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Structural basis for the unfolding of anthrax lethal factor by protective antigen oligomers

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Abstract

The protein transporter, anthrax lethal toxin, is comprised of protective antigen (PA), a transmembrane translocase, and lethal factor (LF), a cytotoxic enzyme. Following assembly into holotoxin complexes, PA forms an oligomeric channel that unfolds LF and translocates it into the host cell. We report the crystal structure of the core of a lethal toxin complex to 3.1-Å resolution; the structure contains a PA octamer bound to four LF PA-binding domains (LF_N). The first α helix and β strand of each LF_N unfold and dock into a deep amphipathic cleft on the surface of the PA octamer, which we call the α clamp. The α clamp possesses nonspecific polypeptide binding activity and is functionally relevant to efficient holotoxin assembly, PA octamer formation, and LF unfolding and translocation. This structure provides insight on the mechanism of translocation-coupled protein unfolding.

Protein secretion and degradation are essential cellular processes that allow for protein trafficking, organelle biogenesis, protein quality control, and cell-cycle regulation^{1–3}. Since folded proteins are thermodynamically stable under typical conditions, these processes often require complex, energy-consuming molecular machines^{3–6}, which catalyze a series of unfolding and translocation reactions^{7–13}. Anthrax toxin^{5,14}, a three-protein virulence factor secreted by *Bacillus anthracis*, is an example of such a transmembrane protein delivery system (Supplementary Fig. 1 online). This bacterial toxin follows the classical two-component *AB* paradigm, where the *A* component is an active enzyme that localizes to and enters cells by forming complexes with the cell-binding, or *B* component. Anthrax toxin is composed of two *A* components, LF (91 kDa) and edema factor (EF, 89 kDa), and one *B* component, PA (83 kDa). Therefore, two different toxic complexes can form: lethal toxin (LT, PA plus LF) and edema toxin (ET, PA plus EF). LT (which we focus on herein) causes macrophage lysis¹⁵, immune system suppression¹⁶, and death¹⁴.

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Accession codes. The structure factors and coordinates for the PA₈(LF_N)₄ complex have been deposited in the PDB (accession code 3KWV).

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AUTHOR CONTRIBUTIONS

G.K.F. crystallized, solved, and refined the PA₈(LF_N)₄ structure. G.K.F., K.L.T., H.J.S., A.F.K., S.G.G., and I.I.T. obtained functional data. G.K.F., K.L.T., H.J.S., A.F.K., E.R.W., and B.A.K. prepared the manuscript.

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The authors declare that they have no competing financial interests.

For LT to inflict its cytotoxic effects, PA and LF must assemble into active holotoxin complexes, which can translocate LF into host cells (Fig. 1a). Proteases present either on host-cell surfaces or in blood serum potentiate LT assembly by proteolytically nicking PA, yielding n PA^{17–19}. Dissociation of a 20-kDa amino-terminal fragment from n PA exposes LF-binding sites, permitting assembly. The resulting LT complex contains multiple copies of LF bound to either a ring-shaped PA homoheptamer, PA₇ (refs. 18–21), or homooctamer, PA₈ (ref. 19). Octameric PA forms more robust LT complexes than heptameric PA under physiological conditions²². The crystal structures of the individual PA and LF monomers^{20,23} and the assembled PA heptamer²⁴ and octamer¹⁹ are known. However, an atomic-resolution X-ray crystal structure of a lethal toxin co-complex has not been described.

After the LT complex is endocytosed, the PA oligomer transforms into a transmembrane, β -barrel channel²⁵ through which LF translocates to enter the cytosol. Due to the narrowness of the channel, LF unfolds during translocation. The acidic endosomal pH conditions required for toxin action¹⁵ not only aid in the destabilization of LF²⁶ but also drive further LF unfolding⁹ and translocation by means of a proton motive driving force⁷. This driving force is comprised of a proton gradient (Δ pH) and membrane potential ($\Delta\psi$). Efficient coupling of the Δ pH requires a catalytic active site in the channel, called the ϕ clamp, composed of a narrow ring of phenylalanine residues^{7,8}. The ϕ clamp forms a narrowly apposed substrate clamping site in the central lumen of the PA channel⁸, and it allows the channel to catalyze unfolding⁹ and translocation⁸ presumably by forming transient interactions with the unfolded translocating chain⁸.

Many, but not all, protein processing machines that translocate, unfold and/or refold proteins utilize analogous polypeptide clamping features to denature a protein and engage with its unfolded structure. The features that bind to unstructured or unfolded polypeptides include hydrophobic/aromatic pore loops^{8,11,27–29}, polypeptide clamping sites^{8,30}, and other substrate binding clefts or adapters^{31–33}. Some of these machines utilize tandem polypeptide binding sites^{8,9,31}: one site is a substrate docking site, which feeds into a second hydrophobic site found deeper within the pore. Questions surround the mechanism of these clamping sites and their interactions with unfolded substrates. How do these sites unfold proteins? How do they process the wide chemical complexity and configurational flexibility contained in an unfolding substrate? These questions have remained unanswered, in part because atomic resolution structures of unfolding intermediates in complex with these clamps have not been described. Here we report a structure of a partially unfolded substrate, the PA-binding domain of LF, in complex with its unfolding machine, the PA oligomer.

