Prepore Stability Controls Productive Folding of the BAM-independent Multimeric Outer Membrane Secretin PulD*

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Members of a group of multimeric secretion pores that assemble independently of any known membrane-embedded insertase in Gram-negative bacteria fold into a prepore before membrane-insertion occurs. The mechanisms and the energetics that drive the folding of these proteins are poorly understood. Here, equilibrium unfolding and hydrogen/deuteration exchange monitored by mass spectrometry indicated that a loss of 4–5 kJ/mol/protomer in the N3 domain that is peripheral to the membrane-spanning C domain in the dodecameric secretin PulD, the founding member of this class, prevents pore formation by destabilizing the prepore into a poorly structured dodecamer as visualized by electron microscopy. Formation of native PulD-multimers by mixing protomers that differ in N3 domain stability, suggested that the N3 domain forms a thermodynamic seal onto the prepore. This highlights the role of modest free energy changes in the folding of preintegration forms of a hyperstable outer membrane complex and reveals a key driving force for assembly independently of the β-barrel assembly machinery.

Outer membrane proteins (OMPs) allow Gram-negative bacteria to exchange nutrients and macromolecules with their environment. Classically, periplasmic broad-specificity chaperones deliver OMPs to the β-barrel assembly machinery (BAM) that catalyzes OMP OM insertion (1). In contrast, a group of large multimeric OMPs in which all protomers contribute part of their sequence to a single, shared transmembrane pore rely on the lipoprotein outer membrane localization (Lol) system for membrane targeting and on lipid-assisted self-insertion (2). Many OMPs that use this alternative assembly route are secretion portals for virulence factors or for the surface presentation of pili, and thus are potential targets for chemicals that abolish bacterial virulence.

The first OMP shown to assemble independently of BAM was the secretin PulD (3). PulD dodecamerizes to form the OM pore of the Klebsiella oxytoca type II secretion system for the secretion of pullulanase (PulA) (4). Mature full-length PulD (PulD3), comprising amino acids 28 to 660, forms a modular structure (Fig. 1A) with periplasmic flexible arms (N0 to N2) (5), a core structure that is referred to as an inverted cup-and-saucer (N3 in the periplasm and the partly membrane-embedded C domain (Fig. 1B)) (6–8), and a domain S for binding to the fatty acylated pilin PulS (9, 10), which delivers PulD to the OM (11) via the Lol system (12). The PulD core structure is hyperstable and resists denaturation by SDS, urea, and trypsin. The acquisition of these characteristics over time showed that a PulD variant lacking the flexible arms, PulD28–42/259–660 (where the superdomain script denotes the numbers of the included amino acids (Fig. 1A)), forms at least one multimeric intermediate (a prepore) prior to membrane insertion (13). The prepore was isolated by a Thr470 to Ile substitution in the C domain of both PulD28–42/259–660 and PulD3 (14). Prepores are only SDS resistant and have a well structured cup but a collapsed outer chamber (14). Genetic dissection demonstrated that PulD cannot multimerize without N3 (15) and PulD28–42/259–660/1322S, a PulD28–42/259–660 variant carrying an I322S substitution on the N3 surface (Fig. 1B), is multimerization defective (16). These results suggest an important role for N3 as a regulator for membrane insertion. To test this, the role of N3 in PulD folding was investigated here by domain exchange of N3 with the homologous N1 and N2 domains, by the effect of the I322S substitution on the structural, energetic, and dynamic properties of N3 and by the formation of mixed multimers between unaltered PulD protomers and PulD protomers containing modifications that affect at least one folding step. The results provide the first insights into the folding energy landscape of an OMP of such large dimensions.

Results

Evaluation of the Effect of the I322S Substitution on PulD28–42/259–660 Folding—Although PulD28–42/259–660/1322S appears multimerization defective upon in vitro synthesis when...
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The Effect of the I322S Substitution Is Specific for the N-subdomain Adjacent to the C Domain—Introducing the I322S substitution into PulD\textsuperscript{B} resulted in a similar effect as observed for PulD\textsuperscript{B}–28–42/259–660 I322S: PulD\textsuperscript{B}I322S predominantly migrated as a monomer, but the amount of multimers increased when it was solubilized from the liposomes with ZW 3–14 prior to SDS-PAGE (Fig. 2B and Table 1). Although some SDS-resistant PulD\textsuperscript{B}I322S multimer was formed \textit{in vitro} and \textit{in vivo}, PulD\textsuperscript{B}I322S did not support efficient PulA secretion in the presence of all the Pul components \textit{in vivo} (Fig. 3A). To validate that the secretion defect was not because of inefficient OM targeting of PulD\textsuperscript{B}I322S, the phage shock protein (Psp) response was measured by the amount of PspA produced upon PulD\textsuperscript{B}I322S production in the presence or absence of Pus. Typically, PspA levels are high when PulD\textsuperscript{B} is suggested to insert into the inner membrane in the absence of PulS, whereas they low when PulS efficiently delivers PulD\textsuperscript{B} to the OM (11). In the presence of PulS, PulD\textsuperscript{B}I322S elicited a smaller Psp response than PulD\textsuperscript{B}WT, consistent with the smaller amount of PulD\textsuperscript{B}I322S multimers produced when compared with the amount of PulD\textsuperscript{B}WT multimers (Fig. 3B). Importantly, the amount of PspA produced in the presence of PulD\textsuperscript{B}WT or PulD\textsuperscript{B}I322S (but not PulS) was more than halved when either of the PulD\textsuperscript{B} variants was produced in the presence of PulS, suggesting that both PulD\textsuperscript{B} variants were targeted to the OM with equal efficiency (Fig. 3, A and B). Hence, the I322S substitution created a folding defect \textit{in vitro} and \textit{in vivo}. 

