

**Structural and Functional Analysis of the Cell Division Protein MinD_{Ng}:
Implications of the C-terminus**

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Abstract

Gram-negative bacteria such as *Escherichia coli* and *Neisseria gonorrhoea* undergo symmetric cell division controlled in part by the Min system constituting of MinC, MinD and MinE. MinD_{Ng} is a peripheral membrane ATPase that functions by recruiting cell division inhibitor MinC_{Ng} to the membrane where it prevents septation. The available crystallographic data of archeal monomeric MinD is not ideal for a dimeric bacterial MinD homolog since there is only 30-40% sequence identity between the two organisms. In addition, the C-terminus of monomeric MinD is disordered in the crystal structures, which lead us to utilize a 13 amino acid truncated mutant of MinD_{Ng} (MinD_{Ng}-13aaCT) and test for the importance of the C-terminus of MinD_{Ng}. Our results indicate that MinD_{Ng}-13aaCT does not oligomerize even in the presence of ATP, in contrast to wild type MinD_{Ng} where we detect some formation of a dimeric form. The loss of dimerization that we observe with MinD_{Ng}-13aaCT could not be attributed to incorrect protein folding since it retained similar stability to the wild-type protein and behaved like a well folded globular protein. By measuring MinD_{Ng} reaction rates over a range of magnesium concentrations, we obtain a sigmoidal shaped curve for wild type MinD_{Ng} with a hill co-efficient of 10 in the absence of MinE and a hill co-efficient of 3 in the presence of MinE, suggesting that cooperativity is an inherent feature of MinD_{Ng}. In contrast, MinD_{Ng}-13aaCT has lost its ATPase activity rendering this mutant catalytically inactive. In addition our *in vivo* analysis illustrates that wildtype and MinD_{Ng}-13aaCT show a range of morphologies from long filamentous cells to minicells with this effect more pronounced in the case of wildtype MinD_{Ng} than compared to the mutant, which suggests that MinD_{Ng}-13aaCT has possibly retained its ability to interact with other Min proteins. In aggregate, our results provide some new insight into the structural and the functional role and importance of the C-terminus of MinD_{Ng}.

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I would like to take this opportunity to express my heartfelt gratitude towards my supervisor Dr. Natalie K. Goto for accepting me into her lab family and making it a valuable learning experience. Thank you for your support, kindness, and your knowledge to lead me this far along! I would also like to thank Dr. Thierry Ducat for his insight and helping me get started on my adventure!

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List of Abbreviations

aa	amino acid
ATP	adenosine 5'-triphosphate
ADP	adenosine 5'-diphosphate
BSA	bovine serum albumin
ddH ₂ O	double distilled water
Ec	<i>Escherichia coli</i>
EDTA	ethylene diamine tetra-acetic acid
IPTG	isopropyl- β -D-thiogalactopyranoside
Kan	kanamycin
kDa	kilodalton
LB	luria bertani
ml	millilitre
mM	millimolar
MTS	membrane targeting sequence
Ng	<i>Neisseria gonorrhoeae</i>
nm	nanometer
OD	optical density
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
μ g	microgram
μ l	microlitre
μ M	micromolar

Introduction

Regulation of symmetrical bacterial cell division

Cell division, simply described, is the process by which genetically identical daughter cells are created after the equal partitioning of genetic and cellular components. Although cell division is vital for the continuity of life, it is a complex process that is still inadequately understood even in relatively simple organisms such as bacteria. Gram-negative bacteria such as *Escherichia coli* and *Neisseria gonorrhoeae* undergo symmetric cell division where the cell pinches itself into two equally sized daughter cells. However, if symmetric cell division is disrupted then one of the products of cell division can be a “minicell”, a small cell that lacks DNA and cannot further propagate itself (Errington et al., 2003; Jaffe et al., 1988). Therefore, in order to maximize progeny production, bacterial cells must ensure that cell division occurs primarily at the midcell position.

In normal symmetric bacterial cell division, a cytokinetic septum is formed only at the midcell. This requires the precise placement of a polymeric structure called the Z-ring, comprised primarily of a protein called FtsZ (**F**ilamenting **t**emperature-sensitive mutant **Z**), at the division site (Errington et al., 2003). GFP and immunofluorescence results have demonstrated that Z-ring formation is a dynamic event in the cell cycle, with FtsZ being the first component of the division complex to assemble at the division site (Errington et al., 2003). However, these studies have raised the question regarding how gram-negative bacteria regulate the positioning and the timing of Z-ring assembly, a process that is known to be regulated by a nucleoid occlusion mechanism, and by the dynamic localization of cell division site placement regulatory proteins known as Min proteins, which were all discovered as *E.coli* mutants (**M**inicells) that could not produce a properly localized septum (P deBoer et al., 1992).

It is thought that potential cell division sites exist along the entire length of the cell and that targeting and activation of the division site in a cell is determined in part by the position of nucleoids (Mulder & Woldringh, 1989; Woldringh et al., 1990). This is supported by localization studies showing that the presence of the nucleoid is enough to locally inhibit Z-ring formation (Yu & Margolin, 1999).

According to the nucleoid occlusion model, nucleation of FtsZ rings can potentially occur both at the poles and mid-cell. After a minimum amount of DNA replication has occurred (e.g. ~70% of the chromosome in *B. subtilis*) septation can commence (McGinness & Wake, 1979). At this point, the nucleoid undergoes a conformational change, becoming bilobed (Figure 1.1B) (Sharpe & Errington, 1998). This bilobation produces a region deficient in DNA, thereby facilitating the accumulation of sufficient FtsZ at mid-cell to allow polymerization to begin (Figure 1.1C). However the cell poles, being polar DNA-free zones, remain available for nucleation of Z rings. This is prevented by the presence of the Min protein system.

The Min proteins play an important role in determining the site of cell division, as shown by studies with *min* operon knockouts in *E. coli*. These mutants underwent cell division at polar as well as midcell sites, creating populations of bacteria with one or two copies of chromosomal DNA and another of 'minicells' containing no DNA (Errington et al., 2003). Hence the major function of the Min system is to ensure that septum formation does not occur at the polar sites, but instead occurs solely at the cell midpoint supporting growth of a viable population.

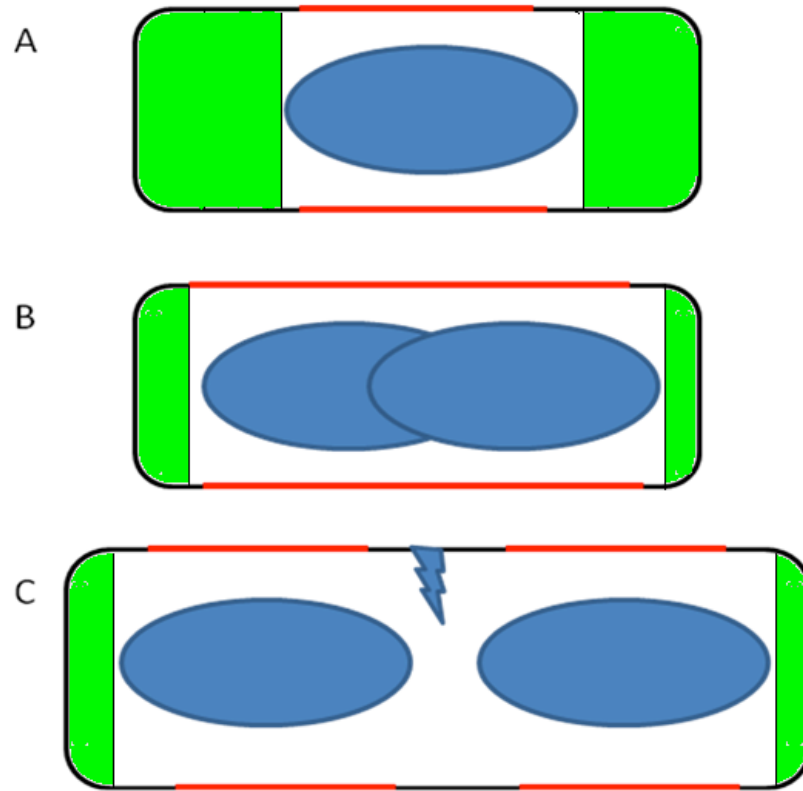


Figure 1.1: Positioning of the nucleoid acts to regulate potential cell division sites. (A and B) The presence of the bulk of DNA (shown in blue) has an inhibitory effect on FtsZ ring formation, thereby reducing the likelihood of cell division occurring at midcell. Green regions represent the cell poles as potential division sites that are far from the nucleoid. (C) Full replication of DNA frees the midcell from the inhibitory effects of the nucleoid, allowing cell division septum formation to occur at this site.

Restriction of cell division to the cell poles is governed by the products of the three genes of the *minB* operon - *minC*, *minD* and *minE* (Hu et al., 1999). MinC, a 25 kDa protein, inhibits division at all potential division sites (Raskin & de Boer, 1999a), but requires localization to the peripheral membrane via interactions with the ATPase MinD, a 30 kDa protein (Raskin & de Boer, 1999b). MinE, a 10 kDa protein, is thought to have two functions: it relieves the inhibition of cell division by MinC and MinD (anti-MinCD function) and causes the MinC and MinD proteins to localize preferentially to the cell poles (topological specificity function) (Raskin & de Boer, 1999a).

At the molecular level, Min proteins regulate placement of the cell division septum through a cycle of interactions that has been elucidated from the results of a number of biochemical studies (Shih et al., 2003; Szeto et al., 2005; Lackner et al., 2003; Hu et al., 2003; Hu et al., 1999) (Figure 1.2A). According to current ideas about this cycle, when present in the cytoplasm, MinD is thought to be in a monomeric ADP-bound state (Shih et al., 2003). Upon nucleotide exchange from the ADP-bound to ATP-bound form, MinD undergoes a conformational change to promote homodimerization and membrane binding (Lackner et al., 2003). MinC, a cell division inhibitor protein, can then associate with the ATP-bound dimer of MinD on the cell membrane, forming the MinCD inhibitory complex (Hu et al., 1999). This complex inhibits FtsZ polymerization and therefore septum formation at that location. However, MinE is also able to bind MinD, displacing MinC and stimulating MinD ATPase activity (Hu et al., 2001). ATP hydrolysis releases MinD from the membrane and regenerates the cytoplasmic ADP-bound monomeric form (Hu et al., 2001). Release of the MinCD complex permits FtsZ polymerization and septum formation can occur at that location.

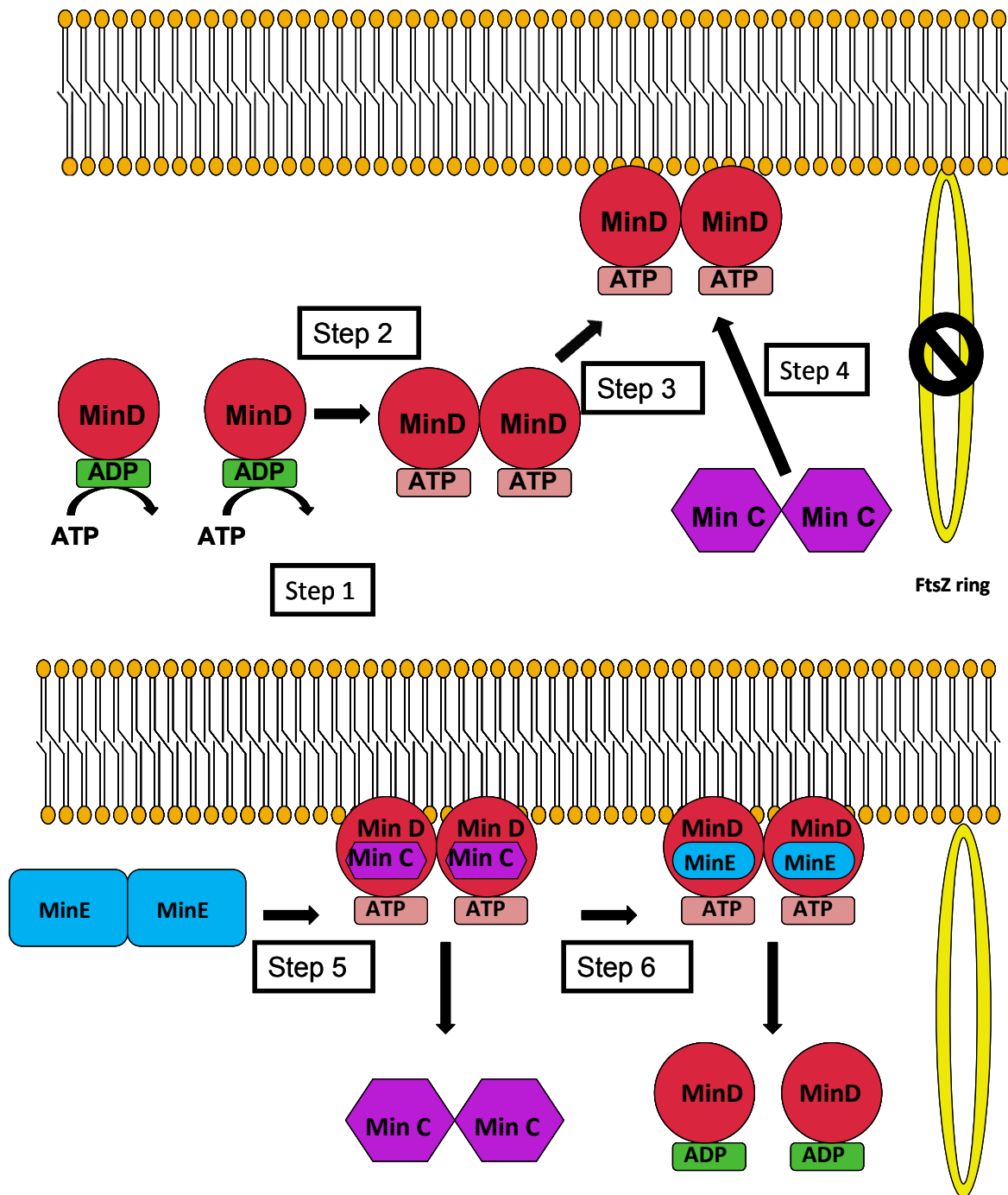


Figure 1.2: a) Model of MinCD inhibitor complex formation to regulate the placement of the Z-ring based on published data. Following nucleotide exchange (step 1) ADP-bound monomers of MinD form an ATP-bound dimer (step 2), which has a higher affinity for the membrane, allowing its localization to the membrane surface (step 3). The MinC dimer binds to membrane-bound MinD, forming the MinCD inhibitor complex that prevents formation of the cell division septum (step 4). **b)** In order to allow Z-ring formation at the midcell, MinE binding to MinD displaces MinC (step 5) from the MinCD inhibitor complex and induces MinD ATPase activity (step 6).

Based on fluorescence microscopy studies, it was revealed that the Min proteins organize into a “membrane-associated helical cytoskeletal substructures” that wind around the cell between the two cell poles (Shih et al., 2003). These coiled structures appear to persist along the length of a cell, and also seem to form a framework upon which the Min proteins oscillate from pole to pole. The observed oscillation appears to take place by redistribution of Min proteins along this helical array, with the MinCD complex appearing at high concentrations on this array at one end of a the bacterial rod before disassembling and subsequently associating with the helical array at the opposite end of the cell to start a new MinCD-rich polar zone. At the leading edge of the larger polar zone is a region of the coil that is rich in MinE, forming a structure known more specifically as the E-ring. As illustrated in Figure 1.3, these results have given rise to a model where dimeric MinD binds to the membrane and recruits MinC along the helical framework, at the polar region of the cell. The E-ring displaces MinC, stimulating MinD ATPase activity at the leading edge of the MinCD-rich polar zone, causing disassembly of the MinCD zone in this region. Movement of the E-ring with the edge of the MinCD zone is followed by disassembly of the E-ring and its reformation at the leading edge of the MinCD zone that has formed at the opposite pole of the cell. It is thought that this oscillatory mechanism results in a high local time-averaged concentration of MinE at the midpoint of the cell, allowing cell septum placement to occur only at that site.

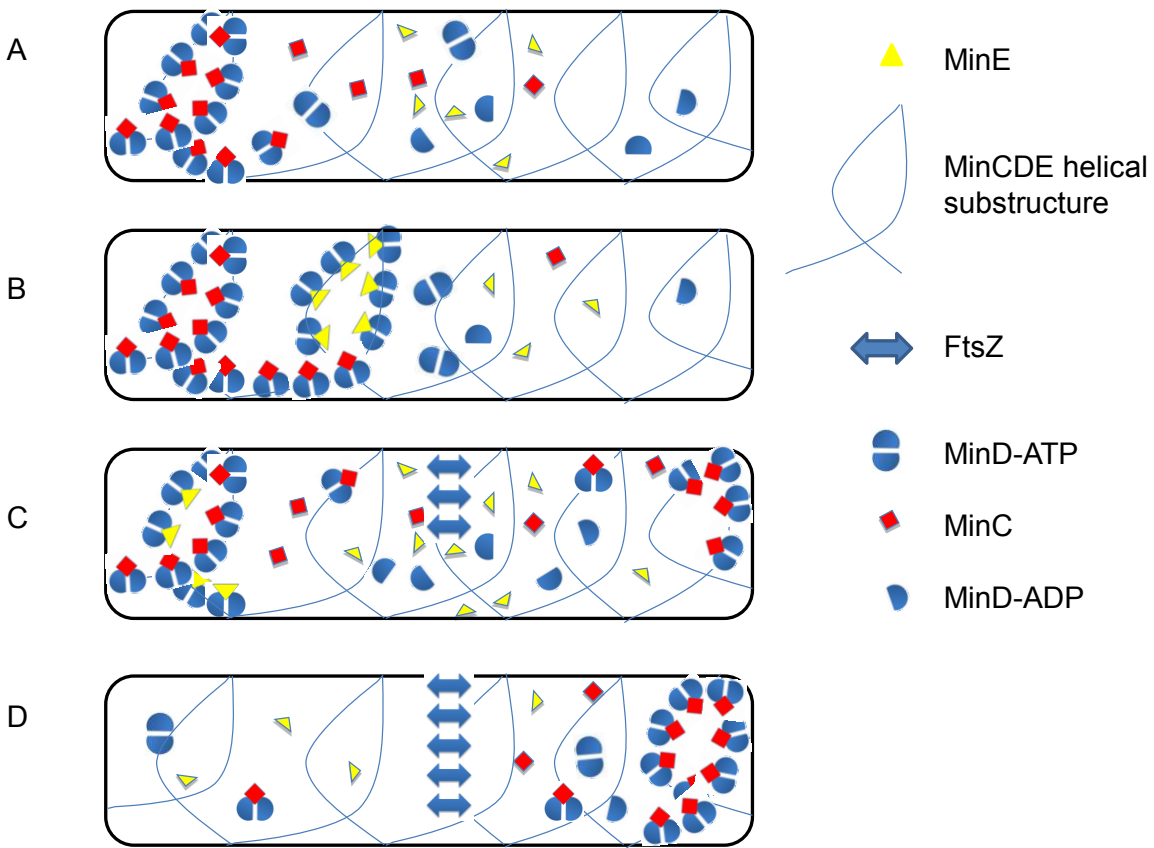


Figure 1.3: Model of MinCDE oscillations depicting the helical array as a permanent scaffold along the cell. (A) The dimeric form of MinD is able to recruit MinC to the helical framework at one polar end of the cell. (B) Once the MinCD inhibitor complex forms, MinE assembles into a ring-like structure. (C) The MinE ring oscillates along the MinCD scaffold and causes disassembly of the MinCD polymer by stimulating MinD ATPase activity. (D) MinD molecules are released from one polar side, diffusing to the other to begin the cycle again. Because of this oscillation, the time averaged concentration of MinC is lowest at midcell, which allows the FtsZ to form a Z-ring precisely in the center.

While much is known about how the Min proteins work together to control the placement of the cell division septum, much less is known about the molecular mechanisms that underlie this cycle. For example, it is not known why MinE binds to MinD on the membrane but not in the cytoplasm, or how MinE binding stimulates MinD ATPase activity. Given the central role that MinD plays in this regulatory cycle, and the potential for disruption of this cycle to serve as a novel target for antibiotic development, the Goto lab has worked to improve understanding of MinD function in pathogenic bacteria. Consequently, the focus of my thesis work has been on the MinD from *Neisseria gonorrhoeae*, the causative agent of Gonorrhea, a common sexually transmitted disease. In the long term, the knowledge that is gained on cell division processes in this organism has the potential to facilitate the development of a new class of antibiotics that target cell division to hinder the spread of this disease. This could be of great utility given that this pathogen has been developing resistance to an increasing number of antibiotics over the past few decades (Tapsall, 2009).