RESULTS

Crystal structure of the PA₈(LF_N)₄ complex

For these crystallographic studies, we focused on the PA₈ oligomer, considering its enhanced thermostability as well as its advantageous fourfold, square-planar symmetry¹⁹. By mass spectrometry (MS), we find that the PA₈LF₄ complex is physiologically relevant, as it assembles from the full-length, wild-type (WT) PA and LF subunits (Supplementary Fig. 2a online). Our best diffracting crystals contain LF_N (LF residues 1–263) and a PA construct lacking its membrane-insertion loop¹⁹, which is superfluous to the known PA-LF_N interaction³⁴. LF_N, the minimal portion of LF that specifically binds PA³⁵, can translocate heterologous domains as amino- or carboxy-terminal fusions into cells^{36,37}. EF contains a homologous PA-binding domain, and the PA-LF_N interaction is likely general to LT and ET complexes³⁸. Homogenous PA₈(LF_N)₄ complexes (Supplementary Fig. 2b online) form crystals in the *P*4₂₁₂ space group that diffract X-rays to 3.1 Å (Table 1). Molecular replacement solutions identify two PA₂ complexes and significant (2.7 σ) unassigned

electron density ($F_o - F_c$) for α helices located proximal to the domain 1' (D1') surface of each PA₂ complex. Rounds of polyalanine-helix modeling and refinement reveal that the novel helical density aligns well with α_2 , α_4 , α_9 , and α_{10} of LF_N. The two occurrences of the PA₂LF_N ternary complex (Fig. 1b) in the asymmetric unit are structurally identical; its PA subunits are structurally similar to the full-length PA monomer²⁰ and the PA subunits observed in the PA₇ and PA₈ prechannel oligomers^{19,24}. Thus the biological unit—the PA₈(LF_N)₄ prechannel complex (Fig. 1c)—is comprised of four PA₂LF_N ternary complexes (Fig. 1d).

Interestingly, LF_N $\alpha 1/\beta 1$ (residues 29–50) unfolds and adopts a novel conformation relative to free LF (1J7N²³). LF_N $\alpha 1/\beta 1$ docks in the cleft formed between adjacent PA subunits and aligns well with the experimental electron density (Fig. 2a,b). We can assign this unique conformation of $\alpha 1/\beta 1$ since it extends from LF_N $\alpha 2$ as a contiguous stretch of electron density contoured at $\sigma=1$ (Supplementary Fig. 3a online). LF_N's carboxy terminus also reveals well-defined electron density (Fig. 2c). Overall, LF_N excludes 1900 Å² of solvent accessible surface area (SASA) on the PA dimer. This surface is comprised of two discontinuous LF_N-binding subsites (Fig. 1b) formed by adjacent PA subunits, termed PA_N and PA_C (to reflect whether the PA subunit interacts primarily with the amino terminus or carboxy terminus of LF_N, respectively). The details of these respective subsites, called the α -clamp binding subsite and the carboxy-terminal binding subsite, are depicted in Figure 3a,b. Thus upon binding the PA oligomer, LF_N partially unfolds, whereby its first α helix and β strand (i) separate from the main body of the protein, (ii) dock into the cleft between two adjacent PA subunits (Fig. 1b), and (iii) orient toward the center of the PA oligomer lumen (Fig. 1c).

The carboxy-terminal binding subsite

At the carboxy-terminal subsite, LF_N's carboxy-terminal subdomain excludes ~900 Å² on PA_C (Fig. 3b). The structure reveals a hydrophobic interface, involving PA_C Phe202, Pro205, Ile207, and Ile210 and LF Val232, Leu235, His229, Tyr223, Leu188, and Tyr236. In particular, LF Tyr236 is well packed against PA_C Ile210 (Fig. 2c) and its phenol hydroxyl forms a hydrogen-bonding network with PA_C His211 and Asp195 near the center of the hydrophobic interface (Fig. 3b). Additional electrostatic interactions surround this hydrophobic core. The carboxyl side chain of PA_C Glu190 forms a pair of hydrogen bonds with both the γ hydroxyl and amide nitrogen of LF Thr141; PA_C Lys197, Lys213, Lys214 and Lys218 form salt bridges with LF Asp182, Asp187, Asp184, and Glu142, respectively; and PA_N Arg200 forms a salt bridge with LF Glu139. PA and LF residues localized in this binding subsite are corroborated by mutagenesis studies, probing binding (Fig. 3c,d), assembly/binding (Supplementary Fig. 4a online),^{34,38–41} and cytotoxicity⁴¹ (Supplementary Fig. 4b,c online).

