Dissecting the role of conformational change and membrane binding by the bacterial cell division regulator MinE in the stimulation of MinD ATPase activity

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The bacterial cell division regulators MinD and MinE, together with the division inhibitor MinC, localize to the membrane in concentrated zones undergoing coordinated pole-to-pole oscillation to help ensure that the cytokinetic division septum forms only at the mid-cell position. This dynamic localization is driven by MinD-catalyzed ATP hydrolysis, stimulated by interactions with MinE’s anti-MinCD domain. This domain is responsible for restriction of the cytokinetic cell division septum at the opposite side of the cell.

The MinE–membrane interactions induce a structural change into a state resembling the open conformation. However, MinE mutants lacking the MinE membrane-targeting sequence stimulated higher ATP hydrolysis rates than the full-length protein, indicating that binding to MinD is sufficient to trigger this conformational transition in MinE. In contrast, conformational change between the open and closed states did not affect stimulation of ATP hydrolysis rates in the absence of membrane binding, although the MinD-binding residue Ile-25 is critical for this conformational transition. We therefore propose an updated model where MinE is brought to the membrane through interactions with MinD. After stimulation of ATP hydrolysis, MinE remains bound to the membrane in a state that does not catalyze additional rounds of ATP hydrolysis. Although the molecular basis for this inhibited state is unknown, previous observations of higher-order MinE self-association may explain this inhibition. Overall, our findings have general implications for Min protein oscillation cycles, including those that regulate cell division in bacterial pathogens.

The Min protein system is composed of MinC, MinD, and MinE, and, together with the nuclear occlusion mechanism, it is responsible for restriction of the cytokinetic cell division septum to the mid-cell region of Gram-negative bacteria (1–3). Min proteins undergo coordinated changes in localization that give rise to dynamic oscillation of concentrated zones of membrane-associated Min proteins from pole to pole (4–9). The timing of this oscillation is critical for proper functioning of the Min system (4), providing a mechanism for the self-regulation of cell length (10). In Escherichia coli, it has been shown that oscillation rates that are too slow can inhibit normal cell division, giving rise to elongated rods (11), whereas oscillation rates that are too fast fail to prevent division at the cell poles (4), leading to the generation of achromosomal minicells that cannot undergo further propagation (12). The chemical engine that drives this coordinated oscillation of Min proteins from pole to pole is powered by MinD-catalyzed hydrolysis of ATP (13, 14), which is stimulated by interactions with MinE once MinD has bound to the cell membrane in a dimeric state (15–19). ATP hydrolysis stimulates the release of Min proteins from the cell membrane (19, 20), freeing them to diffuse through the cytoplasm to establish a new zone that is inhibitory to formation of the cytokinetic septum at the opposite side of the cell.

A striking feature of the series of interactions that give rise to the Min protein oscillation cycle is a dramatic conformational change in the MinE regulator of MinD ATPase activity. In the absence of MinD, MinE forms a dimeric 6-stranded β-sheet supported by an antiparallel pair of α-helices on one side, with the other side being buried by interactions with a shorter N-terminal helix in a nearly perpendicular orientation with respect to the sheet (Fig. 1A) (21). The dimeric interface is defined by an extensive network of backbone and side-chain interactions that comprise the hydrophobic core of the dimer, with the first β-strand (residues 19–31) forming a central part of the dimer. However, biochemical and genetic studies monitoring the effect of mutations in this region provided strong evidence that many residues critical for the MinD interaction are located in this central β-strand, raising questions about how MinD could access these residues (21, 22). These apparent contradictions were reconciled in a subsequent crystal structure determined for MinD in complex with MinE that showed that these residues no longer localized to the central β-strand of the dimer interface but instead were excluded from the sheet, adopting an α-helix that made intimate contacts with a hydrophobic groove formed by the MinD dimer interface (Fig. 1B) (23). In this alternate structure, new intersubunit backbone hydrogen bonds are formed between the β3 strands to create a 4-stranded β-sheet, whereas the interaction between antiparallel helices that form the second part of the dimer interface are preserved. This 4-stranded state had also been previously observed in an early solution NMR structure of a MinE fragment that did not contain the first 30 amino acids (24) (Fig. 1C). Taken
together, these data gave rise to a model where MinE undergoes a structural transition from a 6-stranded state that would be able to diffuse through the cytoplasm to the 4-stranded state that was observed in complex with MinD.

Although this model helps to resolve apparent differences in structure and function observed for residues responsible for interacting with MinD, it is still not known how the conformational change from 6- to 4-β–stranded states occurs in vivo. The 6-stranded state is a stable, high affinity dimer, with amide exchange experiments showing persistent intersubunit hydrogen bonds involving βI backbone amide protons (21, 25). To transition from the 6-stranded (closed) state to the 4-stranded (open) state, an extensive network of interactions that defines the dimer interface must be disrupted, representing an energy barrier to the transition that would be expected to make the process too slow to occur on a biologically useful timescale. Moreover, to capture the MinD-bound state in the crystal structure, it was necessary to introduce a mutation into the central β-strand of MinE (I24N) that would put a polar residue into the hydrophobic core of the 6-stranded structure, reducing its stability in a way that favored the 4-stranded state (23). Because a significant kinetic barrier is expected for this conformational transition, other environmental triggers may be required to stimulate this conformational change in vivo.

