

Clp ATPases: structure, function, and modulation of their activity

by

Zachary Spaulding

B.S., University of Missouri – Kansas City, 2020

AN ABSTRACT OF A DISSERTATION

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Department of Biochemistry and Molecular Biophysics
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Abstract

AAA+ ATPases are found across all domains of life and are involved in a broad range of cellular processes, from regulating DNA replication to mediating protein folding and turnover. The Clp/Hsp100 subfamily of AAA+ ATPases are a group of highly conserved chaperones that support cellular protein quality control or proteostasis.

During the course of infection, many host defense mechanisms work to disrupt proteostasis in the infectious microorganisms. These defense mechanisms, including elevated temperatures and increased oxidative stress, lead to the accumulation of proteotoxic aggregates, limiting the viability of the pathogen cell. ClpB, ClpA, and ClpX all work to alleviate proteotoxic stress by physically unfolding proteins and protein aggregates for either subsequent reactivation (in the case of ClpB) or degradation by the protease ClpP. I investigated the application of DBeQ as an inhibitor of *E. coli* Clp chaperones and chaperone-protease complexes using BAP, an engineered variant of ClpB that, like ClpA and ClpX, binds to the protease ClpP, coupling protein unfolding to proteolysis. In this investigation I found that DBeQ is selective for ClpB/BAP and inhibits this protein with a degree of potency that is orders of magnitude greater than either ClpA or ClpX.

Human CLPB, also known as SKD3, is a novel AAA+ protein found in the mitochondrial intermembrane space. Mutations in the *CLPB* gene have been implicated in human disease. I have discovered that CLPB forms nucleotide stabilized dodecamers, a structure that is rarely observed among AAA+ ATPases. Furthermore, using mutations in the Walker A and Walker B motifs of CLPB, I have identified MICU1, a regulator of calcium signaling and inner membrane morphology in the mitochondria, as a *bona fide* substrate of this chaperone.

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List of Abbreviations

AAA+	ATPases Associated with Various Activities
ADP	Adenosine Diphosphate
AMP-PNP	Adenylyl-imidodiphosphate
ATP	Adenosine Triphosphate
ATP γ S	Adenosine-5'-O-(3-Thiotriphosphate)
DBeQ N ² , N ⁴	Dibenzylquinazoline-2,4-diamine
DLS	Dynamic Light Scattering
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
FPLC	Fast Protein Liquid Chromatography
HL-60	Human Promyelocytic Leukemia Cell Line
IMM	Inner Mitochondrial Membrane
IMS	Intermembrane Space
MICOS	Mitochondrial contact site and Cristae Organizing System
mtCU	Mitochondrial Calcium Uniporter Complex
MCU	Mitochondrial Calcium Uniporter
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
SDS	Sodium Dodecyl Sulfate
SPR	Surface Plasmon Resonance
TEV	Tobacco Etch Virus

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Dedication

I would like to dedicate this dissertation to my fiancé, Alison Slivka, who has been my rock during nearly my entire academic journey over the past ten years. They have consistently offered a shoulder to cry on, a listening ear, and has endured many late-night ramblings about my research. Their steadfast presence in my life throughout its many ups and downs has enabled me to retain my sanity on my lowest days and has greatly enhanced the highest. I would not have been able to get here without their immense love, support.

Chapter 1 - Background and Introduction

Proteostasis is defined as the balance of protein synthesis, folding, trafficking, and degradation within the cell. At every step of protein synthesis and their activity thereafter, it is critical that protein stability is properly maintained, especially under unfavorable conditions such as thermal and oxidative stress. To this end, molecular chaperones ensure proteins remain properly folded at each stage of a protein's lifecycle or that they are refolded/degraded in cases where they misfold or are no longer needed. Chaperones have various mechanisms of action, such as blocking sensitive regions of a protein from solvent exposure during folding, ensuring the formation of critical intra-molecular disulfide bonds, and in many cases by physically unfolding and refolding a client protein.

The AAA+ (ATPases Associated with various Activities) superfamily of ATPases is a broad group of proteins that are ubiquitous across all domains of life. As this acronym implies, these proteins are involved in a wide variety of cellular functions. Their primary function is the conversion of energy from ATP (and more rarely GTP) hydrolysis to perform mechanical work upon their substrates. Some well-known examples of these literal molecular machines include DNA helicases, dynein motors, and the 19S regulatory particle in the 26S proteasome. Proteins in this family are characterized by the presence of one or two highly conserved nucleotide binding domains (NBDs), and their tendency to form ring-shaped hexamers. These proteins are further subdivided into Type I and Type II AAA+ ATPases which contain either one or two NBDs, respectively.

Within the NBD, there are several conserved motifs located in the catalytic site formed between subunits of the oligomer; the Walker A motif (GXXXXGKT/S) which initially binds ATP, the Walker B motif (*hhhh*DE where *h* is any hydrophobic residue) which coordinates the

magnesium ion and activates the attacking water for hydrolysis of the beta-gamma phosphoanhydride bond in ATP, and finally the Sensor I and II motifs which “sense” ATP occupancy by interacting with ATP and inducing conformational changes in the protein to facilitate oligomerization and communicate allosteric effects between domains(Guenther et al., 1997; Walker et al., 1982) (Figure 1-1). Conserved arginine residues, dubbed arginine fingers, contact nucleotides in neighboring subunits within the oligomer and help to facilitate inter-subunit coordination in catalyzing ATP-hydrolysis(Ogura et al., 2004). Mutations in the Walker A and Walker B motifs are common tools in the studies of AAA+ ATPases. Substitution of the Walker A lysine with threonine renders the enzyme incapable of binding ATP. Conversely, substitution of the Walker B glutamate with glutamine prevents ATP hydrolysis, creating a “trap” mutant which retains bound substrates rather than processing them.

The NBDs of AAA+ proteins can be divided into seven clades which help to describe their evolutionary relationships and possible function. The core structure of the AAA+ domain remains consistent across all clades, a large subdomain is comprised of a mostly parallel 5-strand core β -sheet sandwiched between groups of α -helices and contains the Walker A, Walker B, and Sensor I motifs. The small α -helical subdomain provides the sensor II motif. Clade 1 deviates little from this core structure, whereas Clades 2-7 contain additional inserts which distinguish them from one another. The proteins described in this work contain NBDs from Clades 3 and 5. Clade 3, the “classical clade”, is the most well studied of the AAA+ NBDs, it bears an additional alpha-helical insert between β -strand 2 and helix 2 of the large subdomain as well as an additional arginine finger. Clade 5, known as the HCLR clade (HslU, ClpA/ClpB/ClpX, Lon, and RuvB), is characterized by a pre sensor 1 (PS1) insert between helix three and β -strand four of the large subdomain.

The present work is focused on the bacterial proteins ClpA, ClpB, and ClpX, as well as the mammalian mitochondrial protein CLPB, also known as SKD3.

Bacterial Clp ATPases

The Hsp100/Clp (Caseinolytic peptidase) subfamily of AAA+ proteins are molecular chaperones that use ATP hydrolysis to mechanically unfold protein substrates by translocating them across the central channel in the hexameric ring. In bacteria, Clp ATPases are critical in multiple areas of regulating proteostasis, from ribosomal rescue due to aberrant protein folding during synthesis to the turnover or reactivation of misfolded proteins under thermal or oxidative stress. They do this by acting as ATP-powered mechanical “unfoldases”. Each of these proteins form rings around the protein substrate in an asymmetric spiral staircase arrangement (Shorter & Southworth, 2019). Pore loops in the NBDs, located in the central channel of the hexamer, contain tyrosine residues which contact the peptide backbone of substrates. This allows them to physically grip and unfold proteins to be subsequently reactivated or delivered to a protease for degradation. ClpA, ClpB, and ClpX are three AAA+ chaperones which have been extensively studied and are the main subjects of this present work.

ClpB, and its yeast homologue Hsp104 are type II AAA+ chaperones which promote the refolding and subsequent reactivation of misfolded proteins with the aid of cochaperones DnaK (Hsp70), DnaK (Hsp40), and the nucleotide exchange factor GrpE (Zolkiewski, 1999). ClpB contains two NBDs, D1 and D2 from the classical and HCLR clades, respectively (Figure 1-1). These domains work to translocate substrates delivered by DnaK across the central channel of the ClpB hexamer in a D1 to D2 manner. This is accomplished by the sequential clockwise motion of D1 and D2 domains throughout the hexamer along the peptide backbone of the substrate protein coupled with ATP hydrolysis, physically gripping and unfolding the protein

(Deville et al., 2019; Rizo et al., 2019; Yu et al., 2018). The N-terminal domain of ClpB has been implicated in substrate recognition and binding, while the D2 small subdomain is required for oligomerization (Barnett et al., 2000). Among Clps found in gram negative bacteria, ClpB uniquely possesses a coiled coil middle domain. The middle domain can be separated into two motifs which are referred to as motif-1 and motif-2. Motif 2 serves as the interacting site of ClpB with DnaK (Rosenzweig et al., 2013; Seyffer et al., 2012; Yin et al., 2021). Motif 2 has been proposed to function in regulation of ATPase activity in D1 of the neighboring protomer in ClpB hexamers, thus, supporting DnaK-induced activation of ClpB (Oguchi et al., 2012).

Other Clp chaperones, chiefly ClpX and ClpA, work to power the protein degradation machinery in bacteria. These proteins work with the protease ClpP to drive protein turnover in a manner not wholly dissimilar to the 19S chaperone and 20S core particle subunits of the 26S proteasome in eukaryotes (Sauer & Baker, 2011). This chaperone-protease system has been studied extensively and is well conserved across bacteria as well as the mitochondria and chloroplasts of eukaryotes. ClpP is a small 23kDa serine protease which forms a stable barrel-shaped tetradecamer comprised of stacked heptameric rings in which the N-termini of each subunit face outwards and features a central channel that leads to its active site (J. Wang et al., 1997). However, this channel is too small for protein substrates larger than small peptides to freely diffuse into, therefore, chaperones are required to unfold and deliver proteins to ClpP's active site for degradation. ClpA and ClpX interact with ClpP via conserved "IGL" or "IGF" loop insertions in the D2 domain of ClpA and NBD of ClpX (Figure 1-2). The loss of ClpP in pathogenic bacteria impacts virulence and reduces cell viability under thermal and oxidative stress, which has made it a promising target for novel antimicrobials (Aljghami et al., 2022). Some strategies have even sought to dysregulate ClpP activity by activating it rather than

inhibiting it by widening ClpP's central pore, leading to uncontrolled protein degradation of native bacterial proteins (Brötz-Oesterhelt et al., 2005; Mabanglo et al., 2019).

ClpX is a type I AAA+ ATPase, it bears a single NBD from the HCLR clade, as well as an N-terminal Zinc-finger domain and a C-terminal domain. ClpX interacts with ClpP through a conserved "IGF" loop in its NBD, the IGF motif binds to a hydrophobic pocket in the N-terminus of ClpP, forming a stacked asymmetric six-member ring over the seven-member ring at either end of the ClpP tetradecamer (Grimaud et al., 1998; Kim et al., 2001a; A. Martin et al., 2007). ClpXP complex formation is ATP dependent, upon binding of ATP the loops align with the hydrophobic clefts on the surface of ClpP, allowing for complex formation, while the ADP-bound state destabilizes the chaperone-protease complex (Amor et al., 2019). ClpXP recognizes the SsrA degron at the C-termini of aberrantly folded proteins released from stalled ribosomes by the SsrA ribosomal rescue system with the assistance of the adaptor protein SSB (Flynn et al., 2001). Additionally, ClpXP is able to degrade other specific proteins such as the bacteriophage λ O DNA replication protein (Wojtkowiak et al., 1993).

ClpA, like ClpB, is a type II AAA+ chaperone, bearing two NBDs, including the small subdomain in D2, and an N-terminal domain. However, it can be distinguished from ClpB by its lack of a coiled-coil domain between D1 and D2. As a chaperone, ClpA recognizes specific substrates such as those generated by the SsrA ribosomal rescue system and N-end-rule substrates which are targeted for rapid turnover (Dougan et al., 2002; Flynn et al., 2001). Moreover, ClpAP is able to unfold and degrade misfolded proteins (Hoskins et al., 2000). ClpS, an adaptor for ClpA, acts as a specificity switch, driving ClpAP towards the degradation of N-end-rule substrates and misfolded proteins and away from substrates with hydrophobic C-terminal tags, including itself. This is hypothesized to serve as a mechanism for the post-

translational regulation of ClpA by controlling the levels of ClpA with activity towards certain substrates (Dougan et al., 2002; Maglica et al., 2008). ClpA is also able to remodel proteins in the absence of ClpP. RepA, a bacterial plasmid replication initiator, is activated by remodeling of inactive RepA dimers into the active monomeric form by ClpA (Pak & Wickner, 1997). While ClpB does not naturally interact with ClpP, a substitution of the ClpA IGL loop into ClpB at a similar site allows for ClpB to act as a chaperone for ClpP, creating BAP (ClpB, like ClpA, binds to ClpP) (Weibezahn et al., 2004). BAP serves as an exciting tool, providing a straightforward readout of substrate translocation via degradation of substrates by ClpP, simplifying assays to observe substrate processing by ClpB.

Previous work by our laboratory has shown that ClpB is essential for cell survival under thermal stress (Glaza et al., 2021; Zolkiewski, 1999). Indeed, Δ ClpB strains of *E.coli* are less tolerant of elevated temperatures as compared to the wildtype, indicating that ClpB is required for the suppression of protein aggregation. Notably, there are no Hsp104 homologues in mammals, though other mechanisms of heat shock tolerance compensate for their absence. Because this system is critical for cell survival of thermal stress, such as that which occurs from a fever during infection of a host by pathogenic bacteria, and its' absence in Mammalia, ClpB may prove to be a promising target for novel antimicrobial drugs. Screening for small molecule inhibitors of ClpB produced one hit, a previously described ATP-competitive inhibitor of another AAA+ protein, p97, N²,N⁴-dibenzylquinazoline-diamine (DBeQ) (Glaza et al., 2021). *In-vitro*, DBeQ serves as an allosteric inhibitor of casein-stimulated ATPase activity by ClpB, as well as luciferase reactivation by ClpB/DnaK/DnaJ/GrpE. Further, DBeQ treatment sensitizes *E.coli* to thermal stress, and DBeQ treated cultures are even more susceptible to heat shock than Δ ClpB strains, suggesting that DBeQ's mechanism of action is beyond that of mere ClpB inhibition.,

The mechanism of action for inhibition of ClpB by DBeQ has not yet been understood. The present study has demonstrated DBeQ's selectivity for ClpB/BAP over ClpA and ClpX, leading us to hypothesize that there is a possible binding site which resides in a region of the protein, which is absent in ClpA and ClpX, such as the middle domain.

Human CLPB – A mitochondrial AAA+ ATPase

SKD3, or CLPB, encoded by the *CLPB* gene in humans, is a ubiquitously expressed AAA+ ATPase localized to the mitochondrial intermembrane space (IMS) (Thevarajan et al., 2020). Both homozygous and heterozygous mutations in *CLPB*, clinically referred to as *CLPB* deficiency, have been identified to cause a range of disease phenotypes in humans, including moderate-to-severe congenital neutropenia, 3-methylglutaconic aciduria type-7 (MGCA7), intellectual disability, brain atrophy, cataracts, and impaired motor function (Warren et al., 2022; Wortmann et al., 2015). Many children born with more severe manifestations of *CLPB* deficiency tend to have shortened lifespans, measured in a few days to a few years. As such, CLPB is an attractive subject for study as a novel Clp ATPase with a role in human disease.

CLPB knockouts in various Acute Myeloid Leukemia (AML) cell lines sensitizes them to pro-apoptotic drugs and causes dramatic changes in cristae morphology in which the cristae junction is widened (Chen et al., 2019). Subsequent work has established that CLPB knockouts indeed lead to increase propensity towards apoptosis in mouse myeloblast like cells during differentiation as well as decreased membrane potential across the inner mitochondrial membrane (Wenta et al., 2025), indicating that CLPB is likely an important pro-survival factor during cell differentiation.

CLPB has a unique domain architecture as compared to other Clp family members. While it shares an HCLR clade NBD which is homologous to D2 in ClpB/Hsp104, it diverges significantly from other members of the Clp family of ATPases. Notably, compared to ClpB/Hsp104, it lacks the more typical N-terminal, D1, and coiled-coil domains. Instead, it bears an N-terminal four ankyrin (ANK) repeat domain which is preceded by a mitochondrial targeting sequence and short PARL cleavage site which are removed in the mature, active protein (Figure 1-3). ANK repeat domains in other proteins are often involved in mediating protein-protein interactions (J. Li et al., 2006). Another unique feature of CLPB is a loop insertion in the NBD that is not found in other Hsp100 family proteins which appears to have an autoregulatory role in CLPB ATPase and aggregate reactivation activities (R. R. Cupo et al., 2022).

Biochemical characterizations of CLPB *in-vitro* have shown that it acts as a general disaggregase that reactivates denatured luciferase and solubilizes alpha-synuclein fibrils similar to the ClpB/DnaK/J/E bi-chaperone system, though CLPB does not require any cochaperones (R. R. Cupo & Shorter, 2020). Analysis of the aggregated fraction of lysed mitochondria isolated from *CLPB* KO cells by mass spectrometry reveals increased amounts of IMS proteins involved in apoptosis, inner membrane remodeling, protein transport, and calcium signaling under both normal and heat shock conditions as compared to the wildtype control (Baker et al., 2024; R. R. Cupo & Shorter, 2020). Together, these results support the hypothesis that CLPB functions as a general disaggregase in the IMS.

Interestingly, CLPB is not found in many members of Eukarya, including other metazoans such as *Drosophila melanogaster*, a common model organism. Many of the proteins that were identified as being insoluble in $\Delta CLPB$ cells are well conserved across eukaryotes. How then do these organisms overcome the lack of this apparently important mediator of

proteostasis in the IMS, especially if they are so unstable as to need a constitutively active chaperone for them to remain soluble?

Investigations into the oligomerization of CLPB have revealed another unique aspect of this protein. Initial experiments using analytical centrifugation, dynamic light scattering, and negative-stain electron microscopy revealed that, surprisingly, CLPB forms dodecamers comprised of stacked hexameric rings (Spaulding et al., 2022) (also see: Chapter 3). Higher resolution structures acquired via cryo-electron microscopy further revealed that these rings are mediated by head-to-head contacts between the ANK domains of each subunit, though these studies also observed particle classes for both hexamers and dodecamers (R. R. Cupo et al., 2022; Gupta et al., 2023; D. Wu et al., 2023). The exact relevance of these higher order assemblies to CLPB's chaperone function is not well understood. Further, truncations of the CLPB ANK demonstrate impaired disaggregase activity, indicating the ANK domain is required for the reactivation of misfolded proteins (R. R. Cupo & Shorter, 2020). One model that has been proposed suggests that CLPB forms a cage around client proteins, creating an environment that is suitable for refolding in a manner similar to the chaperone GroEL (Gupta et al., 2023). This model, however, does not explain how CLPB transitions from extruding unfolded substrates by the C-terminal NBD to capturing them in a cage formed by N-terminal head-to-head contacts, save for the possibility that extruded substrates are then captured and refolded by a separate CLPB cage. Thus, the functional significance of these double-ringed dodecamers is still subject to speculation.

Interestingly, Lon protease, a tandem AAA+ chaperone and protease found in bacteria has also been observed forming similar dodecameric assemblies of N-to-N-terminal contacts between two hexameric rings (Vieux et al., 2013). In this work, the authors conclude that

dodecameric assembly of Lon protease leads to slowed ATPase activity per Lon subunit and a switch in substrate specificity towards the degradation of smaller substrates. If CLPB works by a similar mechanism, then dodecamers may be a potential means of differentiating between substrates under different cellular conditions. Specifically, the fenestrations in the CLPB “cage” might allow smaller proteins to diffuse into the central cavity while excluding larger substrates. Alternatively, dodecamer may be a “storage” or “unarmed” state when CLPB is not needed, the head-to-head ANK domain contacts would then be a means of avoiding aberrant substrate binding. In either case, further investigation of the oligomeric state of CLPB in the presence of native substrates is warranted.

Proteomics experiments utilizing co-immunoprecipitation and pulldowns for both full length wildtype, Walker-A, and Walker-B mutants CLPB, as well as affinity-tag labeled ANK suggest that CLPB may in fact interact with many of the proteins identified as being insoluble in *CLPB*-KO cell lines (Baker et al., 2024; D. Wu et al., 2023). Additionally, proximity labeling with a biotin ligase, BirA, fused to OPA1 and AIFM1 even showed an increase in biotinylated CLPB over the BirA control, suggesting that these proteins interact (Antonicka et al., 2020). These results support the hypothesis that CLPB possibly interacts with natively folded substrates in the absence of or prior to aggregation. We have confirmed several such interactions in our own studies through pulldown assays against immobilized CLPB, which has led us to probe *in-vitro* specific interactions between purified CLPB and proteins that have been identified in proteomics studies.

Main goals of study

Below is the summary of two main directions of research described in the present dissertation.

1. ClpB is a promising target for novel antimicrobial therapies. We have previously reported that DBeQ inhibits ClpB, but the mechanism of ClpB inhibition by DBeQ is not yet known. Notably, unlike in P97, DBeQ appears to act as an allosteric inhibitor of ClpB. Further understanding of the mechanism of action of this inhibitor will be required for designing more potent and selective derivatives of this molecule. After observing that DBeQ induces dissociation of the BAP-ClpP complex (see Chapter 2), my objective became to determine if a similar effect could be found in other bacterial chaperone-protease complexes or if DBeQ shows selectivity towards ClpB/BAP.
2. Mutations in human CLPB/SKD3 have been linked to several disease phenotypes in patients including neutropenia, cataracts, intellectual disability, and progressive brain atrophy, leading to premature death in extreme cases, but the link between CLPB-deficiency and clinical symptoms is unknown. I aimed to characterize CLPB properties and interactions with other proteins *in-vitro* to better understand its contributions to human disease.
 - In Chapter 2, using a broad range of biophysical techniques, I have shown that CLPB forms nucleotide stabilized dodecamers.
 - In Chapter 3, I identified MICU1 and MICU2 as direct interactors of CLPB, and I explored links between CLPB activity and its interactions with MICU1.

Figures

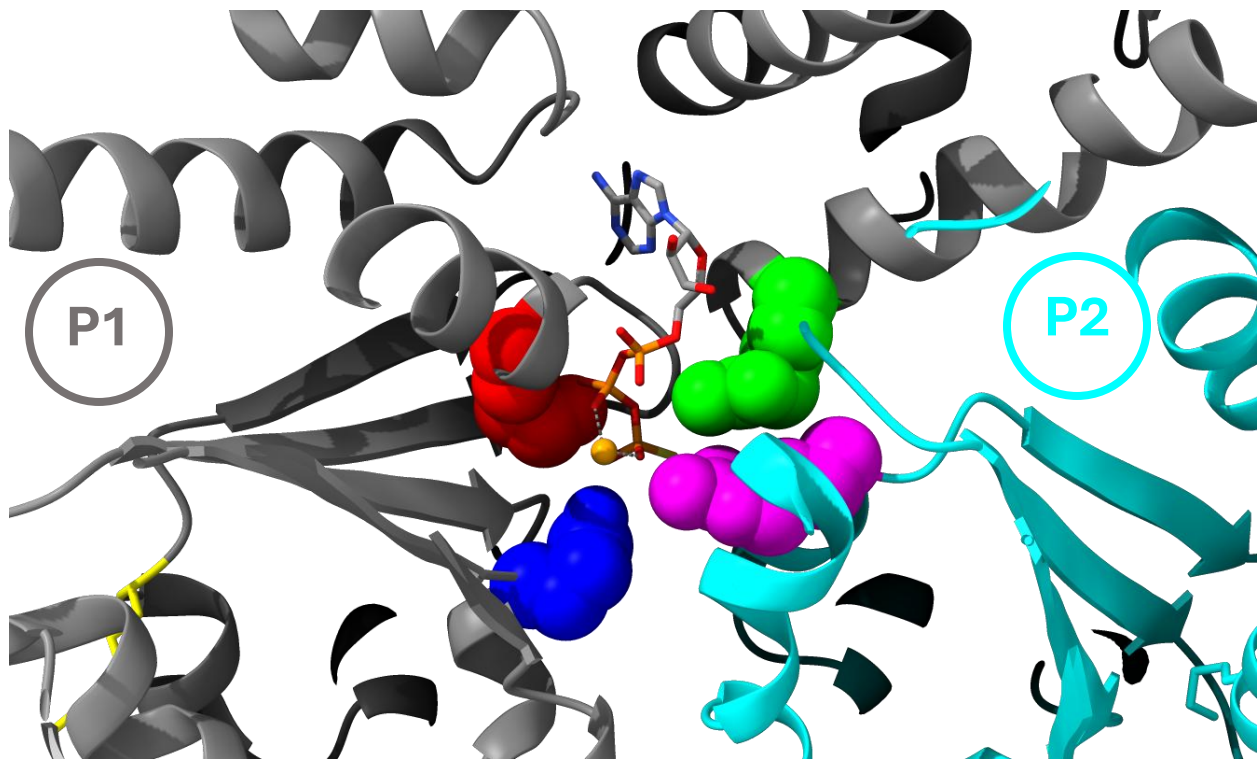


Figure 1-1 Conserved residues in the Clp/Hsp100 ATP binding Site

Shown are several conserved residues in the ATP binding site at the interface of the NBD within two CLPB protomers. Protomer 1 (P1) is in grey and protomer 2 (P2) is in cyan. The Walker A lysine (K357) is shown in red, the Walker B glutamate (E425) is shown in blue. The sensor I and sensor II arginines in protomer 1 and 2 respectively are shown in green and magenta. Between the two protomers is ATPγS, a slowly hydrolysable analogue of ATP.