The α -clamp binding subsite

At the α -clamp subsite, PA_N and PA_C interact with LF_N's unfolded $\alpha 1$ and $\beta 1$ structures (Fig. 3a). Remarkably, hydrogen bonds lost upon LF_N unfolding are reformed on the surface of PA: LF_N $\alpha 1$ maintains a similar helical conformation; and LF_N $\beta 1$ (Ile43 and Lys45) forms parallel β -sheet hydrogen bonds with Leu203 in PA_N $\beta 13$ (Fig. 2b). PA_N Pro205, which is positioned at the end of PA_N $\beta 13$, terminates the parallel-sheet interactions with LF_N $\beta 1$. Overall, LF_N $\alpha 1/\beta 1$ excludes 1000 Å² of SASA on PA. LF_N $\alpha 1$ is docked deep into the α -clamp cleft at the interface of adjacent PA subunits (Figs. 1b and 3a). Reminiscent of calmodulin complexes with peptide helices^{42,43}, PA's twin Ca²⁺-binding sites scaffold the cleft and define its distinct shape and chemical character, including: (i) a delocalized anionic potential created by the excess of negatively-charged PA residues chelating the two Ca²⁺ ions and (ii) a large proportion of SASA contributed by PA backbone atoms. LF_N's side

chains are not well-packed with side chains in the α -clamp cleft, in contrast to the carboxy-terminal binding subsite (Fig. 3a,b). Interestingly, PA contacts the side chains of LF Met40 and His35 through backbone interactions. PA_C Arg178 contacts the hydrophilic face of α 1 at LF His42 while maintaining a hydrogen bond with the backbone carbonyl of PA_N Thr201. Aromatic residues, PA_N Phe236 and Phe464, and aliphatic residues, PA_N Leu187 and Leu203, line the cleft face opposite of PA_C Arg178. Upon binding LF_N, PA_N Phe202 repositions its phenyl group toward LF_N β 1, shielding β 1's backbone hydrogen bonds with PA_N Leu203. The chemical nature of the α -clamp cleft suggests that it is well-suited to bind an unfolded β strand and an amphipathic helix with a positively-charged face.

Both LF-binding subsites are critical for cytotoxicity activity

We initially characterized the PA-LF binding interaction using cytotoxicity assays. Site-directed mutagenesis studies on PA and LF residues involved in either binding subsite reveal defects in LT-induced macrophage cytolysis (Supplementary Fig. 4b,c online). To further address the interaction between LF_N's α 1/ β 1 sequence and the α clamp, we created fusions of the first 20 or 60 residues of LF and the A fragment from diphtheria toxin (DTA), called LF₁₋₂₀-DTA and LF₁₋₆₀-DTA, respectively. When co-administered with PA, we find LF₁₋₆₀-DTA is 100-fold more cytotoxic than LF₁₋₂₀-DTA or hexahistidine-tagged DTA (His₆-DTA, DTA with an amino-terminal, 18-residue leader containing the hexahistidine sequence, Supplementary Fig. 4d online). Interestingly, despite lacking the α 1/ β 1 sequence, His₆-DTA⁴⁴ and LF₁₋₂₀-DTA are cytotoxic when co-treated with WT PA (Supplementary Fig. 4d online); however, all of these DTA constructs are ~1000-fold less cytotoxic when co-treated with the α -clamp mutant, PA R178A (Supplementary Fig. 4e online). Thus the α clamp has broad substrate specificity. However, the role of the α 1/ β 1- α -clamp interaction in toxin function is difficult to surmise from cytotoxicity assays alone, since toxin uptake involves multiple steps (e.g., PA assembly, LF binding, unfolding and translocation).

The role of the α clamp in LT assembly

To determine the role of the α clamp in LT assembly, we performed multiple in vitro PA-LF_N assembly assays. By native PAGE, we find that PA mutations introduced into the LF_N-PA-binding interface disrupt PA co-assembly with LF_N (Supplementary Fig. 4a online). To focus on the role of LF_N α 1/ β 1 in PA assembly, we labeled PA K563C with two different fluorescent probes. A 1:1 ratiometric mixture of these labeled _nPA K563C constructs (_nPA*) produces an increase in fluorescence resonance energy transfer (FRET) upon assembly with LF_N⁴⁵. Using this FRET assay, we find that 5-fold more _nPA* assembles with WT LF_N than with the Δ 47 LF_N amino-terminal truncation (which lacks both α 1 and β 1, Supplementary Fig. 5a online). The circular dichroism (CD) spectra of Δ 47 and WT LF_N are comparable, demonstrating that the assembly defect is not due to the misfolding of Δ 47 LF_N (Supplementary Fig. 5b online). Using electron microscopy (EM), native PAGE, and MS, we find that the percentage of octameric PA oligomers is greatly reduced for Δ 47 LF_N relative to WT LF_N (Supplementary Fig. 5c–e online). By EM, we estimate that ~3% of the PA oligomers produced with Δ 47 LF_N are octameric (10-fold less than that observed with WT LF_N, Supplementary Fig. 5d online). Thus LF_N's α 1 and β 1 structures not only drive PA oligomerization, but also they are critical to the mechanism of PA octamer formation (Fig. 1d).