In this work, we seek to identify the triggers required to induce this structural change in MinE to and determine how these are linked to its ability to stimulate MinD-catalyzed ATP hydrolysis. Our results show that MinE from Neisseria gonorrhoeae (ngMinE) undergoes a structural transition upon binding membranes composed of E. coli lipids with a change in secondary structure that is consistent with the adoption of a 4-stranded structure. However, an N-terminal truncation mutant lacking the membrane-targeting sequence (ΔMTS) stimulated higher rates of MinD-catalyzed ATP hydrolysis than wild-type ngMinE, demonstrating that membrane binding is not required to stimulate MinD and that the MTS has an inhibitory effect on the rate. Instead, the conformational change required for MinD binding is fast relative to the overall rate of the cycle, because a mutant of ΔMTS in the 4-β–stranded state did not change ATP hydrolysis rates. This conformational change appears to depend on Ile-25, although activity could be restored if the same mutation was introduced into full-length ngMinE, suggesting a role for MinE interactions with both MinD and the membrane in the stimulation of MinD activity. Overall, these data provide new insights into the triggers for conformational change and the role of membrane binding by MinE in the MinD ATPase cycle that underlies oscillation in vivo. This gives rise to a new model where MinE interacts with MinD via an encounter complex that facilitates MinE interactions with the membrane, with MinE dissociation from the membrane comprising the rate-determining step in the ATP hydrolysis–Min protein interaction cycle.

Results

Structural and functional impact of MinE–membrane interactions

MinE from Escherichia coli (ecMinE) is capable of undergoing direct interactions with the membrane via its N-terminal amphipathic helix (23, 26–28), called the MTS. Whereas previous studies have shown that a structural change accompanies membrane binding (23, 27), the nature of this change and its relationship to the 4-stranded MinD-bound conformation have not been determined. Using ngMinE (29), which shares 42% sequence identity with ecMinE (Fig. 1D) and can functionally complement the Min system in E. coli (30, 31), we monitored the structural change induced by membrane interactions using CD spectroscopy. As shown in Fig. 2A, the CD spectrum of ngMinE undergoes a change in shape and amplitude in the presence of small unilamellar vesicles (SUVs) composed of E. coli lipids. Secondary structure content based on spectral deconvolution (32) suggests that this change arises from a loss of β-structure and concomitant increase in α-helix (Table 1). As expected, this lipid-dependent change in the CD spectrum is not observed in an ngMinE N-terminal deletion mutant that is missing the MTS (ΔMTS) (Fig. 2D), which has a CD spectrum that is almost identical to that of the full-length protein (Fig. 2C). The loss of β-structure suggests that ngMinE may adopt a 4-stranded state when bound to the membrane, similar to what was observed in the MinD-bound structure.

Based on the lipid-dependent increase in ellipticity at 208 nm in the ngMinE CD spectrum, it was possible to measure the apparent affinity of the MinE–membrane interaction as the lipid concentration required for half-maximal binding (K_{0.5}^{PL}), which was found to be 0.52 ± 0.04 mM for E. coli lipids (Fig. 2B). In contrast with previous affinity measurements on supported lipid bilayers with mixtures of synthetic lipids (28), ngMinE binding was best fit to a cooperative model, with a Hill coefficient of 1.9 ± 0.1, where binding of one subunit to the membrane enhanced the affinity of the second subunit to bind the membrane. However, in our experiments, the interaction was indirectly monitored through the binding-induced change in conformation, and therefore the cooperativity observed by this method suggests that the structural change induced in ngMinE upon lipid binding by the first MTS is sufficient to induce the conformational change in both subunits of the dimer. Consistent with this idea, the same degree of cooperativity was obtained when SUVs composed of the anionic lipid DOPG were used (Fig. 2B). This cooperativity in the lipid-dependent change in the CD spectrum of ngMinE is consistent with a structural transformation from the native 6-β–stranded state to the 4-β–stranded structure that binds MinD.

To assess the impact of the MinE–membrane interaction on its ability to stimulate MinD-catalyzed ATP hydrolysis, activity profiles were acquired for ngMinE in the presence of full-length or ΔMTS ngMinE. As shown in Fig. 2F, there is a cooperative increase in MinD-catalyzed ATP hydrolysis rates with increasing concentrations of ngMinE that could be fit to the Hill equation as described previously (21) to allow determination of the maximal activity (V_{max}) from which it is possible to calculate k_{cat}, the concentration of ngMinE required for half-maximal
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Figure 1. MinE sequence and structure. A, solution NMR structure of the ngMinE dimer (PDB code 2KXO), with each subunit colored either purple or blue. MinD-binding residues 13–30, also referred to as the anti-MinCD domain, are highlighted with darker colors. B, X-ray structure of ecMinD (gray surface) in complex with residues 12–88 from ecMinE with the mutation I24N (PDB code 3R9J). C, solution NMR structure of the dimer composed of residues 31–88 from ecMinE (PDB code 1EV0). Secondary structure element labels all refer to numbering from the corresponding element in the full-length ngMinE structure. D, alignment of MinE sequences from the three homologues for which high-resolution structural data are available performed in ClustalW (69), shown with a schematic diagram of secondary structure elements for ngMinE (top) and functional domains (bottom) that include the MTS, the anti-MinCD domain, and the topological specificity domain. Residues 24 and 25 are indicated with asterisks above the sequence. Identical residues are highlighted in black, and homologous residues are highlighted in gray.

saturation ($k_{0.5}^{\text{MinE}}$), and Hill coefficient ($h$) (Table 2). This analysis indicates that removal of the N-terminal helix leads to an increase in $V_{\text{max}}$ indicating that the interaction between ngMinE and the membrane actually has an inhibitory effect on the Min reaction cycle. The Hill coefficient is slightly greater than 2 in both cases, suggesting longer-range interactions between MinD dimers that allow ATP hydrolysis at one site to enhance hydrolysis at two or more other sites, even in the absence of the MinE MTS. In addition, $k_{0.5}^{\text{MinE}}$ is higher for ΔMTS, which may be interpreted as a lower apparent affinity for ngMinE binding to ngMinD in the absence of the MTS. Overall, the ability of ΔMTS to promote higher levels of ngMinD activity demonstrates that direct interactions between MinE and the lipid membrane are not required for stimulation of MinD ATPase activity.