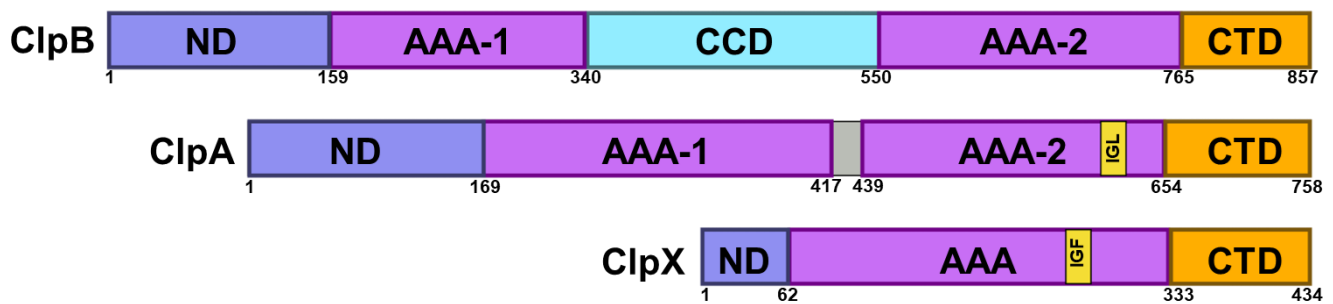


Figure 1-2 Domain Architecture of Bacterial Clps

Alignment of the domains of E.coli ClpB, ClpA, and ClpX. ND = N-terminal domain, AAA-1/2 = D1 and D2 AAA+ domains, respectively, CCD = Coiled-coil domain, CTD = Small D2 C-terminal subdomain. The IGL and IGF loops from ClpA and ClpX are shown in yellow. Made using IBS 2.0 (Xie et al., 2022).

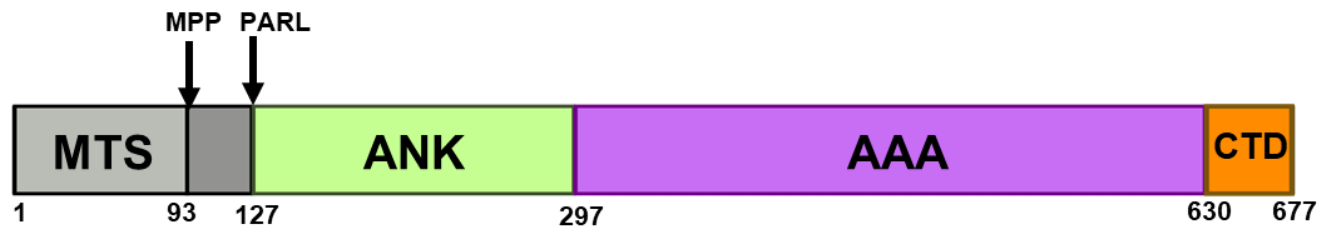


Figure 1-3 Domain Architecture of Human CLPB

MTS = Mitochondrial Targeting sequence. Mitochondrial processing peptidase (MPP) and PARL cleavage sites are shown with arrows. ANK = Ankyrin Repeat Domain, AAA = AAA+ domain, CTD = small subdomain of AAA domain.

Chapter 2 - Targeting Bacterial Clp ATPases with N²,N⁴-dibenzylquinazoline-2,4-diamine (DBeQ)

Introduction

Clp ATPases are a subgroup of a large superfamily of ATPases associated with various activities (AAA⁺) (Khan et al., 2022; Zolkiewski, 2006). A common structural feature of AAA⁺ ATPases is formation of cylinder-shaped hexameric assemblies with a narrow channel located along the cylinder axis (Hanson & Whiteheart, 2005; Khan et al., 2022). Many Clp ATPases, with notable exception of ClpB, ClpG, and ClpL (Bohl & Mogk, 2024), form complexes with peptidases ClpP or ClpQ (HslV) and induce forced unfolding of protein substrates by mediating their translocation through the central channel, followed by their degradation by the associated peptidase (Sauer & Baker, 2011). Substrate translocation is driven by energy from ATP hydrolysis. Such ATPases include ClpA, ClpC, ClpX, and ClpY (HslU) and contain the IGL(F) motif in the C-terminal region that is essential for their interactions with the peptidase subunits (Kim et al., 2001b). Clp ATPase/peptidase complexes are found in prokaryotes as well as in mitochondria and chloroplasts of eukaryotic cells (Gottesman, 2003). Due to their essential function in the cellular protein quality control, ATP-dependent Clp proteases are considered promising targets for development of novel antimicrobials (Illigmann et al., 2021; Motiwala et al., 2022).

Unlike those Clp ATPases that associate with peptidase subunits and mediate protein degradation, ClpB is a stand-alone protein disaggregase that is essential for bacterial survival under severe thermal stress (Bohl & Mogk, 2024; Squires et al., 1991; Zolkiewski, 1999). ClpB binds to aggregated proteins and extracts single unfolded polypeptides from aggregated particles by translocating them through the central channel. The reactivation of aggregates by ClpB

requires the co-chaperones, DnaK, DnaJ, and GrpE (Goloubinoff et al., 1999; Zolkiewski, 1999). A unique structural feature of ClpB, which is not found in ClpA and ClpX, is the coiled-coil domain inserted between the ATP-binding modules and located at the outer surface of the hexameric cylinder (Deville et al., 2017; Lee et al., 2003). The coiled-coil domain is highly mobile, it regulates the ClpB activity and couples its disaggregase activity with aggregate recruitment mediated by DnaK (Rosenzweig et al., 2013; Seyffer et al., 2012). Besides being produced in most of the bacteria, ClpB is found in fungi and plants, but not in metazoans (Erives & Fassler, 2015), which makes it an attractive target for antimicrobial therapies (Kędzierska-Mieszkowska & Zolkiewski, 2021). Indeed, small molecule inhibitors of ClpB identified through high-throughput screens were found to suppress the growth and survival of *Escherichia coli* (I. Martin et al., 2013). We have recently discovered that a previously characterized inhibitor of a mammalian AAA+ ATPase p97, N2,N4-dibenzylquinazoline-2,4-diamine (DBeQ) (Chou et al., 2011) (Supplementary Fig. 1) also efficiently inhibits ClpB in vitro and in *E. coli* cells (Glaza et al., 2021).

To better characterize the mechanism of DBeQ-induced inhibition of ClpB, we employed an engineered ClpB variant BAP (“ClpB which like ClpA interacts with ClpP”) which includes the ClpP-interacting IGL motif (Weibezahn et al., 2004). We discovered that DBeQ induces dissociation of the BAP-ClpP complex, which prompted us to investigate the DBeQ activity towards those ATPases that natively interact with ClpP, namely ClpA and ClpX. We found that among the three Clp ATPases, DBeQ selectively targets ClpB.

Materials and Methods

Chemicals

DBeQ was purchased from Millipore-Sigma (St. Louis, MO) and dissolved in DMSO at 30 mM. All other chemicals were analytical grade and purchased from Millipore-Sigma or Fisher Scientific

DNA Constructs

ClpA from *Escherichia coli* was amplified from pSK24 (Thompson & Maurizi, 1994) with primers to incorporate NdeI and XhoI restriction sites at the 5' and 3' ends, respectively. The PCR product was digested with NdeI and XhoI and subcloned into the pET20b vector to include a C-terminal histidine tag in the expressed protein.

Proteins

Proteins were produced in *Escherichia coli* BL21(DE3) (New England Biolabs, C2527H) from the following vectors: pET20b-ClpA (see above), pET21a-EcClpX (Addgene, Plasmid #111516), pET20b-ClpB (Barnett et al., 2000), pET20b-BAP (T. Zhang and M. Zolkiewski, unpublished results), p6xHis-GFP-SsrA [33], pET37b-ClpP Δ 14aa, and pET37b-ClpP Δ 14aaS111A (gift from Dr. Walid Houry, University of Toronto). Unless otherwise noted, cells were grown in Luria Broth at 37 °C until OD₆₀₀ 0.6-0.8. ClpA expression was induced by supplementing cultures with 0.4 mM IPTG and incubation for 16 hours at 18 °C. ClpP expression was induced with 1 mM IPTG and incubation at 37 °C for 6 hours. Cells bearing pET21a-EcClpX were grown at 37°C until OD₆₀₀ ~ 1. Cells were then chilled to 18 °C and protein expression was induced with 1 mM IPTG

and incubation at 18 °C for 4 hours. Expression of GFP-SsrA was induced with 1 mM IPTG at 25 °C for 16 hours.

Cells were collected by centrifugation, resuspended in lysis buffer (50 mM Phosphate, pH 7.4, 300 mM NaCl, 10 mM Imidazole), and disrupted by sonication followed by centrifugation at 25,000xg for 30 min at 4 °C to remove insoluble material. During purification of ClpA and ClpX, the lysates were supplemented with 0.4% Polyethyleneimine to precipitate nucleic acids followed by an additional centrifugation at 25,000xg for 30 min at 4 °C.

All proteins, except ClpB and BAP, included a 6-His tag and were purified *via* affinity chromatography on Ni-NTA resin (Qiagen). Briefly, the soluble fractions were nutated with Ni-NTA resin equilibrated in lysis buffer at 4 °C for 2 hours. Then, the resin was washed with lysis buffer containing 25 mM imidazole and an additional series of washes with 50 mM imidazole. Unless otherwise noted, proteins were eluted with lysis buffer containing 300 mM imidazole. During purification of ClpP, the resin was additionally washed with 300 mM imidazole, followed by elution with 500 mM imidazole. Collected fractions were analyzed by SDS-PAGE and sufficiently pure fractions were pooled and concentrated with Amicon Ultra 10K MWCO centrifugal filters (Millipore Sigma). ClpA was then dialyzed into HO Buffer (50 mM HEPES-KOH, pH 7.5, 300 mM NaCl, 20 mM MgCl₂, 2 mM DTT, 10% (V/V) Glycerol). ClpP, ClpX, and GFP-SsrA were dialyzed into Buffer P (20 mM HEPES-KOH pH 7.4, 200 mM NaCl, 20 mM MgCl₂, 1 mM EDTA, 1 mM DTT). ClpB and BAP were purified as described before (Barnett et al., 2000). Concentration of purified proteins was measured by absorption at 280 nm. FITC-Casein was Type II fluorescein isothiocyanate labeled casein from Bovine milk (Sigma Aldrich) resuspended in 1x Phosphate Buffered Saline (10 mM Phosphate pH 7.4, 137

mM NaCl, 2.7 mM KCl). Protein solutions were stored at -20 °C. Firefly luciferase was obtained from Promega, α - and κ -casein – from Sigma Aldrich.

α -casein Degradation

Degradation of α -casein by BAP-ClpP was monitored in Buffer Z (25 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 2 mM DTT, 75 mM KCl, 5% DMSO). 250nM BAP₆ and 500nM ClpP₁₄ or ClpP(S111A) were incubated with 5mM ATP for 10 min at 30 °C with or without 50 μ M DBE_Q. The reaction was initiated by adding 4.7 μ M α -casein. Aliquots were withdrawn at various time points and analyzed by SDS-PAGE followed by staining with Coomassie Blue dye.

GFP-SsrA Degradation

Degradation of GFP-SsrA by ClpXP was performed in Buffer X (20mM HEPES-KOH pH 7.5, 20mM MgCl₂, 100mM KCl, 1 mM DTT). 200nM ClpX₆ and 600nM ClpP₁₄ were pre-incubated with 5 mM ATP and 0-100 μ M DBE_Q in 5% DMSO at 30 °C for 10 min. Reactions were initiated by the addition of 3 μ M GFP-SsrA. The reaction was monitored by loss of fluorescence at λ_{ex} = 395 nm λ_{em} = 509 nm measured with an H1 Synergy plate reader (BioTek). Fluorescence intensity was measured every 70 seconds and normalized to the initial reading. Degradation rate was determined from a slope of the linear segment of the fluorescence loss over time corrected for the slope obtained without ClpX and ClpP. Degradation of GFP-SsrA by ClpAP followed an analogous procedure with the following modifications. Experiments with ClpA were performed in HO Buffer due to differences in optimal buffer conditions between ClpA and ClpX [34]. To avoid auto-degradation of ClpA by ClpAP, ClpA was incubated with DBE_Q for 8 min, then supplemented

with 5mM ATP followed by an additional two-minute incubation prior to degradation reactions. Reactions were performed in triplicate.

FITC-Casein degradation

Degradation of FITC-Casein by BAP-ClpP was performed in Buffer X. 200nM BAP₆ and 600nM ClpP₁₄ were incubated with 5mM ATP, and 0-25 μ M DBE_Q in 1% DMSO at 37°C for 10 min. Reactions were initiated by the addition of 10 μ M FITC-Casein and monitored by an increase in fluorescence at λ_{ex} = 340 nm and λ_{em} = 525 nm on an Synergy H1 plate reader (BioTek). Fluorescence intensity was measured every 70 seconds. Degradation rates were determined from slopes of the linear segments of the fluorescence increase over time corrected for the slope in a negative control containing 5 mM ADP. The procedure for FITC-Casein degradation by ClpAP was as described above for GFP-SsrA degradation. Reactions were performed in triplicate.

Peptidase Activity of ClpP

5 μ g/mL ClpP or the inactive S111A mutant was incubated with increasing concentrations of DBE_Q in the presence of L-Leucine-7-amido-4-methylcoumarin hydrochloride (Leu-AMC, Sigma Aldrich) in 25mM Tris/HCl pH 7.5, 10mM MgCl₂, 2mM DTT, 75mM KCl, 5% DMSO. Fluorescence emission was measured at room temperature using λ_{ex} = 380 nm and λ_{em} = 455 nm in Perkin-Elmer LS55 spectrofluorometer.

Reactivation of Aggregated Luciferase

Firefly luciferase (13.75 mg/ml) was diluted 50-fold in the denaturation buffer (30mM HEPES-KOH pH 7.6, 60mM KCl, 1mM EDTA, 10mM MgCl₂, 10mM DTT, 6.9M urea) at room temperature for 2 hours, then stored at -20°C. To initiate the renaturation, denatured luciferase

was diluted 10-fold in the renaturation buffer (30mM HEPES-KOH pH 7.6, 100mM KCl, 1mM EDTA, 10mM MgCl₂, 10mM DTT). Next, the luciferase solution was diluted 10-fold in the renaturation buffer containing 1 mg/ml BAP, 0.065 mg/ml DnaK, 0.05 mg/ml DnaJ, 0.015 mg/ml GrpE, and 5mM ATP. Luciferase activity measurements were performed as previously described (Glaza et al., 2021).

ATPase Activity

The ATPase assay was performed by preincubating 100nM BAP with 0-25μM DBeq in buffer X with 5% DMSO for 10 minutes at 37 °C. Reactions were initiated with the addition of 1 mM ATP, followed by an additional 10-minute incubation at 37°C. ATPase activity was measured by the release of inorganic phosphate using a Malachite Green reagent (Abcam, ab270004). The absorption at 635 nm was measured in a Nunc 96-well optical plate with a H1 Synergy plate reader. The amount of ATP hydrolyzed was determined by comparing the A₆₃₅ of the BAP samples with a standard curve obtained with varying amounts of inorganic phosphate. Background non-enzymatic ATP hydrolysis was determined in samples without BAP. Reaction rates were calculated as nmols of phosphate released *per* μg of BAP *per* hr. The measurements were performed in triplicate.

Determination of the Half-maximal Inhibitory Concentration, IC50

The measured activity was normalized to a positive control without DBeq. The equation $Y=100/(1+(IC50/X)^{n_H})$, where Y the normalized activity, X is log₁₀[DBeq], and n_H is Hill coefficient, was fitted to the experimental data using GraphPad Prism software.

Sedimentation Velocity

Sedimentation velocity experiments were performed with a Beckman Optima XL-I analytical ultracentrifuge equipped with the interference and absorption detection systems and a

four-position AN-Ti rotor. BAP (11 μ M monomer) and ClpP (13 μ M monomer) were diluted in 25 mM Tris/HCl, pH 7.5, 75 mM KCl, 10 mM MgCl₂, 2 mM DTT, 5 mM ATP γ S, 5% DMSO with or without 50 μ M DBE₂Q. 340 μ l of the protein solution and 350 μ l of the buffer were loaded to the right and left sector, respectively, of the double-sector analytical cell. After equilibration at 20 °C at 3,000 rpm, the rotor was accelerated to 30,000 rpm and radial scans of the samples were performed at 1 min interval using the interference detection. Apparent sedimentation coefficient distributions were calculated using the time-derivative method [35] with the Origin software provided by the instrument manufacturer.

Gel Filtration Chromatography

Gel-filtration analysis was performed using Bio-Rad BioLogic LP chromatography system and a 70-cm Superdex 200 column at 0.3 ml/min flow rate. The column was equilibrated with 25 mM Tris/HCl, pH 7.5, 75 mM KCl, 10 mM MgCl₂, 2 mM DTT with 5 mM ATP or ADP, and 5% DMSO. BAP (5 μ M monomer) was mixed with ClpP (8.5 μ M monomer) in the presence of ATP or ADP and injected onto the column. The fractions were collected every 1 min and analyzed with SDS-PAGE followed with staining with Coomassie blue.

Dynamic Light Scattering

Dynamic Light Scattering Experiments were performed using an Anton-Paar Litesizer instrument with an Omega Cuvette (Anton-Parr Mat.No. 225288). 0.1 mg/mL BAP (200 nM BAP₆) was mixed with 0.03 mg/mL ClpP (92 nM ClpP₁₄) and 2 mM ATP γ S with the desired concentration of DBE₂Q in Buffer X containing 1% DMSO. The final particle size distribution was determined by averaging 25 correlation-function decays.

Results

The engineered variant of *E. coli* ClpB, BAP forms a complex with ClpP and mediates degradation of ClpB substrates instead of their reactivation (Weibezahn et al., 2004). Casein is often used as a convenient ClpB pseudo-substrate because its processing does not require the co-chaperones. As shown in Fig. 2-1, BAP with ClpP efficiently degrade casein in the presence of ATP. Casein degradation does not occur when the inactive ClpP variant S111A is used with BAP (Fig. 2-1A) or when ATP is replaced with ADP (Fig. 2-1). Interestingly, casein degradation is also inhibited in the presence of DBE_Q (Fig. 2-1A, B). DBE_Q does not inhibit the intrinsic peptidase activity of ClpP (Supplementary Fig. 2-2), nor it affects the binding of casein to ClpB (Glaza et al., 2021).

To explain the results in Fig. 1, we asked if stability of the BAP-ClpP complex is affected by DBE_Q. The particle size distributions determined from dynamic light scattering (DLS) show that the BAP-ClpP complex formed in the presence of the poorly hydrolysable ATP analog, ATP γ S, is larger than isolated ClpP or BAP (Fig. 2-2A, B, C). DLS data for BAP with ClpP show a mean hydrodynamic diameter of ~17 nm (Fig 2C) which agrees with the apparent size of two BAP hexamers in a complex with one ClpP tetradecamer, as visualized with electron microscopy (Weibezahn et al., 2004). Importantly, the DLS results for BAP-ClpP show a gradual decrease in particle size upon addition of DBE_Q from 1 μ M to 30 μ M (Fig. 2-2D, E, F), which demonstrates dissociation of the BAP-ClpP complexes. With 10 and 30 μ M DBE_Q, the apparent particle size approximates those of isolated ClpP and BAP (Fig. 2E, F), which indicates a significant extent of complex dissociation at those DBE_Q concentrations.

Dissociation of the BAP-ClpP complex in the presence of DBE_Q was corroborated by the DBE_Q-induced decrease in the particle sedimentation coefficient (Supplementary Fig. 2-3A) and

a loss of ClpP co-elution with BAP in size-exclusion chromatography (Supplementary Fig. 2-3B). The sample sedimentation observed between 10S and 15S in the presence of DBeQ and an apparent lack of molecular species sedimenting below 5S (Supplementary Fig. 3A) indicates that DBeQ does not induce dissociation of hexameric BAP into monomers but only weakens BAP's interaction with ClpP.

As shown in Figures 2-1 and 2-2, DBeQ inhibited the BAP-ClpP mediated protein degradation and destabilized the BAP-ClpP complex in a concentration-dependent manner. We also found that DBeQ inhibits the basal and casein-activated ATPase of BAP in the absence of ClpP (Fig. 2-3A) and BAP-mediated reactivation of aggregated firefly luciferase in the presence of DnaK/DnaJ/GrpE (Fig. 2-3B). Importantly, all the above structural and biochemical effects occur within a similar ligand concentration range with the apparent $IC_{50} \sim 7 \mu M$ DBeQ (Fig. 2-3 and 2-4B).

Having observed an unexpected destabilization of the ATPase-peptidase complex in the presence of DBeQ, we asked if that compound might serve as a potent inhibitor of those Clp ATPases that are natively associated with ClpP. We investigated *E. coli* ClpA and ClpX, both of which have been extensively characterized in previous studies (Olivares et al., 2016). ClpA and ClpX natively recognize substrates containing C-terminal SsrA tags, whereas ClpB and BAP do not (Gottesman et al., 1998; Hinnerwisch et al., 2005). We investigated degradation of GFP-SsrA by ClpA-ClpP and ClpX-ClpP in the presence of DBeQ. We found that DBeQ's potency towards ClpA and ClpX was significantly lower than towards ClpB/BAP because less than 50% inhibition of degradation of the SsrA-tagged substrate by ClpX-ClpP was achieved within the limits of DBeQ solubility in a buffer with 5% DMSO, and the apparent IC_{50} for inhibition of ClpA-ClpP was $\sim 65 \mu M$ (Fig. 2-4A). ClpA-ClpP, unlike ClpX-ClpP, efficiently degrades casein

(Mahmoud et al., 2022), which allowed a direct comparison of DBeQ effects on ClpA and BAP. As observed during degradation of GFP-SsrA, DBeQ showed a lower potency towards ClpA than towards BAP during processing of casein, with IC50 values for inhibition of those ATPases differing by a factor of 10 (Fig. 2-4B). The above results indicate that among *E. coli* Clp ATPases, DBeQ shows the strongest preference for ClpB.

Discussion

We have previously described DBeQ as an allosteric inhibitor of ClpB that targets both the ClpB ATPase and its disaggregase activity (Glaza et al., 2021). In *E. coli* cells, ClpB is the main target of DBeQ and a DBeQ-induced loss of bacterial survival is linked to disturbed cellular proteostasis, which highlights a central role of ClpB within the bacterial protein quality control network (Glaza et al., 2021).

In this study, we further characterized the mechanism of DBeQ-induced inhibition of ClpB. By employing the BAP variant of ClpB, we aimed to directly follow substrate translocation across the ClpB channel by observing substrate degradation by the peptidase ClpP attached to the BAP channel exit. Indeed, we found efficient degradation of casein by BAP-ClpP and its inhibition by DBeQ in a dose-dependent manner (Fig. 2-1). Unexpectedly, we discovered that the loss of casein degradation could simply arise from DBeQ-induced dissociation of the BAP-ClpP complex (Fig. 2-2). However, even in the absence of ClpP, DBeQ inhibited the intrinsic activities of BAP, the ATPase and the disaggregase (Fig. 2-3).

The results in Figs. 2-1, 2-2 and 2-3 suggest that DBeQ disturbs a network of allosteric linkages across multiple regions of ClpB/BAP that include the substrate-processing channel, the ATP-binding domains, and the C-terminal region engaged in interactions with ClpP. Interestingly, the BAP-ClpP complex forms in the presence of ATP but becomes destabilized by

ADP (Supplementary Fig. 2-3B and ref. [22]). However, DBeQ-induced destabilization of BAP-ClpP is observed in the presence of ATP γ S (Fig. 2-2 and Supplementary Fig. 3A), which indicates that DBeQ does not simply induce an ADP-like state of ClpB/BAP.

The BAP-ClpP complex has been used as a tool to demonstrate that aggregate reactivation mediated by ClpB involves polypeptide threading through the central channel (Weibezahn et al., 2004). However, it has been suggested that instead of actively translocating substrates into ClpP, BAP merely activates the proteolytic activity of ClpP, which would challenge the accepted model of ClpB-mediated aggregate reactivation by substrate translocation (T. Li et al., 2015). We found that the IC₅₀ values for DBeQ-induced inhibition of the intrinsic activity of BAP as well as the ClpP-linked degradation activity are similar (Figs. 2-1 and 2-3). Thus, using DBeQ as a probe of the BAP-ClpP activity, we demonstrated a direct link between the ATPase engine of BAP (Fig. 2-3A), substrate extraction from aggregates (Fig. 2-3B), and substrate delivery through the channel into ClpP (Figs. 2-1B and 2-4B). Our results support the notion that the activity of BAP in complex with ClpP adequately reflects the native disaggregase activity of ClpB.

Intrigued by the observed DBeQ-induced dissociation of the BAP-ClpP complex, we investigated the DBeQ activity towards other Clp ATPases from *E. coli* which natively interact with ClpP, namely ClpA and ClpX. ClpA, like ClpB/BAP, contains two AAA+ ATP-binding modules, whereas ClpX contains only one (Zolkiewski, 2006). Besides the highly conserved AAA+ modules, each of the Clp ATPases contains less conserved N-terminal domains that participate in binding of substrates and adaptor proteins (Kirstein et al., 2009). Importantly, only ClpB contains the coiled-coil domain inserted into the N-terminal AAA+ module (Lee et al., 2003). Using casein as a model substrate for ClpA and ClpB/BAP, we found that IC₅₀ for

inhibition of casein degradation with DBeQ is more than an order of magnitude higher for ClpA-ClpP than for BAP-ClpP (Fig. 2-4B). During degradation of GFP-SsrA, a common substrate for ClpA and ClpX, the IC₅₀ for DBeQ inhibition of ClpX-ClpP was even higher than for ClpA-ClpP (Fig. 2-4A). Altogether, these results demonstrate that DBeQ does interact with ClpA and ClpX, but displays a significantly lower affinity towards those proteins than towards ClpB/BAP. Thus, DBeQ is a selective inhibitor of ClpB within the family of related ATPases from *E. coli*.

