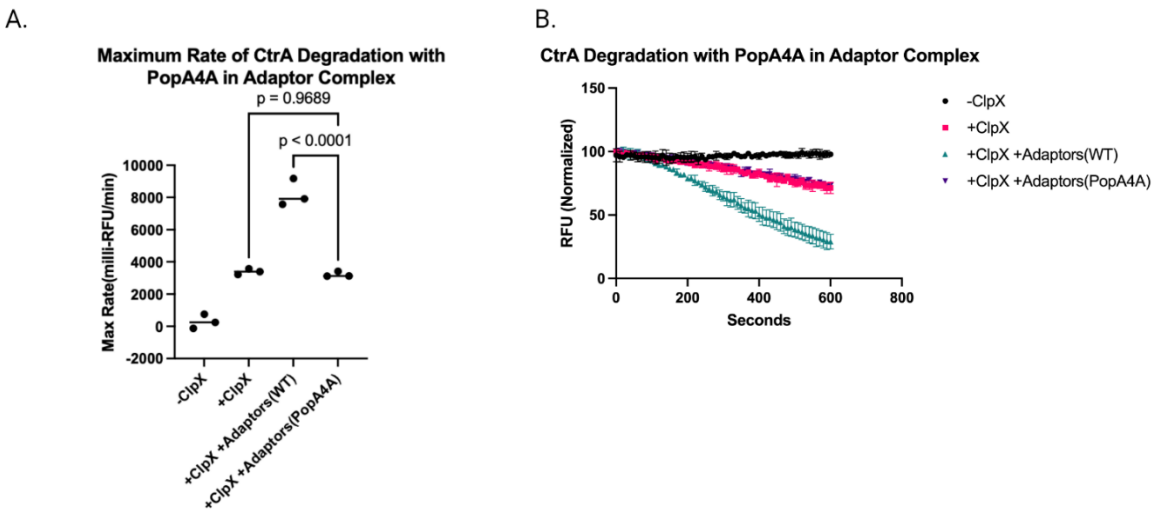


Figure 2.7: CtrA Degradation with PopA4A in Adaptor Complex

A) Maximum rate of CtrA reporter degradation without the addition of ClpX; in the presence of ClpX; in the presence of ClpX and the full WT adaptor complex; and the full adaptor complex with PopA4A in place of WT PopA. B) Normalized CtrA reporter degradation over 10 minutes without the addition of ClpX; in the presence of ClpX; in the presence of ClpX and the full WT adaptor complex; and the full adaptor complex with PopA4A in place of WT PopA.

Seeing that PopA4A does not apparently bind and does not function as a replacement for WT PopA, we next conducted a CtrA degradation assay with excess (4.25uM) PopA4A in competition with WT PopA in the full adaptor complex (Figure 2.8). When compared to GFP-CtrA reporter degradation with and without the presence of the full WT adaptor complex, the addition of excess (4.25uM) PopA4A did not significantly change the degradation rate from the rate where the full WT adaptor complex was present. This agrees with our previous observation that PopA4A has a significant binding deficiency to RcdA.

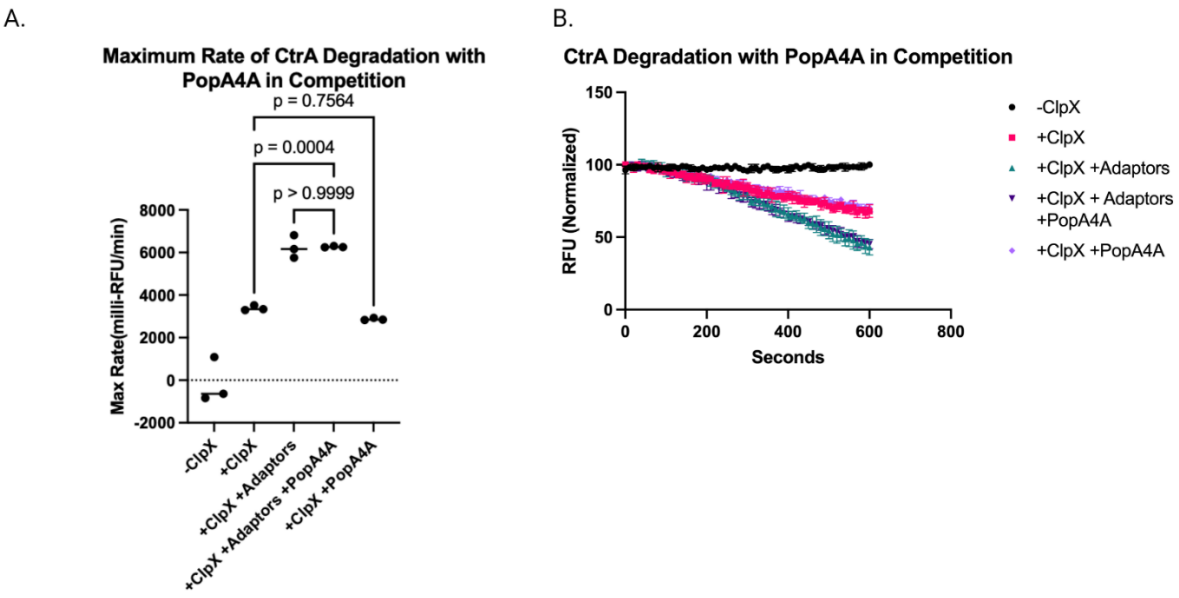


Figure 2.8: CtrA Degradation with PopA4A Competing with PopA

A) Maximum rate of GFP-CtrA reporter degradation without ClpX; with ClpX; with ClpX and the full WT Adaptor complex; with ClpX, the full WT adaptor complex and excess PopA4A (4.25uM); and with ClpX and excess PopA4A (4.25uM). B) Normalized GFP-CtrA reporter degradation over 10 minutes without ClpX; with ClpX; with ClpX and the full WT Adaptor complex; with ClpX, the full WT adaptor complex and excess PopA4A (4.25uM); and with ClpX and excess PopA4A (4.25uM).

2.5 PopA Single-Mutant Binds RcdA but Fails to Deliver CtrA

After successfully narrowing down PopA binding to RcdA and delivery of CtrA to four residues, single-mutants at each residue were created to match the four mutations in the PopA Combo Mutant, F180S, T184D, D187K, and Y188S. Unfortunately, while the cloning and sequencing of the Y188S mutant was successful, it was never able to be expressed and purified successfully. The other three mutants were tested for RcdA binding with Fluorescence polarization anisotropy in comparison with WT PopA (Figure 2.9). The T184D mutant and the D187K mutant both did not demonstrate any binding defects (D187K Kd: 1.4uM, T184D Kd: 1.7uM) however the F180S mutant saw an increase to it's Kd over the WT PopA control of over 3-fold (PopA Kd: 6.9uM, F180S Kd: 22.2uM) indicating a binding deficiency in the F180S mutant.

Despite the D187K mutant showing no binding defect, because it's computational binding prediction implicated R49 from the RcdA3E mutant in its interactions, D187K was further tested to see if it could replace WT PopA in the full adaptor complex in CtrA degradation (Figure 2.10). These results were extremely interesting, as they showed that despite showing no binding defect, D187K could not deliver the GFP-CtrA reporter for degradation any better than if no WT adaptor complex was present at all.

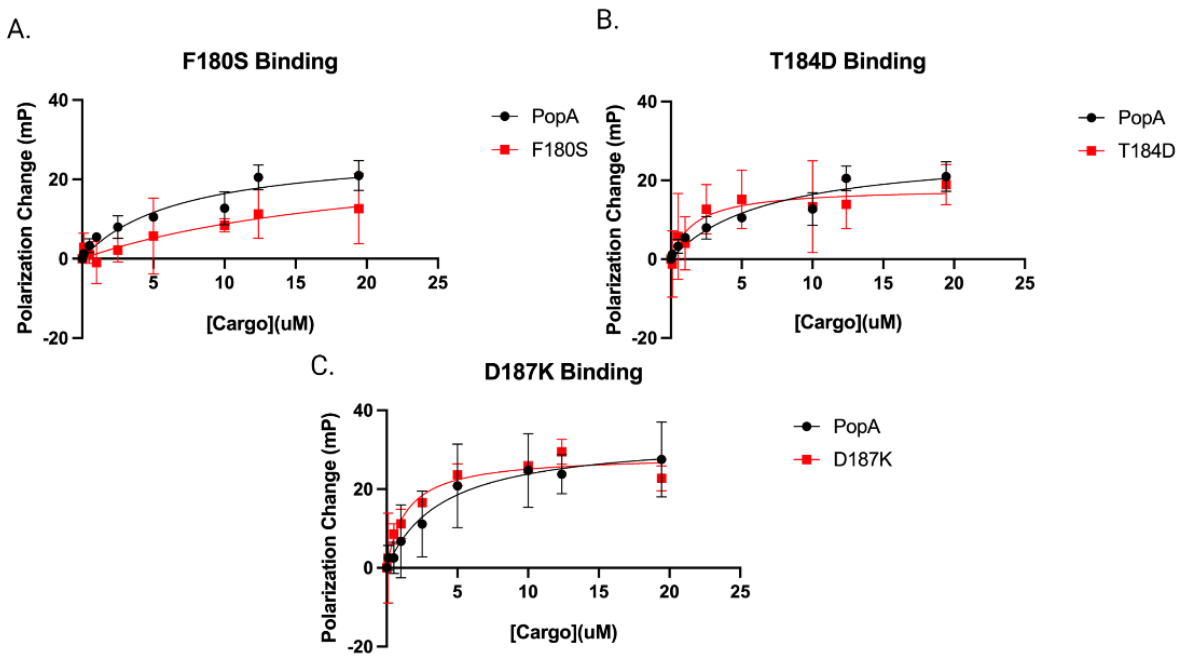


Figure 2.9: PopA Single-Mutant Binding

A) Fluorescence polarization anisotropy showing F180S binding to RcdA L82E. F180S was titrated at increasing concentrations to show a shallower increasing polarization value, indicating inhibited binding to the fluorescently tagged RcdA L82E. B) Fluorescence polarization anisotropy showing T184D binding to RcdA L82E. T184D was titrated at increasing concentrations to show no increasing polarization value, indicating no binding to the fluorescently tagged

RcdA L82E. C) Fluorescence polarization anisotropy showing D187K binding to RcdA L82E. D187K was titrated at increasing concentrations to show no increasing polarization value, indicating no binding to the fluorescently tagged RcdA L82E.