MinE 4-stranded β-sheet structure induced by I24D mutation

Whereas it was surprising that the membrane-MinE interaction was not necessary to stimulate MinD activity, we postulated that the 6- to 4-β-stranded conformational transition might also present a barrier to the activation of MinD. To test this hypothesis, the ngMinE residue Ile-24 was mutated to an aspartic acid (I24D) because the introduction of a negatively charged side chain in the hydrophobic core of the 6-stranded state would be expected to destabilize this structure to favor the 4-stranded state, as was observed with the I24N mutant used to capture the open state of ecMinE by X-ray crystallography (23). As shown in Fig. 3A, the CD spectrum of I24D showed significant differences from the WT spectrum, with some similarities in shape to that of the WT spectrum acquired in the presence of phospholipids. Secondary structure deconvolution suggests that this mutant has helix and β-sheet content that is similar to that of the membrane-bound state of WT ngMinE (Table 1), as would be expected for the 4-stranded state. In addition, size exclusion chromatography showed similar elution profiles for WT and I24D samples, confirming that the mutant remains dimeric, as was seen for ecMinE I24N (23).

Although we were interested in pursuing higher-resolution structure determination to confirm the 4-stranded state in I24D, only low concentrations could be maintained in solution, with the exposed hydrophobic surface of the amphipathic MTS helix probably promoting aggregation. To facilitate higher-resolution structural studies of this mutant, the I24D mutation was also introduced into the more soluble ΔMTS variant. As shown in Fig. 3B, CD spectroscopy showed features similar to those of the full-length I24D mutant, with secondary structure deconvolution also indicating a loss of β-sheet content relative to the WT ΔMTS, as expected for the 4-stranded state. The enhanced solubility of this mutant made it possible to acquire a $^1$H-$^{15}$N HSQC spectrum of I24D-ΔMTS and compare it with that of wild-type ΔMTS (Fig. 3C). The dramatic difference between these two spectra indicates a significant structural difference from the 6-stranded state for I24D-ΔMTS. Broadening was observed for many peaks, with some apparently being broadened beyond detection because only about 65% of the expected backbone amide peaks were observed. This suggests the presence of intermediate-timescale dynamics, raising the possibility that this mutant is not stably folded. However, the thermal denaturation of I24D-ΔMTS monitored by CD spectroscopy showed a reversible, cooperative loss of secondary structure with increasing temperature (Fig. 3D), with a melting
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Table 1

| Sample       | Helical  | Sheet    | Random | Turn  |
|--------------|----------|----------|--------|-------|
| WT           | 32 ± 3   | 20 ± 2   | 17 ± 3 | 32 ± 5|
| WT + E. coli | 35 ± 2   | 15 ± 2   | 16 ± 4 | 34 ± 6|
| ΔMNTS        | 30 ± 3   | 21 ± 1   | 20 ± 1 | 29 ± 2|
| I24D         | 35 ± 4   | 11 ± 3   | 23 ± 1 | 27 ± 1|
| I24D-ΔMNTS   | 36 ± 3   | 15 ± 2   | 22 ± 1 | 27 ± 1|
| I25R         | 30 ± 4   | 22 ± 2   | 20 ± 1 | 29 ± 1|
| I25R-ΔMNTS   | 30 ± 3   | 22 ± 2   | 19 ± 1 | 30 ± 1|

Table 2

| MinE   | $K_{cat}$ | $K_{m}$ | Reaction |
|--------|-----------|---------|----------|
| WT     | $1.1 ± 0.1$ | $0.070 ± 0.008$ | 2.7 ± 0.4 |
| ΔMNTS  | $2.0 ± 0.1$ | $0.100 ± 0.009$ | 2.6 ± 0.3 |
| I24D   | $1.97 ± 0.01$ | $0.10 ± 0.003$ | 2.0 ± 0.5 |
| I24D   | $2.0 ± 0.2$ | $0.14 ± 0.002$ | 2.2 ± 0.3 |
| I25R   | $1.5 ± 0.1$ | $0.18 ± 0.004$ | 1.8 ± 0.2 |
| 12–30  | $2.0 ± 0.1$ | $0.28 ± 0.007$ | 3 ± 1 |

Figure 2. MinE-membrane interaction probed by CD spectroscopy and MinE-stimulated MinD ATPase activity. A, CD spectrum of 15 μg ngMinE in the absence (gray) and presence (green) of 2 μg/ml MinD lipids. B, MinE-lipid binding affinities measured by CD. The lipid-bound fraction of 15 μg ngMinE with SUVs made from DOPG (purple) and E. coli lipids (green) as determined by ellipticity measured at 208 nm and fit to the Hill equation. C, CD spectrums of 1 μg WT (gray) and WT-ΔMNTS (black) ngMinE. D, CD spectrum of 12 μg WT-ΔMNTS in the absence (black) and presence (green) of 0.5 μg/ml SUVs made from E. coli lipids. E, phosphate concentration monitored as a function of time for MinD-catalyzed ATP hydrolysis with WT ngMinE (orange) or 0.04 μg (cyan) and 0.2 μg (red) ngMinE. Theslope of each data series corresponds to the initial reaction rate with the indicated amount of ngMinE. F, initial rates of MinE-stimulated MinD-catalyzed ATP hydrolysis as a function of MinE concentration fit to the Hill equation with WT (gray) and ΔMNTS ngMinE (black). Error bars, S.E. for each data point from three independent replicates. Kinetic parameters obtained from all fits are summarized in Table 2.