Mapping the LF_N-binding interaction with the PA channel

Using electrophysiology, we measure LF_N binding by observing kinetic and equilibrium changes in channel conductance⁸ (Supplementary Fig. 6a–c online); i.e., when LF_N binds to the PA channel, it inserts its amino-terminal end into the channel and blocks conductance. We monitor binding in the absence of an applied $\Delta\psi$ to eliminate its influence on the channel-substrate interaction. Since PA₇ and PA₈ have similar translocation¹⁹ and cell

cytotoxicity²² activities, we use the PA₇ oligomer to maintain consistency with prior reports.^{7–9} To determine the overall thermodynamic contribution of LF_N α 1/ β 1, we made a series of additional Δn LF_N amino-terminal truncations (where n is the number of deleted residues). These Δn LF_N do not block PA channel conductance, as they lack sufficient unfolded/unstructured sequence on their amino termini. We use a competition assay to measure Δn LF_N binding: first we block PA channel conductance with WT LF_N (~100 pM); then we add the competitor Δn LF_N and monitor the restoration of the conductance (Supplementary Fig. 6d,e online). We find that $\Delta 42$ and $\Delta 47$ LF_N reduce WT PA-channel-binding affinity by 3.6–3.8 kcal mol⁻¹ relative to WT LF_N (Fig. 3e). However, since $\Delta 27$, $\Delta 32$, and $\Delta 39$ LF_N destabilize the complex by about 1.2–1.4 kcal mol⁻¹, the α 1/ β 1 interaction is worth ~2.5 kcal mol⁻¹. We assume that downstream interactions within the channel provide the additional ~1 kcal mol⁻¹ of stabilization. We conclude that LF_N α 1/ β 1 binds to the PA channel and provides substantial stabilization of the PA-LF_N complex.

To investigate the details of the interaction between the PA channel and LF_N, we engineered point mutations into residues localized in either LF_N binding subsite and estimated their relative energetic contribution to channel binding (Fig. 3c,d). Several mutations localized in the carboxy-terminal binding subsite, PA R200S, I207S, and H211A, disrupt LF_N binding by 1–1.5 kcal mol⁻¹. These residues form two binding “hotspots”, i.e., locations where point mutations disrupt binding most severely⁴⁶. By contrast, the mutations, F202S and P205S, located between these two carboxy-terminal-site hotspots have minimal effects on LF_N binding, reflecting that LF_N’s carboxy terminus does not make substantial contact with these residues (Fig. 3b). The LF_N Y236A mutant most appreciably perturbs PA-channel binding and represents the LF_N hotspot in the carboxy-terminal subsite interaction. Other adjacent LF_N residues in the carboxy-terminal subsite interaction have minimal effects on PA channel binding.

We then investigated the relative energetic contribution of residues localized in the α -clamp binding subsite (Fig. 3c,d). We find that PA Arg178 comprises the major hotspot site in PA’s α clamp, where the R178A mutation destabilizes the complex by 2.9 kcal mol⁻¹. While the aromatic PA mutant, F464S, destabilizes LF_N binding at the α -clamp site by 0.7 kcal mol⁻¹, the PA F236S mutant does not. Additionally, we find that none of 23 point mutations introduced into LF_N α 1 and β 1 destabilizes the LF_N-PA channel complex. Interestingly, the mutation, LF_N M40A, stabilizes the complex 1.3 kcal mol⁻¹ (Fig. 3d). These results indicate contrasting binding energetic behaviors for the two different LF_N-binding subsites. At the carboxy-terminal subsite, a classical interface is observed, where specific LF_N and PA side chains comprise the respective hotspots on either interface. At the α -clamp subsite, while we identify PA Arg178 as a major hotspot residue, no clear hotspot can be identified on LF_N α 1/ β 1. These observations suggest that the stabilizing interactions in the α -clamp subsite do not involve specific LF_N side chains, but rather the ~2.5 kcal mol⁻¹ of binding stabilization is due to the formation of nonspecific contacts and the more general exclusion of SASA.

The PA α clamp possesses nonspecific binding activity

The robustness of the binding interaction is intriguing given the paucity of specific α -clamp interactions. To test the specificity of the α -clamp interaction, we either replaced the entire LF_N α 1/ β 1 sequence with other non-homologous sequences from LF and EF or introduced multiple mutations into α 1/ β 1 (Supplementary Fig. 7 online). Interestingly, we find that these LF_N α 1/ β 1 replacements bind with similar affinities as WT LF_N (differing by 0.2 to 1.0 kcal mol⁻¹, Fig. 3f). Furthermore, multisite LF_N mutants in which the buried hydrophobic face of α 1/ β 1 is replaced with either four Ser residues (LF_N HS1) or four Phe residues (LF_N Ar1) bind PA with similar affinity as WT LF_N (Fig. 3f), indicating that the α clamp also binds non-amphipathic helices. Finally, we find that these LF_N α 1/ β 1-

replacement constructs bind 1.3–2.4 kcal mol⁻¹ less tightly to PA R178A relative to WT PA (Fig. 3f), thereby confirming that this nonspecific-binding activity is localized to the α -clamp subsite. Thus the α clamp binds a broad array of sequences, providing 1.5–4 kcal mol⁻¹ of stabilization (depending upon the identity of the α 1/ β 1 sequence).