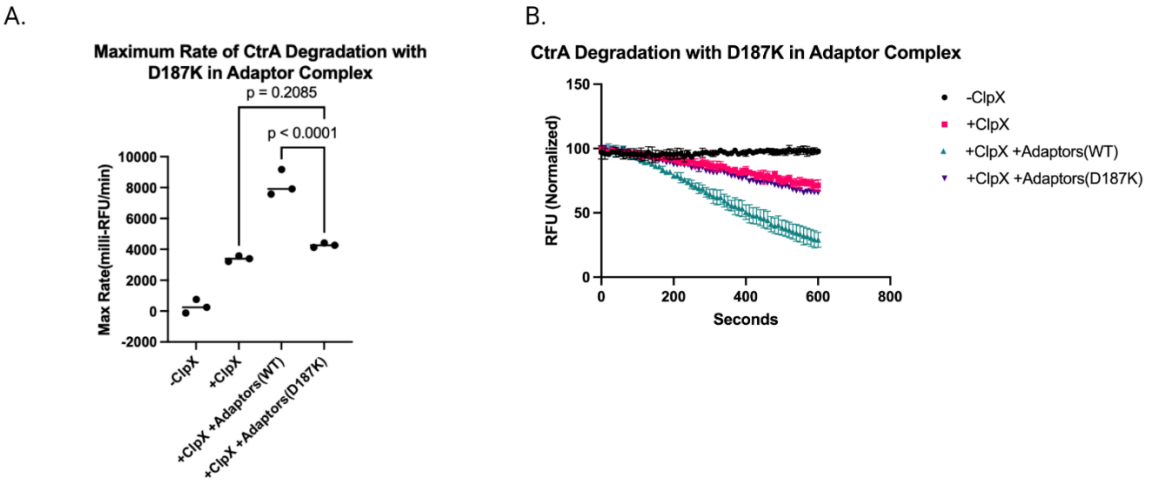


Figure 2.10: CtrA Degradation with D187K in Adaptor Complex

A) Maximum rate of CtrA reporter degradation without the addition of ClpX; in the presence of ClpX; in the presence of ClpX and the full WT adaptor complex; and the full adaptor complex with D187K in place of WT PopA. B) Normalized CtrA reporter degradation over 10 minutes without the addition of ClpX; in the presence of ClpX; in the presence of ClpX and the full WT adaptor complex; and the full adaptor complex with D187K in place of WT PopA.

Finally, because of D187K's apparent ability to bind RcdA but it's inability to deliver CtrA, a degradation assay was performed with D187K in excess (4.25uM) in the presence of the full WT adaptor complex (Figure 2.11). The results of this show that when an excess of D187K is in the presence of the full WT adaptor complex, WT PopA is competed off and the degradation rate drops to a rate not significantly different from the rate of CtrA degradation without the adaptor complex. Performing the same assay with increasing concentrations of D187K also shows the expected behavior of processively slower maximum rate of degradation of CtrA with the full adaptor complex until the rate reaches the same rate as without the adaptor complex.

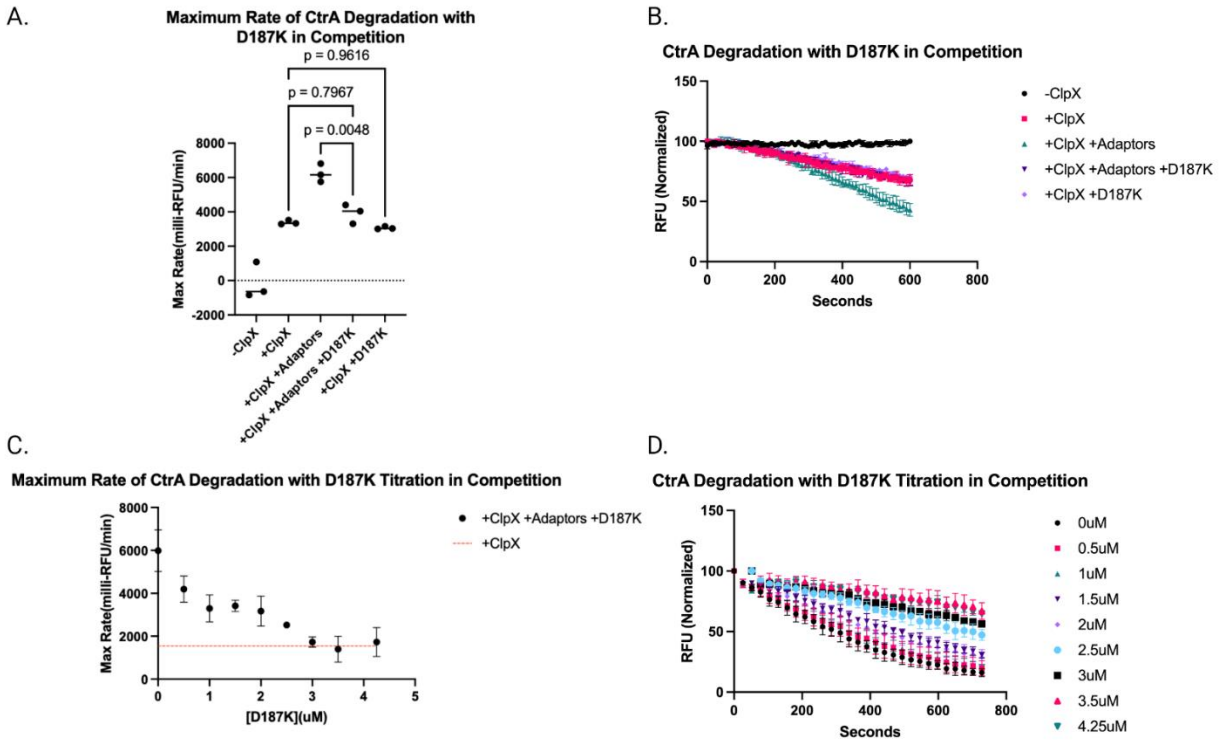


Figure 2.11: CtrA Degradation with D187K in Competition with PopA

A) Maximum rate of GFP-CtrA reporter degradation without ClpX; with ClpX; with ClpX and the full WT Adaptor complex; with ClpX, the full WT adaptor complex and excess D187K (4.25uM); and with ClpX and excess D187K (4.25uM). B) Normalized GFP-CtrA reporter degradation over 10 minutes without ClpX, with ClpX, with ClpX and the full WT Adaptor complex, with ClpX, the full WT adaptor complex and excess D187K (4.25uM), and with ClpX and excess D187K (4.25uM). C) Maximum rate of GFP-CtrA reporter degradation with increasing

concentrations of D187K competing with the full WT adaptor complex. D) Normalized GFP-CtrA reporter degradation over 12 minutes with increasing concentrations of D187K competing with the full WT adaptor complex.

2.6 Discussion

The results of the experiments outlined in this chapter clearly show that it is the REC2 domain of PopA where the binding surface between PopA and RcdA is located. The HDX-MS data and the computational predictions both agree that the binding surface of PopA is located at approximately residues 150-200, lining up with the main fold of REC2. The fluorescence polarization binding data as well as the degradation competition data agree that REC2 is binding to RcdA. When residues were chosen from that 150-200 protected region of PopA that were predicted to interact with specific residues of RcdA by Alphafold 2.0 Multimer to create four-mutants, those PopA mutants could neither bind RcdA, nor deliver CtrA and would not compete with WT PopA in the adaptor complex. Finally, while the F180 was implicated in partially contributing to the inability of PopA4A and PopA Combo Mutant to bind RcdA, the D187K mutant was arguably the most interesting. This is because it had no defect in binding RcdA, but it could not deliver CtrA any better when used in the adaptor complex in place of WT PopA than if no adaptor complex was present. Along with the prediction of RcdA residue R49's interaction with D187 of PopA and RcdA3E's inability to bind PopA or deliver CtrA, this finding implies that it might be possible that RcdA is not only

acting as a scaffolding but is allosterically priming PopA to be able to deliver CtrA via its interaction with D187 of PopA.

2.7 Materials and Experimental Procedures

Protein Expression and Purification. BL21(DE3) pLYS cells containing pET23b and pET28b expression plasmids for diverse proteins were cultivated at a temperature of 37^{o}C until reaching an optical density (OD600) of 0.6-0.8. Subsequently, they were induced with 0.4mM of IPTG, either for a duration of 4 hours at 37^{o}C or overnight at 30^{o}C. The induced cells were subjected to centrifugation at 7,000xg for 8 minutes and then resuspended in a lysis buffer composed of 50mM Tris (pH 8.0), 300mM NaCl, 10mM imidazole, 10% glycerol, 5mM BME, and 1mM PMSF. This cell suspension was frozen at -80^{o}C until the purification process. Subsequently, the cells were thawed and lysed using a Microfluidizer system from Microfluidics (Newton, MA). The resulting lysate was then subjected to centrifugation at 15,000rpm for 30 minutes, and the supernatant was collected. The supernatant was passed through a Ni-NTA column for affinity purification. H6SUMO-tagged proteins were cleaved using Ulp1-his protease.