MinD in Neisseria gonorrhoeae

N. gonorrhoeae is a round-cell bacterium that divides in succession along two alternating perpendicular planes, distinct from rod-shaped bacteria such as *E.coli* cell that divide only once at the midcell. While much less is known about the mechanism underlying cell division for *N. gonorrhoeae*, it is acknowledged that the Min system is involved in regulating symmetric cell division in two perpendicular planes, ensuring equally sized tetrad daughter cells (Westling-Haggstrom, Elmros, Normark, & Winblad, 1977). According to the known model, the *N. gonorrhoeae* homologue of MinD (MinD_{Ng}) undergoes a similar cycle of nucleotide exchange, membrane localization, MinC_{Ng} binding and MinCD complex dissociation by MinE_{Ng} (T. H. Szeto, Rowland, Rothfield, & King, 2002). Previous studies on gonococcal Min proteins have

established that the proteins form a helical framework along the longest axis of the cell as they elongate during their growth cycle producing a slight longitudinal axis (Westling-Haggstrom et al., 1977). Cytoplasmic ADP-bound MinD_{Ng} then diffuses to the opposite end of the cell, binding ATP, relocating to the membrane within the helical framework. MinC_{Ng} forms a complex with MinD_{Ng} in these polar zones, restricting FtsZ polymerization to the midcell. At this point, the DNA replisome carries out DNA replication and segregation to allow full septation. Since gonococcal cells have a bidimensional growth pattern, a new longitudinal axis is created after the first cell division event such that the Min protein helical framework orients along the new axis, giving rise to oscillation in a direction that is perpendicular to the previously formed division septum (Westling-Haggstrom et al., 1977). Meanwhile the DNA replisome localizes to a point in the cell that is directly opposite to the newly developing septa, thereby restricting FtsZ polymerization to the midpoint of the cell.

The similarities in Min protein function in *N. gonorrhoeae* appear to extend to the molecular level with *in vivo* studies showing that MinD_{Ng} can function with *E. coli* Min proteins in *E. coli* (T. H. Szeto et al., 2002). Specifically, over-expression of MinD_{Ng} in *E. coli* has been shown to induce cell division arrest, leading to cell- filamentation, suggesting that this homologue can directly interact with native *E. coli* Min proteins (T. H. Szeto et al., 2002). This has been supported by yeast 2-hybrid studies demonstrating that MinD_{Ng} can associate with both itself and MinD_{Ec} (J. Szeto et al., 2001). In addition, similar to observations with MinD_{Ec} in *E. coli*, a knockout of MinC_{Ng} in *N. gonorrhoeae* leads to aberrant cell division and cell morphology, compromising cell viability. These results all indicate that MinD_{Ng} plays an important role in cell division in *N. gonorrhoeae* that is similar to its function in rod-shaped cells such as *E. coli*.

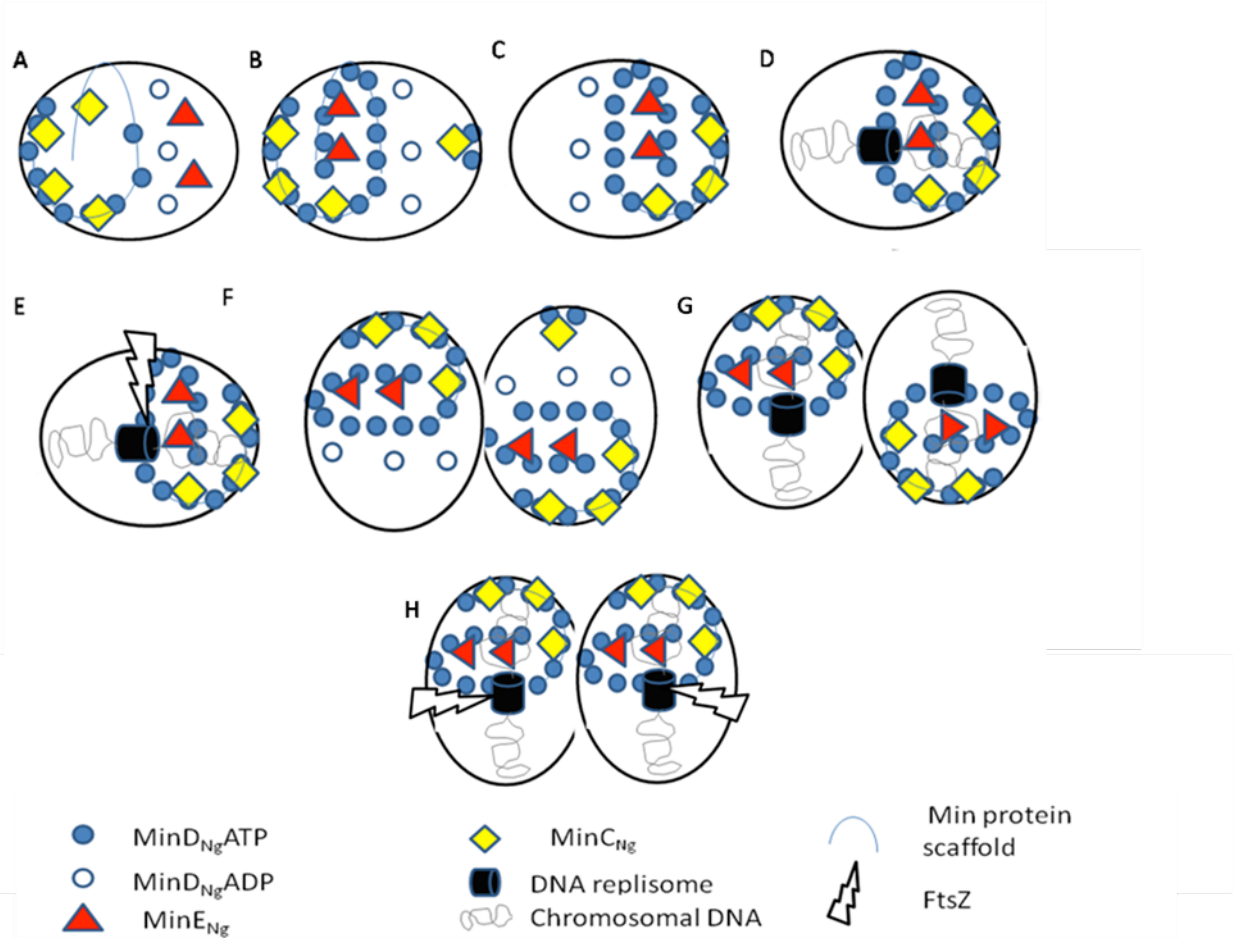


Figure 1.4: Schematic model of cell division regulation in *N. gonorrhoeae*. (A) Dimeric form of MinD_{Ng}-ATP localizes to one end of the cell and recruits MinC_{Ng} to the helical framework that orients along the longest axis of the cell. (B) MinE_{Ng} starts to form a ring-like structure at midcell as the MinCD division inhibitor complex assembles at one half of the cell. (C) The MinE-ring displaces MinC_{Ng} from MinD_{Ng} and stimulates the ATPase activity of MinD_{Ng} that enables it to disassociate from the membrane. (D) Cytoplasmic MinD_{Ng} dissociates from one end of the cell pole to the other, using the helical framework as a track, binds ATP and relocates to the membrane. (E) As a result of this continuous oscillation, MinC_{Ng} occupies the polar region, which allows FtsZ to assemble at the midcell and start the equal partitioning of the cell. (F) DNA replisome undergoes replication and segregation to allow full septation. At this point, the cell reorients itself, producing a new longitudinal axis. (G) Min proteins reorient themselves along the new axis. (H) Redirection of the Min proteins oscillation. The DNA replisome localizes directly opposite to the nascent septum, which restricts FtsZ polymerization between the two cells resulting in cell division that is perpendicular to the first.

Structural homologues of MinD and other members of the ParA family

MinD is a member of the NifH-ArsA-Par MinD subgroup of type A ATPases, which are characterized by a variant form of the nucleotide binding motif, termed the “Walker A ATPase motif” near the N-termini of these proteins (Koonin, 1993; Walker, Saraste et al., 1982). This consensus sequence (XKGGXXK[TS]) (where X is any amino acid) differs from the regular Walker A motif sequence (GXXXXGK[TS]) by the presence of a signature lysine residues at K11. The Walker motif A sequence forms a site for binding nucleotides in many proteins (Ramakrishnan et al., 2002). This deviant motif, however, appears to be unique to bacterial cells where it is thought to play a role in interactions with the phosphate groups of the bound nucleotide (Koonin, 1993).

Crystal structures of MinD homologues from archeal organisms *Archaeoglobus fulgidus*, *Pyrococcus furiosus* and *Pyrococcus horikoshi* (Cordell & Lowe, 2001; Hayashi et al., 2001; Sakai et al., 2001) confirm this role for Walker A motif residues, which form a loop in the active site (Figure 1.5). These structures all show a highly homologous fold of an 8-stranded β -sheet flanked by several helices on both sides. Critical ATP-binding residues are also located in two additional conserved loops called Switch I and Switch II. This nomenclature arises from observations on related ATPases that showed ATP-dependent conformational changes in these loops that influenced interactions with partner proteins. They are thought to be the regions primarily responsible for the communication between the nucleotide-binding pocket and the binding site of the partner proteins (Kintsjes et al., 2007). These loops are also involved in hydrogen bonding interactions to water molecules coordinating an active-site Mg^{2+} ion required for ATP binding and hydrolysis (H. Zhou & Lutkenhaus, 2004).

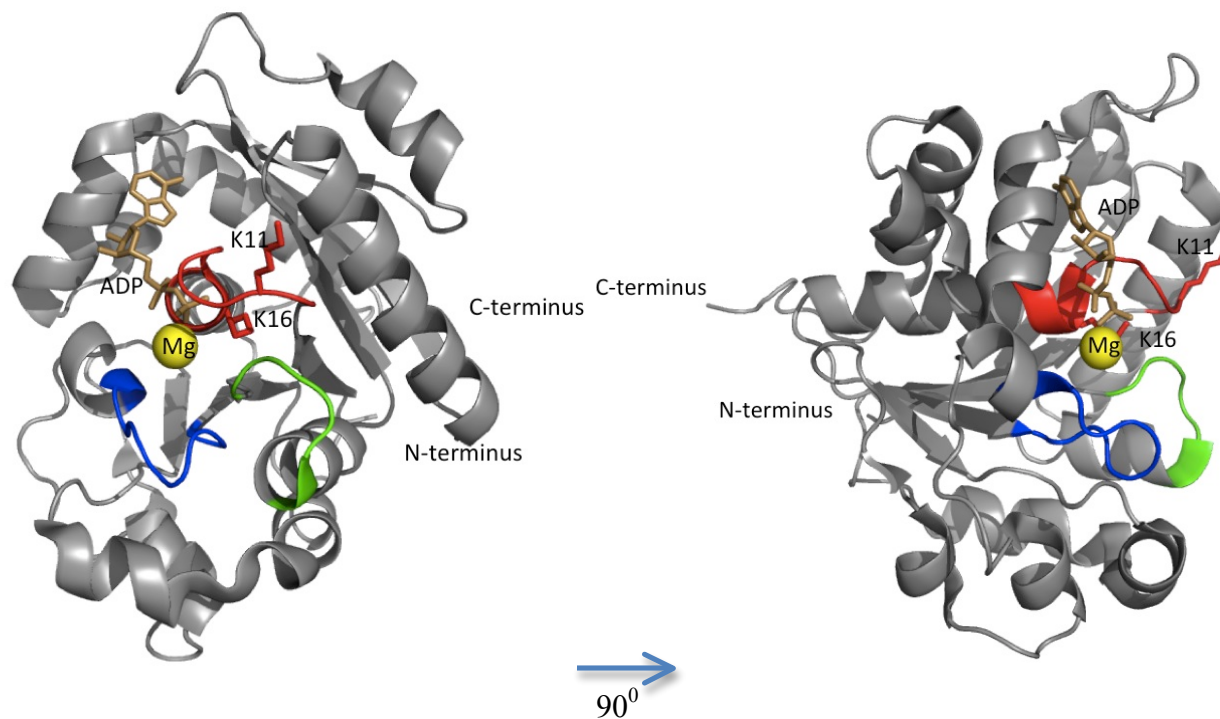
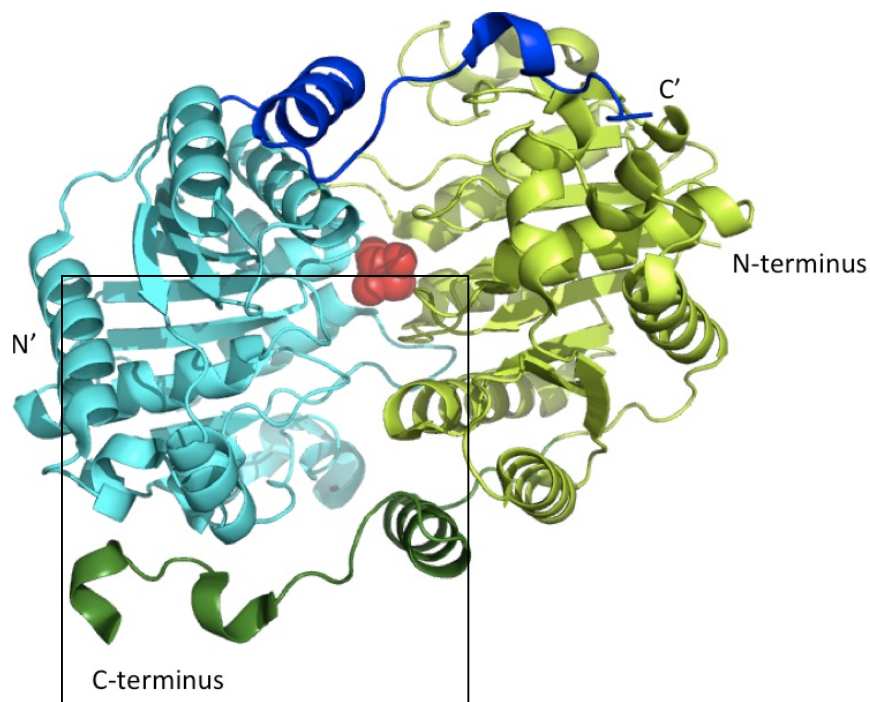


Figure 1.5: Ribbon diagram representation of MinD from *Pyrococcus horikoshii* bound to ADP (PDB # 1ION) (Sakai et al., 2001). Regions of MinD that form the Walker A ATP-binding motif (residues 10-18) are highlighted in red, Switch I (residues 38-46) in blue, Switch II (residues 120-124) in green. The side chain of the signature lysine residue Lys11 (red) characteristic of the “deviant” Walker A motif is also shown. ADP bound to this structure is shown in stick form. Ribbon diagram was generated using PYMOL (The PyMOL Molecular Graphics System, Schrodinger).

All available structures from the MinD sub-family are currently of the monomer, with no dimeric structure yet being reported. However, crystal structures of a dimer form have been obtained for other members of the deviant Walker A motif subfamily, including the NifH nitrogenase, the catalytical subunit of arsenical pump ArsA and the plasmid-partitioning protein Soj. The protein fold of these functionally distinct proteins is remarkably similar although the amino acid identity is on the order of only 20% for any two pairs. For NifH and ArsA the dimer is in a more open conformation in the presence of ADP and contains two nucleotide binding sites at the interface of the two subunits. The binding sites are composed primarily of amino acid residues coming from a single subunit (Figure 1.6) (Schindelin et al., 1997; T. Zhou et al., 2000). Importantly, NifH has also been captured in complex with the transition state analogue ADP-SF₆, which reveals a more closed conformation, with important new intermolecular contacts. The most important of these contacts is made by the signature lysine, which interacts with a phosphate group of the nucleotide that is bound to the opposite subunit (Schindelin et al., 1997).

In 2005, the hydrolysis-deficient mutant of Soj (Soj D44A) was crystallized in the ATP-bound state, showing a nucleotide-centered ‘sandwich’ dimer, similar to that observed for NifH (Leonard et al., 2005). Similarities between the Soj dimer structures include a network of hydrogen bonds between residues on adjacent subunits, with the signature lysine stabilizing the α and γ phosphates of the ATP moiety in the adjacent subunit.

A



B

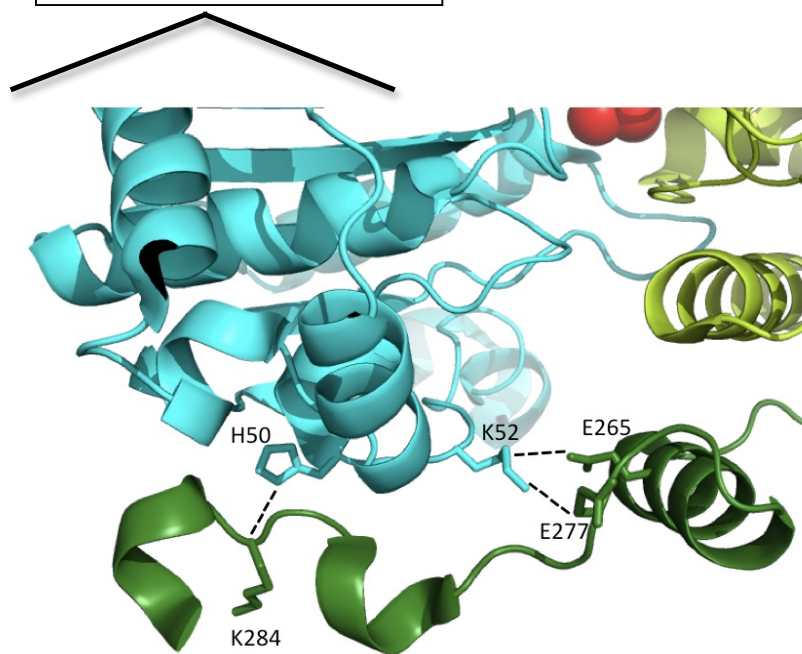
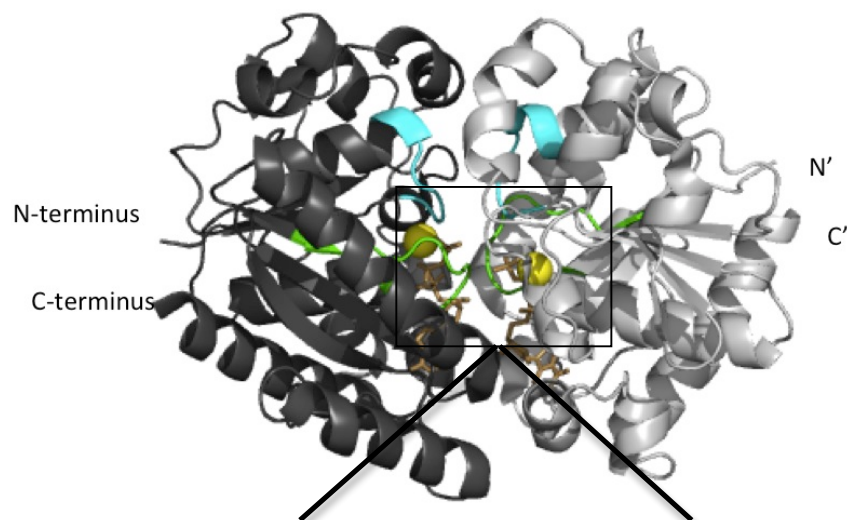


Figure 1.6: Ribbon diagram representation of a NifH dimer from the nitrogenase complex of *Azobacter vinelandii* (PDB # 1G1M). A) Overview of dimer structure of NifH bound to the iron-sulphur cluster (shown in space-filling ball representation). Each subunit is either green or blue, with the C-terminal regions involved in inter-subunit interactions highlighted with darker green or blue. B) Expanded view of the inter-subunit interactions involving the NifH C-terminus, with side chains involved in hydrogen bonding or electrostatic interactions shown.

As shown in Figure 1.7, Soj loops that form part of the nucleotide binding pocket are also involved in inter-molecular interactions. For example, the amide nitrogen atoms of G16 and G17 stabilize the γ -phosphate of the ATP moiety that is bound by the adjacent subunit (Figure 1.7B) (Leonard et al., 2005). Overall the Soj structure highlights the central role that ATP plays in the mediation of inter-molecular interactions, explaining the requirement for ATP-binding to precede MinD dimerization.

Closer inspection of the active sites of Soj and NifH reveals that the nucleotides are bound in two different conformations in the two structures. Specifically, the two ATP molecules housed in the Soj active site adopt a 'kinked' conformation in which the γ -phosphate is bound without a structural rearrangement of the switch II region. Lys15 of the opposing subunit stabilizes the α - and β -phosphates. In contrast, in the NifH active site, ADP adopts an 'extended' conformation in which the conserved Lys10 of the adjacent subunit stabilizes the β -phosphate and the switch II region undergoes a conformational change. This results in a rotation of $\sim 13^\circ$ of each monomer towards the subunit interface, closing the dimer into a more compact structure in the complex (Sakai et al., 2001). In addition, the AlF_4^- molecule that is also contained in the NifH dimer structure allows the transition state of the hydrolysis reaction to be emulated by adopting a planar conformation. Based on this, it has been suggested that the formation of the transition state for Soj also closes the dimer interface in a way that could affect its interactions with Spo0J (Leonard et al., 2005).

A



B

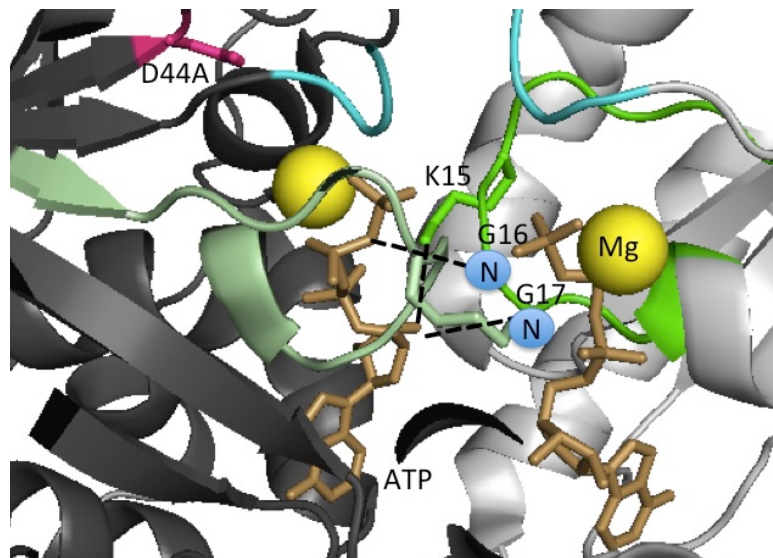


Figure 1.7A: Active site of Soj D44A hydrolysis deficient dimer (PDB # 2BEK). (A) P-loop is shown in green and Switch II in cyan. ATP is shown in brown and Mg^{2+} in yellow. N' and C' termini are of the second subunit. (B) Expanded view of signature Lys 15 stabilizing phosphate of bound nucleotide from one subunit is shown in dark green stick. Amide nitrogens between G16 and G17 (shown as blue spheres) also make inter-subunit contact with the phosphate of bound ATP. D44A is highlighted in pink.