Figure 3. Structural and functional characterization of I24D in full-length and ΔMNTS ngMinE. A and B, CD spectra of full-length (A) and ΔMNTS (B) ngMinE (red) with either WT (gray and black) or I24D (light and dark blue) sequences. The insets show size exclusion chromatography profiles that confirm a dimeric state for each sample. C, 1H-15N HSQC spectrum of WT (black) or I24D (blue) ΔMNTS ngMinE. D, thermostability of ngMinE monitored by CD for ΔMNTS (black) or I24D-ΔMNTS (blue). E and F, 1H-15N HSQC spectrum of TSD ngMinE (green) superimposed on the I24D ΔMNTS ngMinE spectrum (blue) (E), with thermal denaturation curves (F) as monitored by CD spectroscopy.

Figure 3. Structural and functional characterization of I24D in full-length and ΔMNTS ngMinE. A and B, CD spectra of full-length (A) and ΔMNTS (B) ngMinE (red) with either WT (gray and black) or I24D (light and dark blue) sequences. The inset shows size exclusion chromatography profiles that confirm a dimeric state for each sample. C, 1H-15N HSQC spectrum of WT (black) or I24D (blue) ΔMNTS ngMinE. D, thermostability of ngMinE monitored by CD for ΔMNTS (black) or I24D-ΔMNTS (blue). E and F, 1H-15N HSQC spectrum of TSD ngMinE (green) superimposed on the I24D ΔMNTS ngMinE spectrum (blue) (E), with thermal denaturation curves (F) as monitored by CD spectroscopy.

Whereas the severity of peak broadening precluded the assignment of chemical shifts that would be required for structure determination of I24D ΔMNTS, additional evidence that I24D has adopted the 4-stranded state was also provided by NMR spectra of the topological specificity domain (TSD), composed of residues 31-89 (Fig. 3E), previously shown to be a dimer with a 4-strand β-sheet for the E. coli homologue (Fig. 1C). The overall appearance of the TSD spectrum was highly similar to that of I24D ΔMNTS, with most of the broad peaks being almost superimposable with those of the TSD. The subset of higher intensity peaks in the central region of the spectra that would probably correspond to unstructured regions are missing from the TSD spectrum, as would be expected due to

point ($T_w$) of 85.5 ± 0.5 °C (supplemental Table S2). Although this is lower than the 91 ± 1.5 °C that we also determined for ΔMNTS, these data demonstrate that the structure adopted by the I24D-ΔMNTS mutant does contain a stable globular fold, with only a small decrease in stability from the wild-type sequence. In addition, size-exclusion chromatography profiles obtained for I24D mutants showed elution volumes that confirmed a dimeric state, although with a slightly larger size that would be consistent with a loss of globular structure for anti-MinCD residues no longer part of the dimeric interface (Fig. 3B, inset).
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Figure 4. Stimulation of ngMinD ATPase activity in 4-β-stranded ngMinE. Initial rates of ATP hydrolysis are plotted as a function of ngMinE concentration for full-length (A) and ΔMTS (B) WT (gray and black) and I24D (light blue and dark blue) ngMinE sequences.

the absence of anti-MinCD residues. In addition, reversible thermal denaturation yielded a melting point that was within 2 °C of that determined for I24D-ΔMTS (83 ± 1 °C). These similarities in structure and stability between I24D-ΔMTS and the TSD confirm that the I24D mutation has generated a 4-β-stranded state similar to that seen in the crystal structure of the truncated I24N ecMinE in complex with ecMinD (23).

Role of the 6- to 4-β-stranded conformational change in Min activity

The ability of the I24D mutation to trap ngMinE in the 4-β-stranded conformation provided a useful tool to evaluate the role of conformational change in the stimulation of MinD ATPase activity. This was tested with I24D-ΔMTS, which we confirmed to be unable to bind to membranes by CD spectroscopy (data not shown). Stimulation of ATP hydrolysis rates for this mutant was virtually indistinguishable from that obtained with ΔMTS (Fig. 4B), suggesting that the conformational transition between 6- and 4-stranded states is rapid relative to the rate of the overall cycle. Curiously, when the same mutation was tested for ngMinD activation in the context of full-length ngMinE, the maximal rate was not restored to the lower wild-type levels but was the same as that seen for both the WT and I24D ΔMTS mutants (Fig. 4A). Therefore, the rate-reducing effect of the MTS does not seem to play a role in MinD activation by this 4-β-stranded state.