Cloning and Molecular Biology. REC2 was obtained through the process of cloning using the Gibson assembly method. Different variants of PopA were obtained by employing the around-the-horn site-directed mutagenesis technique, which involved amplifying the target plasmid using pET23bH6SUMO-PopA as a reference template. The accuracy of the PopA mutants was verified by Plasmidsaurus through sequencing.

Fluorescence Polarization and Maleimide Labeling. Purified RcdA L82E protein was subjected to labeling using Fluorescin-5-Maleimide (Thermo Scientific™). The purified protein, at a concentration of approximately 8-10mg/mL, was buffer exchanged into a labeling buffer containing 50mM HEPES (pH 7.0), 150mM NaCl, and 2mM TCEP. Fluorescin-5-Maleimide, dissolved in DMSO, was added to the protein at a 20-fold molar excess to cysteine. The labeling process was carried out overnight at a temperature of 4^{o}C. To remove any unbound dye, a buffer exchange was performed using Snakeskin Dialysis Tubing (Thermo Scientific™), with the protein being transferred into a solution containing 20mM HEPES (pH 8.5), 100mM KCl, 10mM MgCl2, and 0.1% Tween. The labeled protein was divided into smaller portions and frozen at -80^{o}C for storage.

Fluorescence polarization binding assays were conducted using 100nM of F5M-labeled L82E protein and varying concentrations of the cargo. The binding reaction was incubated at a temperature of 30^{o}C for 30 minutes to establish equilibrium. Polarization measurements were taken from a 40uL mixture using opaque black 384-well plates and a SpectraMax M5 plate reader (Molecular Devices). The excitation and emission wavelengths were set at 460 and 540, respectively. The binding constants were determined by fitting the polarization data using GraphPad Prism software, utilizing a one-site total and nonspecific binding equation: P = Pmax*[X]/([X] + Kd) + NS*[X] + Background. In this equation, Pmax represents 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 refers to the polarization value when [X] is 0. Error bars were calculated based on the maximum and minimum values obtained from replicates of the experiments.

HDX-MS Analysis. Raw HDX data was acquired, and the peptides were identified and analyzed for uptake plots and charge states using Protein Lynx Global Server (PLGS) and DynamX software (Waters). Differential uptake heatmaps and uptake plots were generated using Deuteros. The PopA structure's surface was rendered

in PyMol (Schrodinger), and depicted regions with protection levels exceeding 15%

***In vitro* Degradation Assays.** Degradation of the GFP-CtrA reporter (GFP-CtrA-RD+15, the Receiver domain of CtrA plus 15 residues tagged to GFP) was monitored with the loss of fluorescence over time. In the reaction mixture, ClpX concentration was 0.4uM, ClpP concentration was 0.8uM, 1x ATP regeneration mix (4mM ATP, 16mM creatine phosphate, 0.32mg/mL creatine kinase), and 2uM of GFP-CtrA-RD+15. When adaptors are present, 1uM of CpdR, RcdA, and PopA (or PopA Mutant) were included as well as 20uM of CdG to a final volume of 20uL. Fluorescence was measured with a SpectraMax M5 plate reader (Molecular Devices) with excitation and emission wavelengths at 488nm and 510nm respectively. Maximum rate of fluorescence loss was calculated in the SpectraMax software using reduction criteria that created a best-fit line using 20 fluorescence readings from 0-600 second.

Bacterial Strains Used in This Study:

Organism	Name	Description	Source
E. coli	TOP10	Cloning Strain	Invitrogen
	BL21(DE3) pLysS	Recombinant protein expression	Invitrogen
	EPC112	BL21DE3 pLysS pET23b Ulp1his protease	(Kuhlman, 2021)
	EPC162	BL21DE3 375 eGFP-His6-CtrARD+15	(Smith et al., 2014)
	EPC1037	BL21DE3 pLysS pet23bh6SUMO-PopA	(Smith et al., 2014)
	EPC1626	BL21 pLYS RcdA L82E	(Kuhlman, 2021)
	EPC1751	Top10 pET23b-h6-SUMO REC2 Domain (PopA)	This Study
	EPC1754	Top10 pET23b-h6-SUMO PopA F180S	This Study
	EPC1755	Top10 pET23b-h6-SUMO PopA T184D	This Study
	EPC1756	Top10 pET23b-h6-SUMO PopA D187K	This Study
	EPC1757	Top10 pET23b-h6-SUMO PopA4A (F180A, T184A, D187A, Y188A)	This Study
	EPC1758	Top10 pET23b-h6-SUMO PopA Combo Mutant(F180S, T184D, D187K, Y188S)	This Study
	EPC1759	Top10 pET23b-h6-SUMO PopA Y188S	This Study

2.8 Acknowledgements

I would like to acknowledge the contributions to this portion of the thesis by Nathan Kuhlman, who provided the raw HDX-MS data that was analyzed that pointed to PopA's REC2 binding to RcdA. The entire body of work rests on this preliminary data.

I would also like to thank Alex Martinez, who helped perform experiments in my last month in the lab. His fast ability to learn where the research was and where it is going is a testament to his ability as a scientist.

I would also like to thank my advisor, Dr. Peter Chien, for his guidance and encouragement to try my own experiments. Without his gentle but firm nudges, I may not have discovered the specific PopA residues that seem so important for RcdA binding and CtrA delivery.

I would finally like to thank Kim Barker and Justyne Oghdal for their help in fine-tuning assays, so the data is clear and understandable.

2.9 References

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4. Kuhlmann NJ, Doxsey D, Chien P. Cargo competition for a dimerization interface restricts and stabilizes a bacterial protease adaptor. Proc Natl Acad Sci U S A. 2021 Apr 27;118(17):e2010523118. doi: 10.1073/pnas.2010523118. PMID: 33875581; PMCID: PMC8092595.

CHAPTER 3

POPA SCAFFOLDING MUTANT SHOWS NECESSITY OF RCDA BINDING FOR CTRA DELIVERY

3.1 Abstract

Bacterial AAA+ proteolysis is regulated by protein adaptors that drive specificity in substrate targeting. In *Caulobacter crescentus*, the master cell-cycle regulator and replication inhibitor CtrA is degraded by ClpXP with the aid of three adaptors processively binding to each other, CpdR, RcdA, and PopA. Elucidation of RcdA's regulatory role in the adaptor hierarchy has not currently been fully understood. Using the SspB-tail known as the XB motif that is recognized by the zinc binding domain of ClpX, we created two PopA mutants that can theoretically bind ClpX without the need for CpdR and RcdA. Using these mutants, we show that, by themselves and in the presence of CdG, they cannot target and deliver CtrA. However, one of the mutants shows a significantly increased degradation rate in the presence of RcdA, indicating that the RcdA-PopA complex may be necessary for CtrA delivery.

3.2 Introduction

This chapter describes our work in attempting to elucidate if RcdA and CpdR are mearly acting as scaffolds for PopA to target and deliver CtrA, or if the

RcdA-PopA interface actively primes PopA for CtrA delivery. Similar research has been done fusing the XB motif of SspB to the C-terminal tail of RcdA and it was shown to be able to deliver substrate for degradation without the need for CpdR. (Joshi et al., 2015). In this study we append the XB motif both N-terminally and C-terminally to PopA (Figure 3.1) and test both to see if they can bind RcdA, if they can replace WT PopA in the adaptor hierarchy, and if they can deliver CtrA by themselves in the presence of CdG or if they need to bind RcdA for CtrA delivery.

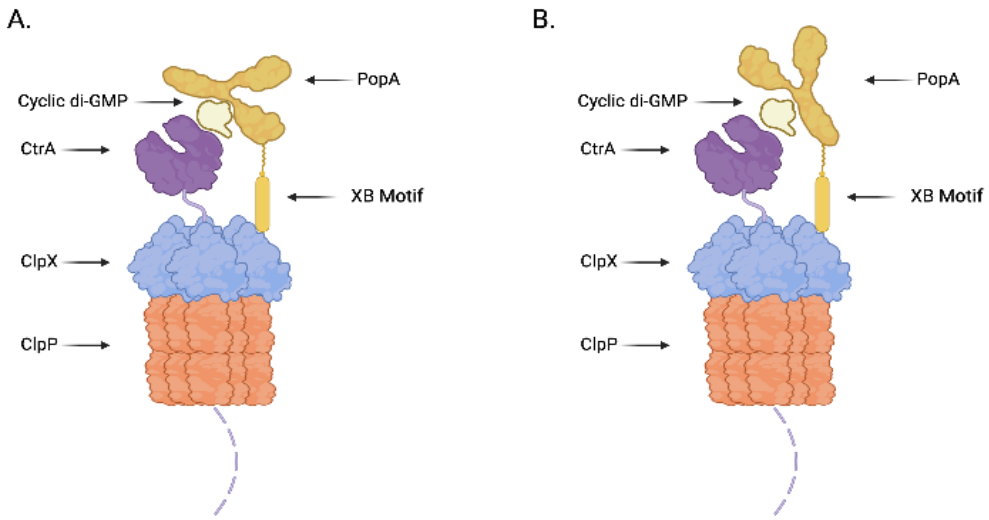

Figure 3.1: PopA XB Mutant Diagram

A) An illustration of the Sspb-tail (XB motif) C-terminally appended onto PopA (PopA-XB), relieving the requirement of CpdR and RcdA in the adaptor hierarchy. B) An illustration of the Sspb-tail (XB motif) N-terminally appended onto PopA (XB-PopA), relieving the requirement of CpdR and RcdA in the adaptor hierarchy.