LF_N must unfold to bind the α -clamp subsite

Our crystal structure and thermodynamic binding data indicate that the α -clamp subsite binds nonspecifically to unfolded protein substrates. This model is well supported by several additional lines of evidence. First, the thermodynamic comparison of WT LF_N and the truncated Δn LF_N mutants is appropriate because these mutants have similar folded secondary structure content as WT LF_N (Supplementary Fig. 5b online). Moreover, the Δ 47 LF_N construct binds similarly to PA R178A as WT PA (Fig. 3f), confirming that the Δ 47 LF_N truncation does not bind at the α -clamp site, as implied by the structure (Fig. 1b). Second, fusions of LF's amino terminus and DTA (LF_{1–60}-DTA and LF_{1–20}-DTA) are sufficient to bind to the α -clamp site, since their affinity for the PA channel is disrupted by the PA R178A mutation (Fig. 3f and Supplementary Fig. 8 online). This result indicates that the α clamp is an independent binding site capable of binding to unstructured sequences at the amino-terminus of a substrate. Third, knowing that LF_N α 1/ β 1 unfolds upon binding PA (Fig. 4a), we engineered the double mutant, LF_N I39C E72C (LF_N^{C39-C72}, which forms a disulfide bond that prevents α 1/ β 1 unfolding). Interestingly, LF_N^{C39-C72} has 10⁴-fold reduced affinity for PA channels under non-reducing conditions (Fig. 4b); however, under reducing conditions (in the presence of dithiothreitol, DTT), LF_N^{C39-C72} binds with the same affinity as WT LF_N (Fig. 4b). We also kinetically observe a DTT-dependent LF_N^{C39-C72} blockade of PA channels (Supplementary Fig. 9a online). Therefore, LF_N must unfold α 1 and β 1 to properly bind the α clamp and interact stably with PA oligomers.

Binding to PA induces strain and disorder into LF_N

We then asked how the unfolding of LF_N α 1/ β 1 on the surface of PA affects the remaining folded structure of LF_N. First, we measured the stability of the Δn LF_N mutants using chemical denaturant titrations probed by CD at 222 nm (CD₂₂₂). The Δn mutants' stabilities are estimated by fitting the CD₂₂₂-probed titration data to a four-state equilibrium unfolding model ($N \rightleftharpoons I \rightleftharpoons J \rightleftharpoons U$)²⁶ (Supplementary Fig. 9b and Supplementary Table 1 online). We find the truncation mutants possess native (*N*), intermediate (*I* and *J*), and unfolded (*U*) states. The truncations, however, destabilize the *N* state by ~1.2 kcal mol⁻¹, where the deletion of the α 1 helix is more destabilizing than the deletion of the β 1 strand (Fig. 4c). Second, we compared the crystallographic atomic displacement parameters (*B* factors) of bound LF_N with free LF_N (1J7N²³). In this analysis, we calculate the relative change in normalized *B* factor (ΔB_{norm}) for each LF_N residue upon binding PA (Fig. 4d). The β 2– β 4 sheet and surrounding helices increase in *B*_{norm} upon binding PA, whereas α 1/ β 1 decrease in *B*_{norm} (Fig. 4d). To corroborate these ΔB_{norm} values, we measure changes in backbone and side chain mobility using fluorescence anisotropy (FA). LF_N mutants with unique Cys substitutions were labeled with thiol-reactive fluorescent probes. Upon binding WT PA₇ oligomers, the fluorescent probes attached to LF_N's α 1/ β 1 structures show gains in normalized relative FA (*FA*_{norm}), and conversely, probes in the β 2– β 4 sheet show losses in *FA*_{norm} (Supplementary Fig. 10a online). Overall, these ΔFA_{norm} values inversely correlate with ΔB_{norm} values (*p* value of 0.04, Fig. 4e), confirming that the more dynamic regions in the crystal are also dynamic in solution. Therefore, we conclude that the ~2.5 kcal mol⁻¹ of stabilization gained when α 1/ β 1 binds to the α -clamp site not only offsets the ~1.2 kcal mol⁻¹ of thermodynamic destabilization imparted by the unfolding of α 1/ β 1 but also accounts for the observed entropic increases in strain and disorder throughout LF_N's remaining folded structure.

The role of the α clamp in protein translocation

To determine the role of the α clamp during protein translocation, we use planar lipid bilayer electrophysiology, which records changes in PA conductance as substrate-blocked channels translocate their substrates and reopen^{7–9}. We examined 37 point mutations in PA and LF_N. Of the 13 PA mutants tested, we find that the α -clamp mutant, PA F202S, slows LF_N translocation 20-fold, or 1.7 kcal mol^{−1} (Fig. 5a). A subset of the LF_N point mutations (H35A, M40A, and H42A), which point toward either face of the α -clamp cleft (Fig. 3a), inhibit translocation 0.8–1.7 kcal mol^{−1} (Fig. 5a). These translocation defects are observed for both PA₇ and PA₈ channels (Supplementary Fig. 11a online). Conversely, other buried α 1 sites (LF_N Leu36, Ile39, and Ile43) are tolerant to substitution and do not affect protein translocation (Fig. 5a). Interestingly, we find that the observed positional translocation defects are restored when a bulky group is placed at position 40 (ref. 9) and a positively-charged residue is placed at positions 35 and 42 (Fig. 5a). All of the LF_N α 1/ β 1 replacements translocate similarly to WT LF_N (Fig. 5a). We conclude, therefore, that efficient LF_N unfolding and translocation are catalyzed by the aromatic α -clamp residue (PA Phe202); however, the LF_N α 1/ β 1 sequence itself has rather minimal charge and steric requirements.