A potential model for the mechanism of MinE-stimulated of MinD ATPase activity was provided by the crystal structure of MinD from *P. horikoshii* (Figure 1.8), which showed the deviant Lys 11 of the Walker A motif interacting with residues away from the nucleotide binding site (Hayashi et al., 2001). As shown in Figure 1.8, Lys 11 interacts electrostatically with conserved residues E146, S148 and D152 in helix 7. Mutagenesis studies established that the elimination of any one of these three residues yields a MinD that cannot be stimulated by MinE, although the corresponding mutants are still capable of binding MinE (H. Zhou et al., 2005). Based on these results it was suggested that MinE binding disrupts these interactions with helix 7 to allow the signature lysine to catalyze ATP hydrolysis (H. Zhou et al., 2005).

No structures for eubacterial MinD are currently available, but since the archeal MinD structures have a 30-40% sequence identity with MinD_{Ng}, a similar fold can be anticipated for the bacterial homologue (Sakai et al., 2001). However, while the archeal structures have provided great insights into MinD function, it is likely that significant differences exist with the bacterial proteins. This is partly because the archeal MinD proteins are not involved in spatial regulation of cell division, and these organisms lack MinC and MinE homologues that could regulate MinD function (T. H. Szeto et al., 2002). In addition, these archeal structures are all monomeric, having been crystallized with ADP (Sakai et al., 2001) or no nucleotides (Hayashi et al., 2001). One structure was determined for a complex of MinD bound to a non-hydrolyzable analog of ATP called adenosine 5'-(beta, gamma-methylene) triphosphate or AMPCP (Hayashi et al., 2001). However, this structure was also monomeric, raising the possibility that archeal proteins do not form dimers in the presence of ATP. In addition, the C-terminus of the archeal MinD was disordered, thus making it clear that in order to gain understanding of the structure of the C-terminus, its functionality would have to be studied further.

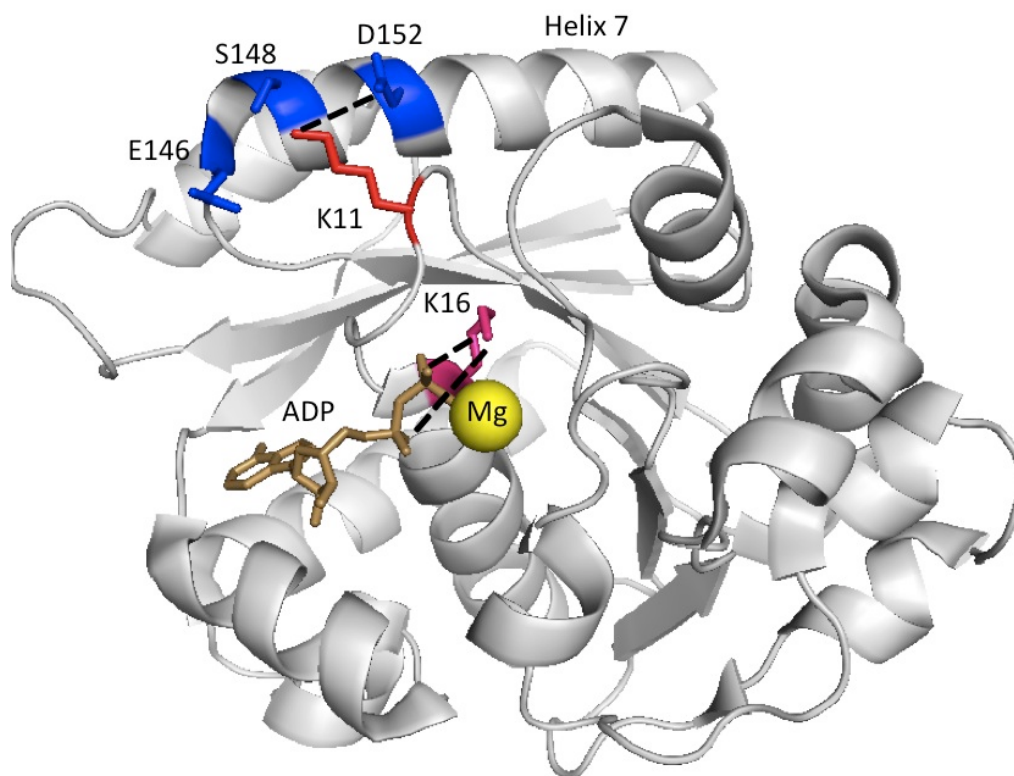


Figure 1.8: Ribbon diagram of MinD from *Pyrococcus furiosus* (PDB # 1G3Q) showing Lys 11 (red) and the interacting triad of residues E146, S148 and D152 (blue-shown in helix 7) (Hayashi et al., 2001). Note that the sidechain of Lys16 (shown in pink) and specifically the nitrogen atoms form hydrogen bonds to the β - and γ -phosphate oxygens of the bound nucleotide.

Functional Importance of the MinD C-terminus

Given the importance of membrane binding for MinD interactions with MinC and MinE, and for its ATPase activity, a number of studies have been done to identify regions of MinD that might be involved in this interaction. For example it has been shown that C-terminal truncation mutants missing 3, 10, 20 or 40 amino acids were unable to localize to the membrane (Hu & Lutkenhaus, 2003). To confirm these *in vivo* results, truncation mutants missing 3 (MinD_{Ec-3aaCT}) or 10 (MinD_{Ec-10aaCT}) residues were tested to see if they could bind to the lipids in an ATP-dependent manner as was previously shown for wildtype MinD (Hu et al., 2003). Since both mutants demonstrated very little vesicle binding, it was concluded that MinD_{Ec-3aaCT} and MinD_{Ec-10aaCT} are deficient in vesicle binding *in vitro*, consistent with the lack of membrane localization observed *in vivo* with the GFP fusions (Hu & Lutkenhaus, 2003).

These studies have shown that the last 8-12 residues are important for MinD membrane localization, giving rise to its designation as a membrane-targeting sequence (MTS). This region of MinD is relatively well conserved, allowing definition of a 12-13 residue MTS consensus sequence of



where X₃₋₄ is a three to four residue insertion found in some MinD proteins (T. H. Szeto et al., 2002). Some sequence conservation also exists outside this region; however, the MTS is the only portion of the C-terminal region that is conserved across the phylum. In fact, MinD is the only member of the ParA superfamily family that has an MTS, and is also the only member known to specifically localize to the membrane (Lutkenhaus, 2007).

Sequence analysis of MTS sequences across a diverse range of species suggests that it has a conserved tendency to form an amphipathic helix (T. H. Szeto et al., 2002). Studies on

MTS sequences from some of these species suggest that both the helicity and the amphipathicity are critical for its membrane targeting function. One particular study demonstrated with the use of GFP tagged truncated mutants, that Lys- 261 to Phe- 268 of the *E.coli* C-terminus is critical for membrane localization in eubacteria, archea and plastids. Subsequently, localization studies of *E.coli* MinD mutants (from the C-terminus) demonstrated the need for both the helicity and the amphipathic nature of the MTS to avoid aberrant cytoplasmic localization of MinD (T. H. Szeto et al., 2002). Since many of the MTSs from Gram-positive bacteria, archea, and chloroplasts contain a three- or four-residue insertion relative to the MTSs from Gram-negative bacteria and the insertion always occurs between residues corresponding to Leu-264 and Lys-265 of the *E. coli* sequence, it was postulated that, if the MTS is helical with 3.6 residues per turn, then a three-to four-residue insertion would maintain the helical phase of the MTS. Smaller or larger insertions would alter the helical phase of the MTS and reduce or eliminate the amphipathicity of the helix, which might diminish the ability of the MTS to target MinD to the membrane.

To test this hypothesis, in one study, mutations were introduced in the MTS that were predicted to disrupt the helical structure, but maintain the pattern of charged and hydrophobic residues in the primary sequence. These mutants were unable to localize to the membrane, and were instead diffusely distributed throughout the cytoplasm (T. H. Szeto et al., 2002). This is not unexpected, since amphipathic helices mediate the association of a wide range of proteins with biological membranes (T. H. Szeto et al., 2002). These helices are often partial to anionic phospholipids because of the large number of positively charged residues on the polar face of the helix. This allows the polar face to engage in interactions with anionic phospholipid headgroups

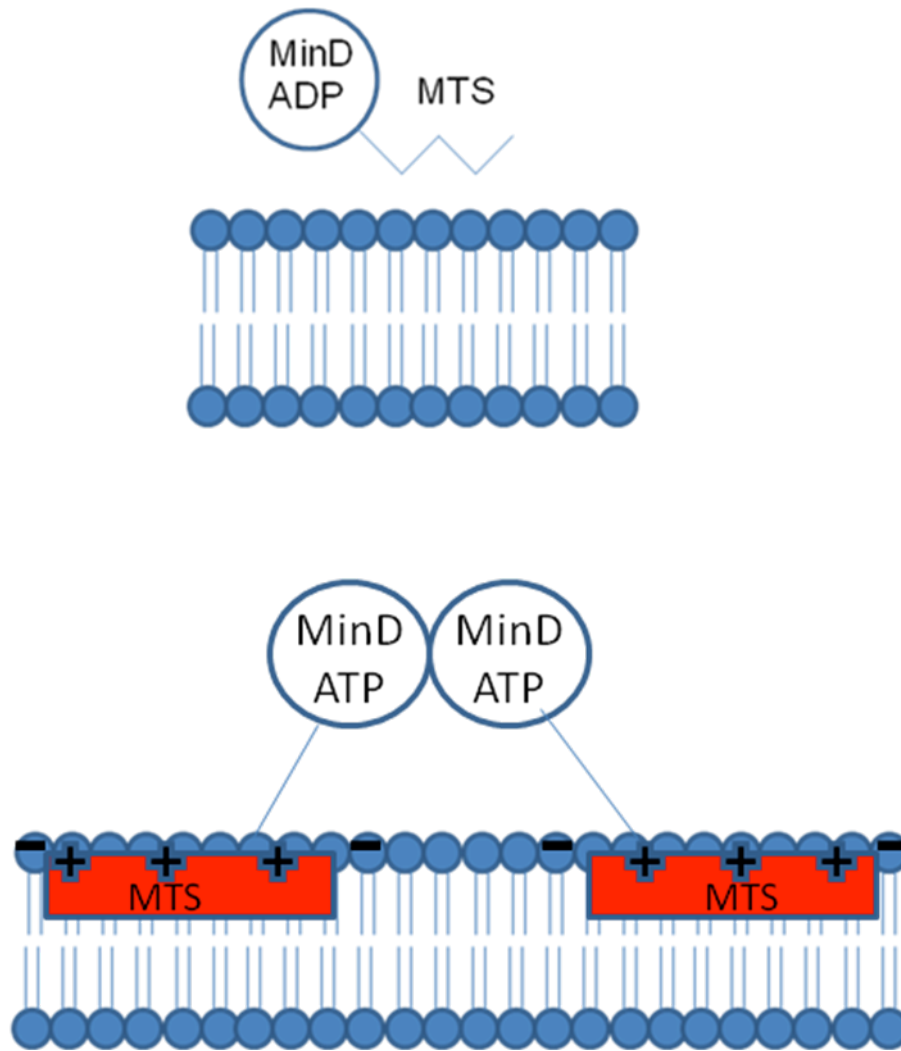


Figure 1.9: Model of MinD-membrane attachment (T. H. Szeto et al., 2002). The C-terminus forms an amphipathic helix that interacts with lipid bilayers and likely orients itself parallel to the membrane. Partial insertion of the helix would allow its hydrophobic residues to interact with lipid acyl chains and the cationic residues on the polar face of MinD MTS (indicated by +) to interact with the anionic phospholipids (indicated by -).

while the hydrophobic face becomes buried in the interior of the bilayer. It is thought that the MinD MTS binds the membrane in this way, potentially undergoing a conformational change from an unstructured, free state to the helical, membrane-bound state (Figure 1.9).

The cycle of membrane attachment and dissociation that MinD undergoes is thought to take place continuously throughout the cell cycle. Since oscillation of the MinCD polar zone occurs within about a minute, this membrane-binding-dissociation cycle must occur on a faster timescale (T. H. Szeto et al., 2002) giving rise to the idea that MinD may only superficially bind to the membrane (T. H. Szeto et al., 2002). The importance of a transient association with the membrane was illustrated by experiments with a chimeric MinD protein with a 43-amino acid hydrophobic transmembrane anchor from cytochrome b5 replacing the MinD MTS. This chimeric protein localized to the *E.coli* cell membrane, but was not able to form the membrane-associated helical cytoskeletal structures normally formed by MinD with MinE (Taghbalout, Ma, & Rothfield, 2006). More significantly, MinD containing the cytochrome b5 membrane anchor lost its ability to form polar MinCD zones in *E. coli* cells in the presence of MinE, which could suggest that the more hydrophobic cytochrome segment may have prevented redistribution of the membrane-associated MinD by increasing the strength of its membrane association (Taghbalout et al., 2006).

Although there is evidence that the MTS is necessary for membrane localization, this sequence alone may not be sufficient for this interaction. In fact the C-terminal 40 residues of MinD_{Ec} was not able to promote membrane localization of a fused GFP tag in vivo suggesting that other regions of MinD may also be involved in this interaction (T. H. Szeto et al., 2003). This study also suggested that the MTS from MinD_{Ec} is not a transplantable membrane- targeting motif. This stands in stark contrast to MinD from the gram-positive rod-shaped bacterium

Bacillus subtilis (BsMinD), which does not oscillate in the cell but is consistent with circular dichroism experiments showing that the *E.coli* MTS has a weaker affinity for phospholipid bilayers than the *B.subtilis* MTS. However, an alternative explanation for the inability of a single MTS tag to bind the membrane alone is provided by the zipper model of MinD binding. According to this model, ATP-dependent polymerization of MinD increases the number of MTS sequences in the complex (T. H. Szeto et al., 2003; H. Zhou et al., 2005). This creates multivalent binding that leads to membrane interactions of a higher affinity for the MinD oligomer than would be observed for the MinD dimer, since binding of the second MTS to the membrane would effectively be an intramolecular association. This model suggests that the multivalent MinD polymer thus acts like a zipper that grows and allows the MTS to attach to the membrane, further stabilizing the membrane-bound MinD polymer (T. H. Szeto et al., 2003). Meanwhile the functionally autonomous MTS of MinD from *B. subtilis* can bind the membrane monovalently may be responsible for the different localization patterns and biological roles of *B. subtilis* MinD. BsMinD does not oscillate and it remains anchored at the cell poles by a protein DivIVA until it is recruited to the nascent septum at a late stage in assembly of the division machinery. In contrast, MinD_{Ec} undergoes rapid pole-to-pole oscillation that appears to be hindered by an MTS with a high affinity for lipid membranes.

Although the MTSs from different species all share amphipathic features that suggest interactions with lipid bilayers, there is some evidence that the different sequences promote preferential interactions with different types of anionic phospholipids. These differences may be teleological, since the membrane composition of different bacteria can vary significantly. For example, the MTS from *B. subtilis* has a distinct preference for PG over CL, consistent with the fact that there are significant levels of PG (~16% of total phospholipid) but hardly any CL (<1%)

in *B. subtilis* cell (T. H. Szeto et al., 2003). Thus, the polar surface of each MTS α -helix may be evolutionarily “tuned” for interaction with specific phospholipids that are present in the inner membrane of the bacterium in which it resides (T. H. Szeto et al., 2003).

Putative MTS sequences have also been identified in archaeal MinD homologs for which x-ray crystal structures were determined (*A. fulgidus*, *P. furiosus*, and *P. horikoshii*) (T. H. Szeto et al., 2003). Unfortunately, these structures do not provide insight into the mechanism by which MinD associates with the membrane since, all three structures are incomplete in the C-terminal region with no electron density observed for the C-terminal 30 and 8 residues of *A. fulgidus* and *P. furiosus* MinD, respectively (T. H. Szeto et al., 2003). Although electron density was more complete for the *P. horikoshii* MinD structure, the C-terminal residues had very high B factors, indicative of significant structural variability in this part of the structure. On the basis of these crystal structures, it was speculated that the C-terminal region of MinD might be structured only when associated with lipid (Hu & Lutkenhaus, 2003).

These crystal structures suggest that the MTS could be a dynamic structure, an idea that has been reiterated in the ‘dimer trigger’ model (Hu & Lutkenhaus, 2003; Hu et al., 2003a). This model suggests that MinD binds ATP and dimerizes, which in turn alters the C-terminal helix in a way to allow interactions with the membrane. However, the observation that ATP bound MinD can oligomerize *in vitro* in the absence of lipids, forming short, thin protofilaments (Hu & Lutkenhaus, 2003) raises the possibility that short filaments rather than dimers may nucleate the cooperative assembly of MinD on the membrane. Overall, the central tenets of neither ‘dimer trigger’ nor the ‘zipper’ model depend on the oligomerization state of the fundamental unit of the membrane-bound MinD polymer. Nonetheless, it will be important in future studies to determine

whether dimerization is an essential prerequisite for the cooperative polymerization of MinD on the membrane.

Enzyme Kinetics

At the heart of the Min protein cycle is the MinD enzymatic activity that catalyzes the hydrolytic cleavage of ATP to form ADP and inorganic phosphate (H. Zhou et al., 2005). Measuring the rate of this reaction in the presence and absence of MinE has the potential to reveal insights into the molecular mechanism of MinE stimulation of MinD ATPase activity. Enzyme kinetics can also be used to characterize the functional effect of mutations in MinD. For these reasons, we have used the measurement of ATP hydrolysis rates for MinD as a probe for MinD function, and to investigate the role of the C-terminal MTS in MinD from *N. gonorrhoeae*.

One of the most common approaches in the study of enzyme kinetics is to utilize the equation for the initial reaction rate (v_0) provided by the steady state approximation that gives rise to the Henri-Michealis-Menten equation:

$$v_0 = \frac{V_{\max} [S]}{K_m + [S]}$$

where, $V_{\max}$ is the maximal activity, $[S]$ is the substrate concentration, and K_m is the substrate concentration at half maximal activity, also known as the Michaelis constant. This equation was derived using the following two assumptions: (i) the system is at steady-state, which assumes that the concentration of the substrate-bound enzyme is constant and (ii) that the products do not inhibit the reaction, and do not undergo the backwards reaction (initial conditions). For these reasons, to observe Michaelis-Menten behavior, reaction conditions must satisfy these two conditions, typically by assaying early stages of a reaction after steady state conditions have been established (i.e. <30% depletion of the substrate) (Endrenyi et al., 1975).

Enzymes that follow Michaelis-Menten behaviour will show a hyperbolic relationship between initial reaction rate and substrate concentration (Figure 1.10A). However, this requires that the active sites of these enzymes behave independently, which is not necessarily the case if oligomeric complexes are formed in a way that functionally links active sites. Given the polymeric structure known to be adopted by MinD in the presence of lipids and ATP, it is not likely that MinD would follow straightforward Michaelis-Menten behaviour, but would instead expect to show cooperativity.