3.3 Functional PopA XB Mutant Requires RcdA to Deliver CtrA

Direct RcdA binding was first tested with the PopA mutants with the N-terminally and C-terminally linked XB motif (henseforth known as XB-PopA and PopA-XB respectively) to test if they were properly folded (Figure 3.2). In the case of both, the binding curve appeared shallower, however at higher concentrations the change in polarization was like WT PopA. This could mean that there is a binding defect in the mutants, or that a population of the mutants in the sample is misfolded. Either way, both mutants appear to at least partially be able to bind RcdA.

Next, both XB mutants were tested in degradation assays to see if they could replace WT PopA in the full adaptor complex (Figure 3.3). Unexpectedly, neither mutant could deliver CtrA for degradation significantly faster than CtrA degradation without the adaptor complex.

Additionally, to confirm RcdA binding, GFP-CtrA reporter degradation assays were performed with both XB mutants titrated to excess (0uM-4.5uM) to verify that if they will not behave like WT PopA but bind RcdA, that they will compete off WT PopA and inhibit degradation (Figure 3.4). While the amount required to compete off WT PopA was greater for both XB mutants than the other mutants tried, it was still able to compete off WT PopA.

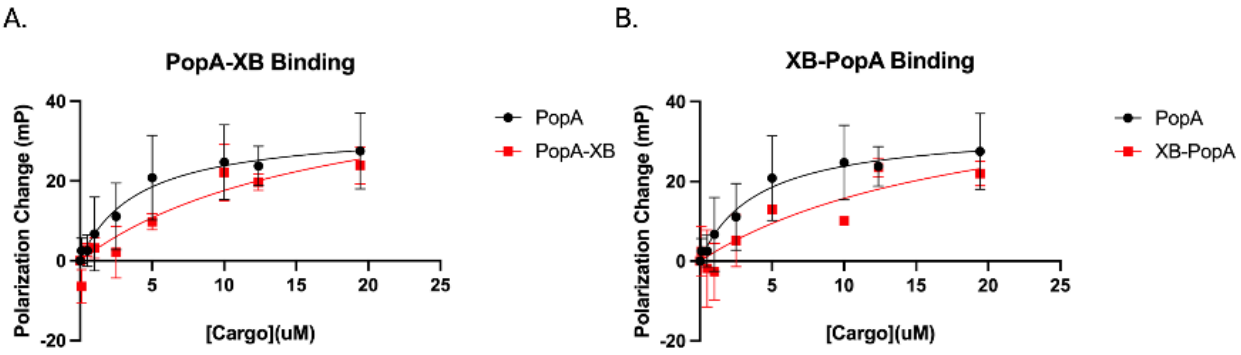

Figure 3.2: PopA XB Mutant Binding

A) Fluorescence polarization anisotropy showing PopA-XB binding to RcdA L82E. PopA-XB was titrated at increasing concentrations to show a shallower increasing polarization value, indicating inhibited binding to the fluorescently tagged RcdA L82E. A) Fluorescence polarization anisotropy showing XB-PopA binding to RcdA L82E. XB-PopA was titrated at increasing

concentrations to show a shallower increasing polarization value, indicating inhibited binding to the fluorescently tagged RcdA L82E.

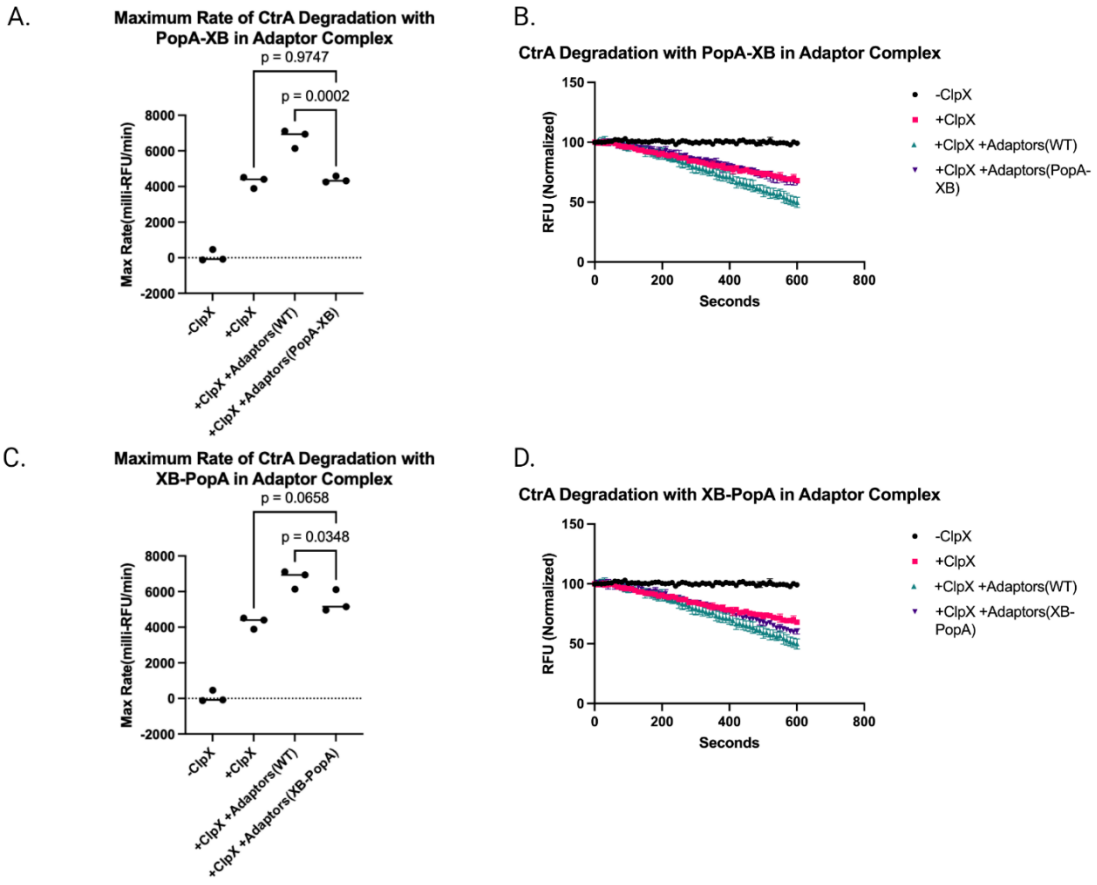

Figure 3.3: CtrA Degradation with PopA XB Mutants in Adaptor Complex

A) Maximum rate of CtrA reporter degradation without the addition of ClpX; in the presence of ClpX; in the presence of ClpX and the full WT adaptor complex; and the full adaptor complex with PopA-XB in place of WT PopA. B) Normalized CtrA reporter degradation over 10 minutes without the addition of ClpX; in the presence of ClpX; in the presence of ClpX and the full WT adaptor complex; and the full adaptor complex with PopA-XB in place of WT PopA. C) Maximum rate of CtrA reporter degradation without the addition of ClpX; in the presence of ClpX; in

the presence of ClpX and the full WT adaptor complex; and the full adaptor complex with XB-PopA in place of WT PopA. D) Normalized CtrA reporter degradation over 10 minutes without the addition of ClpX; in the presence of ClpX; in the presence of ClpX and the full WT adaptor complex; and the full adaptor complex with XB-PopA in place of WT PopA.

Finally, GFP-CtrA reporter degradation assays were performed to test if the XP PopA mutants and RcdA alone without CpdR would cause an increased rate of degradation like the full adaptor complex (Figure 3.5). The assays were performed with the addition of RcdA and each XB mutant alone alongside the XB and RcdA replicates. While the addition of RcdA, XB-PopA, PopA-XB, and XB-PopA with RcdA did not significantly change the degradation rate of the CtrA reporter, PopA-XB with RcdA did significantly increase degradation rate, however it was still significantly slower than the degradation rate of CtrA in the presence of the full WT adaptor complex.