The number and location of DBeQ binding site(s) in ClpB are currently unknown. Given the apparent selectivity of DBeQ towards ClpB, we propose that DBeQ's inhibitory activity is mediated by interactions with the weakly-conserved regions, including the N-terminal domain and the coiled-coil domain. We note that the N-terminal and the coiled-coil domains of ClpB display a significant degree of mobility and conformational flexibility that support the ClpB function (Nagy et al., 2010; Oguchi et al., 2012; Seyffer et al., 2012). Targeting the flexible and mobile protein regions with small-molecule ligands might disrupt the dynamics of inter-domain allosteric linkages within the protein, as indeed observed by us for DBeQ-induced inhibition of multiple functionalities in ClpB/BAP.

Figures

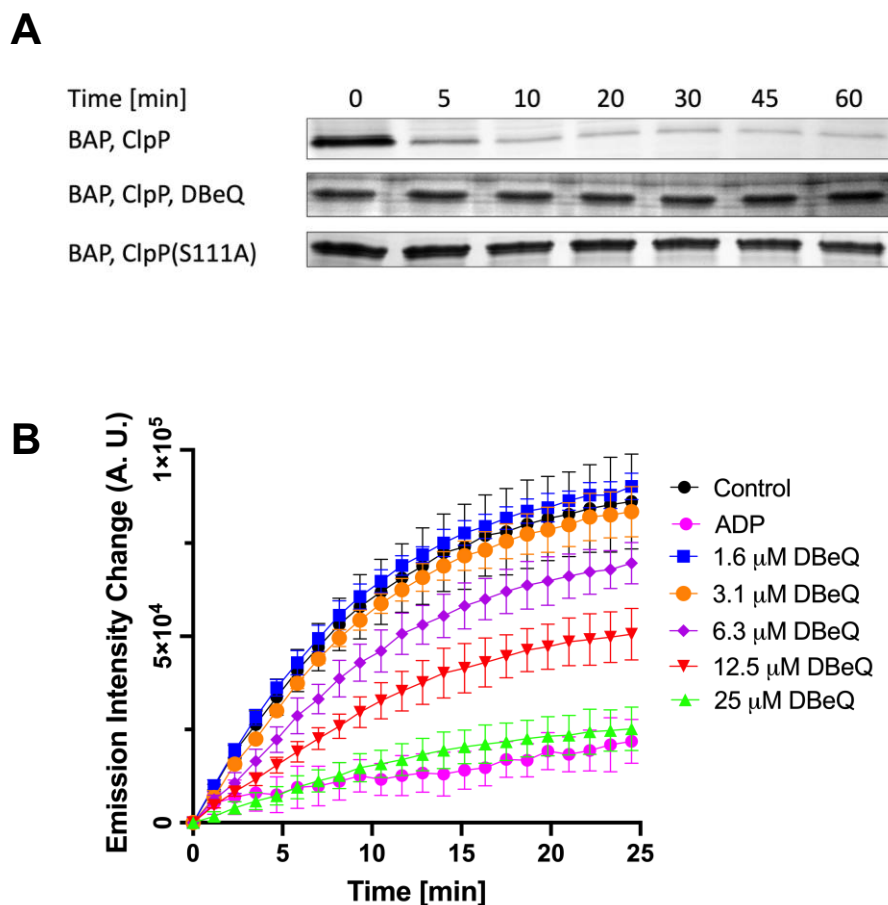


Figure 2-1 DBEIQ inhibits degradation of casein by BAP-ClpP

A) Shown is a representative SDS-PAGE gel image of the α -casein band from analysis of degradation of α -casein with BAP and ClpP or ClpP(S111A) with 5 mM ATP, in the absence and presence of 50 μ M DBEIQ. Each lane corresponds to a sample collected at the indicated reaction time. **(B)** Relative changes in the fluorescein emission intensity during degradation of FITC-casein with BAP-ClpP, 5 mM ATP or ADP, without DBEIQ (Control), or with DBEIQ at the indicated concentrations.

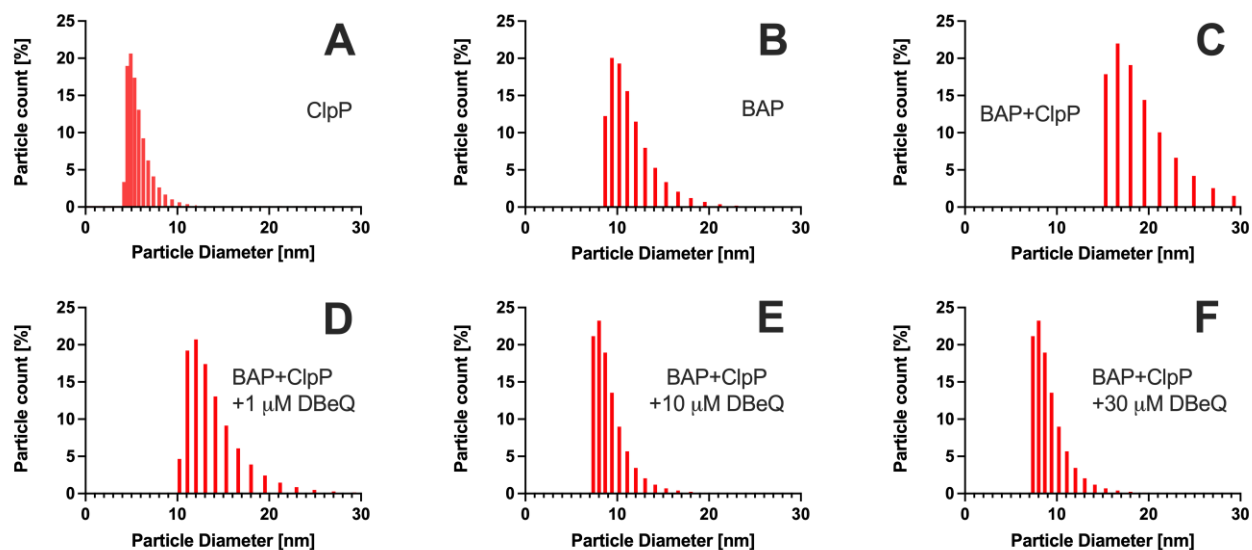


Figure 2-2 – DBeQ induces dissociation of the BAP-ClpP complex

Particle-size distributions measured with dynamic light scattering for ClpP (A), BAP (B), BAP with ClpP (C), BAP with ClpP and 1 μ M DBeQ (D), BAP with ClpP and 10 μ M DBeQ (E), BAP with ClpP and 30 μ M DBeQ (F), in the presence of 2 mM ATP γ S.

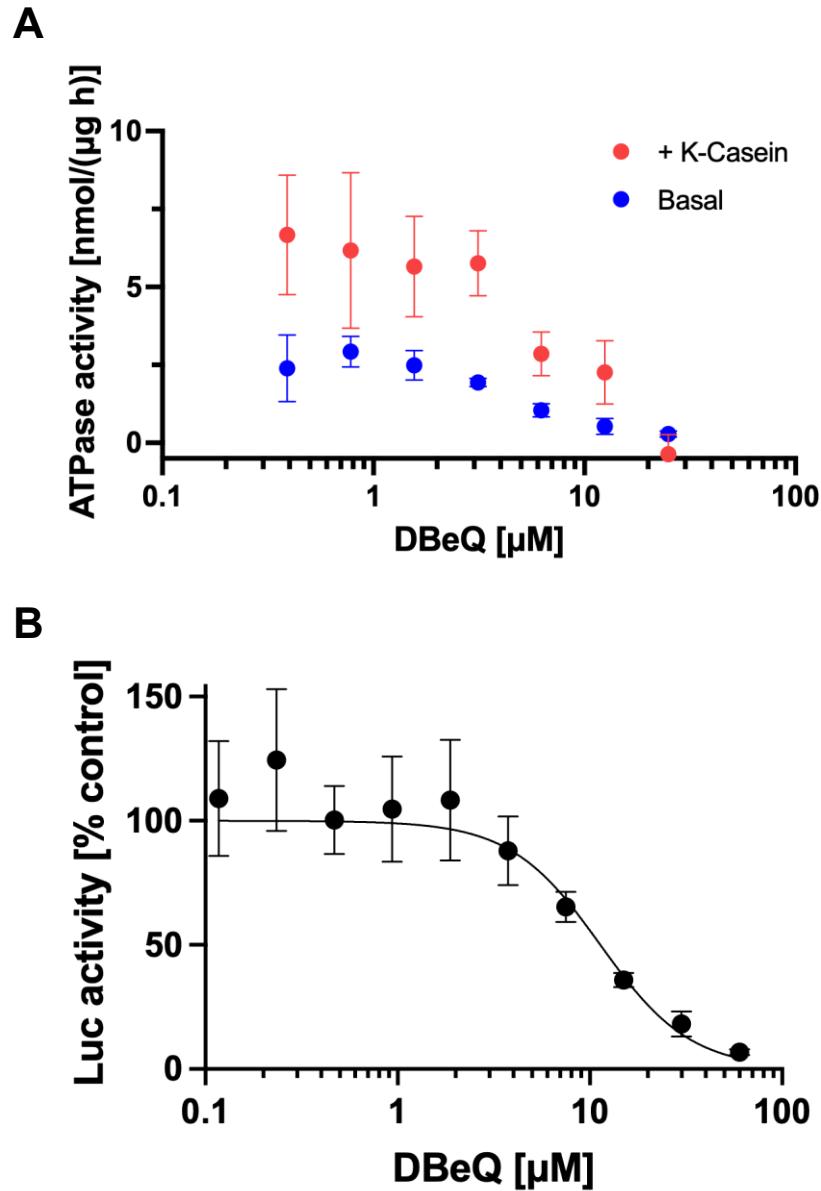


Figure 2-3 – DbeQ inhibits the ATPase and disaggregase activities of BAP

Effects of increasing concentrations of DBeQ on the rate of ATP hydrolysis catalyzed by BAP in the absence and presence of κ -casein (A) and on the reactivation of aggregated luciferase in the presence of BAP, DnaK, DnaJ, and GrpE (B).

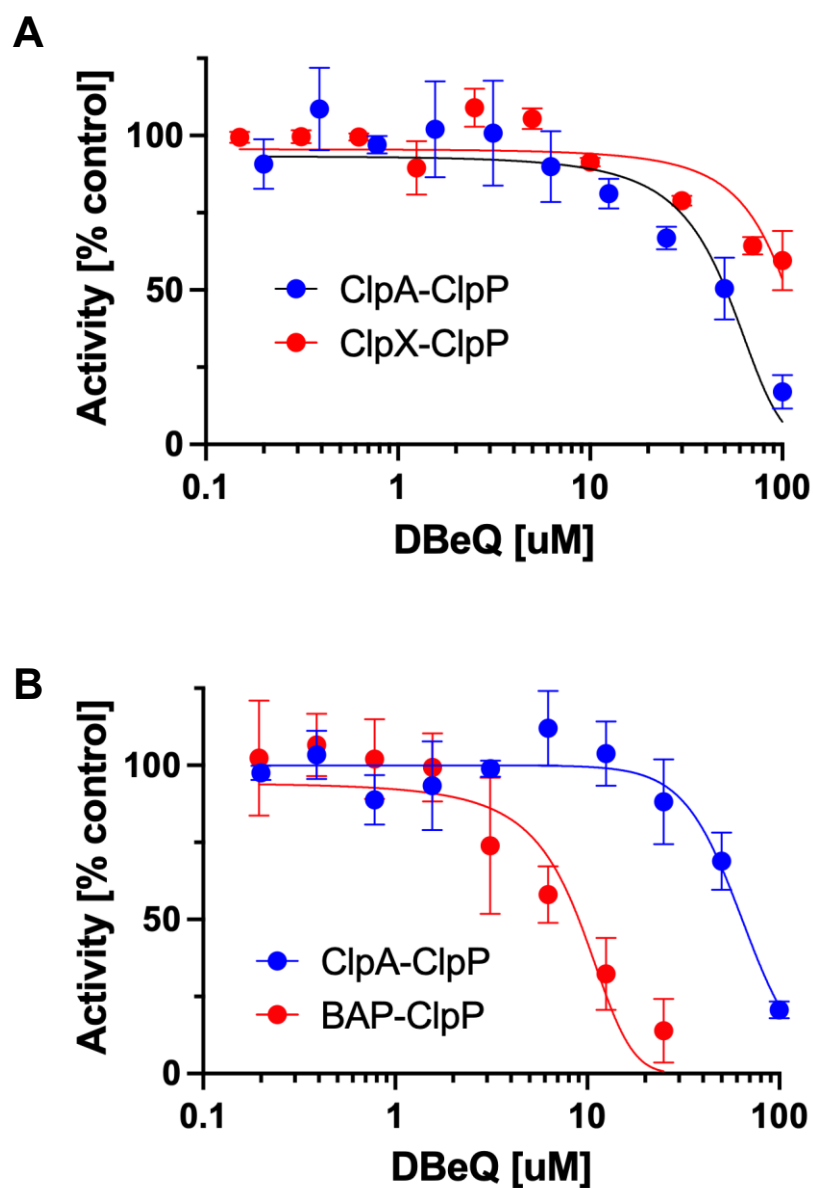
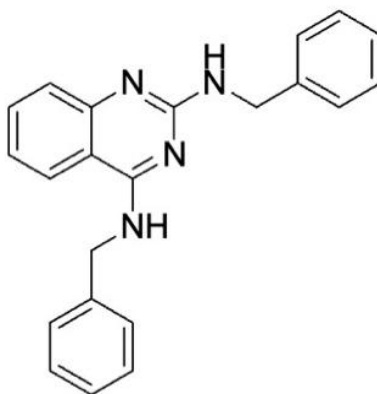


Figure 2-4 – Effects of DBeQ on the activities of ClpA and ClpX

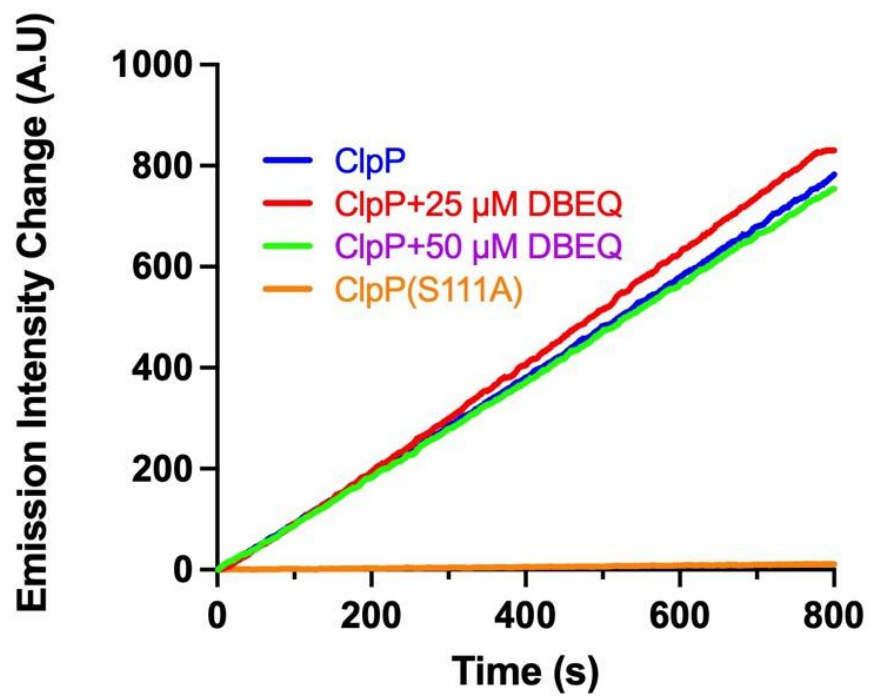
Relative changes in the rate of degradation of SsrA-GFP with ClpA-ClpP and ClpX-ClpP as the function of DBeQ concentration. (B) Relative changes in the rate of FITC-casein degradation with ClpA-ClpP and BAP-ClpP as the function of DBeQ concentration.

Supplementary Figures



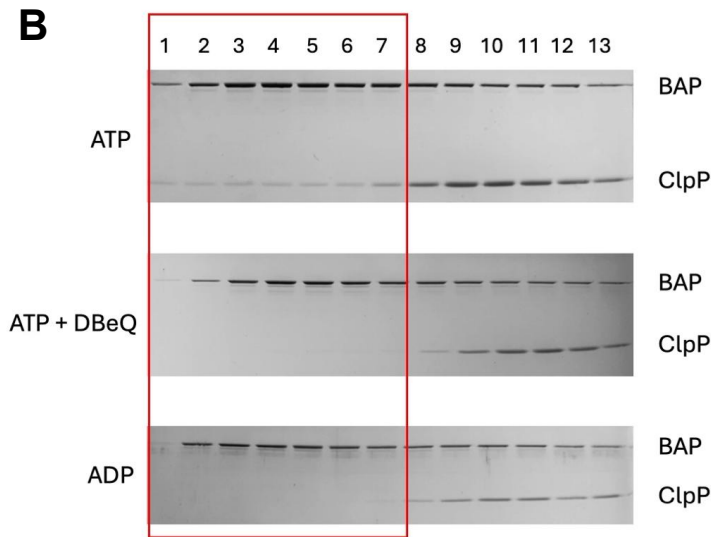
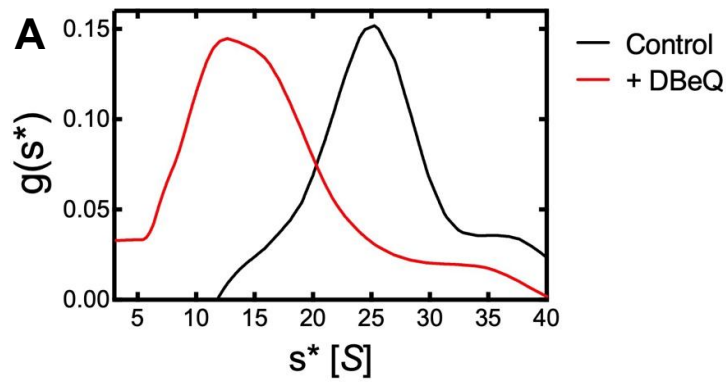
Supplementary Figure 2-1

The structure of N²-N⁴ Dibenzylquinazoline-Diamine (DBeQ)



Supplementary Figure 2-2

DBEQ effects on the intrinsic peptidase activity of ClpP. Shown are representative traces of fluorescence emission changes during ClpP-induced degradation of L-Leucine-7-amido-4-methylcoumarin hydrochloride.



Supplementary Figure 2-3

(A) Sedimentation velocity analysis of BAP-ClpP. Shown are the apparent sedimentation coefficient distributions $g(s^*)$ vs. the sedimentation coefficient in Svedberg units (S) for BAP with ClpP and 5 mM ATP γ S (black) and with 50 μ M DBeQ (red). (B) Gel filtration analysis of BAP-ClpP with 5 mM ATP (top), 5 mM ATP and 50 μ M DBeQ (middle), and 5 mM ADP (bottom). The red rectangle indicates the elution range of the BAP-ClpP complex in the presence of ATP.

Chapter 3 - Human Mitochondrial AAA+ ATPase SKD3/CLPB assembles into nucleotide-stabilized dodecamers¹

Introduction

Suppressor of potassium transport defect 3, SKD3, also known as caseinolytic peptidase B homolog CLPB, belongs to the AAA+ family of ATPases associated with various activities (Hanson & Whiteheart, 2005; Neuwald et al., 1999). The function of SKD3/CLPB is not fully understood and its relation to the well-studied protein disaggregases ClpB/Hsp104 has been under debate. While expression of the disaggregases ClpB/Hsp104 is limited to microorganisms and plants (Erives & Fassler, 2015), SKD3 is only found in vertebrates (R. R. Cupo & Shorter, 2020). SKD3 contains the N-terminal mitochondrial targeting sequence, followed by an ankyrin-repeat domain and a single ATP-binding AAA+ module (R. R. Cupo & Shorter, 2020). In contrast, members of the ClpB/Hsp104 family contain two AAA+ modules separated by a characteristic coiled-coil domain, which couples the disaggregation function with aggregate recognition by the co-chaperones DnaK/Hsp70 (Rosenzweig et al., 2013; Seyffer et al., 2012). The coiled-coil domain is missing in SKD3. While ClpB/Hsp104 are primarily cytosolic and

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their isoforms are located in the mitochondrial matrix, SKD3/CLPB localizes to the mitochondrial intermembrane space (IMS)(Thevarajan et al., 2020). The mature form of SKD3/CLPB is produced upon its import into the IMS through proteolytic processing of the N-terminal targeting sequence (Saita et al., 2017).

Hereditary mutations in the human *SKD3/CLPB* gene have been linked to various pathologies with a broad phenotypic spectrum, including 3-methylglutaconic aciduria, congenital neutropenia, cataract, intellectual disability/developmental delay, progressive brain atrophy, and movement disorder (Capo-Chichi et al., 2015; Kanabus et al., 2015; Pronicka et al., 2017; Saunders et al., 2015; Wortmann et al., 2015). Recently, *de novo* *SKD3/CLPB* mutations have been also associated with congenital neutropenia (Warren et al., 2022; Wortmann et al., 2021). Notably, the *SKD3/CLPB* expression is elevated in acute myeloid leukemia (AML) and further upregulated upon acquisition of resistance to apoptosis-inducing treatment (Chen et al., 2019). In AML cells, SKD3/CLPB is involved in maintaining the mitochondrial cristae structure and SKD3 ablation promotes sensitivity to pro-apoptotic drugs (Chen et al., 2019). Since SKD3 reactivates selected aggregated proteins *in vitro*, it was postulated to function as a molecular chaperone/disaggregase in the IMS (R. R. Cupo & Shorter, 2020).

We have previously shown that SKD3 forms ATP-dependent high-molecular weight complexes in the IMS (Thevarajan et al., 2020). The composition and structure of the SKD3-containing molecular assemblies in the IMS are unknown. To begin closing that gap in knowledge, we produced a recombinant mature form of human SKD3 in *Escherichia coli*. We used dynamic light scattering, sedimentation equilibrium, sedimentation velocity, and negative-stain electron microscopy to obtain the first structural information on the oligomeric assembly of human SKD3.

Materials and Methods

Protein expression and purification

The human SKD3/CLPB cDNA fragment corresponding to the residues 127-707 was PCR amplified to include *Bam*HI and *Not*I restriction sites at the 5' and 3' ends, respectively, using the RC203013 vector (OriGene Technologies) as the template. The PCR product was digested with *Bam*HI and *Not*I, and subcloned into the pOP3MT vector (plasmid #112601, Addgene). Recombinant mature SKD3 was produced as a TEV-cleavable fusion with the N-terminal Avi-His8 and maltose binding protein (MBP) tags using a procedure similar to that of Cupo and Shorter (R. Cupo & Shorter, 2020), with modifications described below.

The pOP3MT plasmid containing the sequence of the mature form of SKD3 was transformed into *Escherichia coli* BL21(DE3) (Millipore-Sigma). Cells were cultured in 2 x 5 ml LB broth with 100 µg/ml ampicillin for 16 hours at 30 °C with shaking, then transferred to 1 L of LB with ampicillin in a 4-L baffled Erlenmeyer flask and cultured with shaking at 37 °C for an additional 2.5 h. Induction was performed by supplementing the media with 1 mM IPTG and culturing the cells at 15 °C for 16 hours with shaking. Cells were collected by centrifugation at 4,500 x g for 30 min at 4 °C and then disrupted by sonication in lysis buffer (40 mM HEPES-KOH pH 7.4, 500 mM KCl, 20% (w/v) glycerol, 10 mM MgCl₂, 2 mM β-mercaptoethanol, and a protease inhibitor cocktail (Sigma) in the amount 1 ml/100 ml buffer). Lysates were cleared via centrifugation at 30,000 x g at 4 °C for 20 min. Nucleic acids were precipitated from cleared lysates with 0.04% polyethyleneimine and pelleted by centrifugation at 20,000 x g for 25 min at 4 °C. The supernatant containing SKD3-MBP fusion protein was incubated with 4 ml amylose resin column overnight at 4 °C. The column was washed with 10 bed volumes of wash buffer (40

mM HEPES-KOH pH 7.4, 500 mM KCl, 10% (w/v) glycerol, 10 mM MgCl₂, 2 mM β-mercaptoethanol) at 4 °C. Protein was eluted with amylose elution buffer (50 mM Tris-HCl pH 8.0, 300 mM KCl, 10% (w/v) glycerol, 10 mM MgCl₂, 2 mM β-mercaptoethanol, 10 mM maltose.) SKD3-MBP fusion protein was cleaved with a 6His-tagged AcTEVTM protease (Thermo Fisher) at 4 °C for 96 h in the amylose elution buffer. After the cleavage, the sample was supplemented with 10 mM imidazole and incubated with 1 ml Ni-NTA resin (Qiagen) for 1 hour at 4 °C. In SDS-PAGE analysis, cleaved SKD3 was found in the wash fractions obtained with 20 mM sodium phosphate buffer, pH 7.4, 300 mM NaCl, 25 mM imidazole, 2 mM β-mercaptoethanol. The SKD3-containing fractions were collected and dialyzed into buffer A (50 mM HEPES-KOH pH 7.5, 200 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 2 mM β-mercaptoethanol). Protein concentration was determined by measuring absorbance at 280 nm, using the absorption coefficient 0.796 cm²/mg (calculated with Protparam, <https://web.expasy.org/protparam/>).