The broad substrate specificity of the α clamp led us to ask which PA residues facilitate translocation of full-length LF, a more complex, multidomain substrate. LF has a different rate-limiting step than LF_N and requires a greater driving force⁷; therefore, we measure its translocation kinetics under a Δ pH and $\Delta\psi$. We find the PA α -clamp mutants, F202S and P205S, reduce LF translocation efficiency, ϵ , by ~60% (where $\epsilon = A_{\text{obs}}/A_{\text{exp}}$, A_{exp} and A_{obs} are the expected and observed amplitudes, respectively, Supplementary Fig. 12 online). The PA mutants F236S and F202S inhibit the rate of LF translocation (Fig. 5b). Interestingly, these PA mutants do not appreciably affect LF_N binding (Fig. 3c), and only PA F202S inhibits LF_N translocation (Fig. 5a). Finally, we find PA R178A is defective in LF_N binding but not defective in translocation. We conclude that hydrophobic and aromatic residues surrounding the α clamp (Fig. 5c) catalyze the translocation of LF.

DISCUSSION

Some models^{8,30} propose that nonspecific clamping sites are critical features of unfolding machines. In general, unfoldases are thought to denature proteins by applying mechanical forces⁹ and transiently trapping partially unfolded conformations in nonspecific binding sites⁸. Unfolded protein, however, is inherently more complex than folded protein, especially in terms of its configurational flexibility and combinatorial chemical complexity. Therefore, a translocase channel would have to accommodate an ever-changing array of possible chemistries and configurations as the unfolded chain is translocated. An elegant solution to this problem may be that unfolded sequences adopt a more rigid and uniform α -helical or β -strand conformation upon binding to an unfoldase, as we observe in the PA-LF_N complex (Fig. 3a). Indeed we find that PA's α clamp can bind to a broad array of amino acid sequences (Fig. 3f). This nonspecific binding activity likely reflects the general helical shape complementarity of the α -clamp site, which excludes ~1000 Å² on PA without making specific side-chain-side-chain interactions. Additionally, backbone hydrogen bonds, which are ubiquitous features of polypeptides, can provide nonspecific contact points between the translocase and substrate, as we observe between LF_N β 1 and PA_N β 13 (Figs. 3a and 5c).

Broad peptide-binding specificity has been observed in other systems, including calmodulin^{42,43}; the ClpXP adapter, SspB^{32,33}; the chaperone, GroEL/ES^{47–49}; and the unfoldase, ClpA/Hsp100 (ref. 31). For calmodulin, which is analogous structurally to the PA oligomer's α -clamp cleft, multiple peptide helices are recognized by the cleft formed by its twin Ca²⁺-ion binding sites. The ClpXP adapter, SspB, binds multiple unstructured carboxy-

terminal degradation signal tags in various conformations in a cleft. The chaperone complex, GroEL/ES, can bind to various amphipathic helices and strands. A substrate binding site, identified in the unfolding machine ClpA/Hsp100, is located above the ϕ -clamp-type site and may be analogous to the α -clamp site on the PA oligomer.

Our structure provides new insight on how a nonspecific polypeptide clamp can unfold its substrate. By binding to LF_N in multiple locations using nonspecific interactions [i.e., in the α clamp (Fig. 3a) and ϕ clamp⁸], LF_N can be partially unfolded (Fig. 4a) and maintained in a more strained (Fig. 4d,e) and less stable conformation (Fig. 4c–e). The region of LF_N that is most destabilized upon binding PA (Fig. 4d,e) coincides with LF_N's β 2– β 4 sheet, which was previously reported as the mechanical breakpoint, or structure that is rate-limiting to the unfolding step translocation⁹. Therefore, we infer the α -clamp site stabilizes unfolding intermediates, introduces strain into the mechanical breakpoint, and feeds unfolded structure into the central ϕ -clamp site.

We estimate that the costs associated with binding to the α -clamp site (Fig. 3c–e) may be offset by orienting the substrate toward the central lumen (Fig. 5c), reducing the stability of the substrate (Fig. 4c), and minimizing the diffusional mobility of unstructured regions before (Fig. 4d,e) or during translocation⁸. We expect that nonspecific-clamping sites should lessen the counterproductive diffusive motions expected for large sections of unfolded polypeptide chain by maintaining contact with the unfolded chain and further reducing backbone conformational entropy, thus allowing the $\Delta\psi/\Delta\text{pH}$ driving force to efficiently unfold⁹ and translocate proteins⁷ (Fig. 5a,b). Although the α clamp forms a stable complex with unfolded structure, this intermediate does not represent a thermodynamic trap. Rather populating partially unfolded translocation intermediates would lower a much greater overall rate-limiting barrier expected in the absence of such intermediates, thereby allowing translocation to proceed on a biologically reasonable timescale.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/nsmb/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The structure of LF's PA-binding domain in complex with the PA octamer
(a) An overview of LT assembly and LF translocation. LF [1J7N²³ (pink) with LF_N (red)] and a PA₆₃ subunit [3HVD¹⁹ (light blue) with D1' (blue)]. LF and PA₆₃ co-assemble into either heptameric (PA₇LF₃) or octameric (PA₈LF₄) LT complexes. The PA₈LF₄ complex depicted is verified by mass spectrometry (Supplementary Fig. 2a online) and based upon structural data presented herein. LT is endocytosed; the endosome is acidified, causing PA to form a β-barrel channel²⁵; LF translocates through the channel under a ΔpH/Δψ-driving force to enter the cytosol; and LF then disrupts normal cellular physiology by cleaving mitogen-activated kinase kinases⁵⁰. (The channel depicted is a model intended for illustration purposes only.) **(b)** (Left) Ribbons depiction of the PA₂LF_N ternary complex. PA_C (chain A, blue), PA_N (chain B, green), LF_N (chain C, red), and calcium ions (gray spheres). (Right) Slices through a surface rendering of the two LF_N-binding subsites: (top) the carboxy-terminal binding subsite and (bottom) the α-clamp subsite. **(c)** Axial rendering