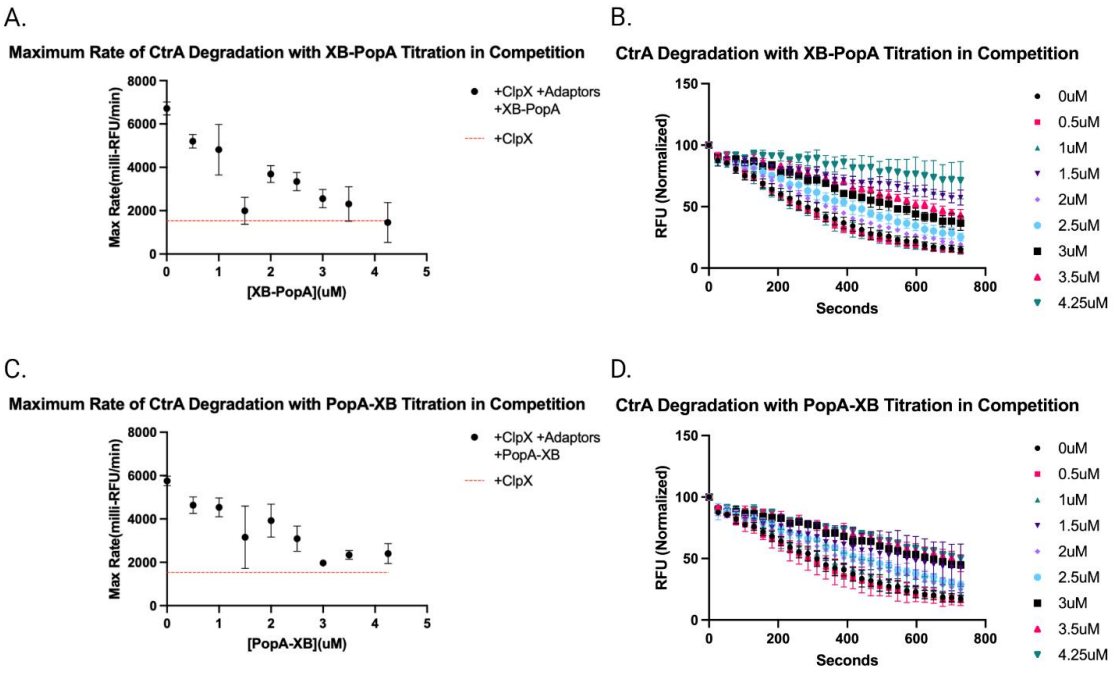

Figure 3.4: CtrA Degradation with PopA XB Mutants Competing with PopA

A) Maximum rate of GFP-CtrA reporter degradation with increasing concentrations of PopA-XB competing with the full WT adaptor complex. B) Normalized GFP-CtrA reporter degradation over 12 minutes with increasing concentrations of PopA-XB competing with the full WT adaptor complex. C) Maximum rate of GFP-CtrA reporter degradation with increasing concentrations of XB-PopA competing with the full WT adaptor complex. D) Normalized GFP-CtrA

reporter degradation over 12 minutes with increasing concentrations of XB-PopA competing with the full WT adaptor complex.

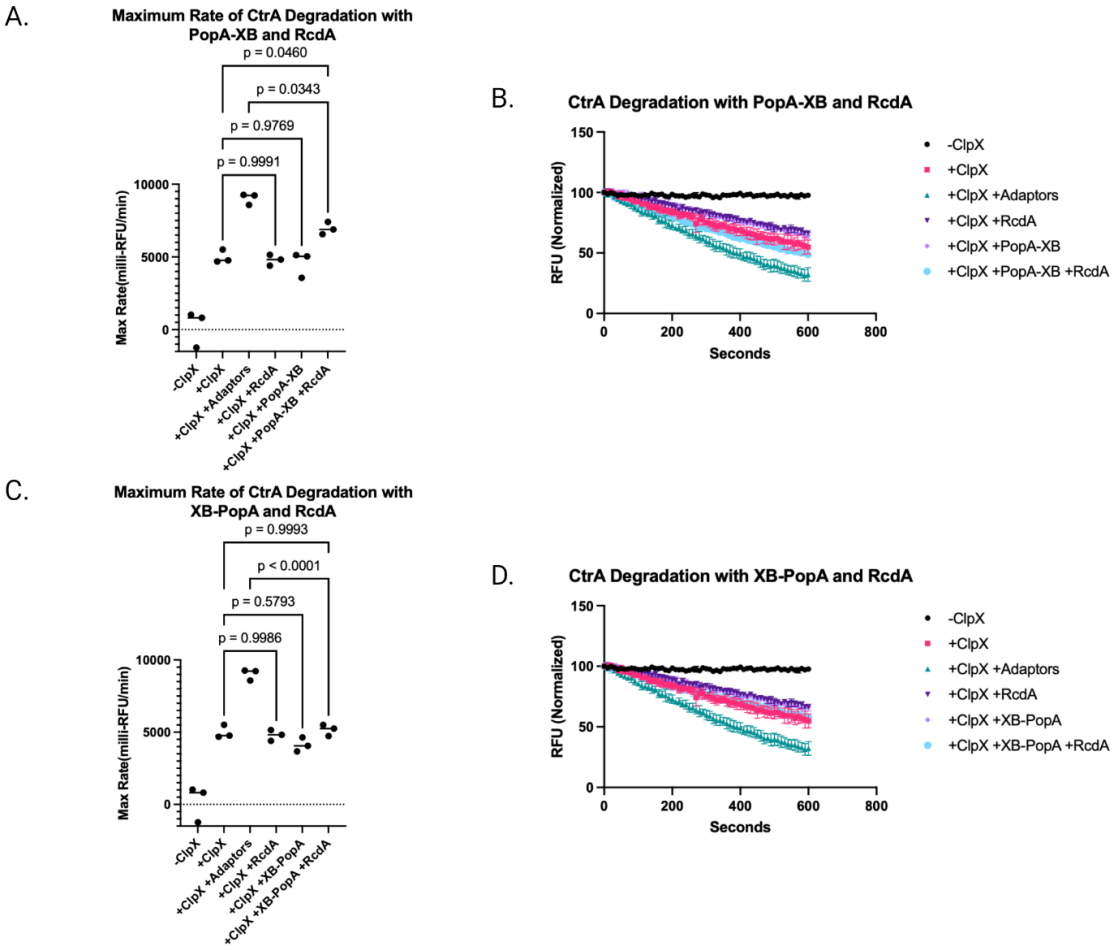

Figure 3.5: CtrA Degradation with PopA XB Mutants in Complex with RcdA

A) Maximum rate of CtrA reporter degradation without the addition of ClpX; in the presence of ClpX; in the presence of ClpX and the full WT adaptor complex; in the presence of ClpX and RcdA; in the presence of ClpX and PopA-XB; and in the presence of ClpX, RcdA, and PopA-XB. B) Normalized CtrA reporter degradation over 10 minutes without the addition of ClpX; in the presence of ClpX; in the presence of ClpX and the full WT adaptor complex; in the presence of ClpX and

RcdA; in the presence of ClpX and PopA-XB; and in the presence of ClpX, RcdA, and PopA-XB C) Maximum rate of CtrA reporter degradation without the addition of ClpX; in the presence of ClpX; in the presence of ClpX and the full WT adaptor complex; in the presence of ClpX and RcdA; in the presence of ClpX and XB-PopA; and in the presence of ClpX, RcdA, and XB-PopA. D) Normalized CtrA reporter degradation over 10 minutes without the addition of ClpX; in the presence of ClpX; in the presence of ClpX and the full WT adaptor complex; in the presence of ClpX and RcdA; in the presence of ClpX and XB-PopA; and in the presence of ClpX, RcdA, and XB-PopA.

3.4 Discussion

The results of the experiments outlined in this section, while promising, are ultimately inconclusive. While it is tempting to believe that the implication of the D187 residue of PopA in CtrA delivery requires RcdA binding, and that the data in Figure 3.5 indicates that PopA-XB is accelerating CtrA degradation when in the presence of CdG and RcdA, several questions remain. If PopA-XB can increase the degradation rate in the presence of RcdA, why was the degradation rate of the PopA-XB in place of WT PopA in the full adaptor complex commensurate with the degradation rate of CtrA without any adaptors? Are the XB PopA mutants even binding to ClpX themselves and are they folded properly? More assays need to be completed to probe the answers to these questions and these will be addressed in the following chapter.

3.5 Materials and Experimental Procedures

Protein Expression and Purification. BL21(DE3) pLYS cells containing pET23b and pET28b expression plasmids for diverse proteins were cultivated at a temperature of 37^{o}C until reaching an optical density (OD600) of 0.6-0.8. Subsequently, they were induced with 0.4mM of IPTG, either for a duration of 4 hours at 37^{o}C or overnight at 30^{o}C. The induced cells were subjected to

centrifugation at 7,000xg for 8 minutes and then resuspended in a lysis buffer composed of 50mM Tris (pH 8.0), 300mM NaCl, 10mM imidazole, 10% glycerol, 5mM BME, and 1mM PMSF. This cell suspension was frozen at -80^{o}C until the purification process. Subsequently, the cells were thawed and lysed using a Microfluidizer system from Microfluidics (Newton, MA). The resulting lysate was then subjected to centrifugation at 15,000rpm for 30 minutes, and the supernatant was collected. The supernatant was passed through a Ni-NTA column for affinity purification. H6SUMO-tagged proteins were cleaved using Ulp1-his protease.

Cloning and Molecular Biology. XB-PopA and PopA-XB were obtained by employing the around-the-horn site-directed mutagenesis technique, which involved amplifying the target plasmid using pET23bH6SUMO-PopA as a reference template. The accuracy of the XB PopA mutants was verified by Plasmidsaurus through sequencing.

Fluorescence Polarization and Maleimide Labeling. Purified RcdA L82E protein was subjected to labeling using Fluorescin-5-Maleimide (Thermo Scientific™). The purified protein, at a concentration of approximately 8-10mg/mL, was exchanged into a labeling buffer containing 50mM HEPES (pH 7.0), 150mM NaCl, and 2mM TCEP. Fluorescin-5-Maleimide, dissolved in DMSO, was

added to the protein at a 20-fold molar excess to cysteine. The labeling process was carried out overnight at a temperature of 4^{o}C. To remove any unbound dye, a buffer exchange was performed using Snakeskin Dialysis Tubing (Thermo Scientific™), with the protein being transferred into a solution containing 20mM HEPES (pH 8.5), 100mM KCl, 10mM MgCl2, and 0.1% Tween. The labeled protein was divided into smaller portions and frozen at -80^{o}C for storage.