I25R unmasksthe MTS

A previous study of 15N spin relaxation by solution NMR provided evidence for conformational exchange where a small population of the MTS amphipathic helix dissociates from the β-sheet (21), a model that has been recently supported by hydrogen–deuterium exchange MS experiments (25). This would be expected to facilitate the interaction of the MTS with the membrane by exposing the hydrophobic face that is normally buried by interactions with the β-sheet in the closed structure. In addition, side chains from residues that are required to bind to MinD, including Arg-21 and Ile-25, are also obscured by the MTS in the closed structure, suggesting that the first step in the conformational transition to the open state would involve dissociation of the MTS from the main body of the structure. To establish the functional implications of this conformational equilibrium involving the MTS, an arginine residue was introduced at Ile-25 (I25R), which should disrupt the interaction between MTS and the β-sheet to shift the equilibrium and favor a more open intermediate state that exposes the MTS to the solvent while preserving the 6-stranded structure.

As had previously been observed for the analogous mutant made in ecMinE (22, 23), the solubility of I25R was much lower than that of the WT protein, possibly due to an increase in solvent-exposed hydrophobic surface area caused by MTS dissociation. Although it was necessary to purify this mutant under denaturing conditions and go through a final refolding step to obtain sufficient amounts for characterization, CD spectra acquired on refolded I25R show that its secondary structure content is very similar to that of the WT protein (Fig. 5A and Table 1), consistent with the preservation of the 6-stranded state. The ability of I25R to stimulate ATP hydrolysis by MinD was significantly different from WT MinE, with a ~2-fold increase in the amount of MinE required to reach half-maximal activity (Fig. 5C and Table 2). This reflects the disruptive effect of the arginine in the MinD interaction, because residue 25 comprises a central part of the interface between MinE and MinD. Despite this lower-affinity interaction, it was still possible to achieve a maximal ATPase activity that was larger than the WT maximum.
A critical role for Ile-25 in the stimulation of MinD activity

The fact that I25R did not reach maximum stimulation levels seen for ΔMTS mutants suggests that it should be possible to further increase the activity of this mutant by removing its MTS. As shown in Fig. 5B, the CD spectrum of I25R ΔMTS is virtually superimposable with that of WT ΔMTS confirming minimal structural perturbation from the I25R mutation. However, when the ability of this mutant to stimulate ngMinD-catalyzed ATP hydrolysis was tested, no activity was observed (Fig. 5D). Even when excess I25R ΔMTS was used (i.e. 40-fold higher than the concentration required to obtain maximal stimulation with full-length I25R), no ATP hydrolysis could be detected.

Although the CD data provide strong evidence that the structure of this inactive mutant is the same as that of the WT ΔMTS, given its surprising inability to activate MinD, we sought to characterize its structure at higher resolution using solution NMR. As shown in Fig. 6, the assigned 1H-15N HSQC of ΔMTS (black) and I25R-ΔMTS (red) show that the average amide chemical shift differences between WT and I25R sequences of ΔMTS mapped onto the solution NMR structure. Note that residues comprising the MTS have been removed from the structure for the purpose of clarity. Residues showing average amide chemical shift differences that are greater than the average difference plus 2, 3, and 4 S.D. values from the mean are colored in yellow, orange, and red, respectively. The mutation site (Ile-25) is highlighted in magenta. C, thermostability of ngMinE monitored by CD ellipticity changes at 218 nm measured for ΔMTS (black) and I25R-ΔMTS (red).

Discussion

**MinD binding is sufficient to trigger conformational change in MinE**

The ability of MinE to bind lipid membranes has raised questions about the role of this interaction in the Min protein oscillation cycle. In particular, membrane binding could facilitate the structural transition from the closed 6-β-stranded state to the 4-β-stranded state seen in complex with MinD. Indeed, under conditions that shift the equilibrium toward the membrane-bound state (i.e. at high lipid concentrations), our CD spectra do reveal a structural transition in ngMinE with secondary structure content that is consistent with a 4-stranded state. However, when expressed in *E. coli* in the absence of MinD, WT ecMinE does not localize to the membrane (8, 22, 33, 34), although membrane localization is observed if mutations are introduced that promote exposure of the hydrophobic face of the MTS helix (22, 23). These include mutations that would be expected to destabilize the 6-stranded state (e.g. L22S and I24N) and the I25R mutation that promotes dissociation of the MTS while retaining the 6-stranded state. Therefore, the inherent membrane-binding ability of MinE is not significant until the conformational transition that unMASKS the membrane-binding surface of the MTS has occurred. Because MinE stimulation of MinD activity does not require MinE–membrane interactions, MinE binding to MinD appears to be sufficient to stimulate this structural transition, which would then prime MinE for binding to the membrane.

One of the implications of MinD activation by MinE constructs lacking the MTS is that the binding energy of the MinE–MinD interaction alone appears to be sufficient to stimulate the transition from the 6-β-stranded to 4-β-stranded state. This suggests that there are an adequate number of solvent-exposed residues that can initially bind to MinD to trigger this conformational change. As shown in Fig. 7, residues from MinE that