Dynamic light scattering

DLS experiments were performed at room temperature with an Anton Paar Litesizer 500 instrument equipped with a Univette 50-μl sample compartment. SKD3 stock solution was diluted in buffer A to the desired concentration. For the experiments with nucleotides, the samples were incubated for 10 min at room temperature after adding the nucleotide. The final particle-size distributions were obtained from averaging of 25 correlation-function decays.

Analytical ultracentrifugation

Sedimentation equilibrium and sedimentation velocity experiments were performed with a Beckman Optima XL-I analytical ultracentrifuge equipped with the interference and absorption detection systems and a four-position AN-Ti rotor. For sedimentation equilibrium experiments,

100 μ l of SKD3 solution and 120 μ l of buffer A were loaded to the right and left sectors, respectively, of the 6-channel analytical cell. The samples were equilibrated at 4 °C at the speed indicated in Figure 2. To monitor the progress towards equilibrium, radial scans were performed every 6 hours. The final data collection was performed after 50-60 h. The data analysis was performed using the single-species model built into the Origin software supplied with the instrument. Partial specific volume of SKD3 (0.737 ml/g) and the buffer density were calculated using Sednterp software by John Philo (<http://www.jphilo.mailway.com>). For sedimentation velocity experiments, 300 μ l of SKD3 solution and 320 μ l of buffer A were loaded to the right and left sector, respectively, of the double-sector analytical cell. After equilibration at 20 °C at 3,000 rpm, the rotor was accelerated to 35,000 rpm and radial scans of the sample were performed at 1 min interval. Apparent sedimentation coefficient distributions were calculated using the time-derivative method (Stafford, 1992) with the Origin software provided by the instrument manufacturer. Observed sedimentation coefficient values were converted to the values corresponding to the density and viscosity of water at 20 °C ($s_{20,w}$) using Sednterp software.

Electron microscopy

The grids for negative staining electron microscopy were prepared according to previously published protocols (Peisley & Skiniotis, 2015). In brief, 3 μ l of SKD3 with 1 mM ATP was applied to glow-discharged 400 mesh formvar carbon film grids with copper supports (Electron Microscopy Sciences, EMS) and stained with a 5 mg/ml uranyl formate solution. For transmission electron microscopy, a total of 45 micrographs were manually acquired using a JOEL JEM-1400 transmission electron microscope with an acceleration voltage of 100 kV. Images were collected at 30,000x magnification with a physical pixel size of 3.897 Å and

nominal dosage of $35 \text{ e}^-/\text{\AA}^2$. Image processing was performed using Cryosparc v3.2.0 (Punjani et al., 2017). The 45 micrographs were CTF-corrected, and 121 particles were manually picked to build an initial 2D set. Using this template, a total of 138,410 unique particles were picked and subjected to 2D classification

Results

Purified recombinant SKD3 corresponding to the mature IMS form (residues 127-707, monomer molecular weight 65,810 Da) displayed a robust ATPase activity of $27 \pm 3 \text{ nmol ATP per nmol SKD3 per min}$, which agrees with the previously reported activity (R. R. Cupo & Shorter, 2020) and is several-fold higher than the ATPase activity of *E. coli* ClpB (Glaza et al., 2021). The SKD3 ATPase did not respond to 0.1 mg/ml α -casein, a well-known pseudo-substrate and activator of ClpB/Hsp104, again in agreement with the previous results (Wortmann et al., 2015). The lack of casein-induced activation the SKD3 ATPase suggests that its substrate-recognition mechanism may differ from that of the disaggregases ClpB/Hsp104.

The particle-size distributions determined with dynamic light scattering (DLS) showed that the apparent hydrodynamic (Stokes) diameter of SKD3 particles did not exceed 1 nm at protein concentration below 0.1 mg/ml (Fig. 3-1A). However, the apparent SKD3 size increased to over 10 nm in diameter at a higher protein concentration (Fig. 3-1B) or in the presence of nucleotides: ATP, ADP, or the non-hydrolysable ATP analogue adenylyl-imidodiphosphate (AMP-PNP) (Fig. 3-1C, D, E). We conclude that SKD3 undergoes self-association into oligomers, which are stabilized by adenine nucleotides. The SKD3 oligomer stabilization does not require ATP hydrolysis and is induced by the diphosphate as well as the triphosphate nucleosides.

To determine the molecular weight of the SKD3 species detected with DLS, we performed sedimentation equilibrium experiments at low and high protein concentration, in the absence and presence of the nucleotides. Since hexameric assemblies are considered canonical for the AAA+ family (Hanson & Whiteheart, 2005), we simulated protein concentration profiles for a putative SKD3 hexamer at the applied centrifugation speed (Fig. 3-2, broken lines), but the simulated profiles deviated strongly from the experimental data in Fig. 3-2A, B, and C. As shown in Fig. 3-2A, the protein concentration gradient for 0.08 mg/ml SKD3 in the absence of nucleotides was consistent with monomeric SKD3. In the presence of 1 mM AMP-PNP or ADP, the protein molecular weight increased approximately 12-fold (Fig. 3-2B, C). The protein concentration gradient for SKD3 at 1.2 mg/ml without a nucleotide could not be approximated with a single-species model (data not shown), which suggests that the oligomers formed at high protein concentration in the absence of nucleotides are less stable than those produced in their presence. Importantly, the results in Fig. 3-2B and 3-2C show, for the first time, that SKD3 forms dodecameric assemblies in the presence of nucleotides.

To obtain further insight into the structure of the SKD3 dodecamer, we performed negative-stain electron-microscopy imaging of SKD3 in the presence of 1 mM ATP. The sample images showed uniformly distributed large particles, which was consistent with the stabilizing effect of the nucleotide (Fig. 3-3, top panel). Image-class analysis and averaging visualized cylinder-shaped particles with an open central channel along the cylinder axis (Fig. 3-3, bottom panels). The SKD3 particle top view showed a ring structure with the diameter of ~13 nm and a central pore with the diameter of ~2 nm. The top-view ring dimensions determined for SKD3 agree with those found for the hexameric assembly of bacterial ClpB (Akoev et al., 2004; Lee et al., 2003) and other ring-forming hexameric AAA+ ATPases (Vale, 2000). Interestingly, the

SKD3 side view showed a ~15 nm-tall and apparently triple-tiered structure with a top-bottom symmetry (Fig. 3-3, bottom panels).

The SKD3 oligomer molecular weight as determined by sedimentation equilibrium (Fig. 3-2B, C) indicates that the particle visualized in Fig. 3 contains twelve SKD3 subunits. Given the particle diameter, which approximates the diameter of a canonical hexameric AAA+ ring, the twelve SKD3 subunits are likely arranged in two ring-shaped hexamers stacked against each other. The side-view symmetry suggests that the association of two SKD3 hexamers occurs either “head-to-head” through the N-terminal ankyrin-repeat domains or “tail-to-tail” through the C-terminal AAA+ domains. Furthermore, in previous imaging analyses of hexameric AAA+ ATPases, the number of side-view tiers corresponded to the number of distinct ring structures formed by individual protein domains (Lee et al., 2003, 2007; Lopez et al., 2020; Yokom et al., 2016). Thus, a double-hexamer sandwich of a two-domain SKD3 might appear as a four-tier particle. Unexpectedly, in the SKD3 dodecamer side view, we observed three distinct structural rings instead of four, which suggests that the ring stacking might involve an overlap or intertwining of the SKD3 domains from the opposite rings. The detailed SKD3 domain arrangement in the dodecameric complex will need to be refined with future structural studies at higher resolution.

To further characterize the SKD3 dodecamers, we performed sedimentation velocity experiments for SKD3 with 1 mM ATP or without nucleotides. The maximum of the apparent sedimentation coefficient distribution curve in Fig. 4 defines the sedimentation coefficient of the predominant molecular species in solution (Stafford, 1992). In the presence of ATP, SKD3 sedimented as a 16.2 S particle (Fig. 3-4A). The predicted sedimentation coefficient for a solid spherical particle with the molecular weight of the SKD3 dodecamer (792 kDa) is 27.9 S, which

is significantly higher than the observed value. A reduction of the sedimentation rate could arise from a significant deviation from a spherical shape. However, the height/diameter ratio for the particles visualized in Fig. 3 is ~ 1.15 , indicating that the shape of an SKD3 dodecamer does not strongly deviate from a sphere. Alternatively, sedimentation rate may be strongly reduced if protein complexes form hollow particles with internal solvent-filled voids. Such hollow particles produce anomalously high frictional forces, which oppose the force of centrifugation. Indeed, a top view of the SKD3 dodecamer reveals a hollow channel/cavity (Fig. 3-3). Thus, a strong reduction of the sedimentation coefficient for the SKD3 dodecamers, as compared to that of a solid sphere with the same molecular weight, could be explained by the voids in their molecular structure.

The sedimentation coefficient distribution for nucleotide-free SKD3 (Fig. 3-4B) differs significantly from that found in the presence of ATP (Fig. 3-4A). The distribution is broadened, and its maximum indicates that the predominant species sediments as a 23.2 S particle, which is significantly higher than the sedimentation coefficient of 16.2 S in the ATP-bound state. This result demonstrates a significant conformational change in the SKD3 oligomers induced by ATP. The sedimentation rate of SKD3 oligomers formed in the absence of nucleotides better approximates the sedimentation of solid spherical particles than the rate observed in the presence of ATP. It is also possible that the SKD3 oligomers formed in the absence of nucleotides are heavier, i.e., contain a higher number of subunits, as was observed previously for the bacterial ClpB (Akoev et al., 2004).

Discussion

In this study, we obtained the first structural information for human SKD3/CLPB. We discovered that SKD3 forms nucleotide-stabilized dodecamers and provided the first EM image analysis for the dodecamers in the most physiologically relevant nucleotide-bound state. While hexameric structures have been observed often among other AAA+ ATPases (Hanson & Whiteheart, 2005; Jessop et al., 2021), the apparent double-hexamer sandwich found for SKD3 is uncommon within this protein family. The functional significance of the non-canonical structure of SKD3 remains to be determined.

In spite of its non-canonical double-hexamer assembly, the dodecameric SKD3 contains a characteristic structural feature that is essential for biological activity of AAA+ ATPases: an open central pore/channel (see Fig. 3-3). Among the variety of AAA+ functions, an insertion or translocation of substrates inside the central channel appears to be a common mechanistic principle of this diverse protein family (Gates & Martin, 2020; Hanson & Whiteheart, 2005; Jessop et al., 2021). An identity of IMS-localized macromolecules that are processed in the SKD3 channel, a mechanism of SKD3-substrate interaction, and biological consequences of SKD3-driven substrate processing remain to be discovered.

Figures

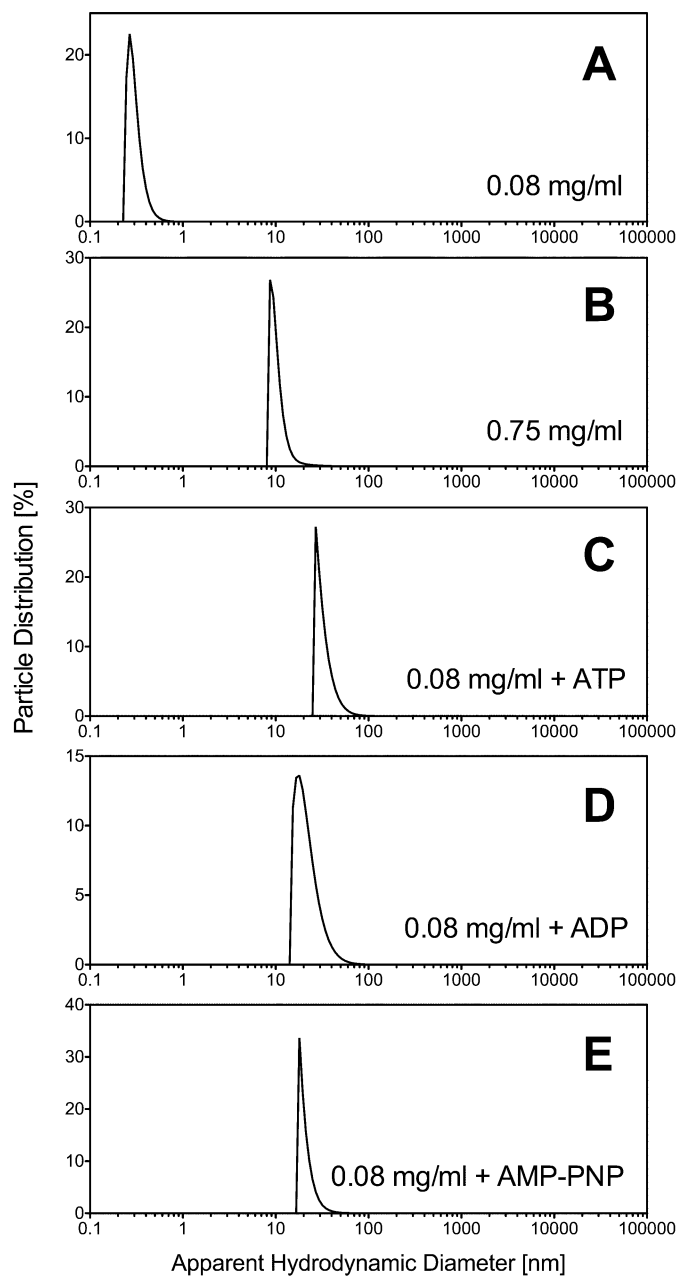


Figure 3-1 Dynamic Light Scattering Analysis of SKD3/CLPB samples

The SKD3 particle-size distributions were measured at the indicated protein concentration without nucleotides or with 1.7 mM ATP, ADP, or AMP-PNP. Shown are representative results from 2 repeated experiments.

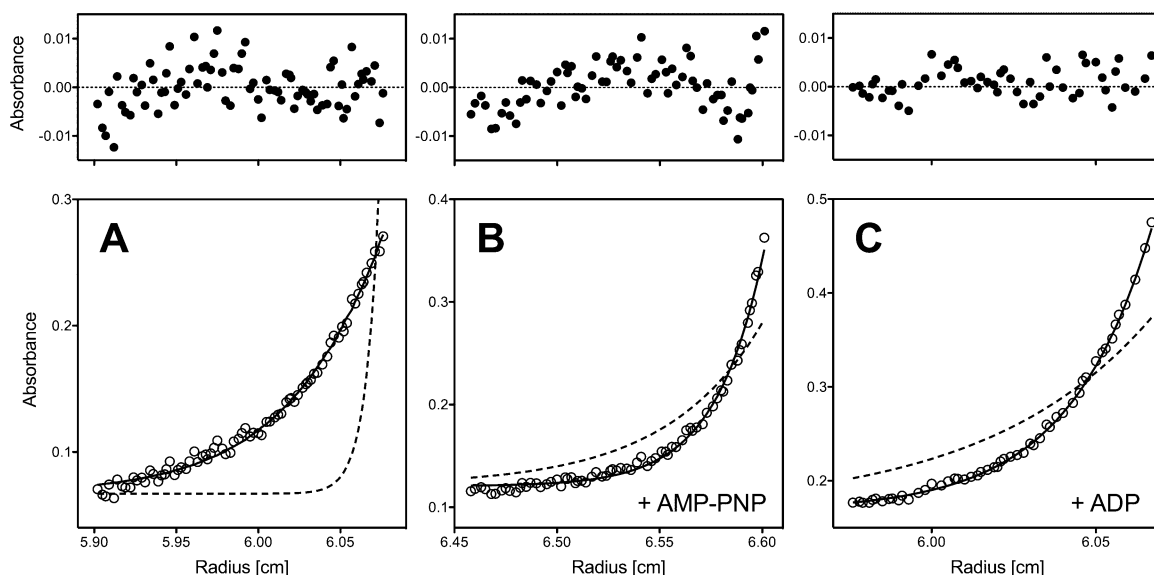


Figure 3-2 Sedimentation equilibrium analysis of SKD3/CLPB

Bottom panels: shown are the protein concentration gradients (open circles), the fits corresponding to single-species model (solid lines), and the simulated profiles for a putative hexameric SKD3 (395 kDa, broken lines). Top panels: shown are the residuals of the fits ($A_{\text{exp}} - A_{\text{model}}$). The concentration gradients were measured at 4 °C after the sedimentation equilibrium was reached at 20,000 rpm with 0.08 mg/ml SKD3 (**A**), at 8,000 rpm with 0.63 mg/ml SKD3 and 1 mM AMP-PNP (**B**), or at 8,000 rpm with 1.2 mg/ml SKD3 and 1 mM ADP (**C**). The protein absorption was measured at 235 nm (**A**) or 295 nm (**B**, **C**). The single species fit gave an apparent molecular weight of 65,780 Da with the non-ideality coefficient of $1.4 \cdot 10^{-5}$ (**A**), 779,160 Da with the non-ideality coefficient $-9 \cdot 10^{-10}$ (**B**), and 788,230 Da with the non-ideality coefficient $-6 \cdot 10^{-9}$ (**C**).

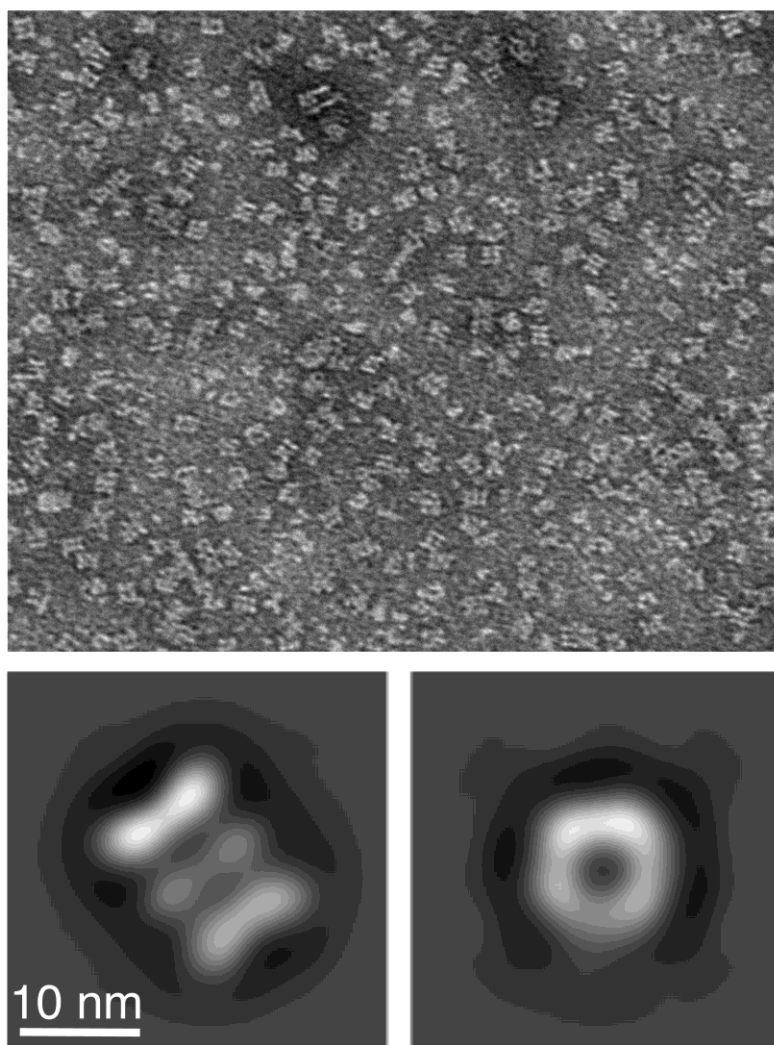


Figure 3-3 Negative-stain electron microscopy images of SKD3/CLPB.

The top panel shows a raw electron micrograph of negatively stained 0.6 mg/ml SKD3 with 1 mM ATP at 30,000-fold magnification. The bottom panels show the side view (left) and top view (right) of the image-class analysis and averaging from the total of 12,548 particles.

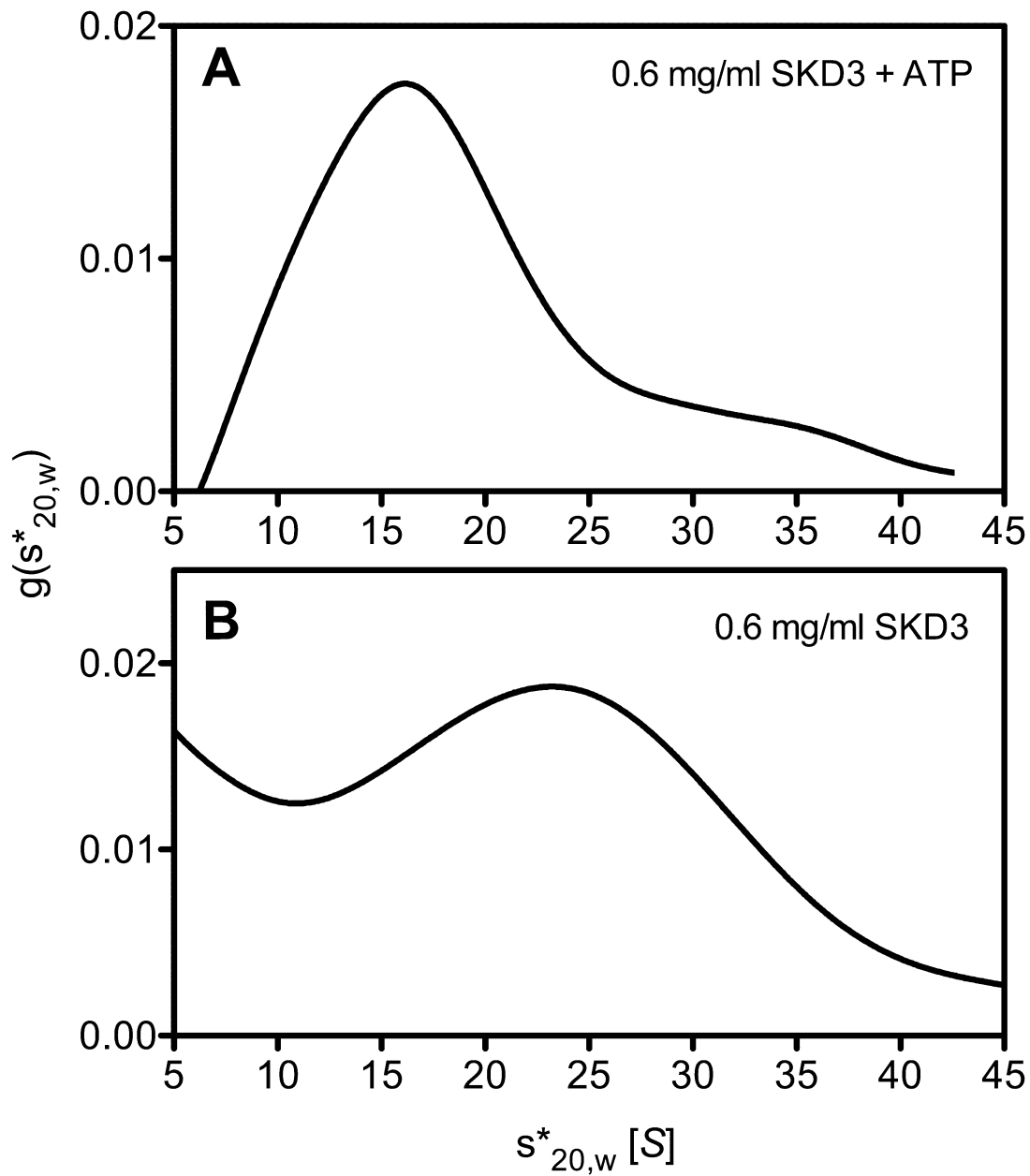


Figure 3-4 Sedimentation velocity analysis of SKD3/CLPB

Shown are the apparent sedimentation coefficient distributions $g(s^*_{20,w})$ vs. the sedimentation coefficient $s^*_{20,w}$ in Svedberg units (S) obtained from the time-derivative analysis [18]. The sedimentation experiments were performed at 20 °C and 35,000 rpm. The progress of SKD3 sedimentation at 0.6 mg/ml was monitored with the interference optics in the presence of 1 mM ATP (A) or with absorbance at 280 nm in the absence of the nucleotide (B).

Chapter 4 - Human CLPB interacts with components of the mitochondrial contact site and cristae organizing system (MICOS)

Introduction

CLPB: a AAA+ chaperone implicated in human disease

CLPB, encoded by the *CLPB* gene, is a distantly related member of the Clp/Hsp100 family of ATPases which originates in metazoa and is localized to the mitochondrial inter-membrane space (IMS) (Thevarajan et al., 2020). Members of the Hsp100 family are characterized by the conversion of chemical energy from ATP hydrolysis into mechanical work by threading protein substrates through a central channel of a homo-hexameric ring. Recent studies demonstrate that CLPB functions as a disaggregase for misfolded model substrates luciferase and alpha-synuclein (R. R. Cupo & Shorter, 2020; Gupta et al., 2023; Lee et al., 2023). Furthermore, CRISPR knockouts of CLPB in multiple cell types lead to the aggregation of several proteins in the IMS, suggesting a disaggregase model for CLPB's primary function (Baker et al., 2024; R. R. Cupo & Shorter, 2020; D'Angelo et al., 2025a).

Both biallelic and monoallelic mutations in *CLPB* lead to a broad spectrum of pathologies in humans, including neutropenia, cataracts, intellectual disability, and impaired motor function (Pronicka et al., 2017; Warren et al., 2022; Wortmann et al., 2015, 2021). These pathologies, are often concurrent and the clinical severity of disease can vary among patients. The precise physiological effects of *CLPB* deficiency and reasons of the apparent variability in disease severity and affected tissues, are not well understood.