Fluorescence polarization binding assays were conducted using 100nM of F5M-labeled L82E protein and varying concentrations of the cargo. The binding reaction was incubated at a temperature of 30^{o}C for 30 minutes to establish equilibrium. Polarization measurements were taken from a 40uL mixture using opaque black 384-well plates and a SpectraMax M5 plate reader (Molecular Devices). The excitation and emission wavelengths were set at 460 and 540, respectively. The binding constants were determined by fitting the polarization data using GraphPad Prism software, utilizing a one-site total and nonspecific binding equation: P = Pmax*[X]/([X] + Kd) + NS*[X] + Background. In this equation, Pmax represents 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 refers to the polarization value when [X] is 0. Error bars were

calculated based on the maximum and minimum values obtained from replicates of the experiments.

***In vitro* Degradation Assays.** Degradation of the GFP-CtrA reporter (GFP-CtrA-RD+15, the Receiver domain of CtrA plus 15 residues tagged to GFP) was monitored with the loss of fluorescence over time. In the reaction mixture, ClpX concentration was 0.4uM, ClpP concentration was 0.8uM, 1x ATP regeneration mix (4mM ATP, 16mM creatine phosphate, 0.32mg/mL creatine kinase), and 2uM of GFP-CtrA-RD+15. When adaptors are present, 1uM of CpdR, RcdA, and PopA (or PopA Mutant) were included as well as 20uM of CdG to a final volume of 20uL. Fluorescence was measured with a SpectraMax M5 plate reader (Molecular Devices) with excitation and emission wavelengths at 488nm and 510nm respectively. Maximum rate of fluorescence loss was calculated in the SpectraMax software using reduction criteria that created a best-fit line using 20 fluorescence readings from 0-600 second.

Bacterial Strains Used in This Study:

Organism	Name	Description	Source
E. coli	TOP10	Cloning Strain	Invitrogen
	BL21(DE3) pLysS	Recombinant protein expression	Invitrogen
	EPC112	BL21DE3 pLysS pET23b Ulp1his protease	(Kuhlman, 2021)
	EPC162	BL21DE3 375 eGFP-His6-CtrARD+15	(Smith et al., 2014)
	EPC1037	BL21DE3 pLysS pet23bh6SUMO-PopA	(Smith et al., 2014)
	EPC1626	BL21 pLYS RcdA L82E	(Kuhlman, 2021)
	EPC1752	Top10 pET23b-h6-SUMO XB-PopA	This study
	EPC1753	Top10 pET23b-h6-SUMO PopA-XB	This study

3.6 Acknowledgements

I would like to acknowledge my advisor, Dr. Peter Chien, for his help in devising the experiments found in this chapter and his tireless help in analyzing the data from them.

I would also like to thank Justyne Ogdahl and Tommy Tashjian for their help in making sure my illustrations follow a standard color scheme.

Finally, I would like to thank Alex Martinez for his help in completing these assays during my final weeks in the lab.

3.7 References

1. Joshi KK, Bergé M, Radhakrishnan SK, Viollier PH, Chien P. An Adaptor Hierarchy Regulates Proteolysis during a Bacterial Cell Cycle. Cell. 2015 Oct 8;163(2):419-31. doi: 10.1016/j.cell.2015.09.030. PMID: 26451486; PMCID: PMC4600535.

CHAPTER 4

IMPACT OF WORK, FUTURE DIRECTIONS, AND ADDITIONAL WORK

4.1 Overview

In this chapter I will explore the impact that this body of work will have on the larger field of *Caulobacter crescentus* protease adaptors, as well as future directions that should be explored to further probe these areas, and finally additional work done during my thesis that didn't fit well into the other thesis chapters. The fundamental, mechanistic and residue level insights that my thesis is focused on will overturn an established finding in the field of ClpXP proteolysis in *Caulobacter crescentus* as well as provide a clear avenue for further investigation into questions about the CtrA degradation adaptor hierarchy.

4.2 Impact of Thesis work on Protease Adaptor Field

My thesis built upon several bodies of work that laid the foundation for both the questions addressed and the methods to answer those questions (Joshi et al., 2015, Kuhlman et al., 2021,). However, my thesis also stands in direct contradiction to a finding published in my field of investigation in the last decade (Ozaki et al., 2014). While this revelation might be interesting enough in itself,

the further elucidation of the RcdA/PopA complex and discovery of the importance of residue D187 of PopA in CtrA delivery offers new insight into how the RcdA/PopA complex might be allosterically primed to target and deliver. The work presented in Chapter 2 overturned previously held beliefs about the binding interface of PopA with RcdA and it was the first direct demonstration of PopA acting as an active primer for CtrA delivery. This piece of information is extremely amenable to future study as more about the exact mechanism of regulation the D187 residue of PopA has on CtrA delivery can be uncovered. An immediate question would be does the D187K mutant of PopA deliver the other PopA substrates KidO and GdhZ (Bergé et al., 2020, Beaufay et al., 2015) for degradation? *In vivo* studies using allelic swap with PopA4A, D187K and other PopA mutants discovered in this thesis would not only be able to answer this question, but the question of whether the promising *in vitro* data we have seen can be recapitulated in a living organism. Additionally, if the PopA D187K mutation is inhibiting CtrA delivery because the charge swap prevents it from interacting with RcdA R49, then the WT degradation rate may be rescued by using an RcdA R49E mutant with the PopA D187K mutant in *in vitro* degradation assays.

However, it is the data presented in Chapter 3 that while promising, needs the most additional inquiry. To determine if the XB PopA mutants are indeed

binding to ClpX, a degradation assay with the SspB adaptor degrading ssrA should be conducted with the XB PopA mutants in excess to determine whether they can compete off the WT SspB. Furthermore, differential scanning fluorimetry assays can be used to compare the melting temperature of the XB PopA mutants compared to WT PopA to determine if they are properly folded.

The uncovering of the regulation of CtrA delivery via PopA by it's D187 residue specifically will hopefully provide a new understanding for how PopA can behave as an adaptor and can possibly elucidate how CtrA is degraded in other bacteria that lack PopA but retain ClpXP, CpdR, and RcdA.

My thesis provides direct insight into how PopA and RcdA interact and how that interaction leads to CtrA delivery to the protease. My specific studies of the residues responsible for PopA binding to RcdA and delivering CtrA are critical to understanding how CtrA is degraded not only in *Caulobacter crescentus* but possibly in other organisms that share a common mechanism.

4.3 X-Ray Crystallography of RcdA-PopA Complex

While the evidence collected over the course of this thesis is convincing, the final evidence that is needed is direct observation of the RcdA/PopA complex. This will require co-crystallization and analysis with X-Ray crystallographic

methods. Previous unpublished attempts to co-crystallize PopA with RcdA have caused a homodimer of RcdA to crystallize. In my research, I attempted, without success, to co-crystallize PopA with RcdA L82E, the RcdA mutant that lacks the ability to dimerize. Having this structural data will verify the exact interactions that were computationally predicted over the course of my research and possibly provide new residues to investigate.

4.4 Characterization of Additional CtrA Binding Residues

A final computational prediction involving PopA, RcdA, and CtrA together seemed also to implicate CtrA residue D44 in the binding complex of all three proteins. Interestingly, CtrA D44 is shown bound to the opposite side of RcdA R49, sandwiching RcdA R49 between PopA D187 and CtrA D44. This interaction, possibly made possible by the resonance structures of Argenine spreading out it's positive charge to its oppsite sides, might possibly explain why the D187K mutant shows an inability to deliver CtrA. Without D187 of PopA holding R49 of RcdA in place, D44 of CtrA cannot properly engage with the whole complex. A future GFP-CtrA-RD+15 mutant should be made with D44 mutated to both Alanine and Lysine. Performing degradation assays with these D44 CtrA mutants with WT adaptors could cause a defect in CtrA delivery if this hypothesis is correct. However, it's possible that utilization of the D44K CtrA Mutant with the D187K

PopA mutant and the RcdA3E mutant could cause a recovery of the delivery defect.

4.5 PopA Partial Proteolysis

The following section covers experiments I performed attempting PopA partial proteolysis to separate PopA into stable fragments and determine which fragment binds to RcdA. I first settled on trypsin for the protease for the partial proteolysis and was able to select a set concentration and duration to consistantly create three fragments of PopA that were between 10-15kDa (Figure 4.1, A.). After quenching the trypsinization reaction, I was able to perform a single point fluorescence polarization anisotropy assay that showed that one of the fragments was indeed binding (Figure 4.1, B.). After analyzing the trypsinization product with MS and correcting for post-translational modifications caused by the serine protease inhibitor AEBSF used to quench the reaction, it was found that the three fragments were a fragment of the REC2 domain (residues 137-262), a fragment of the REC1 domain (residues 36-151), and a fragment of the GGDEF domain (residues 269-354).