Of the three domains that precede N\textsubscript{3} in PulD\textsuperscript{B} (Fig. 1A), N\textsubscript{1} and N\textsubscript{2} are structurally homologous to N\textsubscript{3} (Fig. 2D, (17)), but neither are required for \textit{in vitro} folding and \textit{in vivo} assembly of PulD (18). Consistent with this, creating the homologous substitutions to I322S in N\textsubscript{1} and N\textsubscript{2} by the substitutions L168S and L241S, respectively, resulted into SDS-resistant multimers (Fig. 1C and Table 1). Next, N\textsubscript{3} was replaced by N\textsubscript{1} or N\textsubscript{2} in PulD\textsuperscript{B}–28–42/188–260/342–660 and PulD\textsuperscript{B}–28–42/124–190/342–660, respectively (Fig. 1A), to evaluate the effect of introducing L241S and L168S into these domains while positioned adjacent to C. Only PulD\textsuperscript{B}–28–42/188–260/342–660 formed SDS-resistant multimers, albeit much less than PulD\textsuperscript{B}–28–42/259–660 (Fig. 2C and Table 1). Introducing L241S into PulD\textsuperscript{B}–28–42/188–260/342–660 yielded monomers upon SDS-PAGE, even with ZW 3–14 treatment (Fig. 2C). Thus, it appears that the substitution typified by I322S only induces misfolding when it is present in the N domain that directly precedes C.

Although homologous, replacing N\textsubscript{2} with N\textsubscript{3} and N\textsubscript{1} did not result in efficient PulD folding. A striking difference between the three domains is the length of the L\textsubscript{2}-loop, which is shorter in N\textsubscript{2} than in N\textsubscript{3} and is only a few amino acids in N\textsubscript{1} (Fig. 2D). Deletion of L\textsubscript{2} in N\textsubscript{2} still allowed PulD\textsuperscript{B}–28–42/259–660 \Delta L\textsubscript{2} to
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The secondary structure elements correspond to those found in the model of N3. The N domains were aligned using MAFFT version 7 with the highest gap penalty. The position of Ile322 is highlighted by the asterisk.

Samples were solubilized in SDS-loading buffer (S) in zwittergent 3–14 (ZW) or dissociated in phenol as a total synthesis control (P). Equal amounts of the samples were migrated on the SDS-PAGE. Mo, monomer; Mu, multimer; fl, full-length; tr, 28–42/259–660; WT, unmodified mature PulDfl, unaltered, PulDfl without further modification. Numbers indicate the molecular weight of the markers and Mu<sup>A</sup> and Mu<sup>B</sup> between brackets; D, alignment of homologous N domains in PulD. The secondary structure elements correspond to those found in the model of N3. The N domains were aligned using MAFFT version 7 with the highest gap penalty. The position of Ile<sup>322</sup> is highlighted by the asterisk and the deleted L<sub>2</sub>-loop by the rectangle. The sequence similarity of N<sub>3</sub> with N<sub>1</sub> and N<sub>2</sub> is 36 and 42%, respectively, and sequence identity is 16 and 22%, respectively.

### TABLE 1
Quantification of PulD multimer formed upon in vitro synthesis in the presence of lecithin liposomes

| PulD<sup>Δ</sup> | Without ZW | With ZW |
|---------------|------------|---------|
| Unaltered     | 0.81 ± 0.23| 0.79 ± 0.22|
| I322S         | 0.12 ± 0.12| 0.62 ± 0.28|
| ΔL<sub>2</sub> | 0.93 ± 0.10| 0.94 ± 0.06|
| ΔL<sub>1</sub> | 0.58 ± 0.28| 0.70 ± 0.22|
| WT            | 0.83 ± 0.06| 0.89 ± 0.05|
| I322S         | 0.14 ± 0.10| 0.48 ± 0.08|
| L168S         | 0.77 ± 0.15| 0.82 ± 0.10|
| L2I322S       | 0.41 ± 0.14| 0.34 ± 0.15|
| L2             | 0.10 ± 0.05| 0.82 ± 0.10|

### TABLE 2
Quantification of mixed PulD multimers formed upon in vitro synthesis in the presence of lecithin liposomes

The multiomer fraction was determined by densitometry of the multimer and monomer bands on immunoblots after SDS-PAGE analysis as shown in Fig. 2. Errors represent standard deviations over 3 independent measurements.

| PulD<sup>Δ</sup> | SDS resistant | Urea resistant |
|-----------------|---------------|---------------|
| WT              | 0.90 ± 0.13   | 0.96 ± 0.03   |
| I322S           | 0.85 ± 0.15   | 0.82 ± 0.14   |
| T470I           | 0.80 ± 0.29   | 0.82 ± 0.15   |
| WT              | 0.83 ± 0.24   | 0.71 ± 0.24   |
| I322S           | 0.85 ± 0.24   | 0.86 ± 0.05   |
| ΔL<sub>1</sub>  | 0.87 ± 0.07   | 0.81 ± 0.06   |
| ΔL<sub>2</sub>  | 0.32 ± 0.10   | 0.37 ± 0.10   |

PulD<sup>Δ</sup> was indistinguishable from PulD<sup>Δ</sup> when examined by EM (Fig. 1C). PulD<sup>Δ</sup> exhibited shortened periplasmic vestibules near the rim of the cup, suggesting that the N<sub>3</sub> domains occupied less defined positions or were partially unstructured (Fig. 1C), a feature that it shared with PulD<sup>Δ</sup> (Fig. 1C). Both PulD<sup>Δ</sup>L<sub>2</sub> and PulD<sup>Δ</sup>L<sub>2</sub> were targeted to the OM in vivo and secreted PulA (Fig. 3). The relatively high amount of PspA produced in the presence of PulD<sup>Δ</sup>L<sub>2</sub> and PulD<sup>Δ</sup>L<sub>2</sub> is likely due to the increased susceptibility of the variant to proteolysis of the S domain (Fig. 3).