Cooperativity can be demonstrated in a plot of v_0 versus $[S]$, which in this case would generate a sigmoidal curve. When data follows this trend it is possible to fit it to the Hill equation:

$$v_0 = \frac{V_{\max} [S]^n}{K_{0.5}^n + [S]^n}$$

where, n is the hill coefficient (Endrenyi et al., 1975). This expression has similar terms to the Michaelis-Menten equation ($K_{0.5}$ = substrate concentration at half-maximal activity, $V_{\max}$ = maximal activity), but the range of substrate concentrations over which the most significant change in activity is observed becomes narrower as the cooperativity increases. The cooperativity is measured in terms of the Hill coefficient (n) and indicates the degree to which the binding and reaction of substrate to one active site affects the binding and reaction of substrate to the other active sites. The Hill coefficient can also be considered to reflect the minimum number of active sites that are functionally linked. Since in the case of MinD, dimerization is known to occur in an ATP dependent manner, the binding of ATP to one active site is expected to increase the affinity for ATP binding at the other active sites (Taghbalout et al., 2006). For this reason, positive cooperativity with respect to ATP binding should be observed

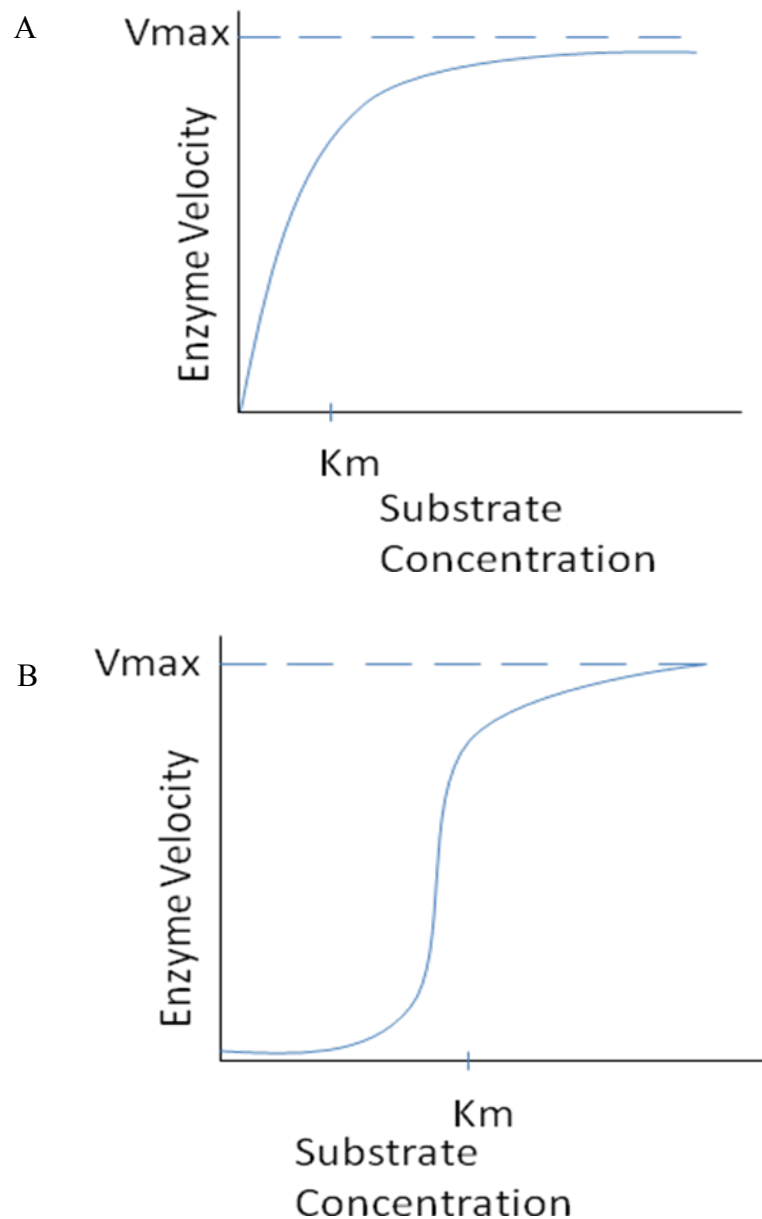


Figure 1.10: Initial velocity versus substrate concentration plot for an enzyme following Michaelis-Menten versus cooperative kinetics. The maximal activity (V_{max}) and the Michealis constant (K_M) can be obtained by fitting the experimental data to this equation.

for MinD.

In this thesis the molecular mechanism by which MinE binding promotes ATP hydrolysis was investigated using kinetic studies on MinD_{Ng}. These studies should help to determine whether MinE binding stimulates MinD activity by increasing the apparent affinity of MinD for its substrate ($K_{0.5}$), by increasing its catalytic efficiency (V_{max}) or by increasing the cooperativity of the reaction (n). In addition, the kinetic profile of a mutant version of MinD_{Ng} with a truncation of the last 13 amino acids on the C-terminus was studied in order to determine the functional role of the MTS.

Characterization of Protein Structure by Circular Dichroism (CD) Spectroscopy

An important consideration in the study of a mutated version of any protein is whether its structural integrity has been affected. CD can provide a structural window into the effect of a mutation since it is a measure of the total secondary structure content of a protein. This allows the identification of mutants with altered structure, and perhaps more importantly, mutants with native structure that nonetheless show compromised activity levels to highlight functionally important sites. For this reason, the structural characteristics of the mutant studied in this thesis were also investigated by CD.

In general, circular dichroism arises when a chromophore absorbs left-hand circularly polarized light to a different extent than right-hand circularly polarized light. The difference between the absorption of the left and right-hand circularly polarized light is what is known as CD (Whitmore & Wallace, 2008). In proteins, circular dichroism in the far-UV region (190-250 nm) arises for α -helices and β -sheets and gives rise to distinctive spectra in this region. As shown in Figure 1.11, the CD spectrum of an α -helix will absorb characteristically with a minima at 222 nm and 208 nm and a maximum at 192 nm.

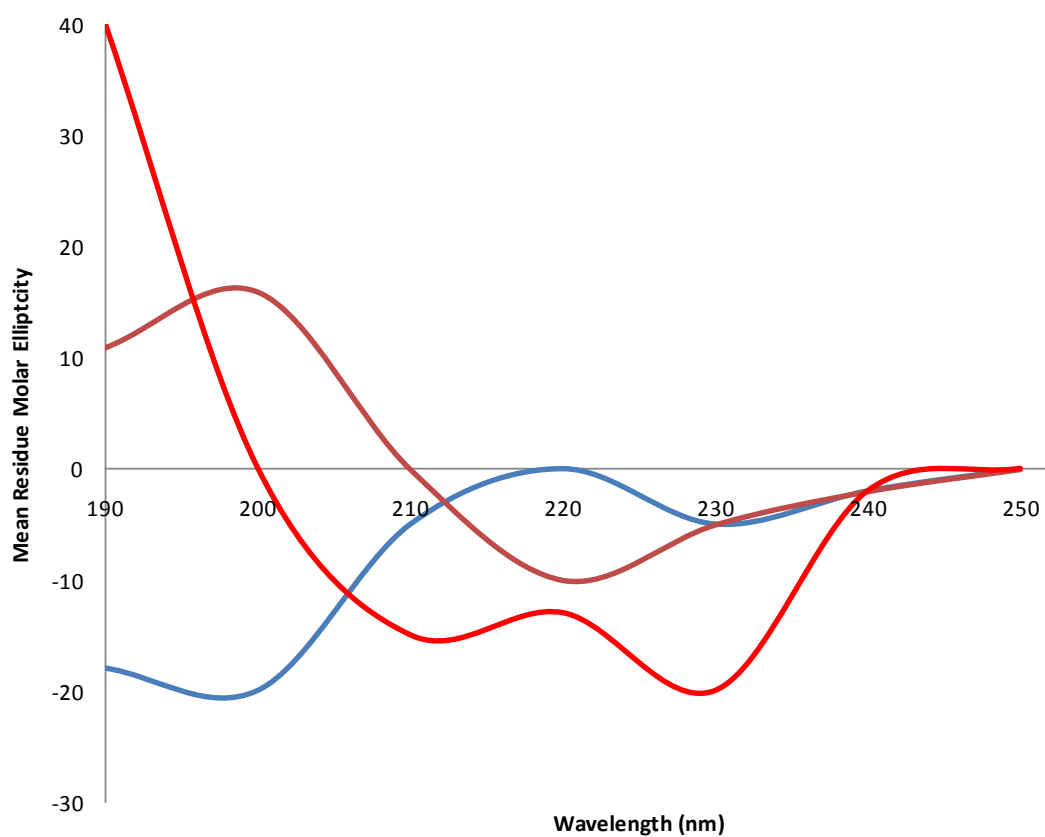


Figure 1.11: Examples of characteristic CD spectra for a α -helix (red), β -sheet (brown) and random coil (blue) measured in mean residue molar ellipticity over a range of wavelength from 190 nm-250nm.

β -sheets on the other hand, absorb characteristically at 215 nm and a 198 nm. In addition, protein structures with random coil conformations commonly show a minimum at 205 nm and a maximum at 226 nm (Figure 1.11). These characteristic spectra can be used to extract secondary structure estimations by spectral deconvolution programs such as Dichroweb to provide an approximation of the secondary structure content of a protein. These programs typically use CD spectra from multiple sets of reference proteins as a base for the estimation of secondary structure content for a CD spectrum from a sample of interest (Johnson, 1999).

Thesis Objectives

Given the apparent importance of the MTS for membrane binding in MinD from *E. coli*, we wanted to focus our studies on the structure and function of C-terminus of MinD from *N. gonorrhoeae*. For this purpose I have studied the functional and structural properties of a 13 amino acid C-terminally truncated mutant of MinD_{Ng} (MinD_{Ng-13aaCT}) and compared this to wild-type MinD_{Ng}. The objectives of this work were the following:

- Generate MinD_{Ng}, MinD_{Ng-13aaCT}, and MinE_{Ng} samples
- Determine the oligomeric state of MinD_{Ng} and MinD_{Ng-13aaCT} to probe the role of the MTS in dimerization
- Investigate the functional role of the MinD_{Ng} MTS through kinetic studies on WT and MinD_{Ng-13aaCT} mutant
- Acquire kinetic profiles for MinD in the presence and absence of MinE to determine the mechanism of MinE activation of MinD ATPase activity
- Characterize the *in vivo* function of MinD_{Ng} and MinD_{Ng-13aaCT}

Overall, the results of these studies should improve our understanding of the function of the MinD C-terminus and the mechanisms that regulate cell division, which in the long term can facilitate in the production of new antibiotics against *N. gonorrhoeae*.

Materials and Methods

Strains and growth conditions

The strain and plasmids that were utilized in this study are listed below in Table 1. *E. coli* BL21 (DE3) was used as the host for all studies. For morphology studies, *E. coli* BL21 (DE3) was grown at 37°C in 1L M9 minimum medium, whereas to overexpress C-terminal His-tagged wild-type and mutant MinD_{Ng} with a 13aa truncation at C-terminus, *E. coli* BL21 (DE3) was grown at 37°C in Luria-Bertani (LB) medium. In both of the growth conditions, 50 µg/ml kanamycin was used for selection and induction of protein expression was achieved using 500ul of 1M IPTG.

Table 1: Strains and plasmids used in this study.

Strain/plasmids	Relevant genotype	Source/Reference
<i>E. coli</i> BL21(DE3)	<i>E. coli</i> B dcm ompT hsdS(r _B ⁻ m _B ⁻) gal F ⁻ ompT gal dcm lon hsdS _B (r _B ⁻ m _B ⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Invitrogen
<i>E. coli</i> DH5α	suppE44 ΔlacU169 (80lacZΔM15) hsdR17 endA1 gyrA96 thi-1 relA1	Gibco
<i>Plasmids for expression studies/template DNA</i>		
pSC9	Kan ^R pET30a; P _{T7} ::minD _{Ng-6XHis}	Szeto <i>et al.</i> (2004)
pSA2	Kan ^R pET30a; P _{T7} ::minD _{Ng-13aaCT-6XHis}	Acharya (2006)
pEC1	Kan ^R pET30a; P _{T7} ::minE _{Ng-6XHis}	Ramirez-Arcos <i>et al.</i> (2002)

MinD_{Ng} Overexpression

A plasmid encoding a C-terminal hexahistadine tag fused to MinD from *N.gonorrhoeae* (PSC9 (MinD_{Ng})) was transformed into *E. coli* BL21 (DE3) cells for over-expression. Briefly, 1-2 µl of 13 ng/µl of pSC9-MinD_{Ng} plasmid was added to 20 µl of competent *E.coli* BL21 (DE3) cells. To ensure selective growth, kanamycin (Kan) was added at a concentration of 50 µg/ml. For the purpose of inoculation, one colony was used in 150 mL of LB broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 1 mL of 1 N NaOH), and shaken overnight at 220 rpm, 37°C. This overnight culture was transferred into 1L of fresh LB broth and grown at 37°C with continual shaking. When the optical density at 600 nm (OD₆₀₀) in an Ultrospec 2100 pro UV/Visible spectrophotometer (Biochrom) reached 0.6-0.8, 500 µl of 1 M IPTG (isopropyl-beta-D-thiogalactopyranoside) was added to induce MinD_{Ng} expression. After incubation for 4 hours at 37°C (OD₆₀₀ was ~ 1.8 or higher) the cells were pelleted by centrifugation in an Avanti[®] J-E centrifuge (Beckman Coulter) at 4000 g at 4°C for 10 minutes and stored at -20°C until purification. MinD_{Ng} with its last thirteen amino acids truncated (MinD_{Ng}-13aaCT) was also utilized for oligomerization studies following the same protocol as described above.

Protein purification

All plasmids that were used in this study were verified by the DNA sequencing by the University of Ottawa Core DNA Sequencing and Synthesis Facility. Protein purification was carried out by nickel affinity chromatography based on previously published protocols (Invitrogen). The bacterial pellets from a 1L culture were resuspended in 40 mL of Lysis buffer (50 mM Tris at pH 8.0, 300 mM NaCl, and 10 mM imidazole). Benzamidine (15 mg) was also dissolved into the resuspension, which was then placed on a rocking platform (VWR) for 20 min followed by sonication on ice to lyse the cells for 1 min twice with 50% duty cycle at 50%

amplitude. The cell suspension was centrifuged at 16,000 g for 20 minutes at 4°C. To carry out the purification, 2.5 mL of nickel-nitrilotriacetic acid (Ni-NTA) resin (Novagen) was loaded on to a column, washed with double distilled water and equilibrated with lysis buffer. The soluble fraction of the lysed pellet was applied to the column, which was then washed with 50 mL of lysis buffer, 50 mL of wash buffer (50 mM Tris at pH 8.0, 300 mM NaCl, and 20 mM imidazole) and 40 mL of elution buffer (50 mM Tris at pH 8.0, 300 mM NaCl, and 250 mM imidazole). In order to evaluate the success of the purification, a 20 µL aliquot was drawn and mixed with 20 µL of 2X SDS-PAGE loading buffer to analyze on an SDS- PAGE gel.

SDS-PAGE

Samples prepared in loading buffer were boiled and loaded on to a gel consisting of stacking gel phase (5% (w/v) acrylamide:bisacrylamide (37.5:1) (Bio-Rad), 125 mM Tris at pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate, and 0.1% (v/v) 1,2-di-(dimethylamino)ethane (TEMED) (EMD)), and a resolving gel phase (15% (w/v) acrylamide:bisacrylamide (37.5:1), 390 mM Tris at pH 8.8, 0.1% (w/v) SDS, 0.1 (w/v) ammonium persulfate, and 0.04% (v/v) TEMED). 5.0 µL of prestained protein ladder (Invitrogen) was also loaded to gain an estimate of molecular weights. The gel was run in an SDS-PAGE apparatus (Bio-Rad) with running buffer (25 mM Tris, 250 mM glycine, and 0.5% SDS) at 180 V for 45 min. The resolving gel was stained with Coomassie blue stain consisting of 50% methanol, 40% water, 10% acetic acid, and 0.1% (w/v) Coomassie brilliant blue for 50 min and destained in 50% methanol, 40% water, and 10% acetic acid for 40 min.

Bradford protein assay

The concentration of proteins samples were determined by performing a Bradford protein assay with bovine serum albumin (BSA) as the protein standard. Five standards including 1

µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml of BSA and diluted proteins were prepared in microtubes. 200 µL of Bradford reagent (BioRad) was added to 1 ml of each protein sample with 100X dilution followed by vortexing. Reaction mixtures were incubated at room temperature (~23°C) for at least 5 minutes to allow for colour development. Protein concentrations were measured at 595 nm on a Ultrospec 2100 pro UV/Visible spectrophotometer.

Size exclusion chromatography

In order to study the oligomerization state of MinD_{Ng}, nickel affinity-purified samples were analyzed by gel filtration chromatography. In these experiments a Superdex 75 column was used since its resolving power is best for proteins in the 10 - 70 kDa range (Figure 2.1). 500 µl of purified MinD_{Ng}, with or without 1 mM ATP, 2 mM MgCl₂ were applied to a 100 mL column of Superdex 75 (Amersham Pharmacia) equilibrated with 50 mM Tris-HCl, 200 mM NaCl, 0.5 mM EDTA, final pH adjusted to 8.5. The column was equilibrated with either 1 mM ATP or 1 mM ADP and 1 mM MgCl₂. The column was run at a flow rate of 0.5 ml/min and 0.5 mL fractions were collected for SDS-PAGE gel analysis. Protein elution profiles were compared to those of Amersham gel-filtration protein standards for molecular mass estimation (Figure 2.1).

For samples to be used in ATPase assays, HiPrep 26/10 desalting column was used for buffer exchange, particularly since imidazole from the nickel affinity- purified samples can also catalyze ATP hydrolysis. The desalting column was equilibrated with 110 mL of elution buffer containing 48 mM Tris, 260 mM NaCl, pH 8.5. 35 mL of eluate from the size-exclusion chromatography step was divided in 3 parts and loaded onto the desalting column using a superloop.

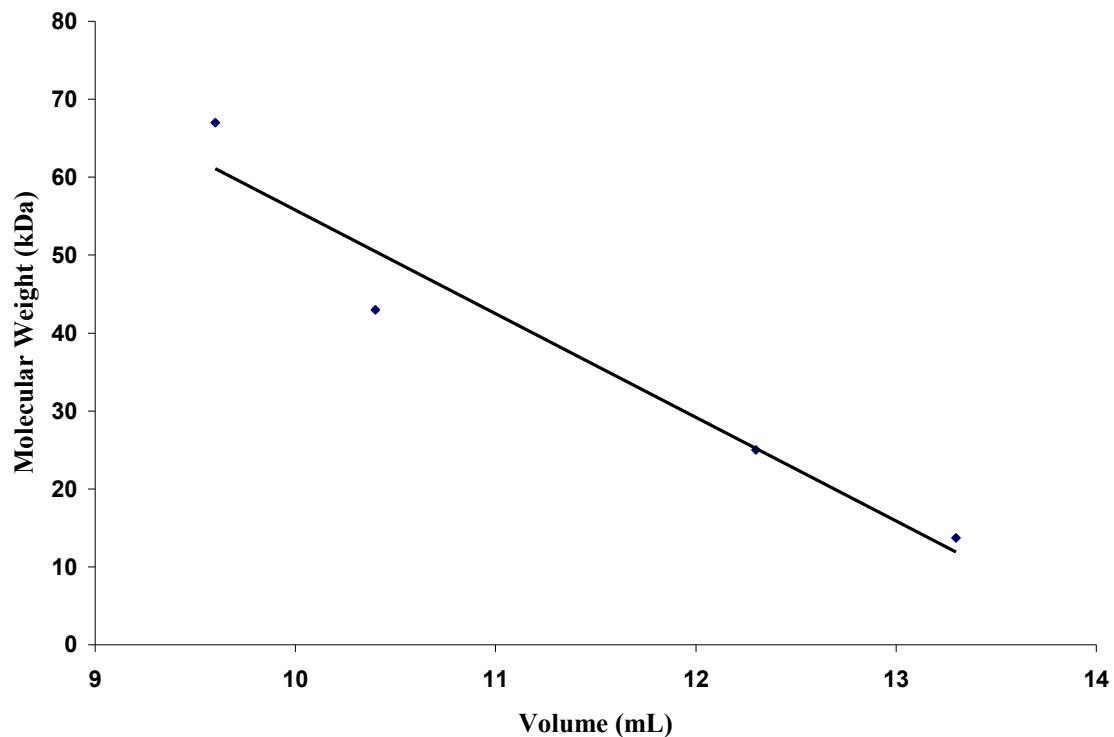


Figure 2.1: Calibration curve for the Superdex 75 column for the estimation of molecular weights from elution volumes. 250 μ l of each protein standard (ribonuclease A (13.7 kDa), chymotrypsin (25 kDa), ovalbumin (43 kDa), BSA monomer (67 kDa) from Amersham Bioscience) was loaded onto the column at a flow rate of 0.5 mL/min in 25mM Tris (pH= 8.0), 200 mM NaCl, and 5 mM β -mercaptoethanol.

In order to aid in the removal of imidazole in our samples, a Nap-10 column (Pharmacia) was also utilized for buffer exchange for samples that underwent the *in vitro* ATPase assays, by equilibrating with 10 ml of the same elution buffer as used above. 1 mL of purified MinD_{Ng}, MinD_{Ng-13aaCT}, and MinE_{Ng} were loaded into separate Nap-10 columns and eluted fractions collected in 250 μ l aliquots.

In vitro ATPase activity assays

ATPase activity was assessed by measuring the release of free phosphate from ATP hydrolysis using the malachite green method (Fisher & Higgins, 1994). In a 300 μ l reaction mixture, the following components were added in sequence: ddH₂O, 10 X reaction buffer (containing 500 mM KCl, 250 mM Tris, pH 8.5), 3 μ M MinD_{Ng}, 1 mM ATP, 0.5 mg/ml *E.coli* phospholipids, 2 mM MgCl₂, and 10 μ M MinE_{Ng}. In addition, control reactions were prepared with no MinE_{Ng}, no MinD_{Ng} or no phospholipids were also prepared. Elution buffer containing 45 mM Tris, 260 mM NaCl, 0.5 mM EDTA, pH 8.5 was added to the reaction mixture in place of any absent protein in negative controls. Flicking the reaction tube mixed the combination in our reaction and subsequently 70 μ L aliquots were removed to be measured at specific time points (t=2 minutes, 15 minutes, 28 minutes, 41 minutes). The aliquots were centrifuged at 16,000 x g for 5 minutes and 25 μ l of each supernatant was carefully removed and loaded in a microtiter plate well containing 50 μ l of a malachite green solution (made by adding one volume of 4.2 % (w: v) ammonium molybdate in 4N HCl in 3 volumes of 0.045% (w: v) aqueous malachite green solution with 0.03% Tween-20). Reaction mixtures were incubated at room temperature (~23°C) for 15 minutes and the absorbances were measured at 620 nm using a Spectramax Plus 384 (software Softmax PRO). A linear time-dependent increase in phosphate concentration was observed, the slope of which was determined using linear regression to give

the reaction rate. Rates were converted from absorbance units into inorganic phosphate concentrations using a standard curve obtained from serial dilutions of KH_2PO_4 in reaction buffer. All experiments were performed at least two times and the average inorganic phosphate release was determined.