CLPB ablation in several cell types leads to impaired cellular respiration, decreased mitochondrial membrane potential, and an increased sensitivity to pro-apoptotic stimuli (Chen et al., 2019; Warren et al., 2022; Wenta et al., 2025). Further, CLPB ablation appears to cause a drastic change in mitochondrial morphology, presenting widened cristae (Chen et al., 2019; D'Angelo et al., 2025a). Cytochrome c is stored within the cristae lumen and is required for electron transfer between Complex III and Complex IV of the electron transport chain. Additionally, its release from the cristae lumen into the intermembrane space, and subsequently into the cytosol in response to pro-apoptotic stimuli initiates apoptosis (Zhou et al., 2024). Therefore, careful maintenance of the cristae junction at the outermost edge of the cristae is required to avoid aberrant cytochrome c release and subsequent activation of the apoptotic pathway. Additionally, proper folding of the IMM facilitates efficient interactions between the different complexes of the electron transport chain by tightly packing them together, promoting ATP production (Mannella, 2020). In CLPB knockout cells, several proteins responsible for maintaining IMM morphology and regulating apoptosis become insoluble, including OPA1, HAX1, MICU1, and MICU2 (Baker et al., 2024; R. R. Cupo & Shorter, 2020). Understanding CLPB's potential interactions with these proteins will be critical for gaining insight into mechanistic links between CLPB and the observed knockout phenotypes, will help in understanding the etiology of CLPB deficiency-linked pathologies.

The increased insolubility of IMS proteins found in the absence of CLPB suggests that they may be prone to misfolding even under non-stress conditions. However, multiple organisms that lack *CLPB* such as *Drosophila melanogaster* bear homologues of those proteins, including MICU1 and OPA1. Thus, CLPB is not required to maintain solubility of IMS proteins in all organisms. Further, as previously mentioned, clinical manifestations of CLPB deficiency do not

affect all tissues, and disease phenotypes vary across patients. Therefore, the stability and solubility of the IMS proteins in mammalian cells may depend on physiological context. Alternatively, it is possible that CLPB's function involves interactions with the soluble forms of IMS proteins, and that the misfolding of mitochondrial proteins is incidental and occurs downstream of the events triggered by a loss of CLPB in mitochondria.

MICU1 and MICU2: regulators of mitochondrial calcium signaling

MICU1 (mitochondrial calcium uptake 1) is classically regarded as a regulator of the mitochondrial calcium uniporter complex (mtCU) by serving as a “gatekeeper”. MICU1 functions in tandem with its paralog, MICU2 to form heterodimers which occlude the face of the mtCU, only opening the channel when a sufficient threshold of $[Ca^{2+}]$ is met (Y. Wang et al., 2020). While MICU1 does indeed interact with the mtCU, the “gatekeeper/occlusion” model has been questioned, as recent studies suggest that MICU1 and MICU2 may in fact stimulate Ca^{2+} transport across the mtCU in response to increased cytosolic $[Ca^{2+}]$ (Garg et al., 2021). Additionally, several studies have found that MICU1 has an additional role in mitochondrial calcium signaling that is independent of mtCU. In multiple human cell lines, MICU1 has been found to operate as a component of the mitochondrial contact site and cristae organizing system (MICOS), functioning as a sensor to facilitate changes in cristae organization in response to calcium signaling (Gottschalk et al., 2019, 2022; Tomar et al., 2023).

In this study, we adopted a candidate-based approach in search of proteins that bind directly to CLPB. We have identified MICU1 and MICU2 as putative binding partners of CLPB and further characterized the CLPB-MICU1 interaction.

Materials and Methods

Purification of Recombinant CLPB-StrepII

Proteins were purified following previously published methods with modifications (Cupo and Shorter, 2020). All variants of CLPB-StrepII (wild-type, E425Q, and K357T) were produced in the Rosetta2(DE3) strain of *E. coli* transformed with a pMAL-c4x vector with inserts encoding the amino acid sequence for the mature form of CLPB isoform 2 (Uniprot Q9H078-2 amino acids 127-677), with an N-terminal Maltose Binding Protein tag followed by a TEV cleavage site as well as a C-terminal StrepII tag.

Starter cultures of transformed bacteria were grown overnight at 37 °C in Terrific Broth supplemented with ampicillin (100 µg/mL), chloramphenicol (34 µg/mL), and 0.2% (W/V) glucose, then diluted 100x into larger flasks of the same media. Cultures were then grown at 37 °C until an OD₆₀₀ of 0.3-0.6 was reached (~2 h). The shaker was then rapidly cooled to 15°C over the course of 20 min and expression was induced by supplementing the media with 1 mM IPTG, followed by overnight incubation. Cells were then collected by centrifugation at 6000xg for 30 min at 4 °C and pellets were resuspended in CLPB Wash Buffer (40 mM HEPES-KOH pH 7.4, 500 mM KCl, 20% (W/V) glycerol, 2 mM β-mercaptoethanol). Cells were then disrupted by sonication, followed by centrifugation at 25,000xg for 30 min at 4 °C to remove insoluble material.

The cleared lysates were then applied onto amylose resin (NEB) equilibrated in CLPB Wash Buffer and nutated for 4 h at 4 °C. The resin was then centrifuged at 800xg for 5 min, and the flow through was carefully discarded, followed by washing with 14 bed volumes (BVs) of CLPB Wash Buffer by repeated centrifugation and resuspension of the resin. The resin was then further washed with 4 BVs TEV Cleavage Buffer (50 mM Tris-HCl pH 8.0, 300 mM KCl, 10% (W/V)

glycerol, 2 mM β -mercaptoethanol). CLPB was eluted by cleavage of the Maltose Binding Protein tag via the addition of 5 mg TEV protease, followed by slowly nutating the resin at 34 °C for 4 h. The resin was then centrifuged at 800xg for 10 min, and the supernatant was collected. The supernatant was then loaded on a HiLoad 26/600 Superdex 200 column (Cytiva) equilibrated in CLPB size exclusion buffer (50 mM Tris-HCl pH 8.0, 500 mM KCl, 10% (W/V) glycerol, 1 mM DTT). Fractions were analyzed by SDS-PAGE and sufficiently pure fractions were pooled and spin-concentrated using Amicon Ultra 10K MWCO centrifugal filters (Millipore Sigma) to a final concentration of 2.4-3 mg/mL, then stored at -20 °C. Protein concentrations were determined by measuring the absorbance at 280 nm.

Purification of Recombinant MICU1, MICU2, and MICU1 Δ C

Proteins were produced in the Rosetta2(DE3) strain of *E. coli* transformed with a pET-Duet vector containing inserts encoding human MICU1 (aa 94-476), human MICU2 (aa 68-434), or human MICU1 Δ C (aa 94-444).

Starter cultures of transformed bacteria were grown overnight with shaking at 37 °C in Terrific Broth supplemented with ampicillin (100 μ g/mL) and chloramphenicol (34 μ g/mL), then diluted 100x in the same media. The diluted cultures were then grown at 37 °C until an OD₆₀₀ of 0.6-0.8 was reached (~2.5 h). Protein overexpression was induced by supplementing the cultures with 0.3 mM IPTG, followed by an additional 4-hour incubation at 37 °C. Cells were then collected by centrifugation at 6000xg for 30 min at 4 °C, resuspended in MICU lysis buffer (25 mM HEPES-NaOH pH 7, 500 mM NaCl, 25 mM Imidazole, 2 mM β -mercaptoethanol), and then disrupted by sonication, followed by an additional centrifugation at 25,000xg for 30 min at 4 °C to remove insoluble material.

All proteins were then purified *via* affinity chromatography on Ni-NTA resin (Thermo Fisher) at 4 °C using an N-terminal 6x-histidine tag. Briefly, the supernatant was applied to the resin by gravity, followed by washes with 30 bed volumes of MICU lysis buffer, then eluted with 500 mM imidazole in the same buffer. Collected fractions were then analyzed by SDS-PAGE and sufficiently pure fractions were pooled and spin-concentrated with Amicon Ultra 10K MWCO centrifugal filters (Millipore Sigma). Proteins were then further purified by gel filtration on a HiLoad 26/600 Superdex 200 column (Cytiva) equilibrated in MICU SEC buffer (25 mM HEPES-NaOH pH 7, 300 mM NaCl, 5 mM DTT, 10% glycerol) at room temperature, with a flow rate of 2.5 mL/min. Peak fractions from gel filtration were analyzed by SDS-PAGE. Sufficiently pure fractions were pooled and spin concentrated to 3 mg/mL for MICU2 and MICU1 Δ C, and 6 mg/mL for MICU1. Protein concentrations were determined by UV-absorption at 280 nm. All proteins were then stored at -20 °C.

Mitochondrial Isolation from HL-60 cells

HL-60 cells were cultured as described in Wenta et al. (2025). Mitochondria were isolated from 135×10^6 HL-60 cells collected by centrifugation at 300xg for 5 min. The resulting pellets were washed with DPBS-/- followed by an additional centrifugation step. Cells were disrupted by suspending the pellets in hypotonic buffer (10 mM NaCl, 1.5 mM MgCl₂, and 10 mM Tris-HCl pH 7.4), incubating on ice for 5 min, then breaking with 40 strokes in a dounce homogenizer. Homogenates were then immediately diluted 2.5x with buffer containing 525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl (pH 7.4), and 25 mM EDTA. The homogenate was centrifuged at 600xg for 5 min, the supernatant was then transferred into 1.5 mL Eppendorf tubes and centrifuged at 15,000xg for 15 min at 4 °C. The resulting pellets containing isolated mitochondria were then resuspended in lysis buffer (30 mM Tris-HCl pH 6.8, 150 mM NaCl, 10

mM MgCl₂, 0.5% Triton X-100, 5 mM ATP, and protease inhibitors) to be used in pulldown assays.

Pulldown Assays Against Immobilized Recombinant CLPB

Four columns of Streptactin-XT Sepharose resin (IBA Life Sciences) equilibrated in ST-XT pull-down buffer (30 mM Tris-HCl [pH 6.8], 150 mM NaCl, 10 mM MgCl₂, 0.5% Triton X100, 5 mM ATP) were prepared. 3 mg of each CLPB variant were applied to the columns, followed by incubation with rocking for 5 min to ensure binding. CLPB was then allowed to flow through the column by gravity, and the unbound material was discarded. The columns were washed with 20 column volumes of ST-XT pulldown buffer and the resin was then resuspended in 2 mL of mitochondrial lysates isolated from HL-60 cells. The columns were then incubated at 4 °C with rocking for 2 h followed by the collection of unbound material by gravity flow. Each column was then washed with 2x100 bed volumes of ST-XT pulldown buffer. CLPB and any bound proteins were eluted using 3 column volumes of ST-XT pulldown elution buffer (30 mM Tris-HCl pH 6.8, 150 mM NaCl, 10 mM MgCl₂, 8 M Urea), mixed 2:1 with 3X Laemmli buffer, heated to 95 °C for 5 min, and resolved by SDS-PAGE. Western blotting was performed as described in Wenta et al. (2025).

MICU1 and MICU2 Direct Interaction with CLPB on Streptactin-XT Magnetic Beads.

CLPB-StrepII variants (wild-type, and E425Q and K357T mutants) were diluted to 0.3 mg/mL in MagStrep wash buffer (30 mM Tris-HCl pH 6.8, 150 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 5 mM ATP, 0.05% Tween 20) and incubated for 10 min at 4 °C. Then, 0.3 mg of the protein was applied onto MagStrep Streptactin-XT beads (IBA Life Sciences). Binding to the beads was performed by nutating slowly for 10 min, followed by 3 washes with 100 bed volumes of the ST-

XT wash buffer to remove unbound material. Binding of MICU1 and MICU2 was then evaluated by applying 0.5 mg of each protein diluted in ST-XT wash buffer to CLPB-containing beads, as well as beads with no CLPB as a negative control. Binding was performed by nutating for 1 hr, followed by extensive washing (200 bed volumes) with wash buffer. CLPB-MICU1 and CLPB-MICU2 complexes were eluted in 5 bed volumes of ST-XT pulldown elution buffer. Eluates were mixed 3:1 with 4X laemmli buffer, heated at 95 °C for 5 min, and analyzed by SDS-PAGE and staining with Coomassie Brilliant Blue R-250.

Measurement of MICU1 binding to CLPB by Surface Plasmon Resonance

Sensor chip surfaces for the immobilization of CLPB^{E425Q}-StrepII were first prepared using an Streptactin-XT twin-strep capture kit (IBA Life Sciences). In brief, Streptactin-XT was diluted to 40 µg/mL in immobilization buffer (10 mM CH₃COONa pH 4.5) and immobilized on a CMD200M sensor chip (Xantec) by standard amine coupling at a flow rate of 10 µL/min for all steps. In brief, each flow cell on the sensor chip surface was equilibrated in water, followed by three 60-s pulses of 50 mM NaOH. The chip surface was then activated by a 10 min pulse of an equal volume mixture of 0.1 M N-hydroxyl succinimide (NHS) and 0.4 M ethyl(dimethylaminopropyl) carbodiimide (EDC). Next, Streptactin-XT was injected onto each flow cell for 10 min, followed by an immediate 10 min injection of ethanolamine to quench the remaining reactive groups. The final immobilization levels of Streptactin-XT on each flow cell were 1,445; 1,515; 1,436; and 1,405 resonance units (RU) for flow-cells 1-4 respectively. Next, CLPB^{E425Q}-StrepII was diluted to 0.6 µM (monomer) in SPR-running buffer (30 mM Tris-HCl pH 6.8, 150 mM NaCl, 10 mM MgCl₂, 2 mM ATP, 1 mM DTT, 0.05% Tween 20) and injected onto flow cells 2 and 4 for 2 min at 10 µL/min, followed by a wash with running buffer

until the baseline was stable, resulting in a final immobilization level of ~3332 RU on flow cell 2, and 3126RU on flow cell 4. Flow cells 1 and 3 were left unbound to use as reference flow cells in each experiment.

To evaluate CLPB binding to MICU1 and MICU1 Δ C, both MICU1 proteins were first exchanged into running buffer using Centri-Spin 10 desalting columns (Princeton separations) and then serially diluted 1:1 to a range of concentrations from 13.28 nM to 3.4 μ M of the monomer. The samples were injected over immobilized CLPB^{E425Q} for 3 min followed by a 5 min dissociation phase. Each injection was followed by a 30 s regeneration step with a 10 mM glycine pH 10, 1 M NaCl solution. The signal obtained from injection over CLPB^{E425Q} was double-referenced by subtracting the signal obtained from a blank injection of running buffer and the signal obtained from injecting each concentration of the analyte over the reference flow cells. Steady state affinity analysis was performed in the Biacore analysis software by selecting the maximal binding signal and K_D as adjustable parameters. All experiments were performed in duplicate.

Analytical Gel Filtration

All gel filtration experiments were conducted on a Superose 6 5/150 gel filtration column (Cytiva) at room temperature that was equilibrated in running buffer (30 mM Tris-HCl, pH 6.8, 150 mM NaCl, 10 mM MgCl₂ 1 mM ATP, 1 mM DTT). To remove potential aggregates, all aliquots of protein stocks were centrifuged at 17,000xg for 15 min at 4 °C prior to mixing. To prepare the samples, 20 μ M CLPB^{E425Q} was mixed with 22 μ M MICU1 Δ C (or an equivalent volume of MICU1 storage buffer) with 2 mM of either ATP or ADP. The samples were incubated at room temperature for 15 min to facilitate MICU1 Δ C-CLPB^{E425Q} complex formation and injected onto the column at a flow rate of 0.3 mL/min. 0.08-s/mL fractions were collected,

mixed at the 3:1 ratio with 4X Laemmli buffer, and analyzed by SDS-PAGE followed by silver staining.

Luciferase Reactivation Assays

Firefly luciferase (13.75 mg/ml) was diluted 50-fold in the denaturation buffer (30 mM HEPES-KOH-pH 8.0, 60 mM KCl, 1 mM EDTA, 10 mM MgCl₂, 10 mM DTT, 6.9 M urea) at room temperature for 2 hours, then stored at -20°C. To initiate the renaturation, denatured luciferase was diluted 10-fold in the renaturation buffer (30 mM HEPES-KOH pH 8.0, 100 mM KCl, 1mM EDTA, 10 mM MgCl₂, 10 mM DTT). Next, the luciferase solution was diluted 10-fold in the renaturation buffer containing 1 μM wild-type CLPB with 5 μM MICU1, MICU1ΔC, or BSA, along with 5 mM ATP. Luciferase reactivation was determined by measuring chemiluminescence on a Synergy H1 plate reader after incubation at room temperature for 90 min. Blanks containing denatured luciferase in the absence of CLPB were subtracted from all measurements, and luminescence was normalized to a control containing CLPB without any additional proteins. The measurements provided are from five independent experiments

ATPase Assays

The ATPase assay was performed by preincubating 0.5 μM CLPB at room temperature in ATPase assay buffer (30 mM HEPES-KOH pH 6.8, 100 mM KCl, 1mM EDTA, 10 mM MgCl₂, 1 mM DTT) for 10min in the presence of 1.8 μM MICU1 or 1 μM κ-Casein, or in the absence of additional proteins. Reactions were initiated by the addition of ATP to a final concentration of 2 mM, followed by an additional 10-min incubation at room temperature. ATPase activity was measured by the release of inorganic phosphate using a Malachite Green reagent (Abcam). The absorbance at 635 nm was measured in a Nunc 96-well optical plate with a H1 Synergy plate reader. The amount of ATP hydrolyzed by CLPB was determined by comparing the A₆₃₅

measured in the presence of CLPB with a standard curve obtained using varying concentrations of inorganic phosphate. Background non-enzymatic ATP hydrolysis was determined in samples without CLPB. Reaction rates were calculated as μmols of phosphate released *per* μmol of CLPB *per* minute. The measurements provided are from four independent experiments.

Results

MICU1 and MICU2 are detected in CLPB pulldown assays

For our experiments, we first expressed and purified variants of isoform 2 of human CLPB in *Escherichia coli* with an added C-terminal StrepII tag (Figure 4-1). These variants included wild-type CLPB, as well as the K357T (Walker A) and E425Q (Walker B) mutants, which either do not bind to ATP or are unable to hydrolyze ATP, respectively. To investigate protein binding partners of CLPB in the neutrophil-like HL-60 cell line, we performed pull-down assays, using purified recombinant CLPB variants immobilized onto Streptactin-XT resin by a C-terminal StrepII Tag. Lysates from isolated mitochondria of HL-60 cells were applied onto columns with immobilized wild-type CLPB, CLPB^{E425Q}, CLPB^{K357T}, or onto a column without CLPB, which served as a negative control. After extensive washing, the proteins were eluted with urea, and the eluates were analyzed by Western blotting in a candidate approach. The selection criteria for candidate proteins were based on protein insolubility in CLPB knockout Hap1 and HEK293 cells in previous studies (Baker et al., 2024; R. R. Cupo & Shorter, 2020). More specifically, we focused on regulators of apoptosis, including HAX1, MICU1, and MICU2 in our initial investigation. Notably, all interactions tested herein are presumably with soluble proteins. HAX1, an intrinsically disordered protein and previously established CLPB interactor (Fan et al., 2022; D. Wu et al., 2023) bound to CLPB with high efficiency, as nearly all of the

protein present in the input was retained by CLPB in our initial pulldown experiments, supporting the validity of this method. MICU1 and MICU2, which negatively regulate calcium transport across the inner mitochondrial membrane (IMM) by gating the mitochondrial calcium uniporter complex (MtCU), also appeared in our screen. MICU1 bound to the wild-type and Walker B variants of CLPB. MICU2 bound all three variants, but showed a weaker interaction with the Walker A mutant than with wild-type and Walker B, suggesting that its interaction with CLPB may be ATP-dependent (Figure 3-2).

Calcium ions are important regulators of apoptosis. Increased $[Ca^{2+}]$ in the mitochondrial matrix induces the opening of the mitochondrial permeability transition pore (mPTP), leading to a loss of mitochondrial membrane potential. A sustained uptake of Ca^{2+} into the matrix ultimately results in cell death (Rizzuto et al., 2012). MICU1 and MICU2 serve to regulate the transport of calcium from the IMS into the matrix by forming a heterodimer that blocks the opening of the MtCU, preventing calcium uptake into the matrix at low $[Ca^{2+}]$ in the IMS. MICU1 and MICU2 have been previously extensively studied in a cellular context and have been reconstituted *in-vitro*, making them good candidates for further studies. Specifically, MICU1 has been shown to be a component of the MICOS complex, which regulates cristae morphology by maintaining the cristae junction in cells (Tomar et al., 2023). The maintenance of this narrow aperture is critical in preventing apoptosis, as cristae widening leads to the release of cytochrome c to the IMS and increased susceptibility for apoptosis. Interestingly, CLPB knockouts in human leukemia cells resulted in widened cristae and increased cytochrome c release (Chen et al., 2019). These observations led us to hypothesize that interactions between CLPB and MICU1 may be critical for protection against apoptosis.

MICU1 and MICU2 bind directly to CLPB

To assess whether the interactions we observed with CLPB were the result of direct binding, rather than being mediated by another factor, we then purified recombinant MICU1 (aa 94-476) and MICU2 (aa 68-434) (simply referred to as MICU1 and MICU2 herein) (Figure 3-3). We initially tested for direct interactions between CLPB and these proteins utilizing a scheme similar the approach used in our initial pulldown assays. As before, CLPB variants were immobilized onto magnetic beads coated with Streptactin-XT. Following this step, the beads were incubated with MICU1 or MICU2, followed by extensive washing and elution using urea-containing buffer. The presence of MICU1 and MICU2 in the eluates was detected by SDS-PAGE and protein staining (Figure 3-4). Interestingly, MICU1 appears to be present in all three eluates (from beads containing all three CLPB variants), as opposed to the eluates from just wild-type and Walker B CLPB beads, unlike our observations in our pulldown experiments using HL-60 mitochondrial lysates (Figure 3-2). We hypothesize that this may be due to the relatively high concentrations of MICU1 in the input, in contrast to the much lower concentrations of the endogenous MICU1 in our initial pulldowns. In addition, the presence of other factors in HL-60 mitochondrial lysates may have also interfered with any apparent ATP-independent binding of MICU1 by CLPB observed in our *in-vitro* experiments on magnetic beads.

We then sought to quantify the affinity of MICU1 and MICU2 for CLPB via SPR. For these experiments, we utilized a similar strategy to our initial pulldown assays, immobilizing the Walker B mutant by its C-terminal Strep-II tag on a sensor chip coated with Streptactin-XT, ensuring that the N-terminal ankyrin repeat domain faced the analyte flowing over the sensor chip surface. We then injected the analyte proteins over the immobilized CLPB. Analysis by SPR using steady state assumptions showed that MICU1 bound to CLPB with an apparent

affinity of $\sim 0.88 \mu\text{M}$, thus confirming our observations in the affinity-based assay using Streptactin-XT (Figure 3-6). Binding of MICU2 by CLPB was not detected by SPR, as MICU2 showed a stronger response in the reference flow cell than to the surface prepared with immobilized CLPB (not shown). MICU1 has been shown to form multiple species of homo-oligomers, ranging from dimers to hexamers, and to larger, poorly-characterized species (Kamer et al., 2017; L. Wang et al., 2014) (see also Figure 3-5). The propensity of MICU1 to form these larger species lead us to hypothesize that the interaction between CLPB and MICU1 may be enhanced by or dependent upon these larger structures.

To investigate the dependence of high molecular weight species of MICU1, we purified MICU1 with a deletion of 32 residues from the C-terminus (MICU1 Δ C). These residues form an alpha-helical tail which mediates interactions between subunits in MICU1 homohexamers (L. Wang et al., 2014). Indeed, this deletion leads to a complete shift towards the homodimeric form of MICU1 Δ C in analytical gel filtration (Figure 3-5). We then utilized our SPR strategy to measure steady-state affinity of MICU1 Δ C binding by CLPB. We found that despite MICU1 Δ C's preference to form almost exclusively homodimers, the protein still bound to CLPB with a similar affinity (Figure 3-7) in our experiments. This suggests that binding of MICU1 by CLPB does not depend on the oligomeric state of MICU1.

Next, we utilized analytical gel filtration to determine the approximate size of CLPB^{E435Q} complexed with MICU1. The full length MICU1 showed a broad elution profile, implying the presence of multiple species of oligomers and making it difficult to assess the apparent molecular weight of any CLPB-MICU1 complexes. Thus, we utilized MICU1 Δ C, which forms a single low-molecular weight peak to better assess the size of MICU1-CLPB complexes. When mixed in the presence of ATP, CLPB and MICU1 Δ C both exhibited a leftward shift in their elution

profiles as compared to the individual proteins, appearing in earlier fractions within the 440-670kDa range, thus forming high molecular weight complexes (Figure 3-8). However, these complexes were not detected in the presence of ADP (Figure 3-9), suggesting that the shift towards high molecular weight complexes is indeed ATP-dependent. This result is consistent with the previously established mechanism of Clp ATPases; namely, the formation of stable, high molecular weight complexes that engage with a substrate is dependent on binding of ATP. (Figures 3-8 and 3-9).

MICU1 is a *bona fide* substrate of CLPB

While we have demonstrated that CLPB interacts with MICU1, the functional consequences of this interaction were unclear. If CLPB acts upon MICU1 as a substrate, then it stands to reason that it should compete with other, putative substrates *in-vitro*. To test this hypothesis, we measured the levels of reactivation of aggregated luciferase by wildtype CLPB in the presence and absence of MICU1 and MICU1 Δ C (Figure 3-10). In these experiments, we supplemented the reactions with saturating concentrations of MICU1 or MICU1 Δ C and normalized the luminescence reading to a control reaction with CLPB alone. Indeed, we observed inhibition of luciferase reactivation in the presence of MICU1 and MICU1 Δ C, as we would expect if a competition between MICU1 and the luciferase pseudo-substrate occurs. Additionally, we used BSA to demonstrate that soluble proteins of similar molecular weights to the MICU1 dimer do not inhibit the CLPB-mediated reaction (Figure 3-10), which suggests that CLPB shows selectivity towards MICU1. However, it is still unclear whether the inhibition of CLPB chaperone activity by MICU1 *in vitro* was due to MICU1 functioning as a regulator of

CLPB chaperone and ATPase activities, or if it was due to MICU1 becoming a substrate of CLPB, analogous to the aggregated luciferase.