The I322S Substitution Destabilizes N<sub>3</sub>—To determine the effect of the I322S substitution on N<sub>3</sub> structure and stability, N<sub>3</sub> domains were purified that carried none, one, or both of the modifications made (Fig. 4A). N<sub>3</sub> domains resisted complete unfolding upon thermal denaturation (Fig. 4, A and B). T470I, N<sub>3</sub> was the least thermostable with thermostability increasing in the order of N<sub>3</sub>N<sub>3</sub>WT > N<sub>3</sub>L<sub>2</sub>N<sub>3</sub>L<sub>2</sub> (Fig. 4B).

Unfolding in urea was reversible and approximated by global fitting to a two-state model with a shared m-value (2.33 ± 0.13 kJ/mol/M), resulting in a free energy of unfolding (ΔG<sup>U</sup>) of −18.82 ± 1.04 kJ/mol for N<sub>3</sub>WT (Fig. 4C). The ΔG<sup>U</sup> between N<sub>3</sub>WT and N<sub>3</sub>I322S was calculated to be 8.33 ± 1.19 kJ/mol, whereas deleting L<sub>2</sub> stabilized N<sub>3</sub>L<sub>2</sub>N<sub>3</sub>L<sub>2</sub> compared with N<sub>3</sub>L<sub>2</sub>N<sub>3</sub>L<sub>2</sub> by −4.69 ± 0.99 kJ/mol (Fig. 4C). N<sub>3</sub>L<sub>2</sub> was more stable than N<sub>3</sub>WT (ΔΔG<sup>U</sup> = −1.40 ± 1.56 kJ/mol) and unfolding was limited even at 8 M urea (Fig. 4C).

The I322S Substitution Increases N<sub>3</sub> Dynamics—Retention of the structure in the N<sub>3</sub> variants was confirmed by the exchange formation SDS-resistant multimers, excluding a role for L<sub>2</sub> in folding (Fig. 2A and Table 1). Surprisingly, introducing I322S in PulD<sup>Δ</sup> yielded SDS-resistant PulD<sup>Δ</sup>ΔL<sub>2</sub>I322S multimers (Fig. 2A and Table 1).
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FIGURE 3. In vivo analysis of PulD<sup>WT</sup> function and outer membrane targeting. A, PulA secretion by <i>E. coli</i> strain Pap105 producing a PulD<sup>WT</sup> variant and all other type II secretion system components and PsPA production in the presence of PulS (as indicated). Secretion was normalized to the amount of PulA secreted by PulD<sup>Mo</sup>, which typically reaches 70–75% (4). PsPA levels were determined by densitometry of the bands shown in B. Errors represent standard deviations over 3 independent measurements; B, comparison of the small amount of PulD<sup>Mo</sup> multimers produced in the absence of PulS with the large amount produced when PulD<sup>Mo</sup> is efficiently targeted to the OM in the presence of PsPA and the levels of PsPA produced in each case as a measure for the phage shock response by PulD<sup>Mo</sup> assembly into the inner membrane (11). OmpF was used as a loading control. WT, PulD<sup>Mo</sup>; I322S, PulD<sup>I322S</sup>; ΔL<sub>2</sub>, PulD<sup>ΔL<sub>2</sub></sup>; ΔL<sub>2</sub>I322S, PulD<sup>ΔL<sub>2</sub>I322S</sup>; PulD<sup>ΔL<sub>2</sub>A</sup>, PulD<sub>ΔL</sub> with cleaved S-domain, which typically occurs in the absence of PulS; Mu<sup>a</sup>, PulD<sup>ΔL<sub>2</sub>S</sup>, multimer; Mo<sup>a</sup>, monomer. Numbers indicate the molecular weight of the markers and of Mu<sup>a</sup> between brackets.

FIGURE 4. Stability of N<sub>3</sub> domains. A, CD spectra of N<sub>3</sub>WT (full black), N<sub>3</sub>I322S (full purple), and N<sub>3</sub>ΔL<sub>2</sub>I322S (full green), and of thermally (dashed purple) and chemically (dotted purple) unfolded N<sub>3</sub>I322S; B, thermal unfolding and C, chemical unfolding (open symbols) and refolding (filled symbols) of N<sub>3</sub>WT (black squares), N<sub>3</sub>I322S (purple circles), and N<sub>3</sub>ΔL<sub>2</sub>I322S (cyan triangles), and of N<sub>3</sub>ΔL<sub>2</sub>I322S (green triangles). Lines represent fits to a two-state equilibrium equation; D, comparison of local free energy changes (∆G<sub>local</sub>) calculated from the three slowest H/DX rates with global free energy changes (∆G<sub>global</sub>) obtained from chemical refolding of the entire domains (the contribution of 2 cis-Pro was subtracted, according to Ref. 43). The full line represents a linear fit to the data and the dotted line shows a linear correlation with a slope of 1. In A–C, MRE, mean residue ellipticity.

of backbone amide hydrogens with deuteriums measured by mass spectrometry (MS) (19, 20). Except for the expected loss of 13 fast-exchangeable amino acids in L<sub>2</sub>, neither the L<sub>2</sub> deletion nor the I322S substitution affected the global hydrogen/deuteron exchange (H/DX) behavior of intact N<sub>3</sub>WT at early time points (supplemental Fig. S3A and Table S1). However, the number of slow exchangeable amide hydrogens was reduced by ∼30% in N<sub>3</sub>I322S and in N<sub>3</sub>ΔL<sub>2</sub> by the I322S substitution, whereas the number of intermediate exchangeable ones increased (supplemental Table S1), indicating a change in protein dynamics.