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**Figure 6. Solution NMR confirms a 6-β-stranded structure for ΔMTS-I25R.** A, assigned 1H-15N HSQC of ΔMTS (black) and I25R-ΔMTS (red). B, average amide chemical shift differences between WT and I25R sequences of ΔMTS mapped onto the solution NMR structure. Note that residues comprising the MTS have been removed from the structure for the purpose of clarity. Residues showing average amide chemical shift differences that are greater than the average difference plus 2, 3, and 4 S.D. values from the mean are colored in yellow, orange, and red, respectively. The mutation site (Ile-25) is highlighted in magenta. C, thermostability of ngMinE monitored by CD ellipticity changes at 218 nm measured for ΔMTS (black) and I25R-ΔMTS (red).
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MTS actually reduces the maximal rate of ATP hydrolysis. To understand the origin of the MTS-mediated reduction in ATPase activity, it is necessary to recall that, as with any steady-state kinetic measurement, the rate of the slowest step in the reaction cycle gives rise to the observed rate, although this may not be the step that generates the read-out of the assay (e.g. the generation of free phosphate). In the case of the Min cycle, there are multiple steps involved in MinE-stimulated ATP hydrolysis by MinD, including nucleotide exchange, protein-protein interactions, and protein-membrane interactions. Our observation that the presence of the ngMinE MTS slows down the maximal hydrolysis rate raises the possibility that membrane dissociation of MinE is the rate-limiting step in the reaction cycle. Although it had previously been suggested that ATP hydrolysis is the slowest step in this cycle (20, 35), this was based on the observation that it is possible to isolate membrane-bound ecMinE and ecMinD when ATP is present. However, the same result would also be expected if Min protein dissociation from the membrane, instead of ATP hydrolysis, is rate-determining. It has also been suggested that the interaction between the MTS and the central MinE β-sheet is inhibitory to MinD binding (25). However, this is not likely to be a rate-determining step in the cycle, because NMR dynamics measurements for ngMinE suggest that conformational exchange is fast relative to the Min interaction/ATPase cycle (i.e. millisecond versus tens of seconds) (21). Moreover, amide solvent deuterium exchange for MinD-binding residues in solvent-accessible regions of ecMinE is close to complete within 15 s (25), suggesting that the MTS-dissociated state was frequently sampled over this time. This suggests that MTS dissociation is at least 1 order of magnitude faster than the ~1-min turnover times measured for ngMinE.

The rate-reducing effect of MinE–membrane interactions on the Min cycle has also been mirrored in fluorescence imaging performed on labeled ecMin proteins on planar lipid bilayers. These studies show concentration gradients of Min proteins that migrate across the surface, with pattern shapes and sizes being determined by a number of factors, including membrane composition, protein concentration, and compartmentalization (26, 36–42). A common feature in many of these dynamic patterns is a concentration gradient of MinD that forms a wave, accompanied by a wave of MinE with maximum intensity that peaks just after the trailing edge of the MinD wave. These fluorescence profiles show that MinE is left behind on the membrane after dissociation of MinD before it undergoes dissociation itself, as would be expected if MinE release from the membrane is rate-determining. In addition, the velocity of wave propagation and the dimensions of the wave are determined in part by the ratio of Min proteins used, with faster propagation being accompanied by a decrease in period, indicating a faster cycle of Min proteins on and off the membrane (26, 36). Because increasing ratios of MinE to MinD also increase ATP hydrolysis rates (13, 21, 43), this suggests that the rate of the ATP hydrolysis cycle determines the scale and dynamics of long-range ordering by Min proteins. It has been shown in similar experiments that the effect of removing the MTS from ecMinE gives rise to faster propagation rates and smaller periods (42), in line with the faster ATP hydrolysis rates.
Membrane binding and conformational change in MinE

An updated version of the Min protein cycle

It is notable that the maximal rates obtained for the more active mutants appeared to hit a ceiling, beyond which further stimulation was not possible. This is highly suggestive of the possibility that the rate-determining step no longer involves the MinE-MinD interaction, but some other step that is independent of MinE for these mutants. This was substantiated by ATPase activity measurements performed on peptide ngMinE 12–30, because they yielded a value for $k_{cat}$ identical to that obtained with ΔMTS (Table 2). One candidate for the new rate-determining step is the dissociation of MinD from the membrane, which could become a limiting factor in the cycle when MinE is no longer able to bind the membrane and potentially undergo higher-order self-association. It has been shown that ecMinD retains membrane-binding affinity when bound to ADP, albeit with a lower affinity than that seen for ATP-bound ecMinD (47). This raises the possibility that loss of key interactions involving the terminal phosphate moiety of ATP (19) upon its hydrolysis could produce a metastable membrane-bound state that is further stabilized by interactions with membrane-bound MinE. Dissociation of the MinE-MinD complex would precede release of MinD from the membrane, with MinE being released at a slower rate. Alternatively, nucleotide exchange of ADP-bound MinD with ATP could instead be rate-limiting, a scenario that would help to prevent rapid reassociation with the membrane and allow its diffusion to the other side of the cell. However, turnover times measured for ATP hydrolysis stimulated by ΔMTS suggest that the lifetime of this state is on the order of ~30 s, which is in better agreement with residence times measured for ecMinD on planar lipid bilayers (38) compared with the 0.3-s residence time measured for ecMinD in the E. coli cytoplasm (48). Whereas measurement of rate constants for these two steps will be required to definitively establish the slower step in the reaction cycle, based on these residence time differences, it seems more likely that MinD dissociation from the membrane comprises the slow step when dissociation of MinE from the membrane is no longer rate-determining.