of the biological unit, the $\text{PA}_8(\text{LF}_\text{N})_4$ complex, colored as in (b). The PA octamer is a molecular surface; and LF_N 's helices and strands are cylinders and planks, respectively. The structure is produced from chains A, B and C, using the C_4 symmetry axis, which is parallel to the c edge of the unit cell at $(-\frac{1}{2}a, 0b)$. **(d)** $\text{LF}_\text{N} \alpha 1/\beta 1$ binds the α -clamp subsite formed at the interface of two PA subunits, driving the assembly of dimeric and tetrameric PA intermediates¹⁹, which in turn form PA_8 complexes.

Figure 2. LF_N electron density in the PA_g(LF_N)₄ complex

A composite simulated-annealing (SA) omit map calculated in PHENIX⁵¹ to 3.1 Å contoured at $\sigma=1$ (gray mesh). The models of PA_N, PA_C and LF_N are rendered in green, blue, and red, respectively. Secondary structure elements and individual residues are labeled. **(a)** LF_N α 1 (residues 31–42) in complex with PA_N. Lysine and glutamate residues are truncated to C β for clarity. **(b)** LF_N α 1 in complex with PA_N β 12–13. LF_N Lys45 is truncated to C β for clarity. **(c)** LF_N's carboxy-terminal binding subsite interaction with PA_C. Additional stereo-pair images of LF_N omit maps following SA refinement are depicted in Supplementary Figure 3 online.

Figure 3. The PA octamer binds LF_N in two distinct subsites

Detailed views of (a) the α -clamp binding subsite and (b) the carboxy-terminal binding subsite are depicted. Highlighted non-covalent interactions are indicated with red dashed lines. Chains and Ca^{2+} ions are colored as in Figure 1b. Changes in equilibrium binding free energy ($\Delta\Delta G$) for PA channel complexes, comparing (c) site-directed mutants of PA, (d) site-directed mutants of LF_N, and (e) Δn LF_N amino-terminal truncation mutants. In (c–e), the reference state is WT LF_N:WT PA. (f) (Left) LF_N $\alpha 1/\beta 1$ -replacement mutant binding to WT PA; $\Delta\Delta G$ values are referenced to WT LF_N. (Right) LF₁₋₂₀-DTA, LF₁₋₆₀-DTA, $\Delta 47$ LF_N and LF_N $\alpha 1/\beta 1$ -replacement mutant binding to PA R178A; $\Delta\Delta G$ values are referenced to WT PA. LF_N $\alpha 1/\beta 1$ -replacement mutants either include multiple point mutations in the

$\alpha 1/\beta 1$ sequence ($^{32}\text{QEEHLKEIMKHIVK}^{46}\text{I}$) or replacements of the $\alpha 1/\beta 1$ sequence with other sequences from LF or EF. The name, replacement sequence, and sequence identity (%) are listed for each: LF $\alpha 14$, SEEGRGLLKKLQI (23%); LF $\alpha 28$, NSKKFIDIFKEEG (23%); EF $\alpha 1$, EKEKFKDSINNLV (31%); hydrophilic sequence 1 (HS1), QEEHSKEISKHSVKS (73%); aromatic sequence 1 (ArS1), QEEHFKEIFKHFVKF (73%). See Supplementary Figure 7 online for alignments and helical-wheel depictions of the $\alpha 1/\beta 1$ -replacement sequences. In (c–f), $\Delta\Delta G = RT \ln K_d^{\text{MUT}} / K_d^{\text{WT}}$, where the equilibrium dissociation constants (K_d) were measured for the mutant (MUT) and WT proteins at pH 7.4, $\Delta\psi = 0$ mV (Supplementary Fig. 6 online); R is the gas constant; and T is the temperature. The error bars are the mean $\pm$ s.d. ($n = 2$ –6).