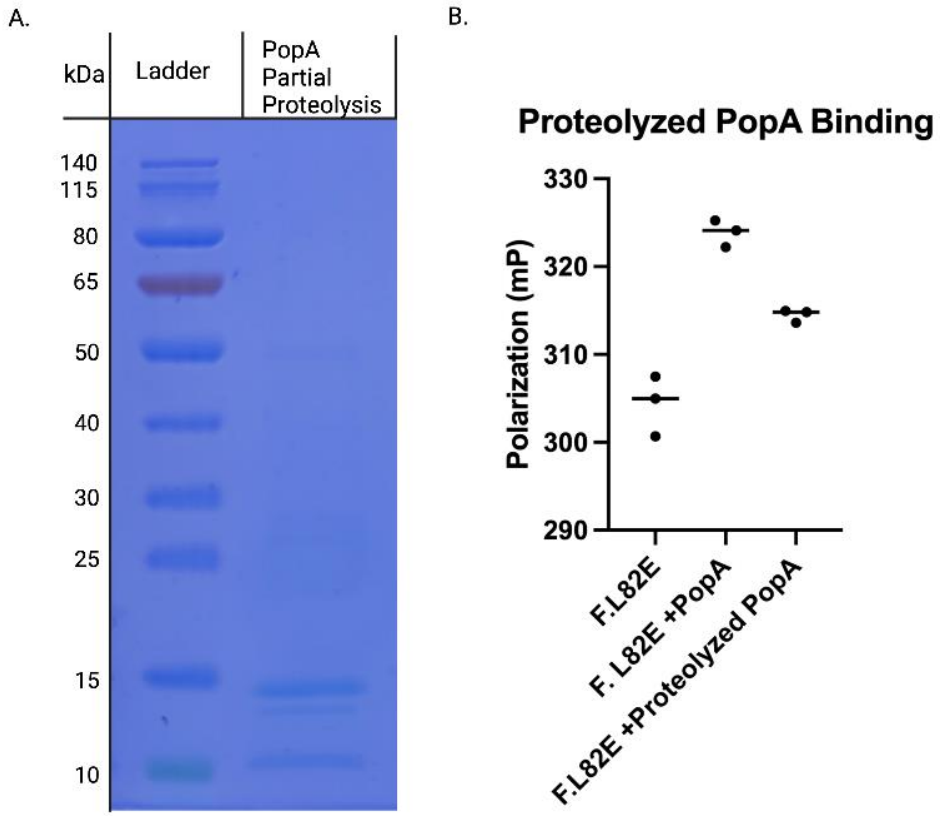

Figure 4.1: PopA Partial Proteolysis Binding

A) Three fragments of PopA after partial proteolysis with 43uM of PopA and 0.1mg/mL Trypsin for 45minutes quenched with AEBSF. The bands are 13.9, 12.6, and 9.8 kDa descending. B) Fluorescence polarization anisotropy of proteolyzed PopA. From left to right, Polarization value of F. L82E alone, F. L82E with WT PopA, and F. L82E with the Proteolyzed PopA.

4.6 CdnL Degradation in the Presence of PPGPP

The following section covers work I did on CdnL degradation in the presense of PPGPP to see if, as preliminary analysis from another lab suggested, PPGPP inhibits CdnL degradation. I performed an *in vitro* degradation assay taking multiple timepoints and ran them each on an SDS-PAGE gel. After gel quantification, the results indicated that PPGPP does not inhibit CdnL degradation by ClpXP.

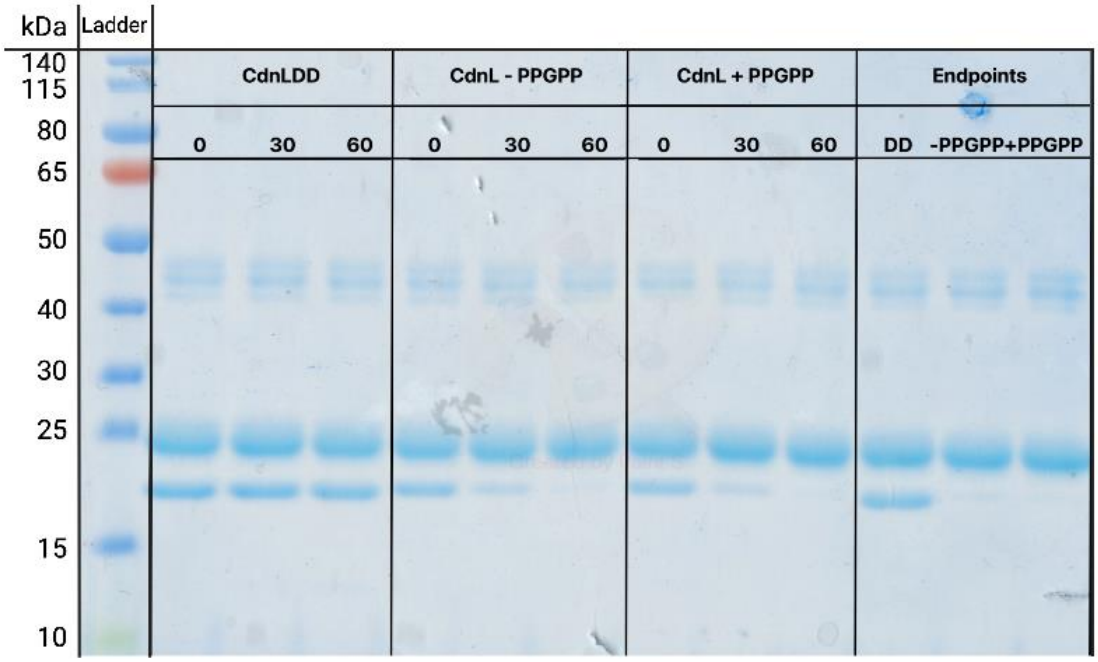

Figure 4.2: CdnL Degradation with PPGPP

In vitro degradation assay with CdnL to test if PPGPP inhibits CdnL degradation by ClpXP. From left to right, CdnLDD (A CdnL mutant with a mutated degron) at 0, 30, and 60 minutes, CdnL alone at 0, 30, and 60 minutes, and CdnL + PPGPP at 0, 30, and 60 minutes.

4.7 Materials and Experimental Procedures

Protein Expression and Purification. BL21(DE3) pLYS cells containing pET23b and pET28b expression plasmids for diverse proteins were cultivated at a temperature of 37^{o}C until reaching an optical density (OD600) of 0.6-0.8. Subsequently, they were induced with 0.4mM of IPTG, either for a duration of 4 hours at 37^{o}C or overnight at 30^{o}C. The induced cells were subjected to centrifugation at 7,000xg for 8 minutes and then resuspended in a lysis buffer composed of 50mM Tris (pH 8.0), 300mM NaCl, 10mM imidazole, 10% glycerol, 5mM BME, and 1mM PMSF. This cell suspension was frozen at -80^{o}C until the purification process. Subsequently, the cells were thawed and lysed using a Microfluidizer system from Microfluidics (Newton, MA). The resulting lysate was then subjected to centrifugation at 15,000rpm for 30 minutes, and the supernatant was collected. The supernatant was passed through a Ni-NTA column for affinity purification. H6SUMO-tagged proteins were cleaved using Ulp1-his protease.

PopA Partial Proteolysis. 500uL of 43uM PopA was incubated with 67uL of 1mg/mL Trypsin and 103uL of H-Buffer (20mM HEPES pH 7.0, 10mM MgCl2,

100mM KCl, 10% Glycerol) at room temperature for 45 minutes and quenched with AEBSF to a final concentration of 5mM.

Fluorescence Polarization and Maleimide Labeling. Purified RcdA L82E protein was subjected to labeling using Fluorescin-5-Maleimide (Thermo Scientific™). The purified protein, at a concentration of approximately 8-10mg/mL, was exchanged into a labeling buffer containing 50mM HEPES (pH 7.0), 150mM NaCl, and 2mM TCEP. Fluorescin-5-Maleimide, dissolved in DMSO, was added to the protein at a 20-fold molar excess to cysteine. The labeling process was carried out overnight at a temperature of 4^{o}C. To remove any unbound dye, a buffer exchange was performed using Snakeskin Dialysis Tubing (Thermo Scientific™), with the protein being transferred into a solution containing 20mM HEPES (pH 8.5), 100mM KCl, 10mM MgCl2, and 0.1% Tween. The labeled protein was divided into smaller portions and frozen at -80^{o}C for storage.

Fluorescence polarization binding assays were conducted using 100nM of F5M-labeled L82E protein and varying concentrations of the cargo. The binding reaction was incubated at a temperature of 30^{o}C for 30 minutes to establish equilibrium. Polarization measurements were taken from a 40uL mixture using opaque black 384-well plates and a SpectraMax M5 plate reader (Molecular Devices). The excitation and emission wavelengths were set at 460 and 540,

respectively. The binding constants were determined by fitting the polarization data using GraphPad Prism software, utilizing a one-site total and nonspecific binding equation: P = Pmax*[X]/([X] + Kd) + NS*[X] + Background. In this equation, Pmax represents 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 refers to the polarization value when [X] is 0. Error bars were calculated based on the maximum and minimum values obtained from replicates of the experiments.

***In vitro* degradation assay.** CdnLDD, CdnL, and CdnL+PPGPP were degraded with ClpXP protease at 30^{o}C for a total of 60 minutes. Samples were taken at 0, 30, and 60 minutes and run on a gel. Concentration of degradation mix was as follows: 0.3uM ClpX, 0.6uM ClpP, 1x ATP regeneration mix (4mM ATP, 16mM creatine phosphate, 0.32mg/mL creatine kinase), 1% BME and 5uM CdnL and when PPGPP was present it was at a concentration of 1uM.