Our results can be incorporated into an updated version of the Min protein interaction cycle as shown in Fig. 8. MinE in the cytoplasm predominantly exists in the closed 6-stranded structure and transiently samples an open state that would enhance the accessibility of loop residues 13–20. In this state, MinE can form an encounter complex with MinD, which triggers the conformational change to expose the remainder of the MinD-binding helix. This stimulates ATP hydrolysis in MinD, with membrane binding by MinE accompanying this conformational transition, anchoring it in a state that is no longer able to stimulate ATP hydrolysis. Although the nature of this inhibited state is not known, it may reflect self-association of MinE dimers via interactions involving MinD-binding residues. MinD may also remain bound to the membrane after ATP hydrolysis but dissociate from the membrane before MinE, allowing it to diffuse to the other side of the cell, where the membrane binding would not be hindered by the presence of membrane-localized MinE. In this updated cycle, there is no requirement for both MinE-binding sites in MinD to be occupied to stimulate ATP hydrolysis as proposed previ-
Figure 8. Updated model of the Min interaction cycle. ADP-bound MinD (gray surface) forms a dimer when bound to ATP that localizes to the membrane surface (15, 16) (1). As suggested by NMR spin relaxation measurements (21), cytoplasmic MinE (ribbon structure) is in equilibrium between a closed state and MTS-exposed state (2). Our work suggests that the MTS-exposed state forms an encounter complex with MinD (3) that triggers the conformational change into the 4-β-stranded open state that binds the membrane while stimulating ATP hydrolysis by MinD (4). Dissociation of the MinDE complex (5) is followed by MinD dissociation from the membrane (6), whereas MinE can also dissociate from the membrane (7) or remain associated with the membrane (8) in a state that inhibits MinD binding and slows membrane dissociation (as shown in the ATP hydrolysis rate measurements in this work), potentially via the self-association of MinE that has been described previously (27).

N. gonorrhoeae Min proteins reveal conserved properties of the Min cycle

Although much of our understanding on the Min system has been derived from studies with E. coli proteins, we have focused on homologues from the coccus N. gonorrhoeae, a sexually transmitted human pathogen that is gaining resistance to an increasing number of antibiotics (50). Despite the fact that cell division in cocci occurs along alternating perpendicular planes to create a tetrad of daughter cells (51), the ngMin protein system plays a similar role in the maintenance of normal cell division, morphology, and virulence as it does in E. coli (29–31, 52). Moreover, there is strong conservation of function between these two homologues, with ngMin proteins oscillating from pole to pole when expressed in E. coli, either together or with the complementary ecMin protein (31). Overexpression of ngMin proteins in E. coli also induced the same irregularities in morphology as seen with ecMin proteins (29, 30). In addition, MinD-catalyzed MinE-stimulated ATP hydrolysis rates on membranes made from E. coli extracts measured for ngMin proteins in this work are very similar to those measured using ecMin proteins (13). Although the lipid mixture used in our experiments was derived from E. coli, membranes from both species are primarily composed of ~70 mol % phosphatidylethanolamine, with ~20 mol % being the anionic lipid phosphatidylglycerol (53, 54). The main difference is that the cardiolipin fraction is much smaller in N. gonorrhoeae (<1 mol % versus ~5 mol % for E. coli), giving rise to a lower charge density predicted for the membrane surface of N. gonorrhoeae. Nonetheless, pattern formation by ecMin proteins on planar lipid bilayers was found to occur over a range of lipid headgroup compositions (26), suggesting that the E. coli lipids serve as a reasonable substitute for those from N. gonorrhoeae. More importantly, the role of the MinE MTS in the Min cycle, as elucidated from our work on ngMin proteins, should have implications for Min protein cycles in general, including those linked to the pathogenicity of Francisella tularensis (55, 56) and enterohemorrhagick E. coli (57) as well as the diverse range of eukaryotic species that are predicted to use mitochondrial and plastid Min proteins (58, 59).

Experimental procedures

DNA plasmids and constructs

Plasmids for the expression of C-terminal His-tagged versions of ngMinD and ngMinE were as described previously (60, 61). ngMinE point mutations were generated using a Quik Change site-directed mutagenesis kit as described by Qiagen (primer sequences in supplemental Table S1). The AMTS ngMinE construct was made from a ngMinE truncation mutant missing 5 amino acids from the N terminus, also generated using site-directed mutagenesis.

Protein expression and purification

ngMinE and ngMinD proteins were prepared and purified as described previously (60, 61). For the purification of I24D, 1× mixture inhibitor (1 mg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 2 μg/ml pepstatin, 1.2 μg/ml E-64, 1 μg/ml bestatin, 1.6 μg/ml phosphoramidon) was also added to the lysis buffer during the purification to prevent proteolytic degradation. In the case of I25R ngMinE, the protein was found to localize to inclusion bodies, and therefore cell lysis and nickel affinity purification were performed under denaturing conditions. The cell pellet was resuspended in 8 m urea, 50 mM Tris, pH 8.5, 250 mM NaCl, and 10 mM imidazole. The insoluble fraction was removed after centrifugation, and the supernatant was applied to a nickel-nitritroltriacetic acid column. Bound protein was then refolded by a stepwise buffer exchange into denaturant-free buffer (urea concentration of 8, 6, 4, 2, and 0 m). The refolded protein was then eluted and further purified as described previously.
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**SUV preparation**

DOPG or *E. coli* total phospholipid extracts (Avanti Polar Lipids Inc.) in chloroform were dried under an argon stream to produce a thin lipid film. To ensure complete removal of chloroform, lipid films were further dried under vacuum overnight. The lipid film was resuspended in buffer containing 25 mM Tris, pH 8, and 50 mM KCl for a final concentration of 10 mg/ml. The lipid suspension was extruded through a 0.1-µm membrane at room temperature for DOPG or first through a 1-µm membrane and then a 0.1-µm membrane for *E. coli* lipids, both at 52 °C, using an Avanti Mini-Extruder.