Circular Dichroism Spectroscopy

Samples for CD spectroscopy were purified by size-exclusion chromatography into a buffer containing 15 mM Tris and 20 mM NaCl, pH 8.5. CD spectras were recorded on a Jasco J-810 circular dichroism spectropolarimeter with a 1 mm path length quartz cell at 25 °C. The spectral scans were recorded from 250-195 nm with a 0.2 nm step resolution, a speed of 10 nm/min and a bandwidth of 1 nm.

Morphology studies of E.coli BL21 cells

For morphology studies, a transformed colony was inoculated in M9 minimum media at 37 °C, and spun at 220 rpm. Four hours post-induction, a 1.0 mL sample was taken from the culture and diluted to O.D 0.3, 20 µl of which was placed onto a glass slide. Cells were heat-fixed by passing through a flame three or four times, and a drop of immersion oil was added to the fixed cells. A coverslip was placed gently on top of the fixed cells and sealed with transparent nail polish on each edge. Samples were visualized by phase contrast microscopy on an Olympus BX-60 microscope under a 100X oil immersion lens.

A histogram of cell lengths was generated based on manually measured cell length measurements with respect to the scale bar for images shown in Figure 3.11, along with additional images acquired for these samples (data not shown). A total of 257 cells were counted in control BL21 cells, 228 in cells expressing MinD_{Ng} and 285 in cells expressing MinD_{Ng-13aaCT}.

Results

Production of purified MinD_{Ng} protein samples

In order to perform our functional and conformational studies, wild-type MinE_{Ng}, MinD_{Ng}, and a 13 amino-acid C-terminally truncated version of MinD (MinD_{Ng-13aaCT}) were over-expressed in *E.coli* as previously described (Szeto et al., 2001). Since all Min proteins used in this study contained C-terminal hexahistidine tags, it was possible to purify all samples using nickel affinity chromatography. Figures 3.1 and 3.2 show an SDS-PAGE gel analysis of fractions taken during the purification of wildtype MinD_{Ng} and wild-type MinE_{Ng}. Both gels show that impurities are removed during lysis and wash steps, and subsequent elution of the His-tagged protein with 250 mM of imidazole yields a sample that is >90% pure, similar to previous observations (J. Szeto et al., 2001). Similar results were also obtained for the MinD_{Ng-13aaCT} mutant; however, there was a significant increase in the expression level and yield of MinD_{Ng} in the elution fractions (Figure 3.3). The fusion of the hexahistidine tag at the C-terminus has been observed to cause higher expression levels of MinD_{Ng-13aaCT} in our lab (Acharya, 2006). This may be due to the high solubility of the poly-His tag being able to counteract the tendency of hydrophobic residues in the C-terminus of MinD_{Ng} to aggregate (Acharya, 2006). This increase in yield is also consistent with previous attempts in our lab to purify this construct, suggesting that the removal of the last 13 amino acids play a role in enhanced MinD_{Ng} yield. The fact that the C-terminus of MinD_{Ng} is involved in membrane binding (Hu & Lutkenhaus, 2003; T. H. Szeto et al., 2002) offers a possible explanation, where our 13 amino acid C-terminal truncated mutant is unable to bind to the membrane, therefore avoiding loss of expressed MinD to the membrane-containing pellet fraction.

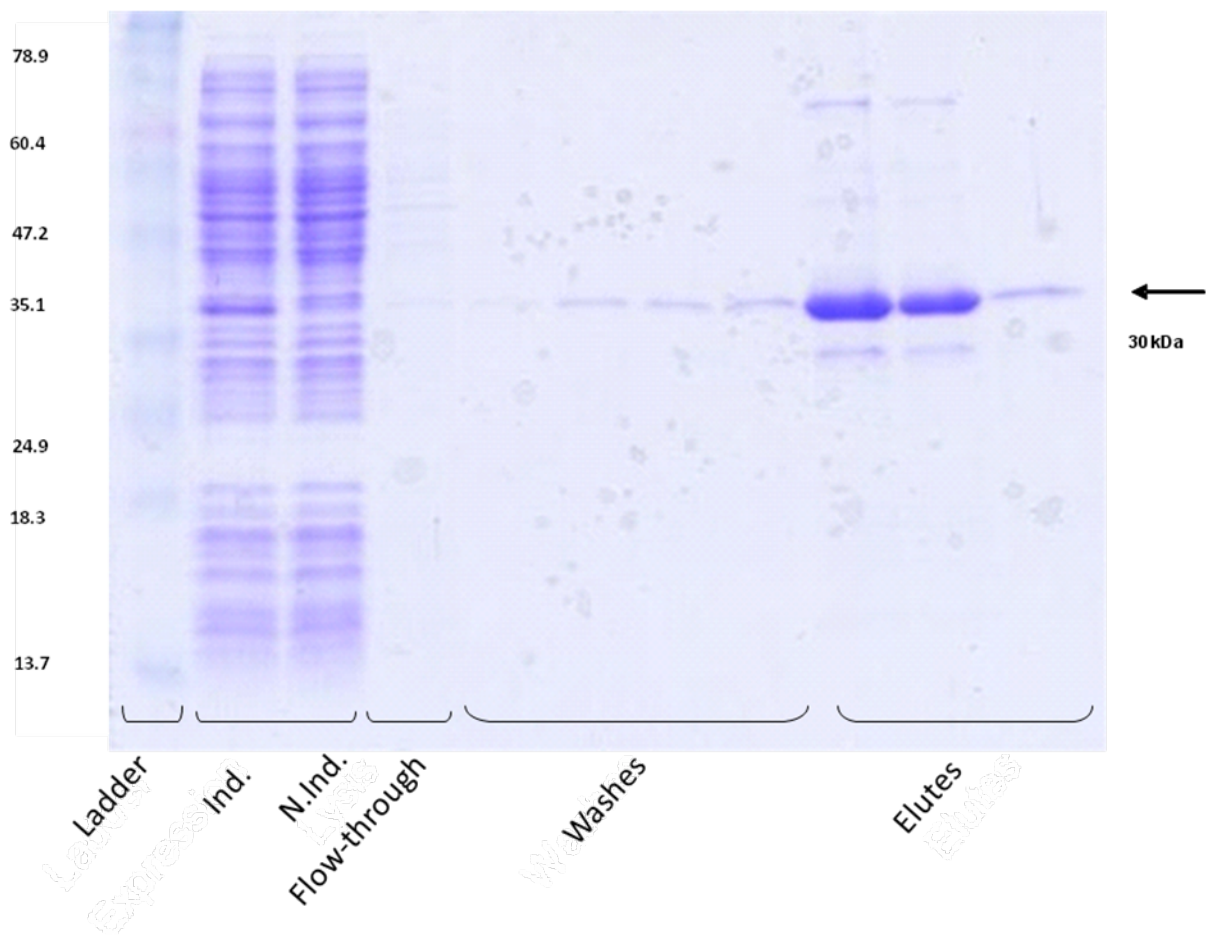


Figure 3.1: Coomassie stained SDS-PAGE gel of WT (wild type) MinD_{Ng} fractions. Whole cell lysates of BL21(DE3) bacteria transformed with the MinD_{Ng} expression plasmid before (N. Ind.) and after induction (Ind.) with IPTG are also shown. Flow through, column washes and elutes were carried out using 10 mM, 20 mM and 250 mM of imidazole respectively. Elute fractions show the main species at ~30 kDa as expected for MinD_{Ng}, as indicated by the arrow.

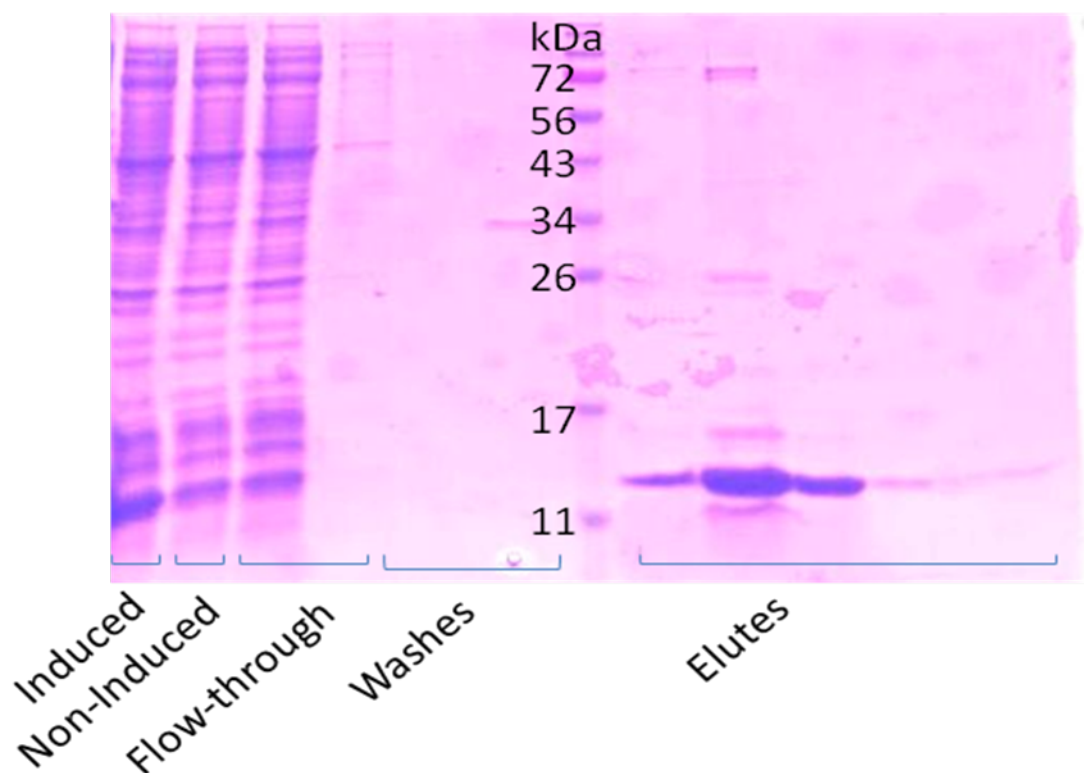


Figure 3.2: Coomassie stained SDS-PAGE gel of WT (wild type) MinE fractions. Whole cell lysates of BL21(DE3) bacteria transformed with the MinE expression plasmid before (N. Ind.) and after induction (Ind.) with IPTG are also shown. Flow through, column washes and elutes were carried out using 10mM, 20mM and 250mM of imidazole respectively. Elute fractions show the main species at ~11 kDa as expected for MinE.

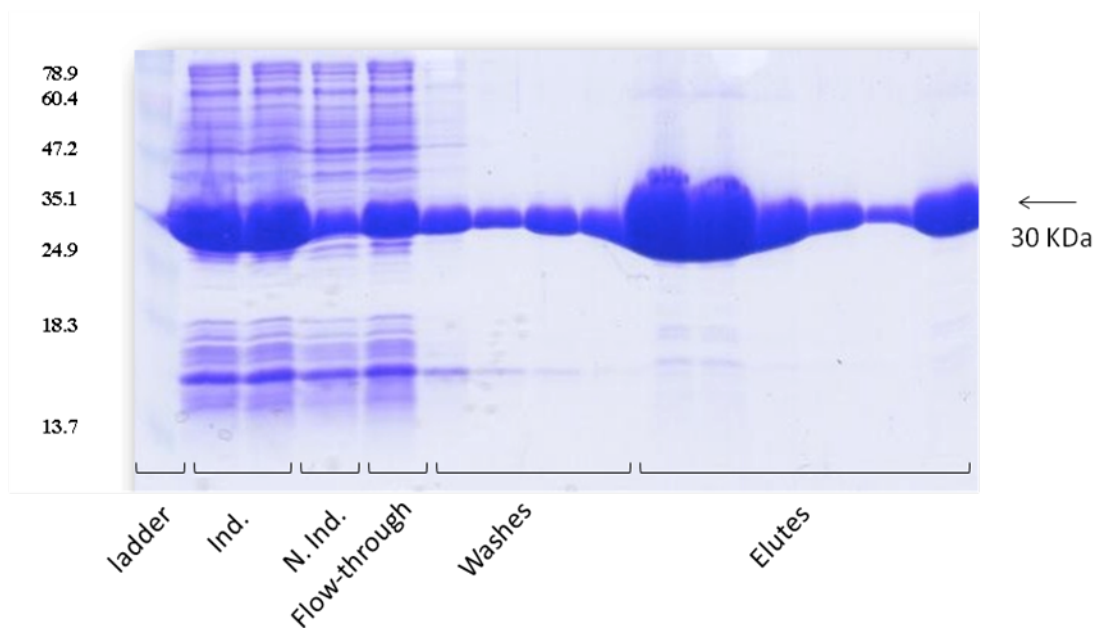


Figure 3.3: Coomassie-stained SDS-PAGE gel of MinD_{Ng-13aaCT} purification. Lysis and wash steps of purification were carried out at an imidazole concentration of 10 mM and 20 mM. In order to obtain higher purity samples, washing conditions were chosen that give rise to some loss of MinD_{Ng}, but nonetheless allowed high yields to be obtained. Elution fractions were collected at an increased imidazole concentration of 250 mM.

MinD_{Ng} oligomerization studied by size-exclusion chromatography

In order to evaluate the molecular weight of MinD_{Ng} and its mutant and probe their oligomerization states, size exclusion chromatography experiments were performed. As shown in Figure 3.4, in the presence of ADP, MinD_{Ng} primarily eluted in a single peak centered around 11.2 mL. According to molecular weight calibration standards (Materials and Methods, Figure 2.1) this elution volume corresponds to a globular protein with a molecular weight of approximately 30 kDa, as would be expected for monomeric MinD_{Ng}. In contrast, when this experiment was performed with ATP in the running buffer a small peak with a smaller elution volume increased in intensity relative to the elution profile obtained with ADP in the profile. The elution volume of this peak corresponds to a molecular weight of ~60 kDa, suggesting that this reflected the presence of dimeric MinD_{Ng} (Figure 3.4). This ATP-dependent dimerization of MinD_{Ng} is consistent with previous observations with the *E. coli* protein.

We were also interested in determining whether MinD_{Ng-13aaCT} dimerization can occur in the presence of ATP. As shown in Figure 3.5, MinD_{Ng} was largely monomeric in the presence of ADP, similar to what was obtained with WT MinD_{Ng} (Figure 3.4). However, in the presence of ATP, no peak corresponding to a dimeric species was observed, in contrast to results obtained with WT MinD_{Ng}. This suggests that the last 13 amino acids in MinD_{Ng} are required for ATP-dependent dimerization to occur.

A

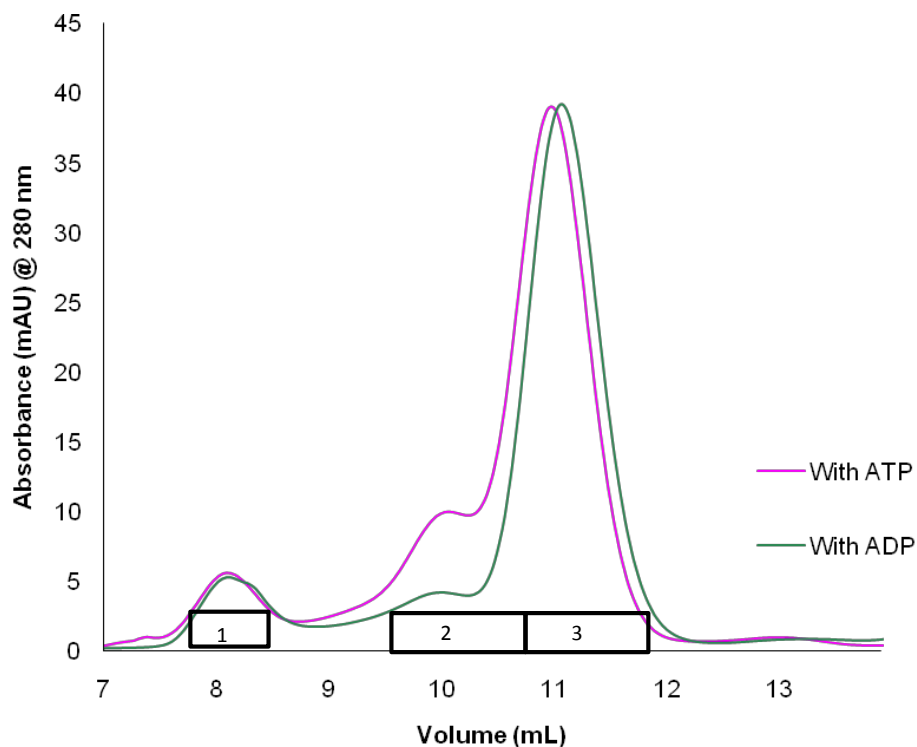


Figure 3.4: Size-exclusion chromatography of Wildtype MinD_{Ng} (A) Superdex 75 size exclusion chromatography profile for 7.5 μ M MinD_{Ng} in 50 mM Tris buffer pH 8.5, 50 mM NaCl, 2 mM MgCl₂ in the presence of 1 mM ATP (pink trace) or 1 mM ADP (green). Region 2 indicates the higher molecular weight species observed in the presence of ATP that elutes at a volume suggesting a molecular weight of approximately 60 kDa, suggesting a dimeric state.

A

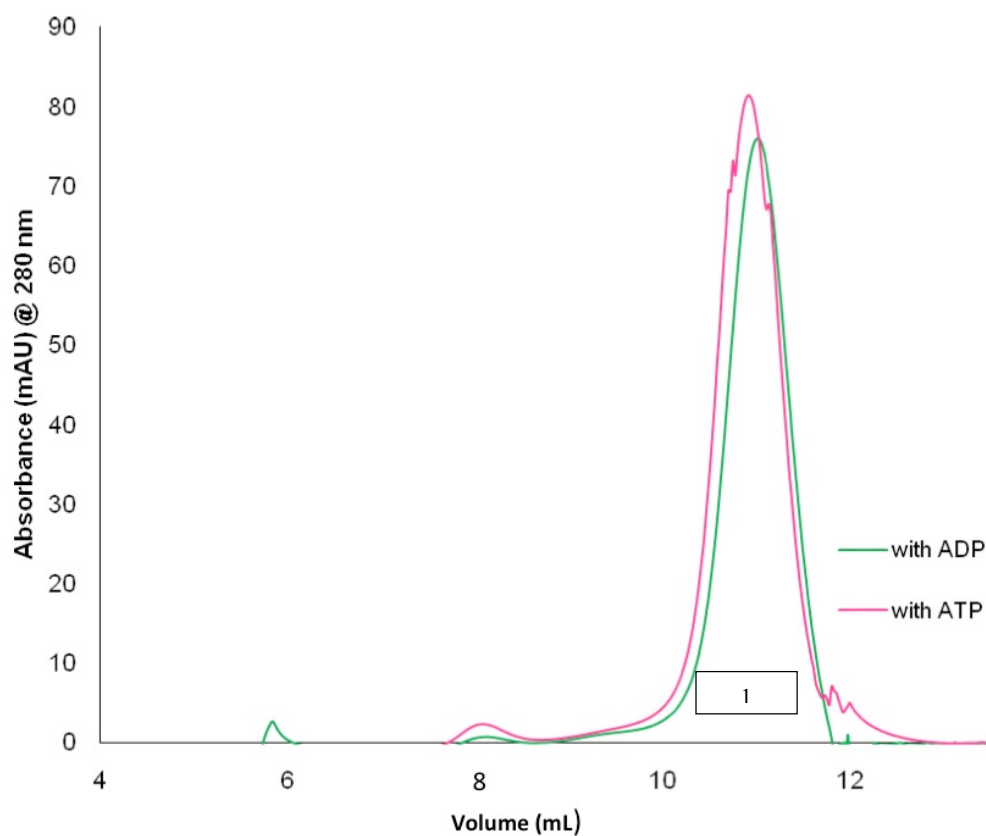


Figure 3.5: Size-exclusion chromatography of MinD_{Ng-13aaCT} (A) Size exclusion chromatography for 13.9 μ M MinD_{Ng} in 50 mM Tris buffer pH 8.5, 50 mM NaCl in the presence of 1 mM ATP and 2 mM MgCl₂ (shown in pink) and 1mM ADP and 2 mM MgCl₂ (shown in green).

Analysis of MinD_{Ng} and MinD_{Ng-13aaCT} secondary structure

Although the elution volume of MinD_{Ng-13aaCT} was similar to that of the wild-type protein, suggesting that removal of the C-terminal residues did not lead to global unfolding, it was possible that the truncation did give rise to improperly folded MinD_{Ng}. To explore this possibility the WT and truncated MinD_{Ng} proteins were analyzed by circular dichroism (CD). CD spectra for MinD_{Ng} are shown in Figure 3.6, demonstrating the presence of a significant proportion of alpha helical content, as characterized by the absorbance minima at 222nm and 208 nm. This is as expected from the MinD structure from *P. horikoshii*, which consists of a β sheet with 7 parallel and 1 antiparallel strands and 11 peripheral α helices (Sakai et al., 2001). More significantly, the CD spectrum of MinD_{Ng-13aaCT} was virtually identical to that of WT MinD_{Ng} providing strong evidence that removal of the C-terminal 13 amino acids did not significantly change the structure of the protein.