4.8 References

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APPENDIX

SUPPLEMENTAL FIGURES

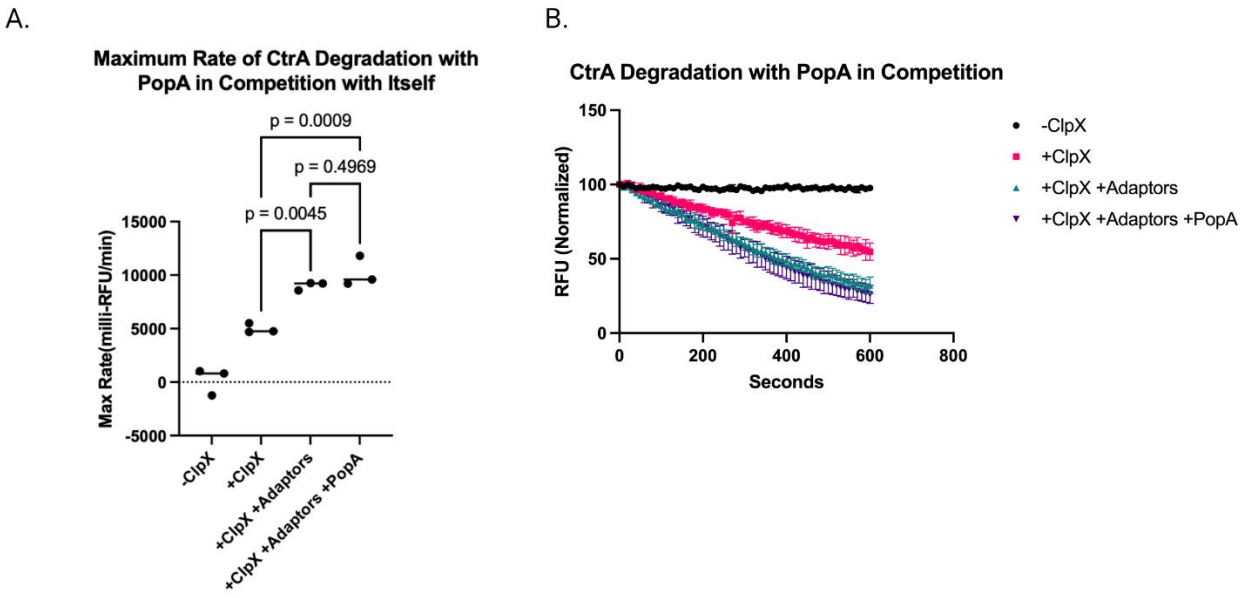

Figure A.1: CtrA Degradation with PopA Competing with Itself

A) Maximum rate of GFP-CtrA reporter degradation without ClpX; with ClpX; with ClpX and the full WT Adaptor complex; with ClpX, the full WT adaptor complex and extra excess PopA (4.25uM). B) Normalized GFP-CtrA reporter degradation over 10 minutes without ClpX; with ClpX; with ClpX and the full

WT Adaptor complex; with ClpX; the full WT adaptor complex and extra excess PopA (4.25uM).

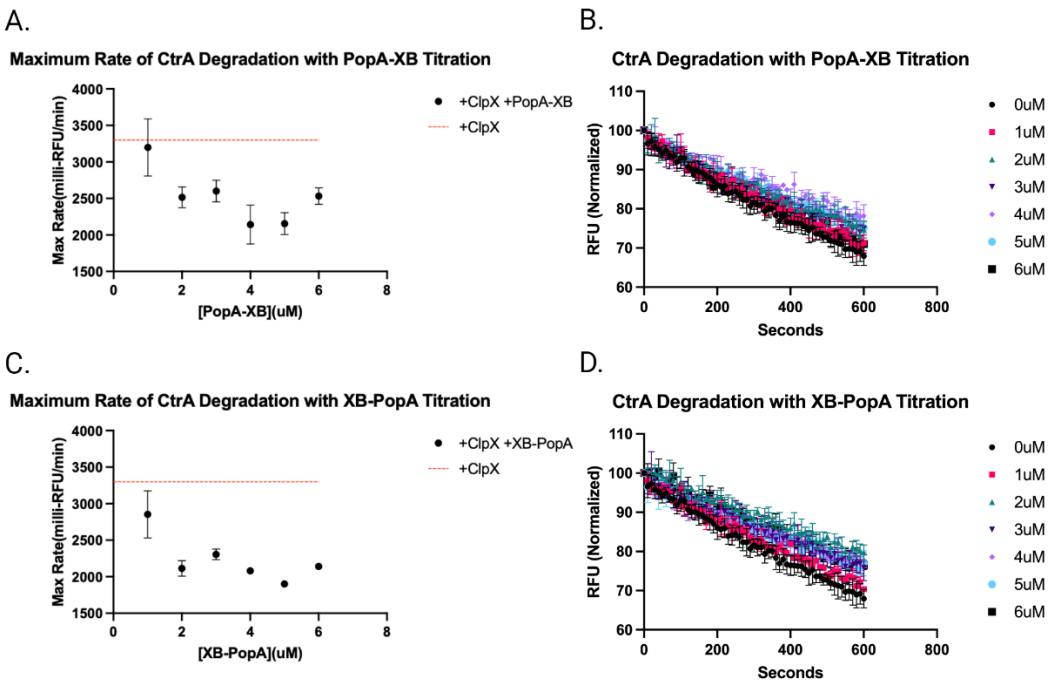

Figure A.2: CtrA Degradation with PopA XB Mutants Titrated Alone

A) Maximum rate of GFP-CtrA reporter degradation with increasing concentrations of PopA-XB. B) Normalized GFP-CtrA reporter degradation over 10 minutes with increasing concentrations of PopA-XB. C) Maximum rate of GFP-CtrA reporter degradation with increasing concentrations of XB-PopA. D) Normalized GFP-CtrA reporter degradation over 12 minutes with increasing concentrations of XB-PopA.

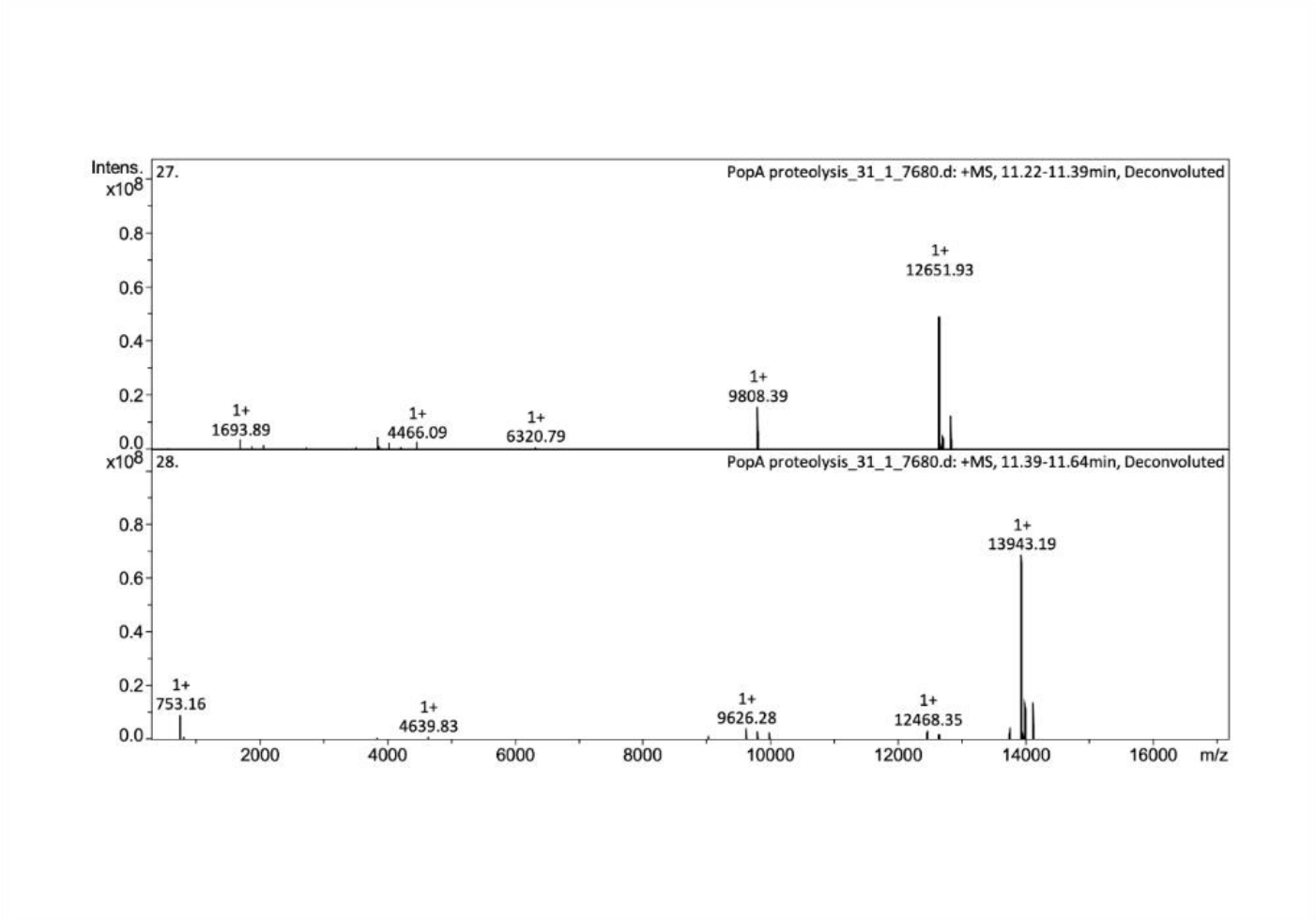

Figure A.3: PopA Partial Proteolysis Mass Spec Data

PopA Proteolysis large peptide Mass Spectrometry data with peaks corresponding to 13.9kDa, 12.6kDa, and 9.8kDa bands seen in the proteolysis gel. The 13.9kDa peak corresponds to REC2 residues 137-262 with one 183Da MetOx. The 12.6kDa peak corresponds to REC1 residues 36-151 with one 183Da MetOx. The 9.8kDa peak corresponds to GGDEF residues 269-354 with two 183Da MetOx.

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