To locate which N<sub>3</sub> segments were affected by the I322S substitution, H/DX in N<sub>3</sub> domains was analyzed by MS after online pepsin digestion (Fig. 5A, supplemental Fig. S4A). Three N<sub>3</sub>I322S segments showed increased deuteron uptake compared with those of N<sub>3</sub>WT. The largest average uptake difference (17%) was observed in a segment represented by peptide Glu<sup>334</sup>–Leu<sup>340</sup>, which forms the C terminus of α-helix 2 (Fig. 5B) and is the most proximal structural element to the C domain in the PulD sequence (Figs. 1 and 2D). The two other segments (e.g. peptides Lys<sup>270</sup>–Leu<sup>282</sup> and Asp<sup>307</sup>–Asp<sup>332</sup>) had an uptake difference of 7% (Fig. 5A) and form a patch on the N<sub>3</sub> structure opposite to the C terminus of α-helix 2 (Fig. 5B). Introducing the I322S substitution in N<sub>3</sub>ΔL<sub>2</sub> affected the same region of the N<sub>3</sub> domain, but uptake differences were smaller or disappeared (for peptide Ile<sup>322</sup>–Leu<sup>333</sup>) (Fig. 5, C–E, and supplemental Fig. S4B). Glu<sup>334</sup>–Leu<sup>340</sup> remained the peptide with the largest uptake difference (9%), but kinetics closely resembled that of N<sub>3</sub>WT (Fig. 5E, far right panel).

H/DX coupled with NMR or MS can correlate local H/DX rates to the global ∆G<sup>0</sup> of a protein or the global ∆G<sup>0</sup> between a protein and its variants (21, 22). The peptides in Fig. 5E represent each of the segments that showed differential H/DX behavior upon the introduction of the I322S substitution. Excluding Ile<sup>322</sup>–Leu<sup>333</sup>, which is not directly comparable as it contains the site of the substitution, all peptides fitted best to a double exponential (Fig. 5E and supplemental Table S1). The slowest exchange rates were found in Glu<sup>334</sup>–Leu<sup>340</sup>, which also had the largest uptake difference, suggesting that the C terminus of α-helix 2 plays a role in maintaining N<sub>3</sub> stability. ∆G<sup>0</sup><sub>local</sub> were calculated from the three slowest exchangeable hydrogens in the peptides. Hence, for all N<sub>3</sub> domains the slow rates of Glu<sup>334</sup>–Leu<sup>340</sup> were used, whereas the slow rates from the three peptides were used for N<sub>3</sub>I322S as each peptide had one slowly exchanging hydrogen (supplemental Table S1). ∆G<sup>0</sup><sub>local</sub> correlated well with ∆G<sup>0</sup><sub>global</sub> obtained by equilibrium unfolding with a slope of 0.9 (Fig. 4D).
Folding Defects Are Overcome in Mixed PulD Multimers—How does N₃ energetics affect PulD multimer folding? Because multiple domains might need to interact to fold the PulD native multimer, it was assessed whether PulD₂₈₋₄₂/₂₅₉₋₆₆₀ I₃₂₂S can form mixed multimers with wild-type PulDfl (PulDflWT) during co-synthesis. As shown before (13), co-synthesis of PulDflWT and PulD₂₈₋₄₂/₂₅₉₋₆₆₀ produces mixed multimers containing from 0 up to 12 protomers from one form and, reciprocally, from 12 down to 0 of the other form. The mixed multimers form a regular ladder between 540 (a PulD₂₈₋₄₂/₂₅₉₋₆₆₀ dodecamer) and 825 kDa (a PulDfl dodecamer) upon SDS-PAGE (Fig. 6A). The biochemical determinants of the native PulD multimer can be used to assign structural states to the mixed multimers: an SDS- and urea-resistant mixed multimer forms a membrane-inserted PulD²₈₋₄₂/₂₅₉₋₆₆₀ pore (13), whereas an SDS-resistant but urea-sensitive mixed multimer forms a prepore (as typified by PulD₂₈₋₄₂/₂₅₉₋₆₆₀ T₄₇₀I (14)). Trypsin resistance of the PulD pore is not useful in this case, because it trims all protomers to the same molecular mass. Ladders produced upon co-synthesis of PulDflWT and PulD₂₈₋₄₂/₂₅₉₋₆₆₀ were SDS- and urea-resistant, indicative of native structures in the mixed multimers (Fig. 6A and Table 2). In the presence of PulD₂₈₋₄₂/₂₅₉₋₆₆₀ I₃₂₂S, SDS and urea resistance required six or more PulDflWT protomers in the multimer (Fig. 6A, B, and Table 2). Thus, native structures can form in the presence of the I₃₂₂S substitution and