Although the structure of the mutant did not seem to be affected, it was possible that the stability of the protein may be compromised by the truncation. To investigate this possibility, we conducted a thermal denaturation experiments for both WT and mutant MinD_{Ng} and monitored unfolding by measuring the CD signal at 222 nm. (Mergny & Lacroix, 2003). Figure 3.7 shows resulting thermal denaturation curves for both proteins, both of which showed a large loss in secondary structure that started around 45°C and reached a plateau at ~60°C. The temperature at which unfolding is 50% complete, also known as the transition temperature, is approximately 55°C for both samples.

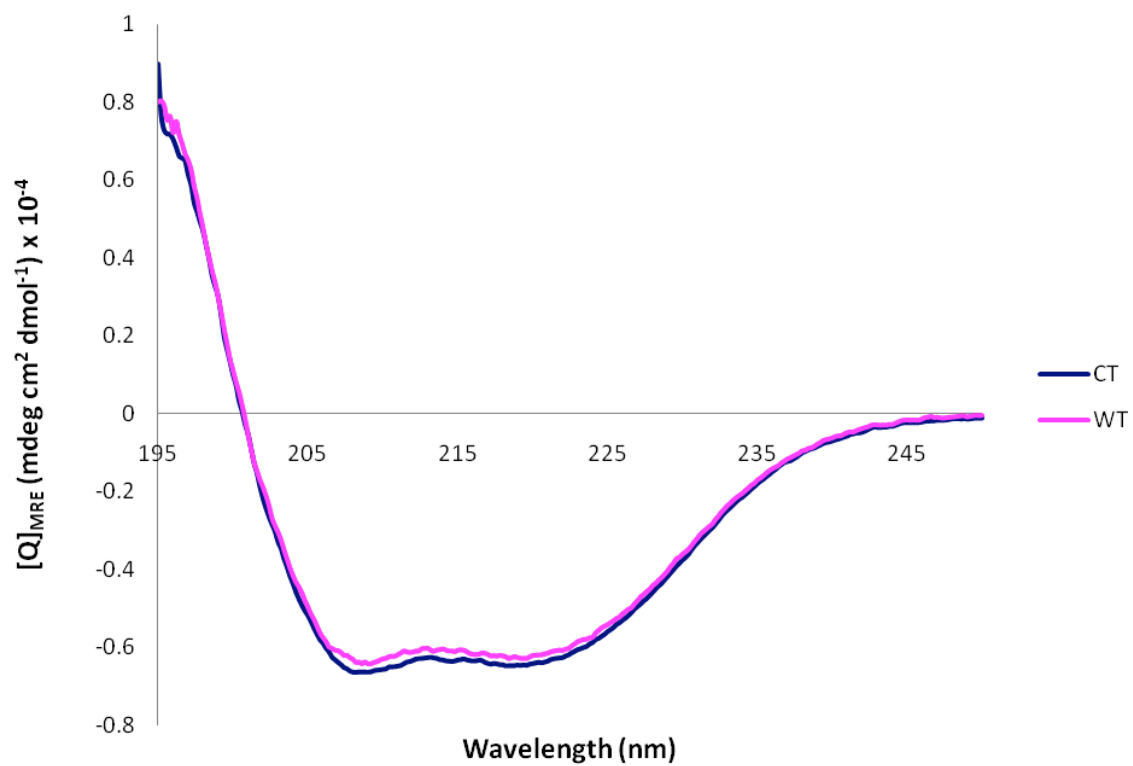


Figure 3.6: CD spectra of WT MinD_{Ng} (pink) and mutant $\text{MinD}_{Ng-13aaCT}$ (blue) in 10 mM Tris, 20 mM NaCl at pH 8.5.

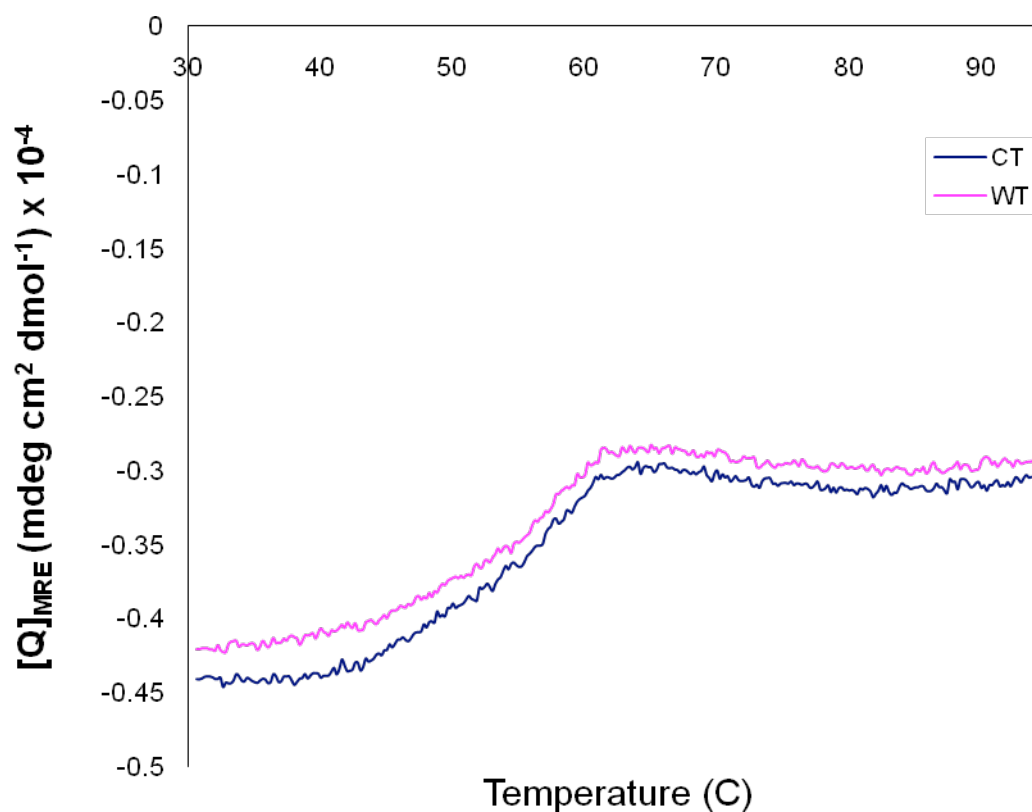


Figure 3.7: Thermal denaturation curves of WT and CT mutant MinD_{Ng} to evaluate the effect of the C-terminal truncation on protein stability. The ellipticity at 222 nm was monitored as the temperature of the sample was increased by 2 °C per minute from 30 °C to 95 °C. Sample conditions were identical to those described in the previous figure.

The sigmoidal shape of these curves is characteristic of thermal denaturation curves of stable proteins, which tend to fold and unfold in a cooperative manner. The fact that the mutant shows unfolding behavior that is virtually indistinguishable from the WT protein indicates that the C-terminus of MinD_{Ng} does not play an important structural role. These experiments rule out the possibility that the inability of MinD_{Ng13aaCT} to dimerize arose from a dramatic change in the structure or stability of the protein.

ATPase activity of purified wildtype MinD_{Ng} protein

To determine whether the C-terminus of MinD_{Ng} was required for its ATPase activity, ATP hydrolysis rates were measured by monitoring the release of inorganic phosphate over time in the presence of MinD_{Ng}, phospholipids, ATP, MgCl₂. Since previous studies have established that the C-terminal His-tagged MinD_{Ng} is still capable of inhibiting cell division in *E. coli* cells the C-terminal hexahistidine tag was not removed for these experiments (J. Szeto et al., 2001). As shown in Figure 3.8, wildtype MinD_{Ng} gave rise to a rapid increase in phosphate concentrations in the presence of MinE_{Ng} that was not present in the absence of phospholipids, ATP, MinE and/or MinD_{Ng}.

For each of these assays, the specific activity of wildtype MinD_{Ng} and MinD_{Ng-13aaCT}, was calculated and averages determined for duplicate runs (Figure 3.9). Similar to previous observations, MinE_{Ng} stimulates wildtype MinD_{Ng} ATPase activity by about 4-fold. In the case of MinD_{Ng-13aaCT}, ATPase activity was not significantly higher than that of the negative control with 'no MinD' suggesting that the removal of the C-terminus leads to a catalytically inactive protein.

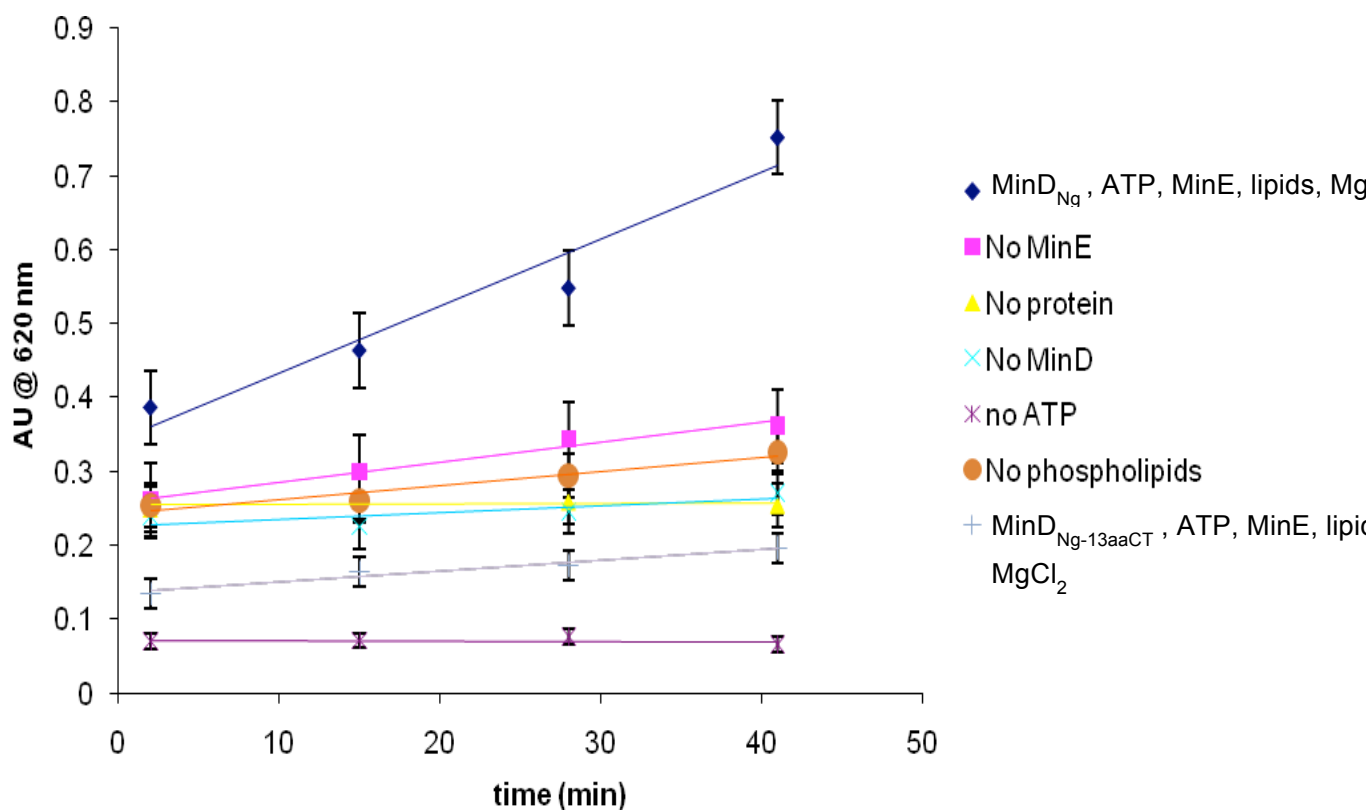


Figure 3.8: ATP hydrolysis monitored with the malachite green assay. Each series shows the malachite green-phosphomolebodium complex absorbance at 620 nm reflecting the amount of inorganic phosphate present at each time point. Each reaction contained 3 μ M wildtype MinD_{Ng} with 1 mM ATP, 0.5 mg/ml phospholipid vesicles, 2 mM MgCl₂ and 10 μ M MinE or with all these reagents except for the reagent indicated in the legend. The slopes of the corresponding lines obtained by linear regression provide the rate of ATP hydrolysis.

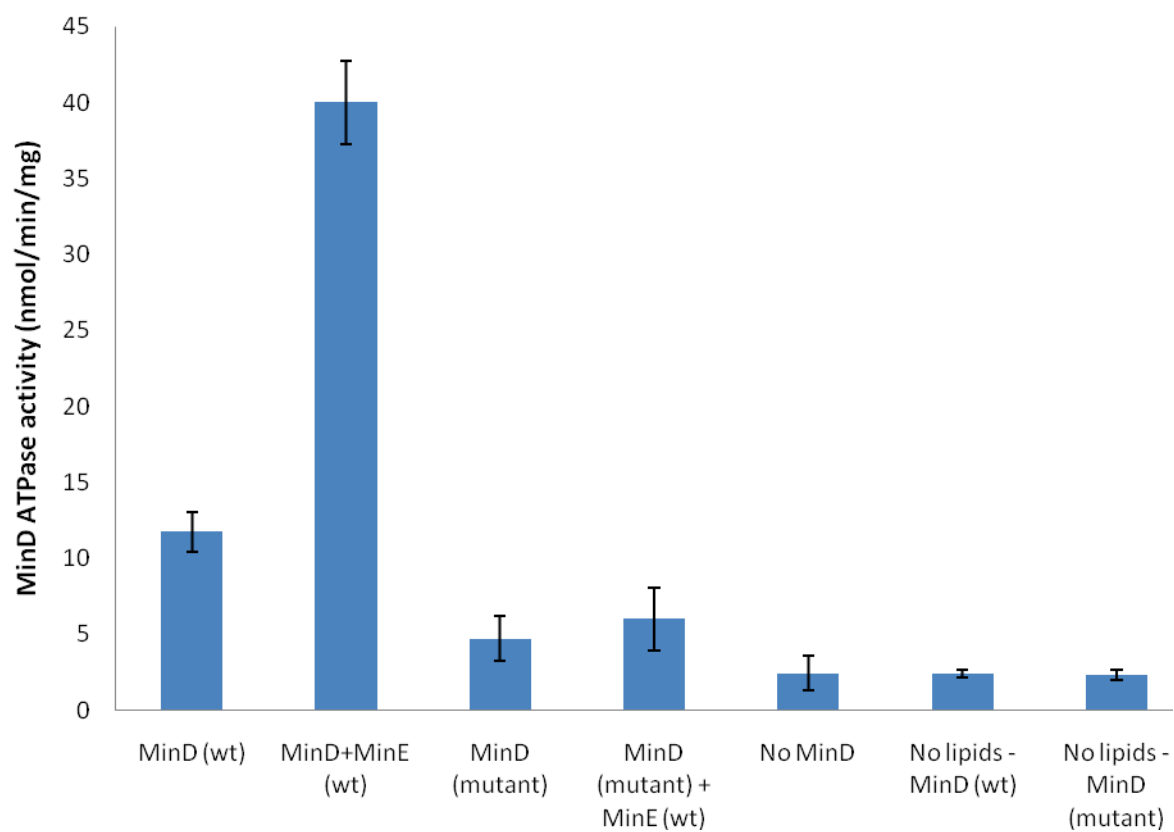


Figure 3.9: Specific ATPase activities for WT and CT mutant MinD_{Ng} in the presence and absence of MinE. Bars represent the average of duplicate trials.

Investigation of cooperativity in MinD_{Ng} ATPase activity

In order to gain some insight into the activation of MinD_{Ng} by MinE, we decided to use this same ATPase assay to investigate the cooperativity of MinD_{Ng} activity by measuring reaction rates over a range of magnesium concentrations to allow a Hill plot analysis. Specifically, a monomeric enzyme with a functionally independent active site is expected to produce a hyperbolic plot of initial rate versus Mg²⁺ concentration, while an enzyme complex with multiple active sites that are functionally linked would be expected to produce a sigmoidal curve in this analysis. Since MinD_{Ng} undergoes ATP-dependent dimerization, and Mg²⁺ is required for ATP binding to occur, wild-type MinD_{Ng} should show cooperativity with respect to Mg²⁺.

As show in Figure 3.10, when the initial rate of ATP hydrolysis was plotted as a function of Mg²⁺ concentration for WT MinD_{Ng} in the presence of MinE_{Ng}, the data followed a trend consistent with significant functional cooperativity. The extent of cooperativity was determined by fitting this data to the Hill equation, which was found to give a hill coefficient of 3. In addition, significant cooperativity was observed if the same experiment was performed in the absence of MinE_{Ng}. Meanwhile, the maximal activity of MinD_{Ng} in the absence of MinE_{Ng} remained low compared to maximal activity obtained in the presence of MinE_{Ng}, while the Mg²⁺ concentration required for half-maximal activity ($K_{0.5}$) was not significantly affected. Together these data show that positive cooperativity is an intrinsic feature of MinD_{Ng} activity, indicating that multiple active sites must be functionally linked both in the presence and absence of MinE_{Ng}.

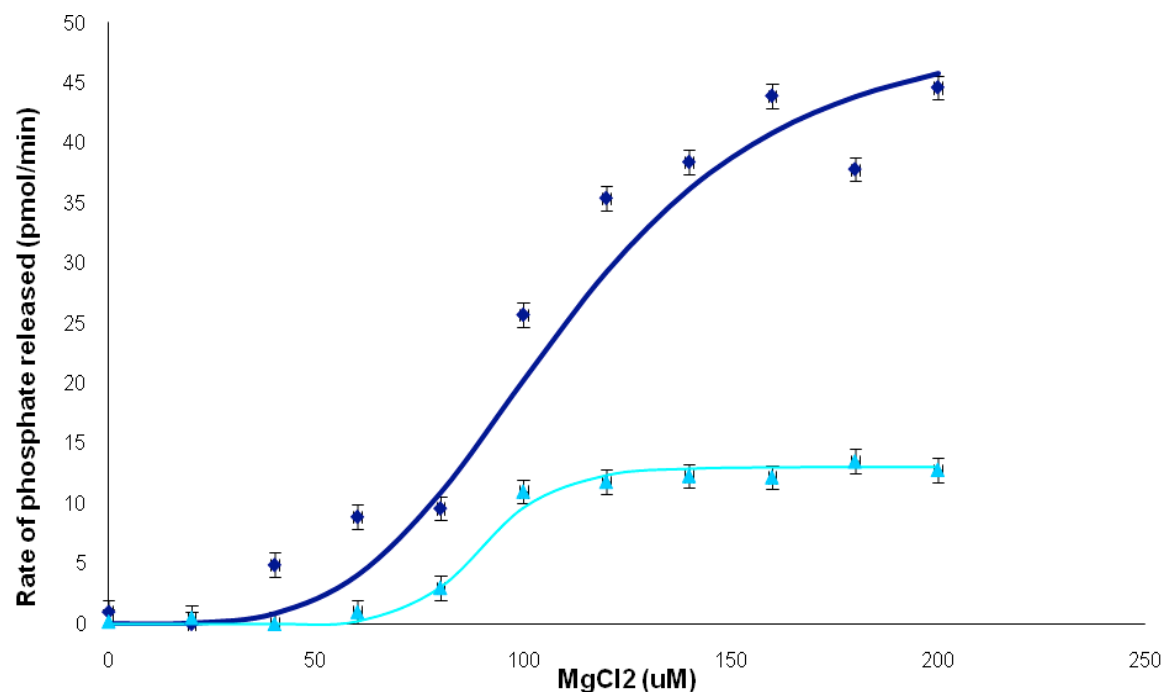
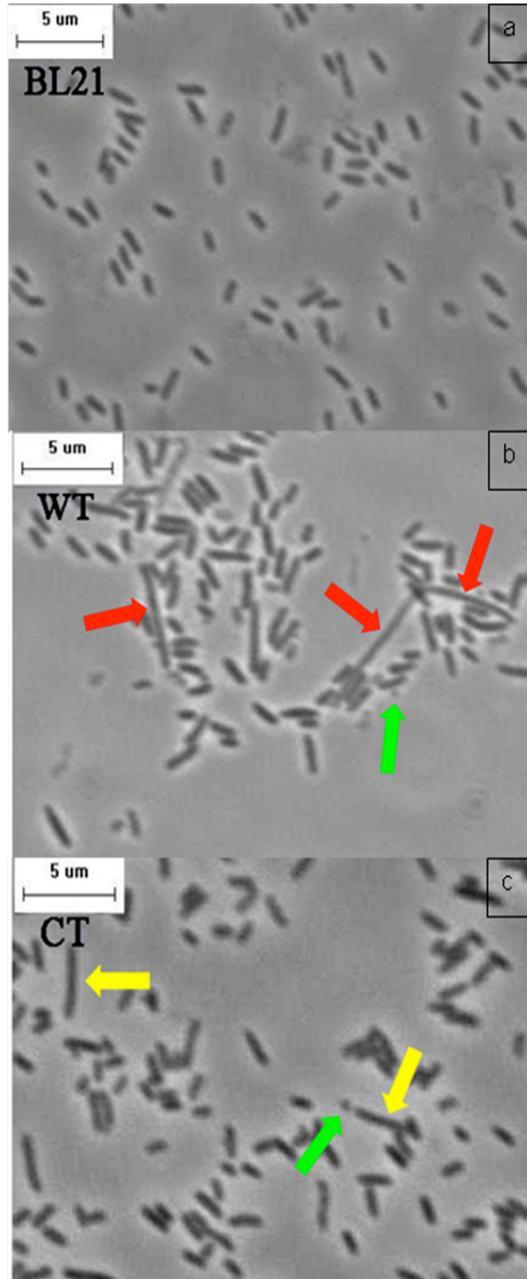


Figure 3.10: Probing Mg^{2+} dependence on ATP hydrolysis rates measured in pmol/min in the presence (Dark blue) and absence (Light blue) of MinE. Line shown here is the best fit to the data points as calculated using the Hill equation with a Hill co-efficient of 3 in the presence of MinE and 10 in the absence of MinE.