**MinD ATPase activity**

2.7 µM ngMinD was combined with 0.5 mg/ml *E. coli* total lipid SUVs in 65 mM Tris, pH 8.2, 80 mM NaCl, 50 mM KCl, 5 mM MgCl₂, and 0.16 mM EDTA with 0–3 µM ngMinE, and 1 mM ATP was added to start the reaction. 40-µl aliquots were removed from the reaction mixture at various time points during the first 30 min of the reaction and immediately immersed in a boiling water bath for 1 min to quench the reaction. Sampling the first 30 min of the reaction and immediately immersed removed from the reaction mixture at various time points during the first 30 min of the reaction and immediately immersed in a boiling water bath for 1 min to quench the reaction. Samples were spun at 16,000 × g for 4 min to separate the reaction mixture from the vesicles. The amount of free phosphate generated was measured using a slightly modified version of the malachite green method (62). Briefly, 15 µl of the supernatant was incubated with 85 µl of 0.035% (w/v) malachite green, 0.6% (w/v) molybdate, 0.15% (v/v) Tween 20 in 0.7 M HCl in a 96-well plate for 15 min at room temperature, and the absorbance at 620 nm was then measured using a Spectramax XS plate reader. Absorbances were converted into free phosphate concentrations using a standard curve generated with potassium phosphate standards made in the same reaction buffer. Linear increases in phosphate concentration were used to calculate initial rates of ATP hydrolysis (ν₀) and plotted as a function of ngMinE concentration ([MinE]). These data were fit to the Hill equation,

\[
\frac{\nu_0}{\nu_{max}} = \frac{[\text{MinE}^h]}{(K_{0.5}^\text{PL})^h + [\text{MinE}^h]} \quad \text{(Eq. 1)}
\]

where \(\nu_{max}\) is the maximal rate of ATP hydrolysis by ngMinD, \(K_{0.5}^\text{PL}\) is the concentration of ngMinE required to reach half-maximal activity, and \(h\) is the Hill coefficient.

**CD spectroscopy**

CD spectra were collected on 10–15 mM samples of ngMinE in 10 mM Tris, pH 8.5, and 130 mM NaCl using a Jasco J-815 CD spectropolarimeter at room temperature with a 0.1-cm quartz cuvette. Spectra were acquired from 200 to 250 nm using eight accumulations at 20 nm/min and a data integration time of 8 s, except for 125R, for which 10 nm/min and 32-s data integration times were used instead. Secondary structure deconvolution of spectra was carried out in CDPro with the CONTIN algorithm and SP43 reference set (63). Errors represent S.E. of at least three independent replicates.

To measure the apparent affinity of MinE-lipid interactions, ~15 µM ngMinE was mixed with increasing concentrations of SUVs, and CD spectra were collected after each increment as described above. The difference between the mean residue ellipticity at 208 nm in the presence of lipids and the absence of lipids (Δθ) was used to calculate the fraction of lipid-bound ngMinE plotted (\(f_0\)), which was a function of phospholipid concentration, and fit to the following,

\[
f_0 = \frac{\Delta \theta}{\Delta \theta_{max}} = \frac{[\text{PL}^h]}{(K_{0.5}^\text{PL})^h + [\text{PL}^h]} \quad \text{(Eq. 2)}
\]

where \(\Delta \theta_{max}\) is maximum difference in molar ellipticity at 208 nm measured for ngMinE in the presence of saturating concentrations of lipids, and \(K_{0.5}^\text{PL}\) is the apparent affinity of ngMinE binding to phospholipids.

**NMR spectroscopy**

NMR spectra were recorded at 298 K on a 600-MHz Bruker AvanceIII spectrometer equipped with a pulsed-field gradient triple-resonance cryoprobe (University of Ottawa NMR Facility). NMR samples were typically 0.5–0.7 mM uniformly 15N- or 15N, 13C-labeled ngMinE in a buffer containing 22.5 mM Tris-HCl at pH 7.2, 45 mM NaCl, 0.1 mM EDTA, 0.2 mM benzamidine, 0.02% NaN₃, and 10% D₂O. 1H, 15N, and 13C backbone chemical shifts were assigned using 15N HSQC, HNCACB, CBCA(CO)NH, and HNCO spectra processed by NMRPipe (65) and analyzed with NMRView (66). Average backbone chemical shift differences between WT and 125R ΔMTS (Δδ) were calculated using the following,

\[
\Delta \delta = \sqrt{\frac{1}{2} (\Delta \delta_{HN}^2 + 0.14 \times \Delta \delta_{N})} \quad \text{(Eq. 3)}
\]

where \(\Delta \delta_{HN}\) and \(\Delta \delta_{N}\) are the chemical shift difference calculated for the amide proton and nitrogen, respectively (67). Secondary shifts from random coil values (68) were calculated for backbone atoms.

**Author contributions**—S. H. A. and N. K. G. designed the study, analyzed the data, and wrote the manuscript. S. H. A. conducted CD and kinetics studies; S. H. A. and A. D. C. performed and analyzed NMR experiments with assistance from A. M. D.; L. J. M. designed, executed, and analyzed membrane affinity measurements; and A. C. Y. F. assisted with experiment design throughout. All authors reviewed the results and approved the final version of the manuscript.

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**References**

1. de Boer, P. A., Crossley, R. E., and Rothfield, L. I. (1988) Isolation and properties of minB, a complex genetic locus involved in correct placement of the division site in *Escherichia coli*. *J. Bacteriol.* 170, 2106–2112