Folding Defects Are Overcome in Mixed PulD Multimers—How does N₃ energetics affect PulD multimer folding? Because multiple domains might need to interact to fold the PulD native multimer, it was assessed whether PulD²₈₋₄₂/₂₅₉₋₆₆₀ I₃₂₂S can form mixed multimers with wild-type PulDfl (PulDflWT) during co-synthesis. As shown before (13), co-synthesis of PulDflWT and PulD₂₈₋₄₂/₂₅₉₋₆₆₀ produces mixed multimers containing from 0 up to 12 protomers from one form and, reciprocally, from 12 down to 0 of the other form. The mixed multimers form a regular ladder between 540 (a PulD²₈₋₄₂/₂₅₉₋₆₆₀ dodecamer) and 825 kDa (a PulDfl dodecamer) upon SDS-PAGE (Fig. 6A). The biochemical determinants of the native PulD multimer can be used to assign structural states to the mixed multimers: an SDS- and urea-resistant mixed multimer forms a membrane-inserted PulD²₈₋₄₂/₂₅₉₋₆₆₀ pore (13), whereas an SDS-resistant but urea-sensitive mixed multimer forms a prepore (as typified by PulD²₈₋₄₂/₂₅₉₋₆₆₀ T₄₇₀I (14)). Trypsin resistance of the PulD pore is not useful in this case, because it trims all protomers to the same molecular mass. Ladders produced upon co-synthesis of PulDflWT and PulD₂₈₋₄₂/₂₅₉₋₆₆₀ were SDS- and urea-resistant, indicative of native PulD structures in the mixed multimers (Fig. 6A and Table 2). In the presence of PulD₂₈₋₄₂/₂₅₉₋₆₆₀ I₃₂₂S, SDS and urea resistance required six or more PulDflWT protomers in the multimer (Fig. 6A, B, and Table 2). Thus, native structures can form in the presence of the I₃₂₂S substitution and
suggest that either neighboring N₃WT domains correct the
conformation of N₃I322S or that the N₃-ring is required
to reach a critical stability. The distribution of protomers in a
multimer is not easily determined (the N₃-N₃ domains in PulDⁿ
are not visible by EM). Hence, to distinguish between these
possibilities, mixed protomers were produced between
PulD²⁸–4²⁵⁹–6⁶⁰ WT and PulDⁿ variants that differ in N₃
stability. PulD²⁸–4²⁵⁹–6⁶⁰ I₃₂₂S formed mixed protomers
with all PulDⁿ variants (Fig. 6A). Importantly, SDS- and
urea-resistant mixed protomers containing PulDⁿΔL₂ (which carries
the most stable N₃ domain) could incorporate up to four extra PulD²⁸–4²⁵⁹–6⁶⁰ I₃₂₂S protomers when produced with
protomers containing PulDⁿWT or PulDⁿΔL₂, I₃₂₂S (Fig. 6A and Table 2). Consistent with the poor multimerization of
PulD²⁸–4²⁵⁹–6⁶⁰ WT and PulDⁿΔL₂, I₃₂₂S, SDS- and urea-resist-
ant mixed PulD²⁸–4²⁵⁹–6⁶⁰ I₃₂₂S/PulDⁿΔL₂, I₃₂₂S-multimers
were low in abundance (Fig. 6A and Table 2). Hence, the results
mirrored the stability and dynamics of the N₃ domains and
support the idea that the stability in the N₃-ring needs to attain
a critical value to enable PulD membrane insertion.

As the ring formed by the 12 N₃ domains is structured in the
PulD prepore (14), it was next investigated whether I₃₂₂S sub-
stitution of N₃I₃₂₂S. The sharp reduction in the amount of mixed
protomers exceeded six (Fig. 6B and Table 2), confirming that the I₃₂₂S substitution affected the
formation of the SDS-resistant PulD-prepore.

Effect of the I₃₂₂S Substitution on the PulD Folding
Mechanism—To fold into the SDS-resistant prepore, the SDS-
sensitive PulD²⁸–4²⁵⁹–6⁶⁰ multimer must overcome a transi-
tion barrier, defined by the Arrhenius activation energy (Eₐ).

Failure to produce an SDS-resistant prepore in PulD²⁸–4²⁵⁹–6⁶⁰
I₃₂₂S either results from an insurmountable Eₐ (which relates
to the reaction rate constant k via k = Aexp(–Eₐ/RT), where
A is a constant that depends on the collision frequency) or from
destabilization of the SDS-resistant prepore to such an extent
that the SDS-sensitive multimer is the more stable structure
(supplemental Fig. S5, A–C). Rate constants for SDS-resistant
PulD multimerization are measured by the intensities of the
multimer bands over time upon SDS-PAGE (13). Although these are easily obtained for PulD²⁸–4²⁵⁹–6⁶⁰ and PulD²⁸–4²⁵⁹–6⁶⁰ T₄⁷₀ I₃₂₂S (0.1 ± 0.04 and 0.07 ± 0.05 min⁻¹,
respectively), the rate constant is not easily determined for
PulD²⁸–4²⁵⁹–6⁶⁰ I₃₂₂S, which remains largely monomeric
upon ZW 3–14 treatment in kinetic experiments (Fig. 7, inset,
and supplemental Fig. S5, D–F). However, PulD²⁸–4²⁵⁹–6⁶⁰
I₃₂₂S monomers decayed with a rate constant of 0.34 ± 0.14
min⁻¹ (Fig. 7, inset), providing an estimate for the multimeri-
zation rate constant. This places the rate constants for all
PulD²⁸–4²⁵⁹–6⁶⁰ variants within 2-fold of each other, indicat-
ing that Eₐ would change by less than 2 kJ/mol/protomer. The
effect of the I₃₂₂S substitution on SDS-resistant prepore stabili-
y was also estimated via the ΔDG = 8.33 kJ/mol between N₃
and I₃₂₂S. The sharp reduction in the amount of mixed
protomers formed with PulDⁿWT when the number of
PulD²⁸–4²⁵⁹–6⁶⁰ I₃₂₂S or PulD²⁸–4²⁵⁹–6⁶⁰ I₃₂₂S/T₄⁷₀ I₃₂₂S
protomers exceeded six (Fig. 6B), suggested that the maximal
destabilization tolerated in the N₃-ring to produce a large
amount of native multimers is ~50 kJ/mol (~6 × 8.33 kJ/mol).
If this would be attributed to an increase in Eₐ, the mixed
protomers would take years to fold (because k_mixed = exp(–ΔEₐ/RT)) or k_mixed = exp(–ΔEₐ/RT).
This is inconsistent with the abundant SDS and urea-resistant
PulD-mixed multimers observed within 6 h. Thus, kinetic and thermody-
namic measurements suggested that the I₃₂₂S substitution
must primarily destabilize the SDS-resistant prepore.