Previously, it has been demonstrated that the presence of putative substrates stimulates the ATPase activity of Clp chaperones, such as ClpB/Hsp104 in bacteria and fungi (Woo et al., 1992). Indeed, others have shown that the presence of κ -Casein, a commonly used pseudo substrate in the study of Clp ATPases, induces moderate stimulation of ATP hydrolysis by CLPB, as compared to its basal activity. If MICU1 inhibits CLPB's disaggregase activity through competition with a substrate, then it should stimulate CLPB's ATPase activity. In contrast, if the inhibition of luciferase reactivation in the presence of MICU1 occurred due to inhibition of CLPB, then MICU1 should most likely inhibit the CLPB ATPase activity. We determined the ATPase activity of CLPB by measuring the release of inorganic phosphate from ATP and compared it to the activity of CLPB in the presence of MICU1 or κ -Casein (Figure 3-11). Consistent with the results from other groups, the presence of κ -Casein led to a nearly two-fold increase in the ATPase activity of CLPB. The presence of MICU1 appeared to cause a similar increase in the rate of ATP hydrolysis by CLPB. Thus, we have concluded that CLPB likely recognizes MICU1 as a substrate, possibly performing mechanical work and acting as an unfoldase in some capacity, though the extent of MICU1 remodeling by CLPB is not yet clear.

Discussion

In this work, we screened potential candidates for CLPB binding partners in the mitochondrial intermembrane space using lysates from HL-60 cells and identified MICU1 and MICU2 as potential interactors. Further, we have characterized MICU1 as a novel direct interactor and a putative substrate of CLPB.

MICU1 shows increased insolubility in CLPB knockout cells, which supports the original hypothesis in the field that CLPB functions primarily as a disaggregase, responding to an increased misfolding of proteins to relieve proteotoxic stress in mitochondria (Baker et al., 2024; R. R. Cupo et al., 2022; D'Angelo et al., 2025b; D. Wu et al., 2023). However, our findings demonstrate that CLPB binds to the soluble form of MICU1, both in pulldowns from cell lysates and in experiments with purified proteins. Previously, pulldowns performed by overexpressing the N-terminal ANK-repeat domain of CLPB with an added twin-strep tag in HEK-293T cells found that CLPB-MICU1 interactions are likely mediated by this region (D. Wu et al., 2023). This supports our hypothesis that MICU1 is possibly a substrate of CLPB, as AAA+ ATPases are known to engage their substrates using their N-terminal domains, mechanically translocate proteins through their central channel in a strictly N-domain to C-domain direction, and release processed substrates from their C-terminal domains.

Other work has reciprocally identified CLPB as a putative interactor of MICU1 through proximity labeling experiments using MICU1-BioID2 fusion as bait, further supporting our findings (Tomar et al., 2023). Analysis of the mass spectrometry data therein reveals that CLPB's proximity to MICU1 in cells is not significantly affected by the presence or absence of mtCU. Additionally, that study showed that a 70 amino-acid truncation of the C-terminus of MICU1 results in a total loss of interaction with other MICOS components in co-immunoprecipitation assays, suggesting a reliance on the formation of high molecular weight complexes for MICU1 localization at the cristae junction. Our own experiments show that a truncation at the C-terminus of MICU1, though shorter than 70 a.a., is not sufficient to eliminate binding between CLPB and MICU1. Together, these results support the hypothesis that interactions between CLPB and MICU1 might not depend on MICU1 association with the

MICOS complex or the mtCU. MICU1 also features a lysine-rich N-terminal polybasic region (KKKKRSGFRDRK), which is retained in our construct. Experiments with *E. coli* ClpB have shown that the protein favors polybasic motifs for binding, and poly-lysine has been shown to stimulate ClpB's ATPase activity (Liu et al., 2002; Ranaweera et al., 2018). Thus, a preference to engage positively charged sequences may be conserved in mammalian CLPB. If so, we can hypothesize that the binding site for CLPB in MICU1 is at or near the N-terminus of the protein, possibly within this polybasic region.

While our data suggests that CLPB binds to MICU1 independently of its oligomerization state, we do not yet know the functional consequences of CLPB-MICU1 interactions. We predict that MICU1 is a *bona fide* substrate of CLPB that might become either partially or entirely unfolded during its processing by CLPB, perhaps to be transformed into a state required for its physiological function. This hypothesis is analogous to the remodeling of natively folded proteins by Clp ATPases in other organisms, such as the reorganization of inactive RepA dimers into their active monomeric form by ClpA (Pak & Wickner, 1997). Future work should aim to determine the extent of unfolding or dissociation of MICU1 induced by CLPB. Additionally, further study is needed to determine if the formation of MICU1-MICU2 heterodimers affects the ability of either of these proteins to bind to CLPB. Previous cryo-EM and analytical gel filtration studies of CLPB have shown that when engaged with the pseudo-substrate casein, CLPB forms primarily hexamers and dodecamers (R. R. Cupo et al., 2022; Gupta et al., 2023; D. Wu et al., 2023). Indeed, in the presence of MICU1 Δ C, CLPB appears to form large-ATP dependent complexes (Figure 3-8). Estimating the approximate size and stoichiometry of the CLPB-MICU1 Δ C complex with higher resolution techniques will be instrumental in developing a deeper understanding of the functional relevance of this interaction. Additionally, to validate

these findings in a cellular context, experiments should be performed to assess the stability of the MICOS complex in CLPB knockout cells.

Figures

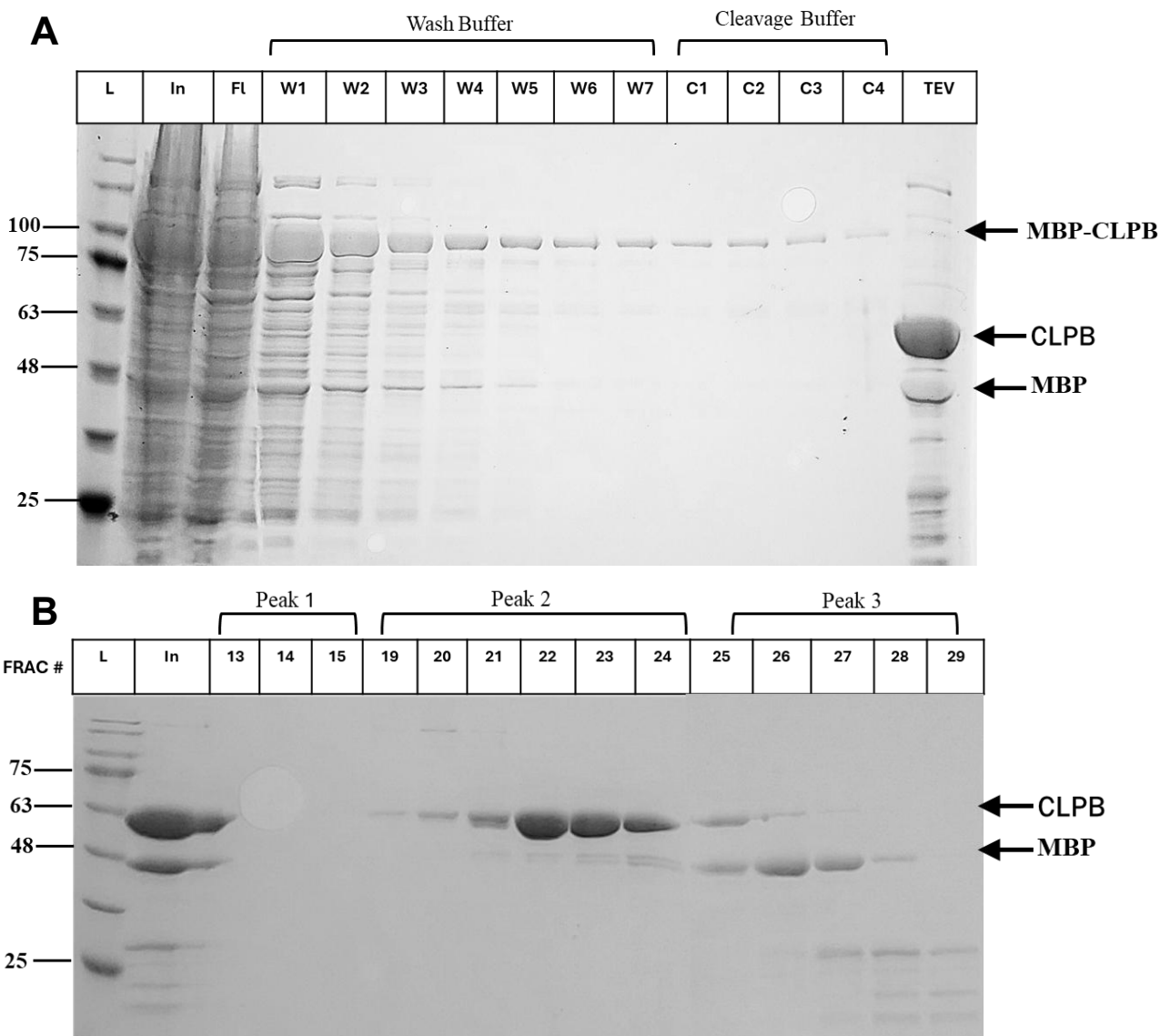


Figure 4-1 Purification of human CLPB expressed in *Escherichia coli*

An SDS-PAGE gel showing purification of MBP-tagged-CLPB on Amylose resin, followed by elution via TEV cleavage of the MBP tag. (A) Further purification of TEV-cleaved CLPB by gel filtration chromatography, analyzed by SDS-PAGE. CLPB elutes as a single peak in fractions 19-24 (B).

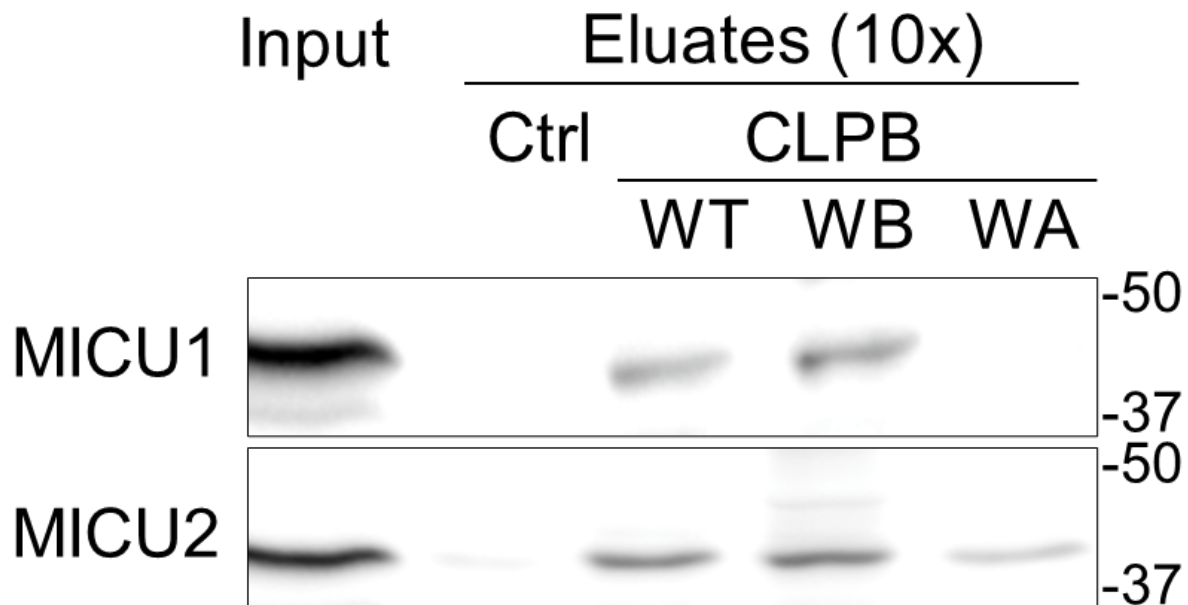


Figure 4-2 CLPB captures MICU1 and MICU2 from mitochondrial lysate

Western blot analysis of the eluates from Streptactin-XT columns containing immobilized CLPB (WT = wild-type, WB = Walker B mutant CLPB_{E425Q}, WA = Walker A mutant CLPB_{K357T}) and incubated with lysates from isolated HL-60 mitochondria. The WT and WB lanes in the top blot show a band for MICU1 at the same molecular weight as the input. In the bottom blot, the WT, WB, and WA lanes all show bands consistent with MICU2 in the input.

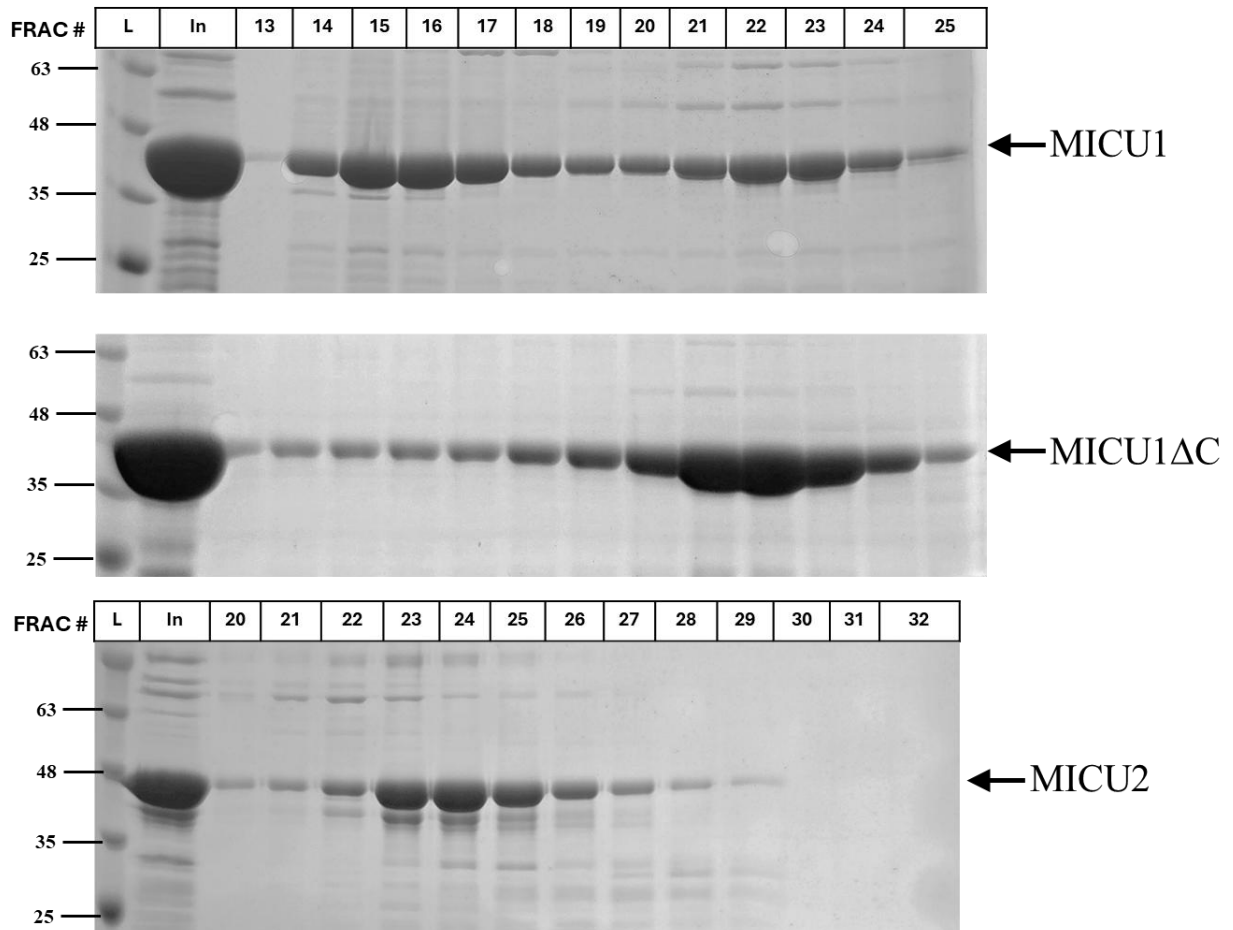


Figure 4-3 Purification of human MICUs expressed in *Escherichia coli*

SDS-PAGE gel analysis of fractions from purification of MICU1 (top), MICU1ΔC (middle), and MICU2 (bottom) by gel filtration chromatography. MICU1 elutes broadly with two major peaks in fractions 14-17 and 21-24. MICU1ΔC and MICU2 each elute as single peaks in fractions 20-24 and 22-26, respectively

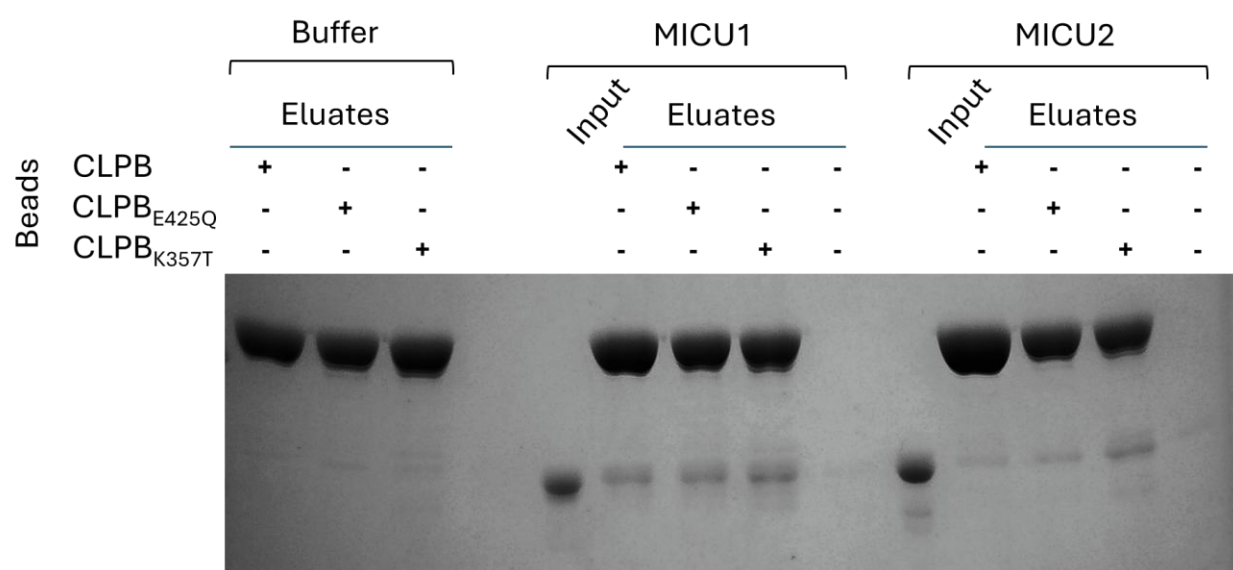


Figure 4-4 MICU1 and MICU2 interact directly with CLPB.

SDS-PAGE gel analysis of the eluates (35% of the total) from Mag-Strep resin containing immobilized variants of CLPB and incubated with purified MICU1 or MICU2. Buffer lanes show eluates from columns containing CLPB alone with no MICU1/2 added. Input lanes show the initial MICU1 and MICU2 samples (5% of the total) applied to columns.

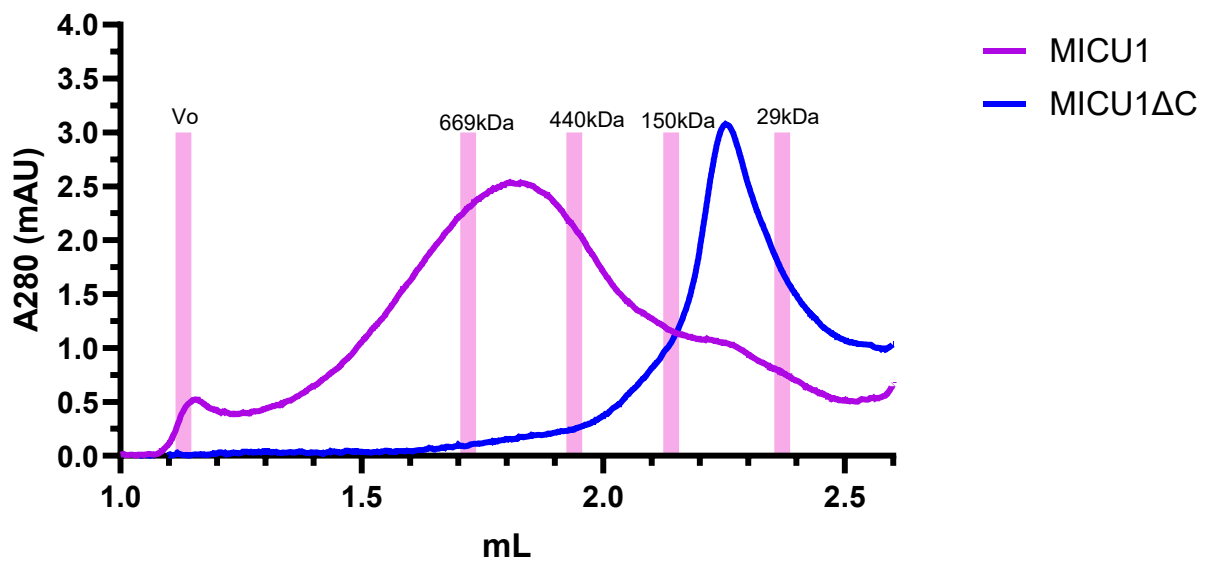


Figure 4-5 MICU1 forms large oligomers, while MICU1ΔC forms homodimers.

MICU1 and MICU1ΔC were resolved by gel filtration chromatography using a Superose 6 5/150 column and with measuring absorbance at 280 nm. MICU1 (purple trace) elutes broadly, with a major peak consistent with high molecular-weight oligomers. The elution profile of MICU1ΔC (blue trace) is consistent with a dimer. The peak elution volumes of protein standards are highlighted in pink.

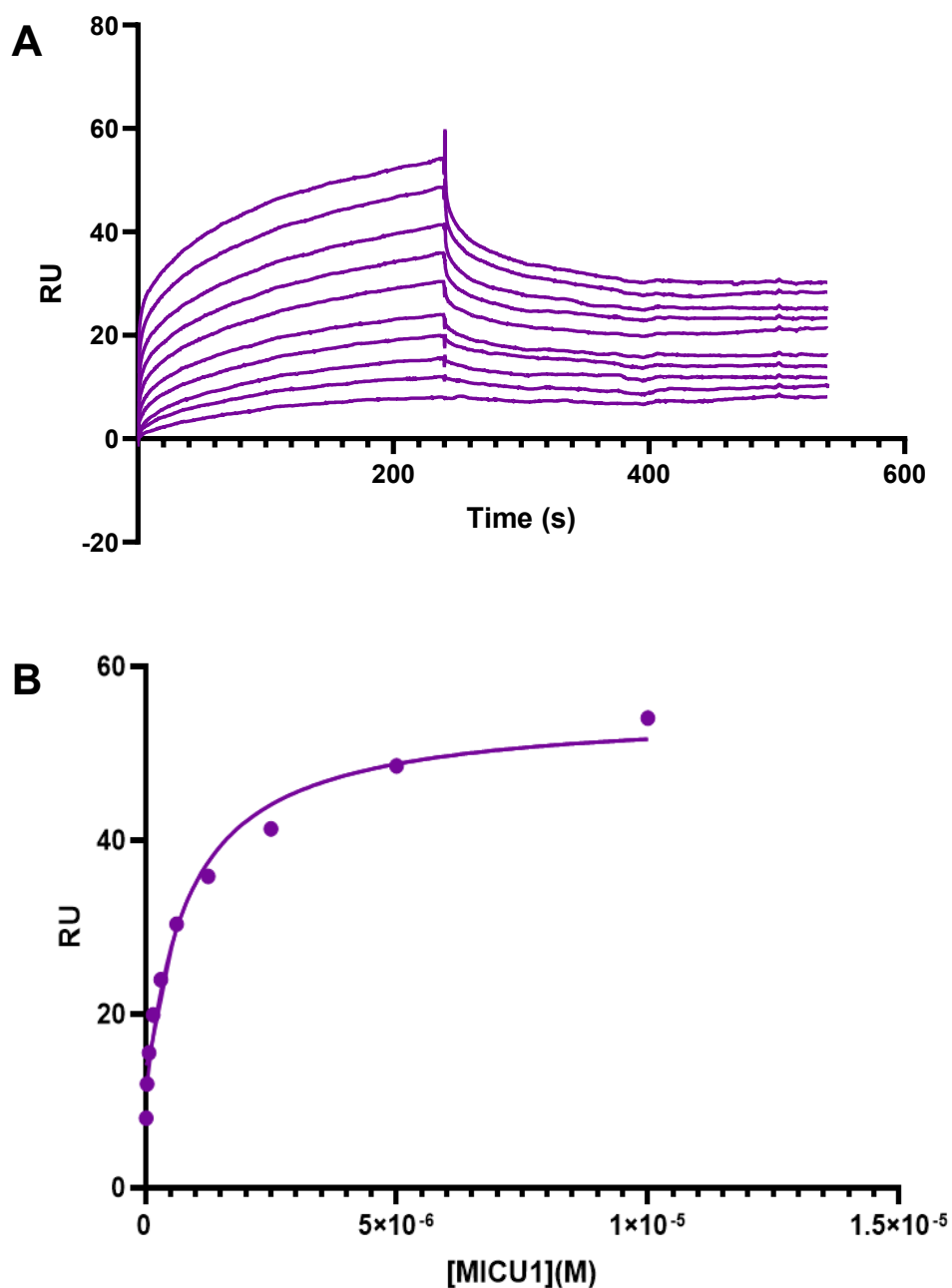


Figure 4-6 SPR analysis of the interaction of CLPB and MICU1.