Phenotype of MinD_{Ng} wildtype and MinD_{Ng-13aaCT} mutant protein expression

In order to assess the in vivo functionality of wildtype and mutant MinD_{Ng}, the morphology of *E. coli* cells overexpressing wild type or mutant MinD_{Ng} was examined using phase contrast microscopy. Previous studies have established that gonococcal MinD is active in *E. coli* cells and can be used to indicate MinD_{Ng} functionality (J. Szeto et al., 2001). Figure 3.11A (a) shows the negative control of untransformed BL21 cells that exhibited a normal *E. coli* short rod morphology with most cells being ~1 micron in length as expected (Akerlund et al., 1993). On the other hand, over-expression of the wild-type MinD_{Ng} resulted in cells with a filamentous morphology (red arrows) (Figure 3.11A (b)), indicative of cell division inhibition at potential division sites, and a minicell morphology (green arrow), indicative of cell division at cell poles. These results confirm previous observations that over-expression of wild-type MinD_{Ng} in *E. coli* leads to a disruption of symmetric cell division at midcell. However, when MinD_{Ng-13aaCT} was over-expressed, cells with relatively shorter filamentous morphology (yellow arrows) compared to those for wildtype MinD_{Ng} were observed (Figure 3.11A (c)), in addition to some minicell formation (green). Interestingly, the effect of long filamentous cells and minicells formation is more emphasized in wildtype MinD_{Ng} than MinD_{Ng-13aaCT} (Figure 3.11B). In addition, the ratio of cells in the range of 1.1-1.5 μM is higher for MinD_{Ng-13aaCT} than for the wildtype MinD_{Ng} and the opposite is true for cell length >1.6 μM (Figure 3.11B). Overall, the range of morphologies caused by the expression of both wild-type and mutant MinD_{Ng} is characteristic of the disruption of cell division inhibition.

A



B

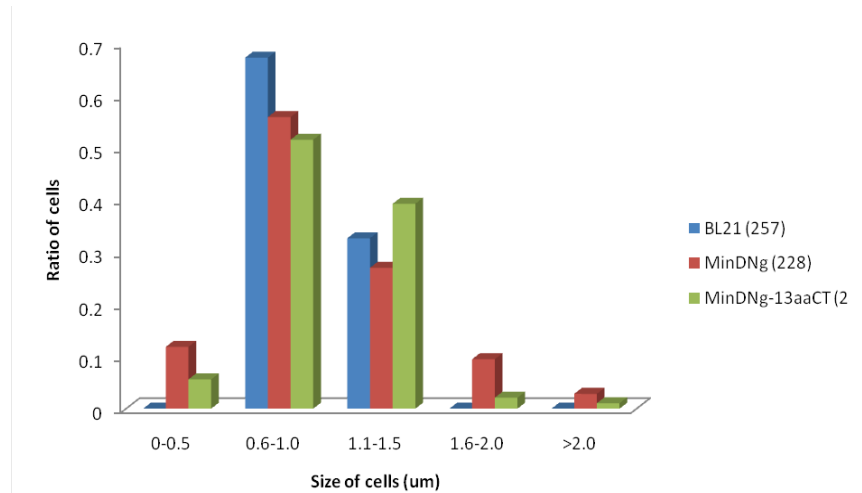


Figure 3.11: Effects of over-expression of wild-type, and MinD_{Ng}-13aaCT in *E. coli* BL21 (DE3) cells. (A) Phase contrast microscopy of *E. coli* BL21(DE3) cells (a) with no plasmid showing normal rod-shaped morphology, or (b) over-expressing wild-type MinD_{Ng} showing filaments (red arrows) and minicells (green arrow), or (c) mutant MinD_{Ng}-13aaCT showing relatively short filaments (yellow arrows) and minicells (green arrow). (B) Histogram of cells represented in the slides showing an average length of ~1 μm (any length longer than 1 μm would be considered a filament). Total number of cells used to calculate ratio is stated in the legend in parentheses. Wildtype MinD_{Ng} cells also demonstrate filaments that are roughly two times longer than those found in MinD_{Ng}-13aaCT.

Discussion

Role of the MTS in dimerization

Many studies have demonstrated the structural and functional importance of the N-terminus of MinD (J. Szeto et al., 2004); however, the same breadth of knowledge has not been established for its C-terminus. It has been suggested that the conserved C-terminal motif on MinD forms an amphipathic helix to serve as a Membrane Targeting Sequence (MTS) responsible for tethering MinD to the membrane (T. H. Szeto et al., 2003). However, only the MinD dimer is able to make direct interactions with the membrane, raising the question as to why dimerization is required for this binding event to occur. This was one of the questions that led us to study the C-terminus in more depth by performing structural and functional experiments on a 13 amino acid C-terminal truncated mutant of MinD (MinD_{Ng-13aaCT}). This mutant had been constructed in our lab by a previous graduate student who was evaluating its potential for high resolution NMR structural studies of MinD_{Ng}, since removal of the MTS gave rise to more favorable solubility properties (T. H. Szeto et al., 2003). Our size-exclusion chromatography experiments that were conducted with MinD_{Ng-13aaCT} indicated that this mutant does not undergo ATP dependent dimerization and remains as a monomeric species, even in the presence of ATP. This is in contrast to what we observed for wildtype MinD_{Ng}, which undergoes ATP dependent dimerization as expected based on previous studies that have been conducted on *E.coli* MinD (MinD_{Ec}) (Lackner et al., 2003).

In our experiments with MinD_{Ng} ATPase activity, we see that wildtype MinD_{Ng} has ATPase activity that seems to increase about four- fold upon stimulation from MinE. Other MinD homologues also show increased activities after binding to a partner protein; e.g. AcsF from *Clostridium thermoaceticum*, NifH from *A. vinelandii*, and HypB from *E. coli* and *Bradyrhizobium japonicum*, and recently, CooC1 from *Carboxydotherrmus hydrogenoformans*

,with HypB and CooC1 also undergoing nucleotide-dependent dimerization (Jeoung et al., 2009; Leonard et al., 2005; Loke & Lindahl, 2003; Schindelin et al., 1997). This is in contrast to what we observe for MinD_{Ng-13aaCT} where the activity is not significantly elevated from what we observe for our negative controls (Figure 3.9). In addition, it does not seem to be stimulated by MinE, suggesting that MinD_{Ng-13aaCT} is a catalytically inactive protein.

The absence of activity in the C-terminal truncation mutant can most easily be explained by its inability to form the ATP-dependent dimer that is required for ATP hydrolysis. Once MinD forms a dimer, MinE is able to bind to it and can stimulate its ATPase activity via a mechanism that is not yet known. Secondary structure deconvolution of CD spectra on MinD_{Ng-13aaCT} using DichroWeb suggests that this mutant has the structure characteristics expected for this protein. Specifically, MinD_{Ng-13aaCT} is predicted from its CD spectrum to contain 30% helical, 12% β -sheet and 57% random coils, which is similar to the expected 40% helical, 18% β -sheet and 42% random coil content in the x-ray structure of MinD from *P. horikoshii* (Sakai et al., 2001; Whitmore & Wallace, 2004). The thermal denaturation profile of the mutant MinD (Figure 3.7) showed a single cooperative thermal transition, which is also consistent with a stable folded protein.

Our size exclusion chromatography, CD spectra and thermal denaturation profiles together provide excellent evidence that MinD_{Ng-13aaCT} forms a stable and properly folded monomeric structure. However, our dimerization results stand in contrast with previous studies on MinD_{Ec-3aaCT} and MinD_{Ec-10aaCT} using size exclusion chromatography which detected a dimer for these mutants in the presence of ATP. Unfortunately, quantitative of ATP-binding and dimerization affinity constants were not performed in those experiments and consequently the available data on these mutants does not help to determine if there was any decrease in the

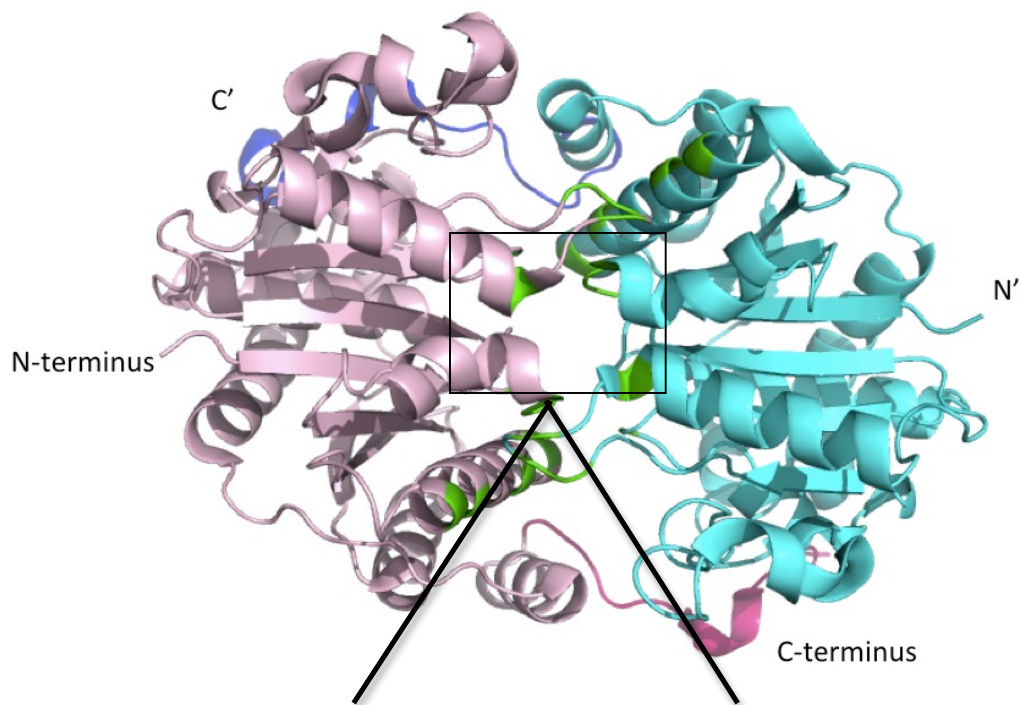
affinity of the interaction caused by the truncations (Hu & Lutkenhaus, 2003). Interestingly, they also established that the ATPase activity of these mutants was not fully stimulated by MinE (2-3 fold stimulation versus 10-fold stimulation for the wild-type protein (Hu & Lutkenhaus, 2001)). Based on their results they concluded that the C-terminal 10 amino acids are not required for dimerization or binding to MinC but do affect the ability of MinD to bind to the membrane. Given the high conservation of the MTS between MinD_{Ec} and MinD_{Ng} (Figure 4.1), it is possible that these results obtained from MinD_{Ec} mutants can be extrapolated to MinD_{Ng} mutants, with an additive effect on MinD ATPase activity for each amino acid that is taken from the C-terminus. This could help to explain why the removal of an additional 3 amino acids to make our 13-amino acid truncation mutant completely abrogated the ATPase activity of MinD.

Modeling the MinD dimer on the NifH and Soj structure

In order to gain insight into how removal of C-terminal amino acids might affect dimerization, it can be helpful to consider models for the dimeric structure of MinD. One view of the interaction can be derived from the crystal structure of dimeric NifH from *Azobacter vinelandii*, which was solved in 1992 and revealed a structurally homologous fold to that of MinD (Hu et al., 2003). However, while MinD_{Ng} and NifH share 16% sequence identity, the NifH sequence is longer on the C-terminal side.

As seen from sequence alignments (Figure 4.1), NifH contains two additional helices at the C-terminus compared to the sequences of MinD. These helices are highlighted in Figure 4.2, along with a number of primarily polar residues that mediate the interactions largely through hydrogen bonding and salt bridge interactions.

A



B

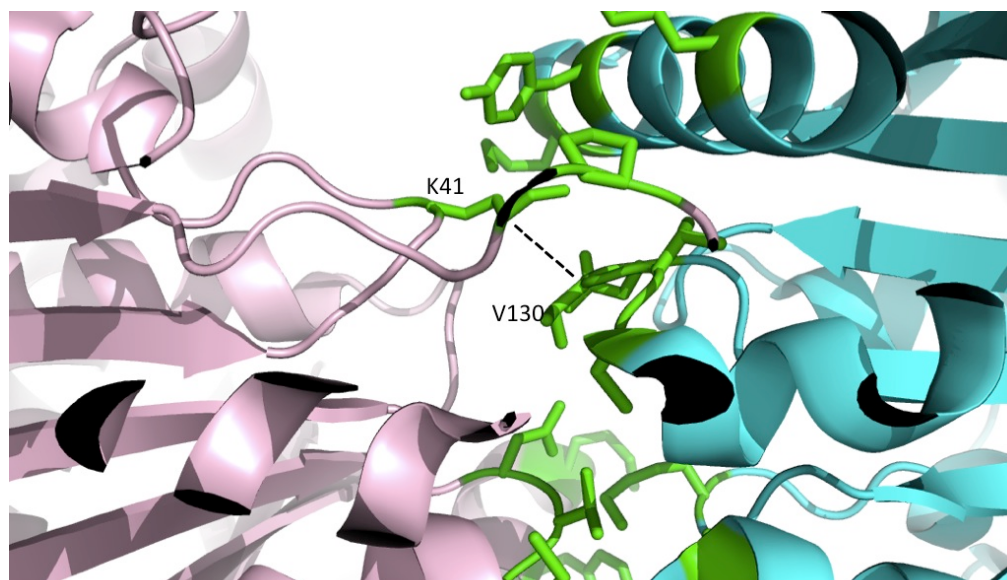
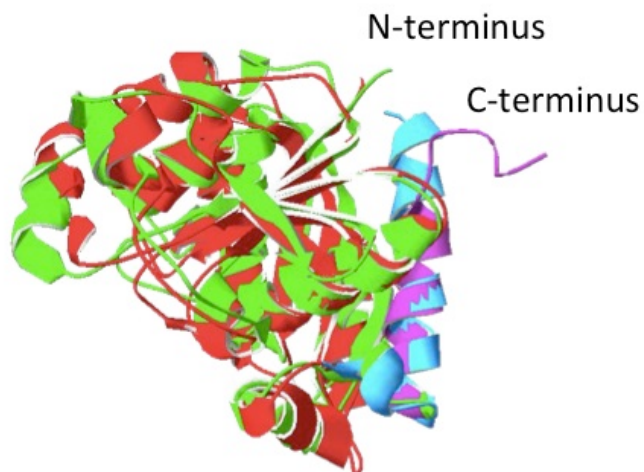


Figure 4.2: Residues of NifH involved in dimerization are highlighted in green color for the two different subunits (PDB # 1G1M). (A) These include K41, E92, P93, V95, A98, D129, V130, V131, C132, M156, Y159, K166 and K170. The C-terminus of the cyan subunit (highlighted in dark blue) and the light pink subunit (highlighted in dark pink) show their proximity to the dimeric interface. (B) Expanded view of the inter-subunit interactions involving residues important for dimerization. The dotted line shows a potential hydrogen bond or salt bridge formation.

Closer examination of the C-terminal region of the NifH structures shows that it forms a helix in the dimer interface (Figure 4.2). This has been speculated to provide an additional degree of intersubunit interaction in NifH as residues from this region wrap around the body of the opposing subunit and enhance the overall stabilization of the NifH dimer (Lahiri et al., 2008). In contrast, the C-terminus of archaeal MinD is unstructured, and highly dynamic, with no electron density for the C-terminal region being observed (Cordell & Lowe, 2001). Although NifH and MinD_{Ng} share 15.3% sequence identity and 52% similarity (Lahiri et al., 2008), the absence of the extra C-terminal helices makes it difficult to construct parallels with the role of the C-terminus in MinD versus NifH dimerization.

A more relevant model for the MinD dimerization may be provided by Soj, which is more closely related to MinD than NifH. Soj and archaeal MinD sequence alignments (Figure 4.3) show 22.5% sequence identity. As shown in Figure 4.4A in a comparison of monomeric *P. horikoshii* MinD and Soj structures, the two proteins are highly similar in structure, with a C α rmsd of 3.77Å for 226 equivalent residues.

A



B

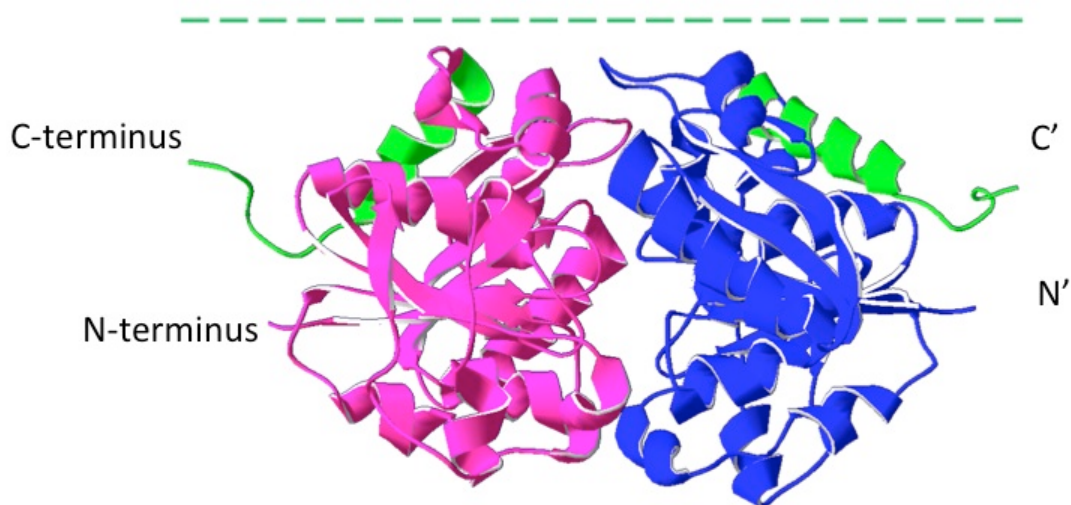


Figure 4.4: Model of MinD dimer based on Soj dimer. (A) Superimposition of MinD (green) from *Pyrococcus horikoshii* and Soj monomer (red) shows the high similarity between the two structures. The C-terminus of MinD is highlighted in purple and Soj is in blue. (B) Model of the MinD dimer made by superimposing the MinD monomer structure onto each subunit (blue and pink) of the Soj dimer. The C-terminus of MinD is shown in green. The potential location of the lipid bilayer is provided by the dotted line to illustrate how dimeric MinD_{Ng} might associate with the membrane.

In order to generate an approximate model of the MinD dimer the MinD_{Ph} structure was superimposed on each subunit in the Soj dimer structure (Figure 4.4B). As shown in this model, there is no clear role for the C-terminal residues of MinD in dimerization. The C-terminus does project away from the structure, providing a view into how MinD might dock to the membrane surface. However, these termini do not straightforwardly localize to a single plane on the surface of the dimer. A significant part of the C-termini are not included in the MinD structures though, and it is possible that these flexible regions of MinD may be able to access the membrane and adopt the amphipathic α - helices proposed by previous researchers (T. H. Szeto et al., 2003).

Overall, these and other types of models can provide only rudimentary insights into the actual structure of the MinD dimer, making it difficult to determine how a 13 amino acid C-terminal truncation could alter ATP-binding and/or MinD dimerization. It is hoped that high-resolution structural data on a MinD dimer will be available in the future to help resolve this issue.

Role of the MTS in membrane localization and cell morphology

When we over-expressed wild-type MinD_{Ng} and MinD_{Ng-13aaCT} in *E.coli* cells, some minicells were observed with wildtype MinD_{Ng}, which is indicative of asymmetric cell division as seen in similar experiments (J. Szeto et al., 2001). In the case MinD_{Ng-13aaCT}, most cells exhibited normal short rod morphology similar to the negative control, although a few filaments and minicells were also observed. It was interesting to note that the filaments were generally 2 times shorter than the filaments obtained when wild type MinD_{Ng} was over-expressed (Table 2). This could suggest that the MinD_{Ng-13aaCT} interacts with the endogenous MinD_{Ec} to interfere with its ability to localize to the membrane, but less effectively than the full length MinD_{Ng}. This would have been facilitated by the very high levels of expression that we obtain for the mutant

MinD. However, previous studies have shown that mutant MinD_{Ec-10aaCT} was still able to interact with MinC_{Ec} and MinE_{Ec}, but not wild-type MinD_{Ec} in the two-hybrid system (Hu & Lutkenhaus, 2003). This raises the possibility that over-expressed protein could still compete with MinC_{Ec} and MinE_{Ec} interactions in the cytosol (Hu & Lutkenhaus, 2003; Taghbalout et al., 2006). This would likely reduce the amount of MinC_{Ec} delivered to the membrane to inhibit asymmetric cell division and reduce the amount of MinE_{Ec} to stimulate MinD_{Ng} ATPase and give rise to the phenotype observed in our studies.