Discussion

Although each PulD protomer only shares parts of the C
domain to form the dodecameric transmembrane pore and the
gate that closes it (6), PulD will not assemble into such a struc-

FIGURE 6. Mixed multimer formation of PulD with its variants. SDS and urea resistance of mixed multimers formed in the presence of the same amount of
one PulDⁿ variant and one PulD²⁸–4²⁵⁹–6⁶⁰ variant. Equal amounts of the samples were migrated on the SDS-PAGE. Mu, mutimer; Mo, monomer; fl,
full-length; tr, 28–42/259–660; WT, unmodified mature PulDⁿ; unaltered, PulD²⁸–4²⁵⁹–6⁶⁰ without further modification. Numbers indicate the molecular
weight of the markers and of Mu²⁸ and Mu⁶⁰ between brackets.
Prepore Stability Controls PulD Membrane Insertion

In vitro, prepore formation can be studied in vitro after detergent solubilization. The detergent-solubilized multimeric PulD structures (with the substitutions that arrest folding at a given step as indicated by the flat arrow) that correspond to each of the multimeric membrane-bound forms and the biochemical characteristics they share with it are also shown. Inset, kinetics of the most abundant PulD-population upon the introduction of substitutions (Mo or Mo) compared with PulD28-42/259-660 is in black, PulD28-42/259-660I322S in purple, and PulD28-42/259-660T470I is in orange.

FIGURE 7. Free energy diagram for PulD folding. Monomers (Mo) assemble into an EP before consolidation into an SDS-resistant LP and subsequent membrane insertion (NMu). Reaction coordinate. Numbers indicate the ΔΔGº (in kJ/mol; see text for details). Schematic of the detergent-solubilized PulD structures (with the substitutions that arrest folding at a given step as indicated by the flat arrow) that correspond to each of the multimeric membrane-bound forms and the biochemical characteristics they share with it are also shown. Inset, kinetics of the most abundant PulD-population upon the introduction of substitutions (Mo or Mo) compared with PulD28-42/259-660 is in black, PulD28-42/259-660I322S in purple, and PulD28-42/259-660T470I is in orange.

Observation that six PulD²WT protomers are required to form a mixed multimer with PulD²²/²⁵⁹-⁶⁶⁰I322S (6 × 8.33 kJ/mol = 50 kJ/mol). This is the equivalent of four van der Waals contacts or a H-bond per monomer. We propose that the N₃-ring forms a seal onto the assembled C domains and must attain a critical stability to facilitate the conformational rearrangements in the C domain that drive pore formation (13), while preventing multimer disruption.

Insights into the folding of OMPs that multimerize into a shared transmembrane pore mainly come from the isolation of intermediates, but the kinetic and thermodynamic relationship with the native structure is mostly unknown (14, 23–25). Previous reports (13, 14, 26) and data obtained here help to define these relationships for PulD by characterizing intermediates that form in the presence of a lipid membrane in detail after detergent solubilization (Fig. 7). Combined, these data indicate that four structures reside in local energy minima along the reaction coordinate: the monomer (Mo), an SDS-sensitive early prepore (EP), the sealed SDS-resistant late prepore (LP), and the pore or native multimer (NMu) (Fig. 7). The largest driving force for PulD pore formation likely comes from membrane insertion of the transmembrane domain, consistent with the remarkable stability of NMu and the ease by which LP is dissociated by moderate urea concentrations (14). CD spectra and EM reconstructions support that PulD traverses the membrane by a β-barrel that is 9 nm in diameter (6, 13, 15). To create such a β-barrel, each PulD protomer should contribute at least 4 β-strands (27). With each β-strand contributing 10 kJ/mol to OMP stability (28), this amounts to an overall folding free energy of at least 480 kJ/mol (Fig. 7). Yet, the balance between EP and LP is attenuated by a modest ΔΔGº of 50 kJ/mol/odocamer. Furthermore, the low multimerization efficiency of PulD²²/²⁵⁹-⁶⁶⁰I322S, as determined by SDS-PAGE, suggests that the ΔΔGº associated with the Mo to EP transition is of the same order of magnitude (= 12 × 8.33 kJ/mol; Fig. 7).

Can this reflect the in vivo pathway? PulD belongs to an expanding group of BAM-independent OMPs that rely on lipid-assisted self-assembly once monomers are delivered to the OM via the Lol system, which in the case of PulD is mediated by the lipoprotein PulS (2, 3, 12, 26). This provides a rationale to study the membrane-associated steps of PulD assembly in a controlled in vitro environment in the presence of liposomes and to compare the results from in vitro studies to the in vivo pathway. Whereas PulS targets PulD to the OM in vivo, PulS accelerates PulD multimerization in vitro (26), providing a first driving force for PulD folding. The binding energy between PulD S domains and PulS was determined to be 40–50 kJ/mol (10). With the Mo to EP and EP to LP transitions each stabilizing the PulD multimer 50 kJ/mol (Fig. 7), the formation of these structures does not provide the energy to dissociate PulD/PulS heterodimers. Interestingly, dissociation is not required to form NMu in vivo (26) and PulS co-purifies with PulD from in vivo sources (29). PulS prevents proteolysis of PulD protomers in vivo (30), which could rationalize the necessity for a prolonged PulD/PulS association as PulD protomers join and break free from growing complexes until the correct conformation is found. Alternatively, the PulD/PulS heterodimer is required to retain PulD folding competence during multimerization.
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Hence, whereas the rough free energy landscape from Mo to EP only provides modest stability, it effectively lowers the entropy associated with PulD multimerization, providing a second driving force for PulD assembly. Lowering entropy ultimately facilitates rapid lipid-assisted membrane insertion, a third driving force in PulD assembly. In this context it is worth noting that a loss of 100 kJ/mol in a PulD28–42/259–660 I322S dodecamer relative to PulD28–42/259–660 is equivalent to the folding free energy of a small to medium-sized BAM-dependent OMP.