Representative SPR sensograms of increasing concentrations of MICU1 titrated over immobilized CLPB (A).

Binding isotherm showing the SPR response as a function of increasing concentrations of MICU1, giving an apparent K_D of $\sim 0.88 \mu\text{M}$ (B).

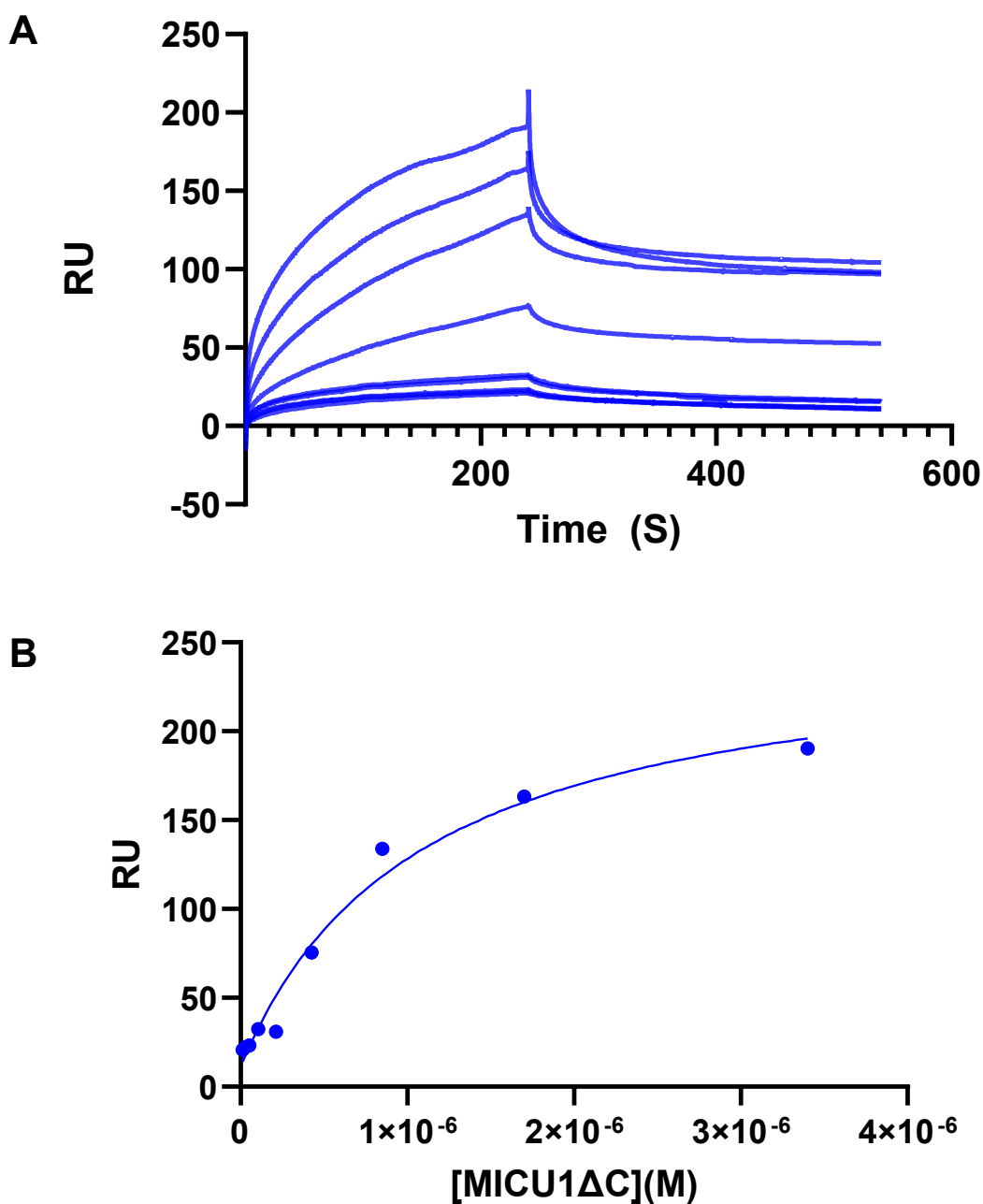


Figure 4-7 SPR analysis of the interaction of CLPB and MICU1ΔC.

Representative SPR sensograms of increasing concentrations of MICU1ΔC titrated over immobilized CLPB (A).

Binding isotherm showing the SPR response as a function of increasing concentrations of MICU1ΔC, giving an apparent K_D of $\sim 1.1 \mu\text{M}$ (B)

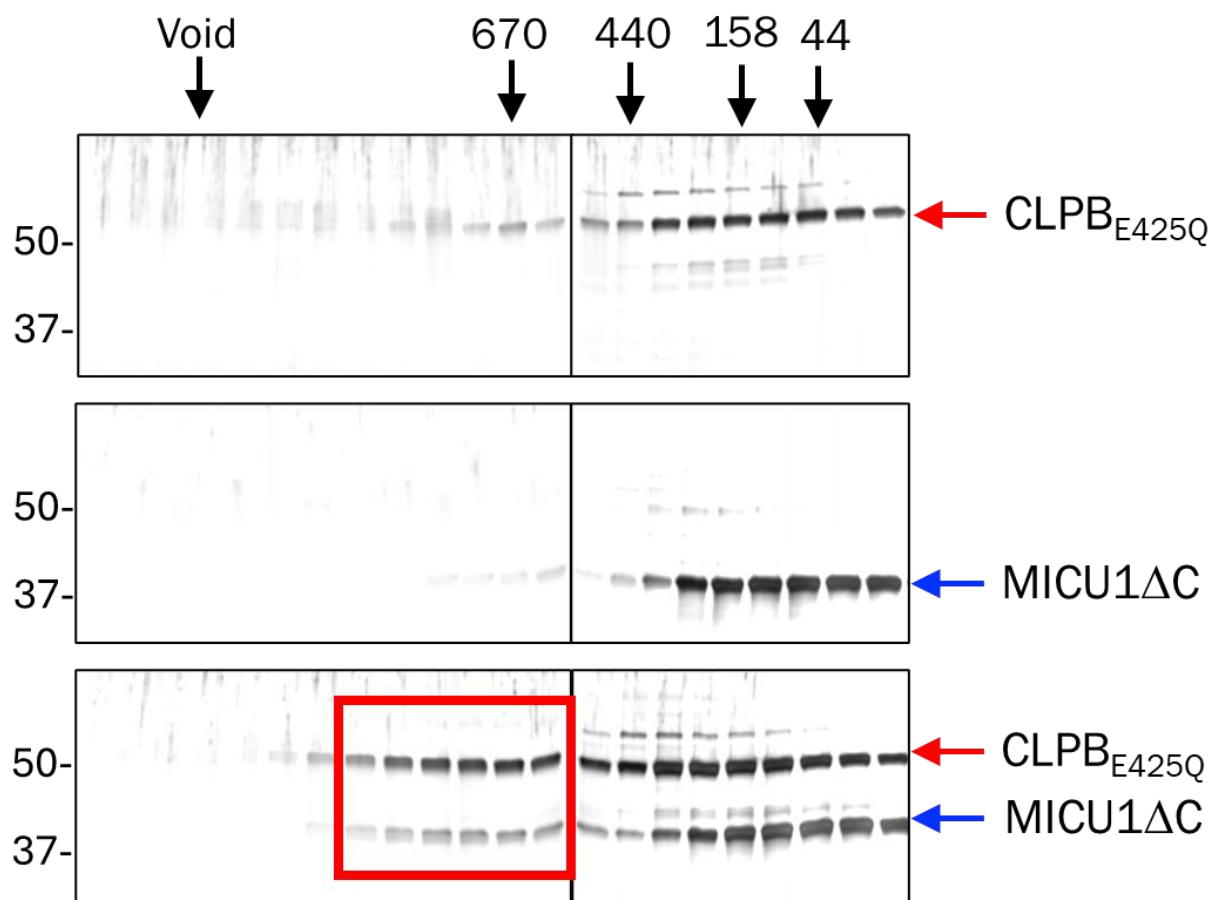


Figure 4-8 MICU1ΔC forms large complexes with CLPBE425Q in the presence of ATP. SDS-PAGE gel showing fractions from gel filtration on a Superose 6 5/150 column with 20 μM CLPB_{E25Q} (top), 22 μM MICU1ΔC (middle), and 20 μM CLPB_{E425Q} with 22 μM MICU1ΔC (bottom), in the presence of 1 mM ATP. Fractions containing high molecular-weight CLPB-MICU1ΔC complexes are marked with a red box

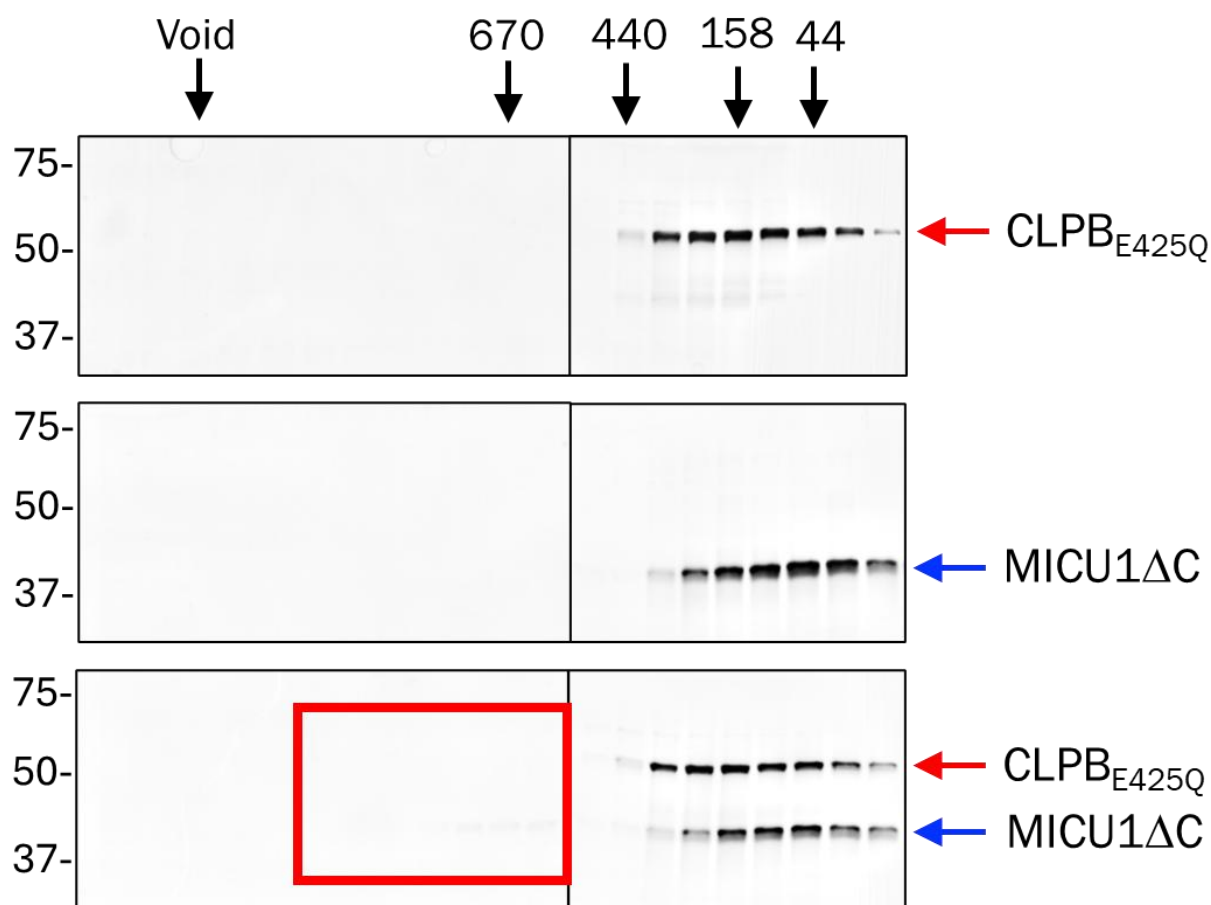


Figure 4-9 MICU1ΔC-CLPBE425Q complex is not detected in the presence of ADP.

SDS-PAGE gel showing fractions from gel filtration on a Superose 6 5/150 column with 20 μM CLPB_{E25Q} (top), 22 μM MICU1ΔC (middle), and 20 μM CLPB_{E425Q} with 22 μM MICU1ΔC (bottom). Experiments were performed in the presence of 1mM ADP. Fractions corresponding to high molecular weight CLPB-MICU1ΔC complexes detected in the presence of ATP in Fig. 7 (red box) do not contain CLPB_{E425Q} or MICU1ΔC.

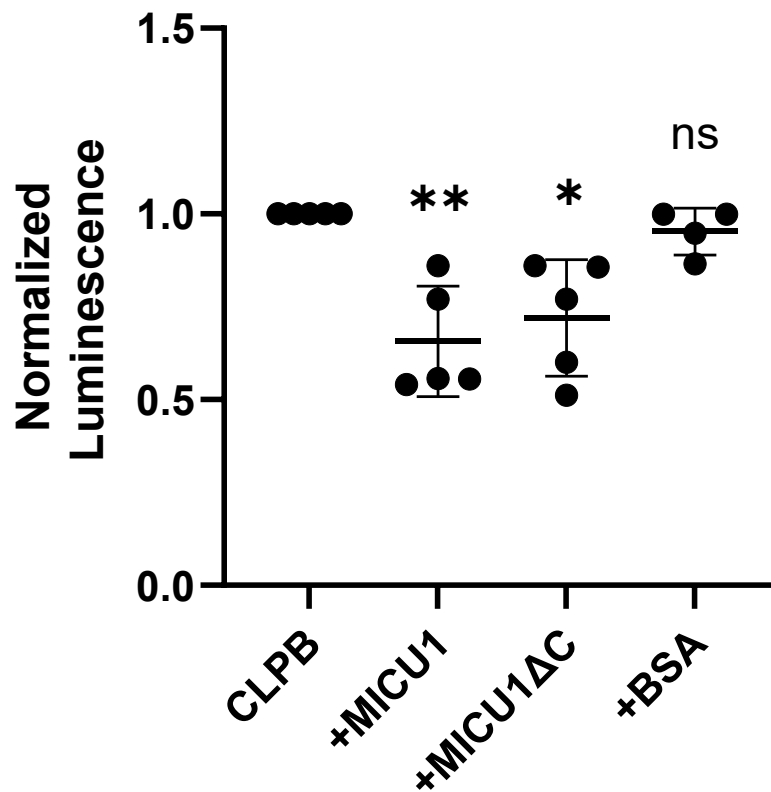


Figure 4-10 MICU1 and MICU1ΔC inhibit reactivation of aggregated luciferase by wild-type CLPB. Comparison of the reactivation of urea-denatured firefly luciferase by CLPB in the presence of MICU1, MICU1ΔC, or bovine serum albumin (BSA), normalized to the luminescence of luciferase reactivated by CLPB alone. Data are shown as means ± S.D. (N = 5). P values were calculated using one-sample t-test and the hypothetical value of 1 (*, $p < 0.05$; **, $p < 0.01$; ns, non-significant).

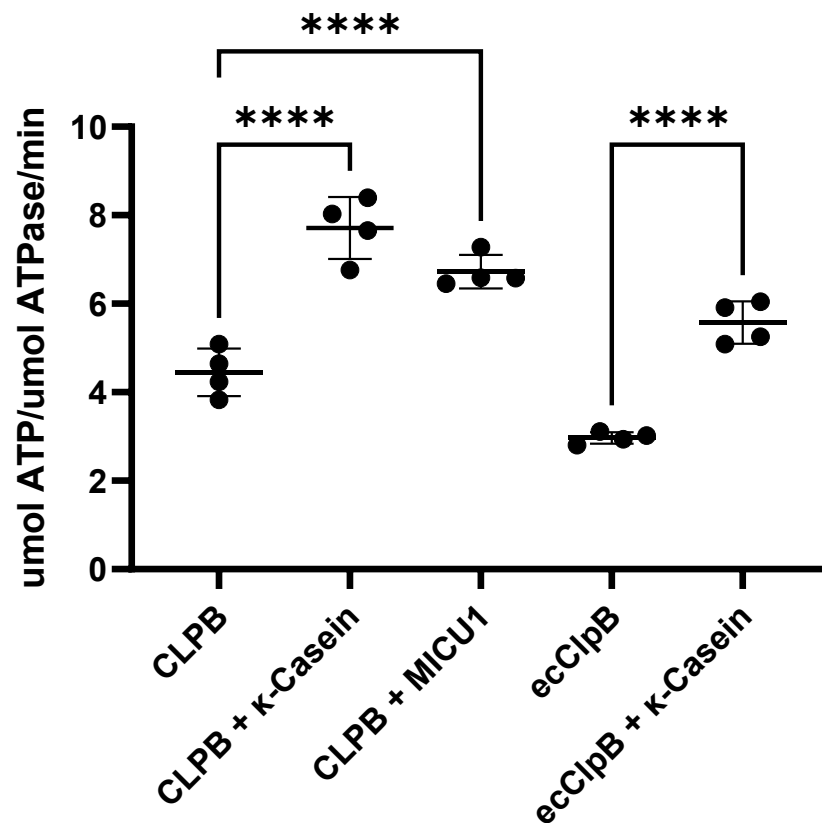


Figure 4-11 MICU1 stimulates the ATPase activity of wild-type CLPB.

Effects of MICU1 on the rate of ATP hydrolysis catalyzed by CLPB, as compared to the basal and κ -casein-stimulated ATPase activity of CLPB. The basal and κ -casein-stimulated ATPase activity of ClpB from *E. coli* are shown as a positive control. Data are shown as means $\pm$ S.D. (N = 4). P values were calculated using one-way ANOVA (****, $p < 0.0001$).

Chapter 5 - Discussion and Future Work

The results for this dissertation demonstrate that DBeQ is a selective inhibitor for ClpB in *Escherichia coli*. This result supports DBeQ's viability as a candidate inhibitor of bacterial proteostasis and a potential antimicrobial compound. We have shown that DBeQ inhibits BAP both alone and with ClpP, with a degree of potency that is orders of magnitude greater than for other bacterial Clp ATPases. This selectivity suggests that DBeQ's mechanism may engage one of the less-conserved regions of ClpB, such as the N-terminal domain or the middle domain. Furthermore, DBeQ's ability to dissociate the BAP-ClpP complex implies an inhibitory effect that leads to substantial conformational changes in the protein which are sufficient to disrupt the BAP-ClpP complex, despite the fact that DBeQ does not disrupt BAP hexamers. Further work is required to identify the binding site for DBeQ in ClpB to better understand its mechanism of action. Ideally, obtaining high resolution structures of the DBeQ-bound ClpB will be instrumental in this, however ClpB from *E.coli* has proven difficult to crystallize. Cryo-electron microscopy may be a more promising method, as multiple high-resolution Cryo-EM structures of ClpB, Hsp104, and ClpA, have all been obtained. Alternative methods to identify the binding site, such as using cross-linkable derivatives of DBeQ in conjunction with mass-spectrometry to identify interacting residues may also be an avenue for further understanding how DBeQ inhibits ClpB. These efforts could also be coupled with mutational analysis of the essential residues to verify if mutations abolish binding of DBeQ to ClpB.

If DBeQ indeed binds to the middle-domain of ClpB, then other potential targets for DBeQ may exist in other bacteria. For example, ClpC is found in Gram-positive organisms and contains a portion of the middle coiled-coiled domain which is homologous to that of ClpB.

ClpC replaces ClpA in Gram-positive bacteria, serving as a chaperone component of bacterial chaperone-ClpP complexes and may become a useful target for inhibitor development.

In summary, the work presented herein provides a deeper understanding of how the drug candidate DBEQ inhibits ClpB, contributing to the potential development of a novel class of anti-microbial drugs. This is in part supported by its apparent selectivity for ClpB and its variant BAP over ClpA and ClpX. Furthermore, the induced separation of BAP and ClpP implies a broader conformational shift in the protein upon substrate binding, helping to elucidate its mechanism of action.

In our research on human CLPB, we have discovered that CLPB forms nucleotide-stabilized dodecamers, a property that is rare among Clp ATPases. The role that this oligomeric structure plays in CLPB function and its physiological relevance are unknown. It is difficult to imagine that the CLPB dodecamer, which is 200nm across the length of the cage, is able to form in the inter-membrane space, as the distance between the inner boundary membrane and the outer membrane is also 200nm. However, the presence of a cavity in the CLPB cage somewhat resembles another chaperone, GroEL, which encloses unfolded proteins to provide an environment conducive to refolding. Observation of the CLPB dodecamer has been independently corroborated by multiple groups after our initial discovery(R. R. Cupo et al., 2022; Gupta et al., 2023; D. Wu et al., 2023). These high resolutions structures confirm our initial findings, however, the ankyrin domain remains poorly resolved in Cryo-EM studies, so the exact means by which it contributes to the formation of dodecamers has not yet been elucidated. The presence of the N-to-N ankyrin contacts between hexameric rings is puzzling, as the general consensus is that Clp chaperones tend to bind to their substrates through their N-terminal domain, then process them through the central channel, finally releasing them from their C-

terminal region. Whether or not this paradigm remains applicable for human CLPB has yet to be seen. Others have observed that CLPB does appear to unfold aggregated luciferase, but further study using physiological substrates will be key to understanding how substrate processing may be linked to the dodecameric structure (Gupta et al., 2023). The cage-like structure of CLPB dodecamer resembles the structure of GroEL, another chaperone which aids in protein reactivation by encapsulating unfolded substrates, providing an environment more suitable for refolding. From this perspective, it is possible that extrusion of single polypeptides from aggregates mediated by hexameric CLPB might be coupled to their capture by another dodecameric CLPB molecule to promote their complete refolding.

Furthermore, we have identified MICU1 and MICU2 as direct interactors of CLPB and have identified MICU1 as a putative CLPB substrate. Surprisingly, CLPB's interactions with these proteins occur while they are in their soluble, natively folded form. The biological significance of this result is currently unknown; however, it may indicate that CLPB is involved in the remodeling of soluble proteins. Alternatively, these interactions may be necessary for a protective effect in response to cellular stress. It is also possible that CLPB acts as a "holdase", enclosing and holding onto substrate proteins until particular conditions are met.

Limitations of this study include the fact that we were unable to determine the effects of other nucleotides or the absence of nucleotides on CLPB's affinity for MICU1, due to considerable baseline drift in SPR, indicating that the interaction between CLPB-Strep II and the immobilized Streptactin-XT was destabilized by the absence of nucleotides, perhaps due to conformational shifts in the protein in its ADP-bound and apo states. Future immobilization strategies should optimize the immobilization conditions, possibly using a different system for CLPB capture. Furthermore, the extent of MICU1 remodeling by CLPB, if any, has yet to be

explored. Future studies might use a trap mutant of GroEL to capture unfolded MICU1 extruded by CLPB. Finally, the capability of CLPB to bind MICU1-MICU2 dimers was not assessed in the present study. This is in part due to MICU2's propensity to bind strongly to carbamidomethyl dextran surface which we observed in our experiments (not shown). Other researchers produced a MICU1-MICU2 chimaera to form stable mono-disperse dimers for crystallography, and this approach could be useful for experiments with CLPB (W. Wu et al., 2020).

To confirm the physiological relevance of CLPB interactions with MICU1, experiments on cell lysates should be performed to assess the stability of the MICOS complex in the absence of CLPB. Additionally, other MICOS complex proteins should be screened for interactions with CLPB in pull-down assays. Moreover, it will be important to probe the CLPB interactions with putative substrates under apoptotic conditions, possibly using tools like Bio-ID for a more quantitative measure of CLPB's proximity to MICU1 and other MICOS components ((Roux et al., 2018).

In summary, we have identified a novel structure for CLPB/SKD3 which has not been observed in other Hsp100/Clp family proteins. We have also identified interactions with putative soluble substrates from the mitochondrial intermembrane space. Taken together, these results may aid in the interrogation of the molecular mechanisms behind CLPB deficiency-linked pathologies, particularly linking the relationship between CLPB and apoptosis, and its subsequent effects on neutrophil maturation arrest.

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Appendix A - Engineering, cloning, and expression of the human CLPB variant, SAP

Introduction

Among bacterial AAA+ proteins, the chaperone ClpA works with the protease ClpP to mediate protein turnover. ClpA mechanically threads SsrA-tagged, N-end-rule, and misfolded protein substrates through its central channel into the active site of the protease ClpP for degradation (Fig. A-1). The ClpAP complex is mediated by contact between mobile loops in ClpA containing the hydrophobic “IGL” motif in the D2 domain and hydrophobic pockets at the N-terminus of ClpP. We hypothesize that a similar mechanism of substrate translocation is employed by human CLPB/SKD3. In this work we have utilized NEBuilder assembly to substitute the ClpA IGL loop into human CLPB in an attempt to verify our hypothesis. The engineered protein is referred to as SAP (SKD3, which like ClpA binds to ClpP).

Results

Design of SAP

We began designing SAP by conducting a sequence alignment of *Escherichia coli* ClpA and human CLPB. We identified a region of the human CLPB sequence with potential to be replaced by the fragment of bacterial ClpA that contains the IGL loops (Figure A-2). Alignment of the NBDs of CLPB and ClpA resolved by cryo-EM verify that the sequences we have identified occupy the same region of the protein, exhibiting a similar helix-loop-helix like structure, though there is an additional insertion of 28 residues in the region as compared to

E.coli ClpA and ClpB (Figure A-3). After determining the location of the fragment to be substituted, we designed our DNA construct using the NEBuilder Assembly Tool (Figure A-4).