Figure 4. Dynamics and thermodynamics of the pre-translocation unfolding of LF_N
(a) Rendering of LF_N's unfolding transition on the surface of the PA_NPA_C dimer (green and blue, respectively). Free LF_N (gold) (PDB 1J7N²³) is C α -aligned to the LF_N in the PA₈(LF_N)₄ complex (red). **(b)** LF_N^{C39-C72} binding to WT PA channels (pH 7.4, 0 mV) in the presence of 5 mM DTT (red $\blacktriangle$) and in the absence of DTT (black $\blacktriangle$). A WT LF_N binding curve ($\circ$) is also shown. Normalized equilibrium currents were fit to single-site binding model to obtain K_d values: WT LF_N, $K_d = 120 (\pm 30)$ pM; LF_N^{C39-C72}, $K_d = 1.2 (\pm 0.1)$ μ M; and LF_N^{C39-C72} + 5 mM DTT, $K_d = 240 (\pm 60)$ pM. **(c)** Equilibrium stability measurements (pH 7.5, 20 $^{\circ}$ C) of amino-terminal deletions of LF_N (Δn LF_N). Equilibrium free energy differences ($\Delta\Delta G_{NU}$) were obtained from denaturant titration data fit to a four-state equilibrium unfolding model²⁶ (Supplementary Fig. 9b online), where $\Delta\Delta G_{NU} = \Delta G_{NU}(\Delta n) - \Delta G_{NU}(\text{WT})$. Error bars are the mean $\pm$ s.d. ($n = 3-4$). Fit parameters are listed in Supplementary Table 1 online. **(d)** Residues in LF_N are colored by their differences in normalized B factor (ΔB_{norm}), which is obtained by comparing the model of free LF_N (1J7N, structure 1) and LF_N in complex with PA (structure 2) using $\Delta B_{\text{norm}} = B_{1,i} / \langle B_1 \rangle - B_{2,i} / \langle B_2 \rangle$. The $\langle B \rangle$ is the average B factor for the entire chain. ΔB_{norm} values indicating increasing and decreasing disorder upon binding to PA are colored red and blue, respectively. **(e)** ΔB_{norm} is plotted against the normalized fluorescence anisotropy (FA) change (ΔFA_{norm}) for 7 different site-specifically-labeled residues (37, 48, 72, 126, 164, 199, and 242) in LF_N. $\Delta FA_{\text{norm}} = FA_{1,i} / \langle FA_1 \rangle - FA_{2,i} / \langle FA_2 \rangle$, where free LF_N and the LF_N-PA oligomer complex are state 1 and state 2, respectively. The linear fit is significant ($p = 0.04$). Raw anisotropy changes upon binding the PA oligomer for these labeled LF_N are shown in Supplementary Figure 10 online.

Figure 5. The role of the α clamp in LF_N and LF translocation

Planar lipid bilayer translocation results for various mutant channels and substrates. **(a)** Differences in translocation activation energy ($\Delta G^{\ddagger}$) for (top) LF_N mutants, (bottom left) LF_N $\alpha 1/\beta 1$ -replacement mutants, and (bottom right) PA mutants are shown. The reference state is WT LF_N:WT PA. $\Delta G^{\ddagger} = \Delta G^{\ddagger}(\text{WT}) - \Delta G^{\ddagger}(\text{MUT})$, and $\Delta G^{\ddagger} = RT \ln t_{1/2} / c$. The $t_{1/2}$ value is the time for half of the protein to translocate, and c is a 1-sec reference constant. All LF_N translocation rates were measured at symmetrical pH 5.6, $\Delta\psi = 40$ mV. A negative value indicates the rate of translocation slowed upon mutation. The relative translocation efficiencies for these LF_N translocations are given in Supplementary Figure 11b online. **(b)** Full-length LF translocation at $\text{pH}_{\text{cis}} = 6.1$, $\text{pH}_{\text{trans}} = 7.4$, $\Delta\text{pH} = 1.3$, $\Delta\psi = 20$ mV. (left)

$\Delta\Delta G^\ddagger$ values and (right) relative translocation efficiencies ($\epsilon_{\text{MUT}}/\epsilon_{\text{WT}}$) for mutant PA channels. Individual LF translocation records are shown in Supplementary Figure 12 online. Error bars in (a–b) are the mean $\pm$ s.d. ($n = 2\text{--}12$). (c) (left) $\text{LF}_N \alpha 1/\beta 1$ (red ribbon) unfolds from the structured carboxy-terminal subdomain (red surface) by binding into the α -clamp site (cyan surface) on the PA oligomer (gray surface). The interaction is comprised of nonspecific interactions. The α -clamp sites orient the unfolded structure toward the central pore, where the protein is translocated. (right) Residues in PA's α -clamp site (cyan) that affect LF_N and/or LF translocation are rendered as sticks. $\text{LF}_N \alpha 1/\beta 1$ (red ribbon) and parallel β -sheet hydrogen bonds (black dotted lines) between $\text{LF}_N \beta 1$ and PA $\beta 13$ are shown.

Table 1

Data collection and refinement statistics

PA₈(LF_N)₄^a	
Data collection	
Space group	<i>P</i> 4 ₂ 1 ₂
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	178.38, 178.38, 240.36
α , β , γ (°)	90, 90, 90
Resolution (Å)	49.8–3.1(3.2–3.1) ^b
<i>R</i> _{p.i.m.}	6.9(46.0)
<i>I</i> / σI	11.4(2.2)
Completeness (%)	92.0(78.0)
Redundancy	7.9(8.0)
Refinement	
Resolution (Å)	49.8–3.1
No. reflections	65, 165
<i>R</i> _{work} / <i>R</i> _{free}	24.9/ 28.1
No. atoms	
Protein	20,397
Ligand/ion	8
Water	4
<i>B</i> -factors	
Protein	100.7
Ligand/ion	53.3
Water	56.7
R.m.s. deviations	
Bond lengths (Å)	0.005
Bond angles (°)	0.610

^aData for this complex were collected from a single crystal.

^bValues in parentheses are for the highest-resolution shell.