Multimeric OMPs (3, 14, 23–25, 31) and bacterial pore-forming toxins and eukaryotic-pore forming complexes (32) that form preprores typically contain at least one domain in the membrane periphery. Folding pathways are not easily obtained, because of the fast assembly rates and the complex environment that these proteins often require. However, multiple sequential pre-integration structures have been described for the bacterial toxins aerolysin (33) and perfringolysin O (34). A salt bridge between neighboring domains in the membrane periphery critically stabilizes an EP into an LP before NMu formation occurs in perfringolysin O, but removing it increases $E_a$ only by 2 kJ/mol/monomer (34). Because a salt bridge is typically stronger, it is possible that it also acts to seal EP into LP. In conclusion, we propose that the energies associated with EP-to-LP transitions driven by domains in the membrane periphery will emerge as key driving forces for membrane insertion of all constitutive and non-constitutive multimeric complexes that assemble independently of a membrane-embedded insertase.

**Experimental Procedures**

**Strains and Growth Conditions**—Cloning and stress response experiments were performed in the *Escherichia coli* strain K-12 PAP105 (Δ(lac-pro) F' (lacPl ΔlacrZM15 proAB+ Tn10)). *E. coli* strain MC4100 PAP7447 (F' lacPl pro+ Tn10)], with pulS, pulA, and pulC-O integrated into malPp and with a large deletion in pulD, was used for in vivo secretion (11). Strains were grown at 30 °C in LB medium with 100 μg/ml of ampicillin and/or 25 μg/ml of chloramphenicol (as appropriate). Expression of the pul genes was induced with 0.4% maltose.

**Plasmid Construction and Site-directed Mutagenesis**—Plasmids and primers used are given in supplemental Table S2. The plasmid encoding for the truncated version of PulD with the N3 domain instead of the N4 domain (PulD28–42/124–190/342–660) was created in 3 steps. In step 1 (a) the DNA fragment from the SphI site on the pIVEX2.3 vector up to the DNA sequence encoding for L2 (amino acids 205 to 308) in N3, and/or 25 μg/ml of chloramphenicol (as appropriate). Expression of the pul genes was induced with 0.4% maltose.

**Gel Electrophoresis and Immunoblotting**—Proteins were separated by electrophoresis on a 4–10% SDS-polyacrylamide gel. Samples were mixed with SDS loading buffer (final concentra-
tion of 62.5 mM Tris-HCl, pH 6.8, 12.5% glycerol, 2% SDS). Following SDS-PAGE, the proteins were transferred onto a nitrocellulose sheet by semi-dry blotting. PulD variants, PspA and OmpF were detected using anti-PulD, anti-PspA, and anti-OmpF antibodies, respectively. Primary antibodies were detected by a secondary antibody coupled to horseradish peroxidase (GE Healthcare). Peroxidase activity was measured by the chemiluminescence produced by ECL2 (Pierce) and integrated on a Typhoon imager (GE Healthcare).

PspA Induction and Pulullanase Secretion—PspA induction was performed in the absence or presence of PulS (pCHAP585). Cultures were inoculated from overnight growths at \( D_{600} = 0.15 \) and grown for an additional 6 h at 30 °C before cell harvesting. PspA production was analyzed by SDS-PAGE and immunoblotting. In cells solubilized in detergent-free buffer. To assess the effect of urea on the multimeric state of PulD28–42/259–660 I_{322S}, diluted with detergent-free buffer. To assess the effect of urea on the multimeric state of PulD28–42/259–660 I_{322S}, diluted with detergent-free buffer.

Folding Kinetics Followed by SDS Treatment—Folding kinetics of PulD28–42/259–660 variants were obtained by mixing aliquots of the synthesis reaction at the time points indicated with SDS-loading buffer to stop folding and incubated on ice before analysis by SDS-PAGE (13). Where kinetics relied solely on integration of the monomer, curves were corrected for linear degradation of the monomer. Curves were fitted to a single exponential equation in Origin 8.0.

Equilibrium Unfolding—N \(_3\) variants were purified as described before (10), diluted to 0.2 mg/ml into 50 m mole sodium phosphate, pH 8.0, and the appropriate amount of urea and incubated overnight to reach equilibrium. CD spectra were taken at 25 °C on an AVIV spectrometer between 190 and 260 nm (where possible) at a rate of 20 nm/min and a bandwidth of 1 nm in a 0.1-cm cuvette. Spectra were averaged over three measurements. For equilibrium unfolding experiments, 100 data points taken at 220 nm were averaged. The data fitted to \( y = (y_N + m_N[\text{urea}]) + (y_U + m_U[\text{urea}]) \exp(-\Delta G_{\text{UN}} - M_{\text{UN}}[\text{urea}]/RT)/(1 + \exp(-\Delta G_{\text{UN}} - M_{\text{UN}}[\text{urea}]/RT)) \), with shared \( y_N, m_U, M_{\text{UN}} \) values. Restrainting the fit further using shared \( y_N \) and \( m_N \) values did not significantly alter the fitting parameters.

Transmission Electron Microscopy (TEM) and Image Processing—His-tagged PulD28–42/259–660 variants were extracted from liposomes by 3% ZW 3–14 in 50 mM Tris, pH 7.5, and 250 mM NaCl and purified using a covalent affinity chromatography (Talon, Clontech). Solubilized PulD28–42/259–660 variants were bound to the resin for 1 h and washed with 5 column volumes of 50 mM Tris, pH 7.5, 250 mM NaCl, and 0.6% ZW 3–14 before elution in the same buffer supplemented with 5 mM EDTA. Eluted PulD28–42/259–660 variants were concentrated and frozen for EM analysis. His-tagged proteins were used as they increased the amount of particles that adsorbed perpendicular on the EM grid.