Role of the MTS in activation of MinD ATPase activity

Our results with MinD_{Ng-13aaCT} showed that the ATPase activity is lost for the C-terminal truncation mutant and cannot be stimulated by MinE (Figure 3.9). As shown for truncation mutants of MinD_{Ec} this is likely due to a reduced ability to bind to the lipid membrane, a known prerequisite for MinD ATPase activity (Hu & Lutkenhaus, 2001; Hu & Lutkenhaus, 2003). However, it is also possible that the C-terminus contains determinants that help to regulate MinD ATPase activity. This hypothesis is based on the structure of the helix appearing closest to the C-terminus in the x-ray crystal structures (PDB 1ION), with conserved hydrophobic residues flanked by acidic residues that are within van der Waals contact of highly conserved N-terminal acidic residues - a pattern which is present in the *E.coli* and *N. gonorrhoeae* MinD proteins as well. It is possible that acidic amino acids that flank Val233 provide charge that is complementary to the conserved Arg3 since they are oriented towards the N-terminus (Figure 4.5). Deletion of the C-terminal MTS could potentially impact nearby residues, including those around Val233, such that interactions with the N-terminal β -strand may also be altered. In fact it has been shown that MinD_{Ng} proteins lacking the first three N-terminal residues or having the

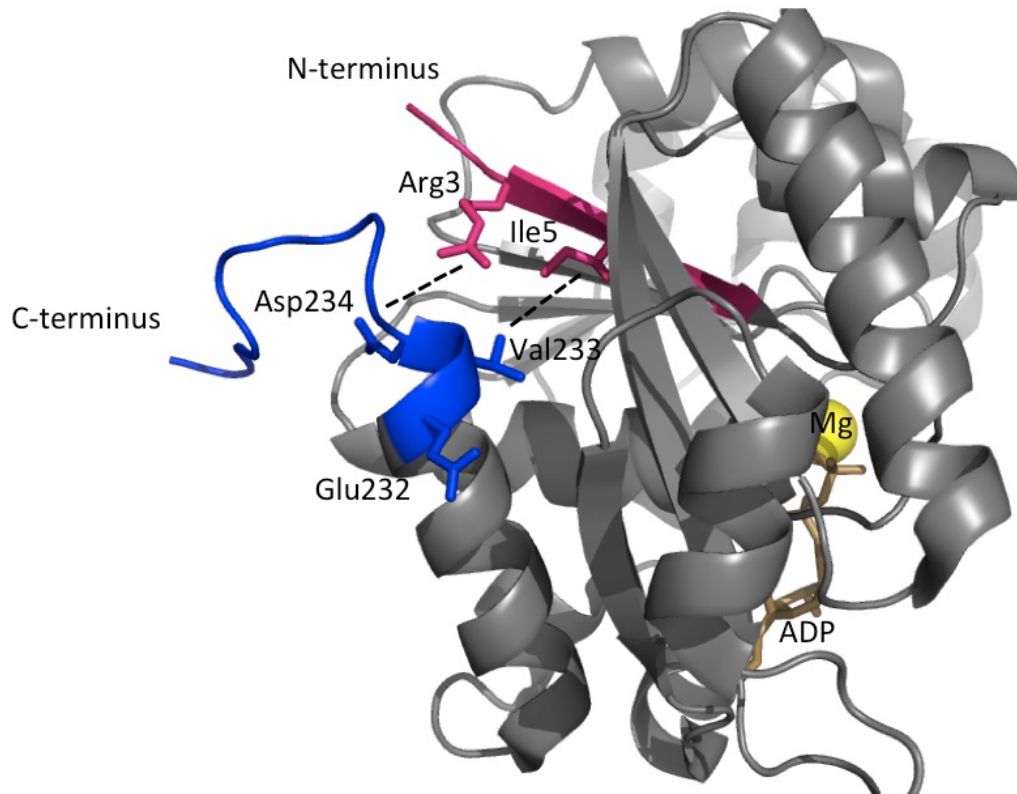


Figure 4.5: Ribbon diagram of MinD from *Pyrococcus horikoshii* (PDB #1ION) demonstrating the proximity of the N-terminus to the C-terminal helix. Archeal structures of MinD contain a conserved patch of hydrophobic residues (specifically Val 233 shown in pink) that could potentially interact with the nearby N-terminus (specifically Arg 3 and Iso 5 also shown in blue) to stabilize the protein fold. Dotted lines represents potential electrostatic interaction.

mutation I5E had increased basal ATPase activity relative to WT that could not be further stimulated by MinE_{Ng} (J. Szeto et al., 2004). While the MTS is not visible in the MinD structures, it is possible that it may interact with the N-terminal residues to stabilize the protein fold (Figure 4.5). In the future this hypothesis could be tested with V233G and E232A mutations to disrupt the interactions between the N- and C-terminal parts of the protein. It would be interesting to see if these mutants were still able to form ATP-dependent dimers, hydrolyze ATP and be stimulated by interactions with MinE_{Ng}. This could allow us to determine if the C-terminus is indeed interacting with the N-terminus and the functional effect of those interactions if they are removed.

Although it is difficult to simply explain how small alterations to the C-terminus could affect its dimerization capacity, there are a few examples of other proteins where even single amino acid mutations in one part of the protein are able to modulate long distance conformational changes. For example, a mutant of T4 lysozyme was recently shown to result in large-scale conformational changes 17–25 Å away from the site of mutation, with these changes being transmitted through the body of the protein (Sagermann et al., 2003).

It is also interesting to note that the C-terminus of the Arabidopsis homologue of MinD (MinD_{At}) also seems to play an important role in chloroplast cell division (Colletti et al., 2000). In particular, it was determined that an Ala to Gly mutant (A296G) results in mislocalization and loss of dimerization capacity (Fujiwara et al., 2004). The mutated Ala 296, is a plant-specific conserved residue predicted to be present in α -helix 11 (secondary elements are based on structural analyses of *P. horikoshii* MinD), which occurs just before the MTS in the amino acid sequence (Figure 4.6).

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gi|MinDEc      YADTVERLLGEE-RPFRFIEEE--KKGFLKRLFGG 270
gi|MinDNg      YKIVTARLLGEN-REMRFLEAE--KKSFFKRLFGG 271
gi|MinDAt      FEQAAMRLVEQDSMKAVMVEEPPKRGFFS-FFGG 326
gi|MinDPh      FIKLAEEVDKLAGIKAKI----- 243
               : . . :

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Figure 4.6: Sequence alignment of the C-terminus of MinD from *Arabidopsis* (MinD_{At}), *E.coli* (MinD_{Ec}), *N. gonorrhoeae* (MinD_{Ng}) and *P.horikoshii* (MinD_{Ph}). Alanine 296, found only in MinD_{At} is shown in green and the corresponding residues in other organisms shown in red. Red bars indicate a helix for both the structure of MinD_{At} and MinD_{Ph} and the red box indicates the aligned residues in other organisms corresponding to A296 in MinD_{At}.

The A296G mutation in MinD_{At} was found to cause aberrant localization inside chloroplasts and was no longer capable of forming homodimers. It is possible that this loss of dimerization capacity is due to a local C-terminal conformational disruption caused by the A296G substitution. This further suggests that the C-terminal region of MinD_{At} could play a role in MinD_{At} dimerization (Fujiwara et al., 2004). Despite this, it is also possible that MinD in plants has acquired new dimerization properties that differ from that of classical prokaryotic MinD (Fujiwara et al., 2004).

Mechanism of MinE-stimulation of MinD activity

Our in vitro ATPase data with wildtype MinD_{Ng} demonstrated its cooperativity with respect to Mg²⁺ concentration. Of particular interest is the apparent difference in Hill coefficients which suggest that there are at least 3 and 10 functionally linked active sites present in the MinD complex in the presence and absence of MinE_{Ng} respectively. This indicates that MinD forms a polymer under these conditions and the degree of polymerization may decrease in the presence of MinE. This high level of cooperativity was somewhat unexpected given previous findings with MinD_{Ec} binding to liposomes, which was found to have a hill coefficient of 2 with respect to the MinD_{Ec} concentration (Mileykovskaya et al., 2003). This suggests that MinD-ATP binds the liposome surface in a dimeric state. However, in our study the impact of magnesium on ATPase activity was being monitored, with cooperativity being measured with respect to Mg²⁺. In fact, we were originally interested in studying the cooperativity of MinD with respect to its substrate ATP, however, there were concerns that background levels of ATP hydrolysis in buffer could interfere with the assay. Consequently, we chose to use magnesium instead, since ATP cannot bind without it. However, this only provides an indirect measure of MinD_{Ng}.

cooperativity with respect to its substrate, and so in the future it will be necessary to examine this more directly.

An interesting result from these in vitro assays was that the Mg^{2+} concentration required for half-maximal activation of $MinD_{Ng}$ ($K_{0.5}$) was not affected by $MinE_{Ng}$. This suggests that $MinE$ binding appears to increase $MinD_{Ng}$ catalytic activity but not its affinity for one of its 'substrates' (Mg^{2+}). In other words, $MinE$ seems to change the properties of $MinD$ such that it can hydrolyze ATP more efficiently potentially by inducing a conformational change in the active site. One possible scenario that could explain $MinE$ stimulation was based on the finding that Asp152 in the well-conserved helix 7 of $MinD_{Ec}$, appears to interact with conserved lysine residues in the Walker A motif, and was also found to be required for its interaction with a $MinE_{Ec}$ (Hayashi et al., 2001; Zhou et al., 2005). Mutation of this residue eliminated $MinE$ stimulation of $MinD$ ATPase activity indicating that this helix may form part of $MinE$ interaction surface or even possibly couple $MinE$ binding to enhanced ATP hydrolysis. It has therefore been suggested that $MinE$ binding can affect the interaction between Asp152 in helix 7 and Walker A motif residues leading to changes in the charge and shape of the active site, providing a possible explanation of $MinE$ stimulated $MinD$ ATPase activity.

Conclusion

In aggregate, our studies on MinD_{Ng} and MinD_{Ng-13aaCT} suggest that the MTS is essential for dimerization, membrane-binding, and ATPase activity. Further study will be required to understand the basis for this loss of activity, particularly given the demonstration that the truncation mutant is stably folded. These insights should ultimately help to determine the role of the C-terminus in modulating MinD dimerization and membrane localization.

In the bigger picture, we can say that these studies have helped us to uncover the role for the C-terminus in MinD dimerization. Further experiments that determine how the C-terminus of MinD can affect oligomerization will be particularly interesting, especially given the critical role that MinD polymer formation plays in the Min protein cycle. In the long run, findings from these studies have the potential to generate a new class of antibiotics that can target MinD oligomerization to disrupt the cell cycle and eradicate the growth of *Neisseria gonorrhoeae*.

References

- Akerlund, T., Nordstrom, K., & Bernander, R. (1993). Branched *Escherichia coli* cells. *Molecular Microbiology*, 10, 849-858.
- Colletti, K. S., Tattersall, E. A., Pyke, K. A., Froelich, J. E., Stokes, K. D., & Osteryoung, K. W. (2000). A homologue of the bacterial cell division site-determining factor MinD mediates placement of the chloroplast division apparatus. *Current Biology*, 10, 507-516.
- Cordell, S. C., & Lowe, J. (2001). Crystal structure of the bacterial cell division regulator MinD. *FEBS Letters*, 492(1-2), 160-165.
- DeBoer, P., Crossley, R., Rothfield, L., (1992) The essential bacterial cell-division protein FtsZ is a GTPase. *Nature*, 359 (6392), 254-256.
- Endrenyi, L., Fajsz, C., & Kwong, F. H. (1975). Evaluation of hill slopes and hill coefficients when the saturation binding or velocity is not known. *European Journal of Biochemistry*, 51, 317-328.
- Errington, J., Daniel, R. A., & Scheffers, D. J. (2003). Cytokinesis in bacteria. *Microbiology and Molecular Biology Reviews*, 67, 52-65.
- Fisher, D. K., & Higgins, T. J. (1994). A sensitive, high-volume, colorimetric assay for protein phosphatases. *Pharmaceutical Research*, 11, 759-763.
- Fujiwara, M. T., Nakamura, A., Itoh, R., Shimada, Y., Yoshida, S., & Moller, S. G. (2004). Chloroplast division site placement requires dimerization of the ARC11/AtMinD1 protein in *Arabidopsis*. *Journal of Cell Science*, 117, 2399-2410.
- Hayashi, I., Oyama, T., & Morikawa, K. (2001). Structural and functional studies of MinD ATPase: Implications for the molecular recognition of the bacterial cell division apparatus. *The EMBO Journal*, 20, 1819-1828.

- Hu, Z., & Lutkenhaus, J. (2003). A conserved sequence at the C-terminus of MinD is required for binding to the membrane and targeting MinC to the septum. *Molecular Microbiology*, 47, 345-355.
- Hu, Z., Mukherjee, A., Pichoff, S., & Lutkenhaus, J. (1999). The MinC component of the division site selection system in *Escherichia coli* interacts with FtsZ to prevent polymerization. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 14819-14824.
- Hu, Z., Saez, C., & Lutkenhaus, J. (2003a). Recruitment of MinC, an inhibitor of Z-ring formation, to the membrane in *Escherichia coli*: Role of MinD and MinE. *Journal of Bacteriology*, 185, 196-203.
- Hu, Z., & Lutkenhaus, J. (2001). Topological regulation of cell division in *E. coli*: spatiotemporal oscillation of MinD requires stimulation of its ATPase by MinE and phospholipid. *Molecular Cell*, 7, 1337-1343.
- Jaffe, A., D'Ari, R., & Hiraga, S. (1988). Minicell-forming mutants of *Escherichia coli*: Production of minicells and anucleate rods. *Journal of Bacteriology*, 170(7), 3094-3101.
- Jeoung, J. H., Giese, T., Grunwald, M., & Dobbek, H. (2009). CooC1 from *Carboxydotherrmus hydrogenoformans* is a nickel-binding ATPase. *Biochemistry*, 48, 11505-11513.
- Johnson, W. C. (1999). Analyzing protein circular dichroism spectra for accurate secondary structures. *Proteins*, 35, 307-312.
- Kintses, B., Gyimesi, M., Pearson, D. S., Geeves, M. A., Zeng, W., Bagshaw, C. R., et al. (2007). Reversible movement of switch 1 loop of myosin determines actin interaction. *The EMBO Journal*, 26, 265-274.

- Koonin, E. V. (1993). A superfamily of ATPases with diverse functions containing either classical or deviant ATP-binding motif. *Journal of Molecular Biology*, 229, 1165-1174.
- Lackner, L. L., Raskin, D. M., & de Boer, P. A. (2003). ATP-dependent interactions between *Escherichia coli* min proteins and the phospholipid membrane in vitro. *Journal of Bacteriology*, 185, 735-749.
- Lahiri, S., Pulakat, L., & Gavini, N. (2008). NifH: Structural and mechanistic similarities with proteins involved in diverse biological processes. *American Journal of Biochemistry and Biotechnology*, 4, 304-316.
- Leonard, T. A., Butler, P. J., & Lowe, J. (2005). Bacterial chromosome segregation: Structure and DNA binding of the Soj dimer--a conserved biological switch. *The EMBO Journal*, 24, 270-282.
- Loke, H. K., & Lindahl, P. A. (2003). Identification and preliminary characterization of AcsF, a putative ni-insertase used in the biosynthesis of acetyl-CoA synthase from *Clostridium thermoaceticum*. *Journal of Inorganic Biochemistry*, 93, 33-40.
- Lutkenhaus, J. (2007). Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z ring. *Annual Review of Biochemistry*, 76, 539-562.
- McGinness, T., & Wake, R. G. (1979). Division septation in the absence of chromosome termination in *Bacillus subtilis*. *Journal of Molecular Biology*, 134(2), 251-264.
- Mergny, J., & Lacroix, L. (2003). Analysis of thermal melting curves. *Oligonucleotides*, 13, 515-537.
- Mileykovskaya, E., Fishov, I., Fu, X., Corbin, B. D., Margolin, W., & Dowhan, W. (2003). Effects of phospholipid composition on MinD-membrane interactions *in vitro* and *in vivo*. *The Journal of Biological Chemistry*, 278, 22193-22198.

- Mulder, E., & Woldringh, C. L. (1989). Actively replicating nucleoids influence positioning of division sites in *Escherichia coli* filaments forming cells lacking DNA. *Journal of Bacteriology*, 171, 4303-4314.
- PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.
- Ramakrishnan, C., Dani, V. S., & Ramasarma, T. (2002). A conformational analysis of walker motif A [GXXXXGKT (S)] in nucleotide-binding and other proteins. *Protein Engineering*, 15, 783-798.
- Raskin, D. M., & de Boer, P. A. (1999a). MinDE-dependent pole-to-pole oscillation of division inhibitor MinC in *Escherichia coli*. *Journal of Bacteriology*, 181, 6419-6424.
- Raskin, D. M., & de Boer, P. A. (1999b). Rapid pole-to-pole oscillation of a protein required for directing division to the middle of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 4971-4976.
- Sagermann, M., Gay, L., & Matthews, B. W. (2003). Long-distance conformational changes in a protein engineered by modulated sequence duplication. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 9191-9195.
- Sakai, N., Yao, M., Itou, H., Watanabe, N., YµMoto, F., Tanokura, M., et al. (2001). The three-dimensional structure of septum site-determining protein MinD from *Pyrococcus horikoshii* OT3 in complex with mg-ADP. *Structure*, 9(9), 817-826.
- Schindelin, H., Kisker, C., Schlessman, J. L., Howard, J. B., & Rees, D. C. (1997). Structure of ADP x AIF4(-)-stabilized nitrogenase complex and its implications for signal transduction. *Nature*, 387, 370-376.

- Sharpe, M. E., & Errington, J. (1998). A fixed distance for separation of newly replicated copies of oriC in *Bacillus subtilis*: Implications for co-ordination of chromosome segregation and cell division. *Molecular Microbiology*, 28, 981-990.
- Shih, Y. L., Le, T., & Rothfield, L. (2003). Division site selection in *Escherichia coli* involves dynamic redistribution of min proteins within coiled structures that extend between the two cell poles. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 7865-7870.
- Szeto, J., Acharya, S., Eng, N. F., & Dillon, J. A. (2004). The N terminus of MinD contains determinants which affect its dynamic localization and enzymatic activity. *Journal of Bacteriology*, 186, 7175-7185.
- Szeto, J., Eng, N. F., Acharya, S., Rigden, M. D., & Dillon, J. A. (2005). A conserved polar region in the cell division site determinant MinD is required for responding to MinE-induced oscillation but not for localization within coiled arrays. *Research in Microbiology*, 156, 17-29.
- Szeto, J., Ramirez-Arcos, S., Raymond, C., Hicks, L. D., Kay, C. M., & Dillon, J. A. (2001). Gonococcal MinD affects cell division in *Neisseria gonorrhoeae* and *Escherichia coli* and exhibits a novel self-interaction. *Journal of Bacteriology*, 183, 6253-6264.
- Szeto, T. H., Rowland, S. L., Habrukowich, C. L., & King, G. F. (2003). The MinD membrane targeting sequence is a transplantable lipid-binding helix. *The Journal of Biological Chemistry*, 278, 40050-40056.
- Szeto, T. H., Rowland, S. L., Rothfield, L. I., & King, G. F. (2002). Membrane localization of MinD is mediated by a C-terminal motif that is conserved across eubacteria, archaea, and

- chloroplasts. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 15693-15698.
- Taghbalout, A., Ma, L., & Rothfield, L. (2006). Role of MinD-membrane association in min protein interactions. *Journal of Bacteriology*, 188, 2993-3001.
- Tapsall, J. (2009). Multidrug-resistant *Neisseria gonorrhoeae*. *Canadian Medical Association Journal*, 180, 268-269.
- Walker, J. E., Saraste, M., Runswick, M. J., & Gay, N. J. (1982). Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *The EMBO Journal*, 1, 945-951.
- Westling-Haggstrom, B., Elmros, T., Normark, S., & Winblad, B. (1977). Growth pattern and cell division in *Neisseria gonorrhoeae*. *Journal of Bacteriology*, 129, 333-342.
- Whitmore, L., & Wallace, B. A. (2008). Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases. *Biopolymers*, 89, 392-400.
- Woldringh, C. L., Mulder, E., Valkenburg, J. A., Wientjes, F. B., Zaritsky, A., & Nanninga, N. (1990). Role of the nucleoid in the toporegulation of division. *Research in Microbiology*, 141, 39-49.
- Yu, X. C., & Margolin, W. (1999). FtsZ ring clusters in min and partition mutants: Role of both the min system and the nucleoid in regulating FtsZ ring localization. *Molecular Microbiology*, 32(2), 315-326.
- Zhou, H., & Lutkenhaus, J. (2004). The switch I and II regions of MinD are required for binding and activating MinC. *Journal of Bacteriology*, 186, 1546-1555.

- Zhou, H., Schulze, R., Cox, S., Saez, C., Hu, Z., & Lutkenhaus, J. (2005). Analysis of MinD mutations reveals residues required for MinE stimulation of the MinD ATPase and residues required for MinC interaction. *Journal of Bacteriology*, 187, 629-638.
- Zhou, T., Radaev, S., Rosen, B. P., & Gatti, D. L. (2000). Structure of the ArsA ATPase: The catalytic subunit of a heavy metal resistance pump. *The EMBO Journal*, 19, 4838-4845.

Claims to original research

- Novel demonstration that the loss of the C-terminus of MinD_{Ng} results in loss of dimerization capacity, even when structurally it remains as a well folded globular protein
- Further confirmation to support that cooperativity is an inherent feature of MinD_{Ng}, while MinD_{Ng-13aaCT} has lost its ATPase activity
- First evidence that polymerization of MinD_{Ng} may be reduced in the presence of MinE_{Ng} and this binding increases MinD_{Ng} catalytic activity but not the affinity for one of its substrate, Mg²⁺
- Initial characterization of MinD_{Ng-13aaCT} *in vivo* to demonstrate that it has retained its ability to interact with other Min proteins

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