Cloning and assembly

The Polymerase Chain Reaction (PCR) used respective primers to amplify three distinct fragments: the 1152 bp CLPB segment upstream of the loop insertion (H1), the 485 bp downstream portion of CLPB (H2), and the 121 bp *Escherichia coli* ClpA loop (A). The PCR products were visualized using agarose gel electrophoresis (Figure A-5a). Simultaneously, a restriction digest was performed on the pOP3MT vector at sites recognized by the restriction nucleases BamHI and Not-I (Figure A-5b). The resulting PCR fragments and the linearized vector were purified from the agarose gel by gel extraction. After concentrating the PCR fragments and linearized vector, we used NEBuilder HiFi Assembly Kit to assemble the recombinant plasmid.

SAP is robustly overexpressed in Escherichia Coli

After assembly of the construct and verification by sanger sequencing, we transformed our resulting plasmid into the Rosetta (DE3) strain of *Escherichia Coli*. Following transformation, the overexpression of SAP under the T7 promoter was induced by supplementing 1mM IPTG. Analysis by SDS-PAGE showed that cultures supplemented with IPTG contained a strong band for a protein with an approximate molecular weight of ~100kDa, which is consistent with the predicted molecular weight of MBP-tagged SAP (Figure A-6)

SAP functions as a disaggregase but does not degrade casein

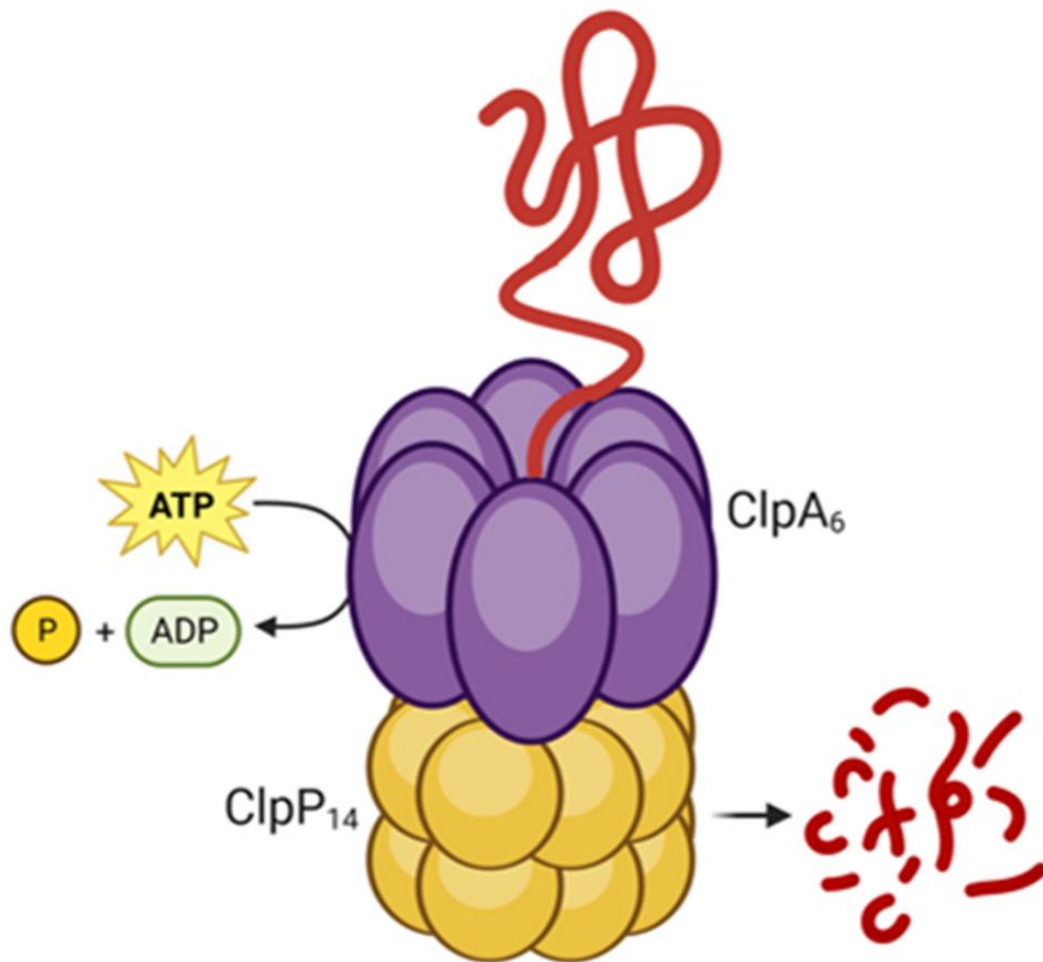
We then purified our recombinant SAP using established methods for the purification of human CLPB (this thesis, see Chapter 4, Figure 4-1). We tested the functional viability of SAP

by its ability to reactivate aggregated luciferase as compared to the wildtype CLPB (Figure A-7). Surprisingly, SAP does appear to be able to reactivate luciferase, though it displayed a considerably lower level of activity than that of the wild type. Following this, we next tested SAP's ability to degrade casein in the presence of ClpP. We found that unlike ClpAP, SAP-ClpP does not appear to degrade casein (Figure A-8).

Discussion

In this work, we created the human CLPB variant SAP, by substituting the mobile IGL loops from ClpA into a homologous region of CLPB. We found that this substitution did not wholly eliminate this protein's ability to reactivate aggregated luciferase. However, the addition of these loops does not appear to introduce the ability to degrade the model substrate casein with the addition of ClpP, suggesting that this may not be a viable tool for our investigation. Recently, a crystal structure of CLPB from the species *Danio rerio* captured by X-ray diffraction was deposited in the Protein Data Bank (PDB). The new monomeric CLPB structure was compared to the structure predicted by AlphaFold. Unlike previous structures of CLPB resolved by cryo-EM, there is a notable difference in the position the ANK domain relative to the NBD. Surprisingly, this structure shows that the ANK domain is oriented towards the region we selected for substitution with the IGL loop from bacterial ClpA (Figure A-11). The partial occlusion of the loop site could provide explanation as to why this construct was functional but did not interact with ClpP, as originally hypothesized.

Figures



Appendix Figure A-1

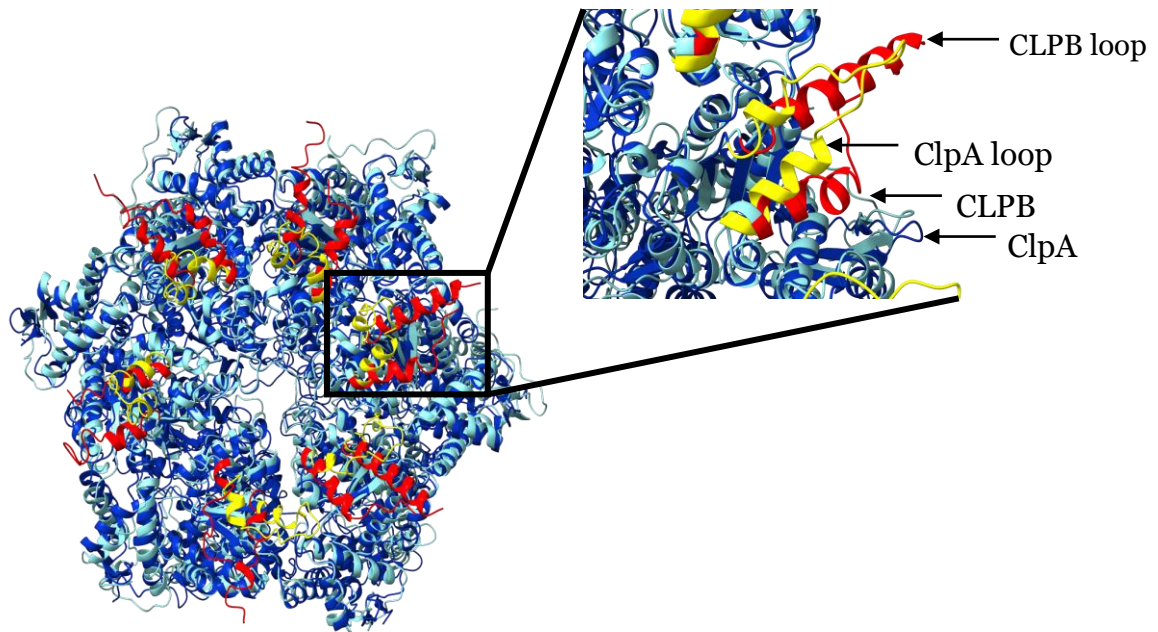
The translocation and degradation of proteins through the ClpA-ClpP complex. (Created using Biorender.com)



Appendix Figure A-2 Sequence alignment of human and bacterial Clps.

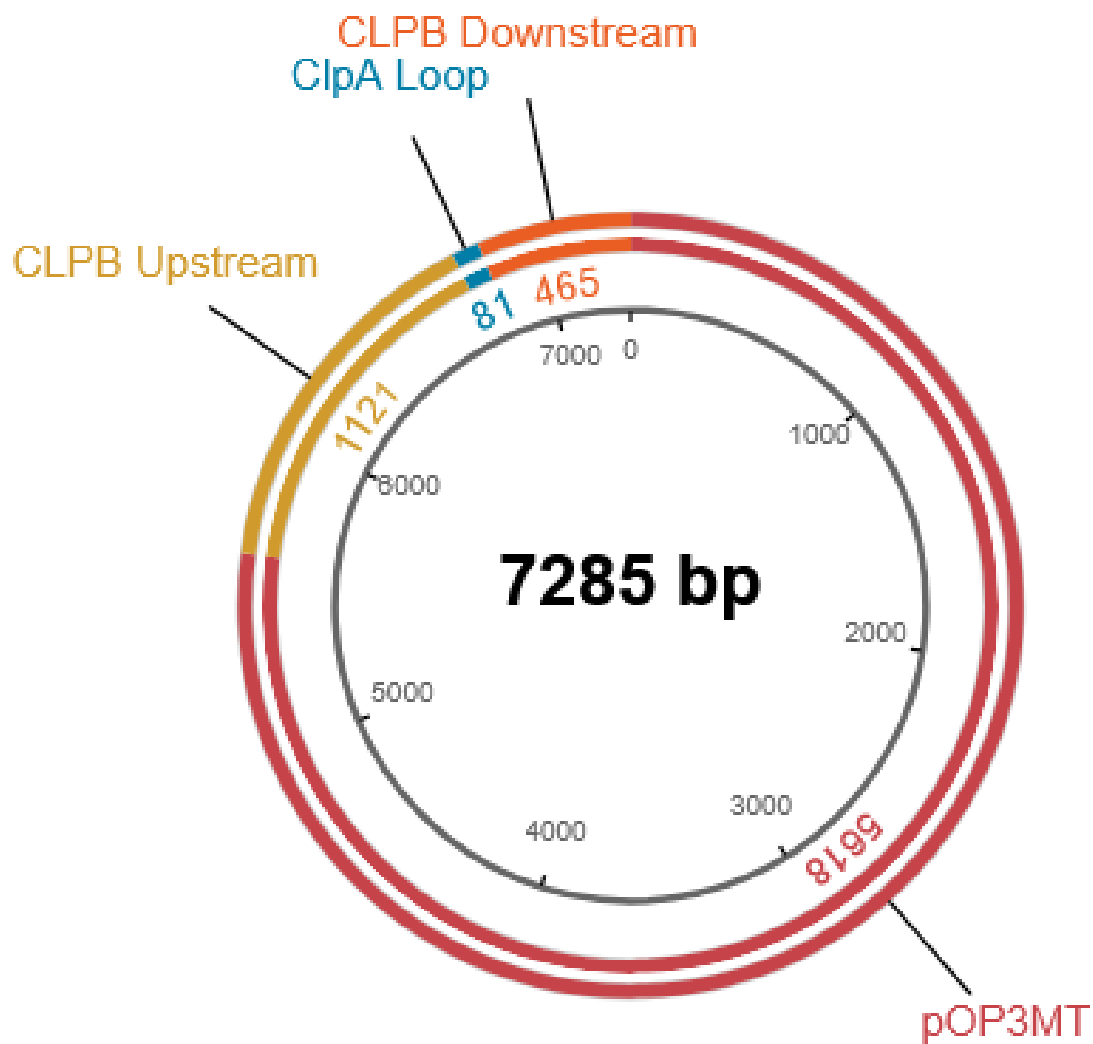
: The sequence alignment of human CLPB, E. coli ClpA, and E. coli ClpB from Clustal Omega.

The arrows indicate the beginning and end of the CLPB region that we aimed to replace with the sequence from ClpA including the hydrophobic IGL motif (black box).



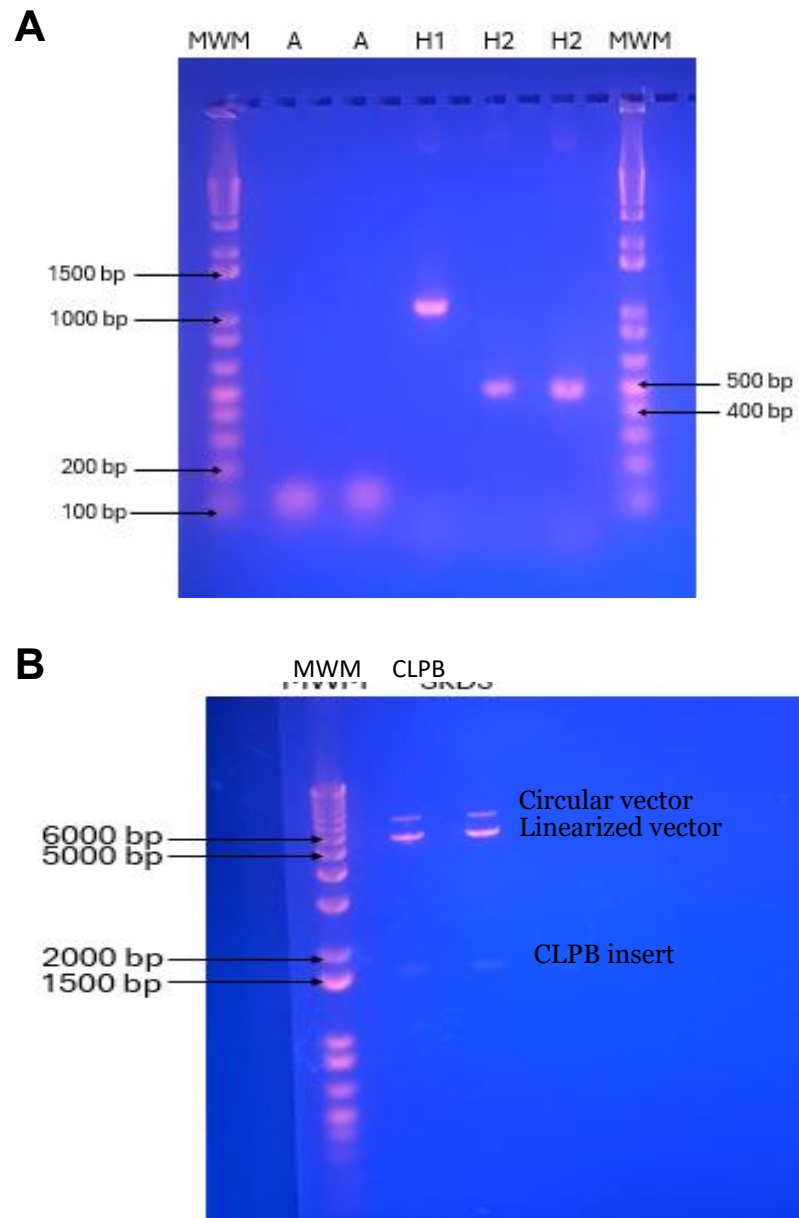
Appendix Figure A-3 Overlay of ClpA and CLPB NBDs

The alignment of the structures of CLPB (PDB 7TTS, cyan) and ClpA (PDB 6W22, dark blue) confirms that the ClpA loop (yellow) containing the “IGL” motif can replace the CLPB loop (red). The alignment of hexameric CLPB and ClpA is shown on the left with to the enlarged fragment on the right

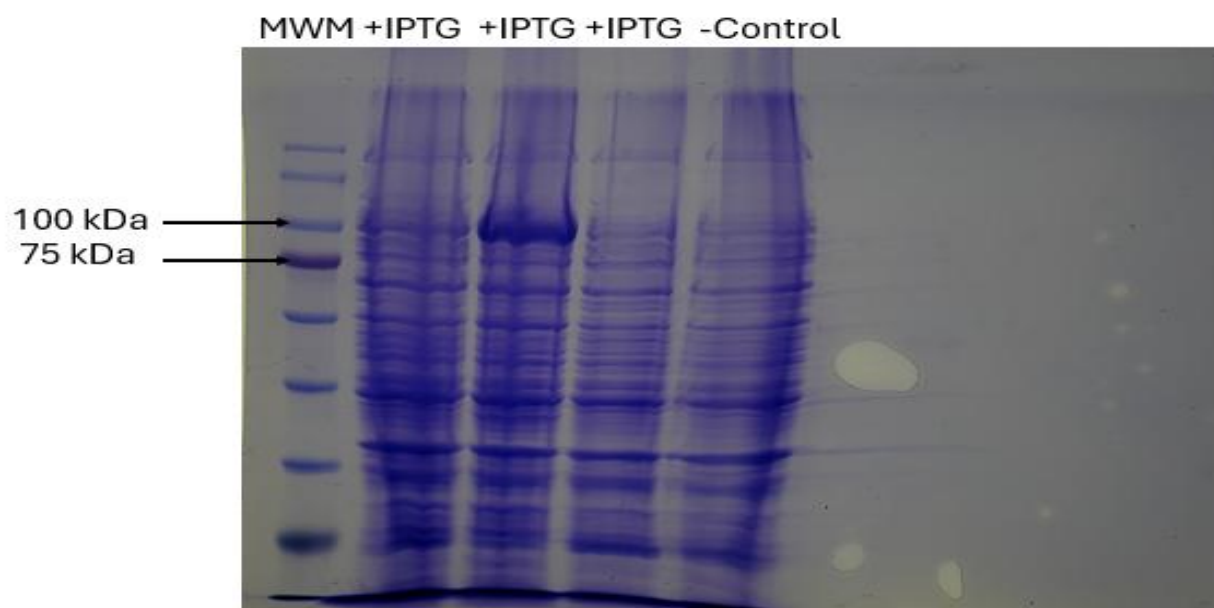


Appendix Figure A-4 Plasmid map of SAP-pOP3MT

The plasmid map for the engineered SAP. The sequence encoding CLPB upstream and downstream of the substituted loop are shown in yellow and orange, respectively. The sequence encoding the IGL loop from ClpA is shown in blue. The pOP3MT vector backbone is shown in red.

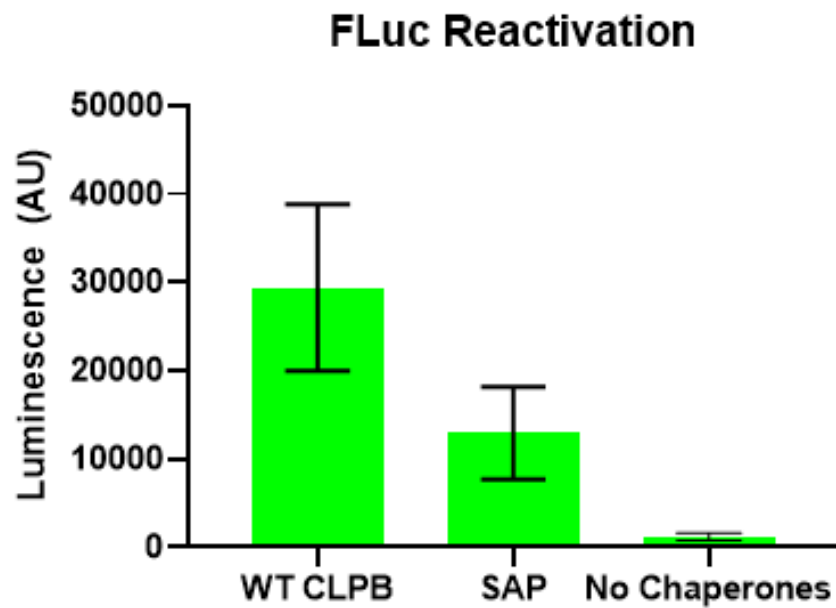


Appendix Figure A-5 – PCR of SAP fragments and preparation of the linearized vector
(A) 1% Agarose gel containing DNA ladder and the PCR Products



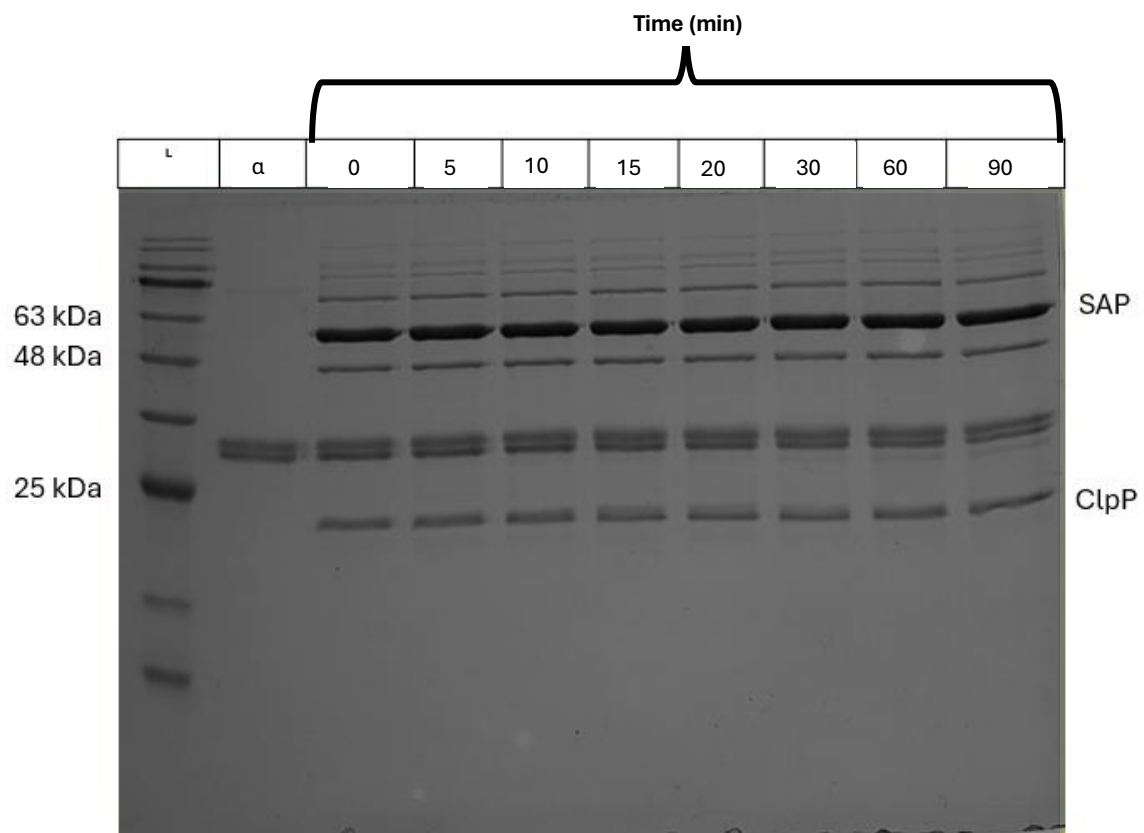
Appendix Figure A-6 Expression of SAP in *Escherichia coli*

SDS-Page gel demonstrating the expression of SAP.



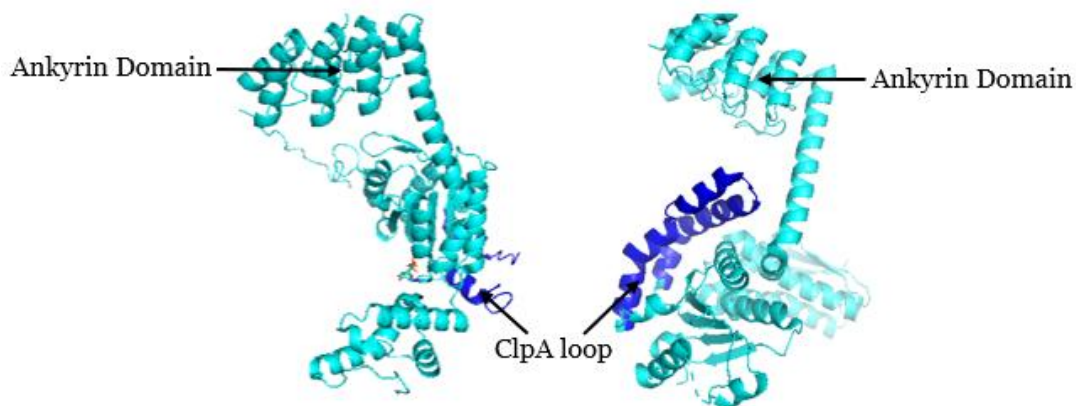
Appendix Figure A-7 Reactivation of Aggregated Luciferase by SAP

A comparison of the measurements of luminescence from the luciferase reactivation assay with without chaperones. samples that wildtype CLPB only, SAP only, and a no-chaperone control



Appendix Figure A-8 Degradation of α -Casein by SAP

The SDS-Page gel showing that the level of α -casein does not change in the presence of SAP. α =
 α -casein



Appendix Figure A-9 – Orientation of the ANK domain in *Danio Rerio* CLPB

The previously modeled CLPB (left) predicted by AlphaFold with the section that was replaced with the ClpA IGL loop (dark blue) demonstrating no interference from the ankyrin domain.

The new crystal structure of CLPB (right) shows the ANK domain occluding the region substituted with the ClpA IGL loop (dark blue) (PDB ID:8ZR7)