A 4-μl aliquot was adsorbed onto a glow discharged carbon film-coated copper EM-grid, washed with three droplets of pure water, and subsequently negatively stained with 2% (w/v) uranyl-acetate. The grids were imaged using a Philips CM10 TEM (FEI Company, Eindhoven, the Netherlands) operating at 80 kV. The images were recorded by the 2k × 2k side-mounted Veleta CCD camera (Olympus, Germany) at a magnification of 130,000. The pixel size at the sample level was 3.7 Å.

Sample Preparation for H/DX-MS—All samples were prepared in triplicate using a fully automated LEAP H/DX Pal robot (LEAP Technologies, Carrboro, NC). Prior to deuterium labeling, N \(_3\) variants (15 μm in 50 mM HEPES buffer, pH 7.0) were equilibrated for 60 min at room temperature and placed at 4 °C in the protein compartment of the robot. H/DX was initiated by diluting 2 μl of each protein (30 pmol) with 48 μl of HEPES buffer made with 99.9% D\(_2\)O, pH 7.0, and at 20 °C. At time points between 10 s and 4 h, 30 μl of the labeling reaction was removed, quenched with 60 μl of ice-cold 1.5% formic acid to lower the pH to 2.5, and immediately analyzed by MS. Fully deuterated samples were prepared manually by 24 h incubation at 60 °C (final D\(_2\)O content of 96%), quenched as described above and frozen until LC-MS analysis.
Guard BEH C4 1.7 μm, Waters, Milford, MA) at 100 μl/min with 95% mobile phase A (0.15% formic acid, pH 2.5) and 5% mobile phase B (acetonitrile, 0.15% formic acid, pH 2.5). Proteins were eluted from the trap column to the mass spectrometer using a 2-min gradient of 5–90% mobile phase B at 100 μl/min. For local H/DX analyses, samples were passed across a Poroszyme-immobilized pepsin column held at 20 °C (2.1 × 30 mm, Applied Biosystems) and the resulting peptides were trapped for 2 min onto a C18 trap column (VanGuard BEH C18 1.7 μm, Waters) at 100 μl/min mobile phase A and 0 °C. Peptides were eluted from the trap column to an analytical C18 column (CSHOT C18, 130 Å, 1.7 μm, 1 × 100 mm, Waters) with a 6-min linear gradient of 8 to 40% mobile phase B at 40 μl/min. After each run, the pepson column was cleaned with 0.8% formic acid, 5% acetonitrile, 1.5 μM guanidinium chloride, pH 2.5. Blank injections were performed between each injection to confirm the absence of carryover.

Mass spectra were acquired in resolution and positive ion mode (m/z 50–2,000) on a Synapt G2-Si HDMS mass spectrometer (37) equipped with a standard electrospray ionization source. Mass accuracy was ensured by continuously infusing a Glu-1-Fibrinogen solution (100 fmol/μl in 50% acetonitrile) through the reference probe of the electrospray ionization source. Peptides were identified in non-deuterated samples by a combination of MS², data-dependent acquisition, and exact mass measurement. Peptide identifications were made by database searching in ProteinLynx Global server 3.0 (Waters) and each fragmentation spectrum was manually inspected for assignment confirmation.

**H/DX-MS Data Processing**—For global H/DX analyses, the centroid mass value of each protein was extracted in MassLynx 4.1 (Waters) and the amount of incorporated deuterium was determined from the mass difference between the deuterated and non-deuterated samples. DynamX software 3.0 (Waters) was used to extract the centroid masses of all peptides selected for local H/DX analyses; only one charge state was considered per peptide. No adjustment was made for back-exchange and the results are reported as relative deuteration levels expressed in either mass units (Da) or relative fractional exchange. Relative fractional exchange data were obtained by dividing the measured deuteration uptake for each peptide by the maximum number of exchangeable backbone amide hydrogens that could be theoretically replaced into each peptide. This maximum number is the number of amino acid residues in the peptide minus the number of proline residues and the N terminus (38). All local H/DX-MS results were analyzed using MEMHDX (39).

**Calculation of Thermodynamic Parameters from H/DX Rates**—Under conditions where the native state is preferentially populated, the exchange rate, \( k_{ex} \), is determined by the product of the internal rate of exchange for the unfolded protein, \( k_{int} \), and the ratio of the rates for opening and closing of the protein fold, \( k_{op}/k_{cl} \), respectively. \( k_{ex} = (k_{op}/k_{cl})k_{int} \) if \( k_{cl} \gg k_{int} \) (40). If \( k_{int} \) is known or calculated, the free energy associated with opening of the protein fold is calculated from the equilibrium constant for opening, \( K_{op} = k_{op}/k_{cl} = k_{ex}/k_{int} \) via \( \Delta G_{op} = -RT \ln K_{op} = -RT \ln (k_{ex}/k_{int}) \) (40). When two protein variants \( a \) and \( b \) are compared, \( k_{int} \) is the same except for the sites where modifications were made. Thus, when comparing peptides originating from two protein variants (excluding those containing modifications), the local difference in free energy between variants \( a \) and \( b \) is calculated via \( \Delta \Delta G_{local} = -RT \ln (k_{ex,a}/k_{ex,b}) \).

**Author Contributions**—G. H. M. H., A. P. P., and I. G. conceived the study; G. H. M. H., I. G., S. B., and M. C. worked on the investigation; analysis was done by G. H., S. B., I. G., and M. C.; software development by V. H.; resources were supplied by O. F., J. C.-R., A. P. P., and G. H. M. H.; and all authors contributed to writing.

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