The substrate-binding cap of the UDP-diacylglucosamine pyrophosphatase LpxH is highly flexible, enabling facile substrate binding and product release

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The abbreviations used are: LPS, lipopolysaccharide; LpxH, UDP-diacylglucosamine pyrophosphatase; LpxI, UDP-galactose:beta-1,3-glucosamine-1-phosphate pyrophosphohydrolase; UDP–DAG, UDP-2,3-bis(3R)-hydroxytetradecanoyl)-α-D-glucosamine-1-phosphate; UDP–DAG, UDP-2,3-bis[O-(3R)-3-hydroxy-myrstioyl]-α-D-glucosamine; LpxH4, LpxH from P. aeruginosa; HILpxH, LpxH from H. influenzae; LpxH–mCherry, E. coli LpxH with C-terminal fusion of mCherry; 3, 1-(5-((4-(3-(tri-(1-fluoromethyl) phenyl) piperazin-1-yl) sulfonyl) indolin-1-yl) ethan-1-one; β-ME, β-mercaptoethanol; DSF, differential scanning fluorimetry; SEM, selemnomethionine; PDB, Protein Data Bank.

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The atomic coordinates and structure factors (code 5WLY) have been deposited in the Protein Data Bank (http://wwpdb.org/).

This article contains Tables S1 and S2, Figs. S1–S8, and Video S1.

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Gram-negative bacteria are surrounded by a secondary membrane of which the outer leaflet is composed of the glycolipid lipopolysaccharide (LPS), which guards against hydrophobic toxins, including many antibiotics. Therefore, LPS synthesis in bacteria is an attractive target for antibiotic development. LpxH is a pyrophosphatase involved in LPS synthesis, and previous structures revealed that LpxH has a helical cap that binds its lipid substrates. Here, crystallography and hydrogen–deuterium exchange MS provided evidence for a highly flexible substrate-binding cap in LpxH. Furthermore, molecular dynamics simulations disclosed how the helices of the cap may open to allow substrate entry. The predicted opening mechanism was supported by activity assays of LpxH variants. Finally, we confirmed biochemically that LpxH is inhibited by a previously identified antibacterial compound, determined the potency of this inhibitor, and modeled its binding mode in the LpxH active site. In summary, our work provides evidence that the substrate-binding cap of LpxH is highly dynamic, thus allowing for facile substrate binding and product release between the capping helices. Our results also pave the way for the rational design of more potent LpxH inhibitors.

The increasing problem of antibiotic resistance necessitates the characterization of new antibiotic targets (1, 2). The Raetz pathway of lipid A synthesis is essential in most Gram-negative bacteria and includes several novel targets (3). In these bacteria, lipid A forms the membrane anchor of lipopolysaccharide (LPS), which forms the outer leaflet of the outer membrane (4). LPS helps to protect the bacteria from environmental stressors such as hydrophobic toxins, including many antibiotics, and in infection, from the host immune system (3, 5–7). Disrupting LPS synthesis can stall growth or compromise the selective barrier of the outer membrane (5, 6, 8, 9). Moreover, lipid A is responsible for overstimulating the mammalian immune system through binding to the Toll-like receptor 4–myeloid differentiation factor 2 complex, causing potentially fatal organ damage from acute inflammation during systemic Gram-negative infections (10–13).

Most of the enzymes in the Raetz pathway have already been structurally characterized. Recently, LpxH was added to the list of structurally characterized enzymes with the publication of structures of LpxH from Haemophilus influenzae and Pseudomonas aeruginosa bound to the product (lipid X) and in the unbound form (14, 15). LpxH is a hydrolase that catalyzes the cleavage of the phosphoanhydride of UDP-2,3-diacylglycosamine (UDP–DAG) utilizing a dimanganese center (8, 16). Approximately 70% of Gram-negative bacteria utilize LpxH, whereas Chlamydia utilize the distantly related LpxG, and most of the rest utilize the nonhomologous enzyme LpxI (17, 18). LpxH has a calcineurin-like metal-dependent phosphoesterase fold with the addition of a unique helical cap, comprising three α-helices, that covers the active site and binds the lipid substrates (14, 15). Although previous structures suggested that the capping domain is stably attached to the rest of the protein and only becomes slightly disordered in the absence
of ligand (15), we provide multiple lines of evidence that the capping domain is highly dynamic in the absence of ligand. These results have implications for the mechanism of substrate binding and product release: although slight disordering of the third capping helix, as observed previously, could allow lipids to enter and exit the active site, a more extensive conformational change would enable facile binding and release of lipids through a wider opening between the capping helices.

Results and discussion

Crystal structure

A solubilized version of Escherichia coli LpxH including four solubilizing mutations (F141H, L142S, L146S, and F147H) and four surface entropy reduction mutations (E14A, E15A, K161T, and E162A) (LpxH4/H110014) was generated to improve protein expression and crystallization. LpxH4/H110014 crystallized in plate-shaped crystals that diffracted to 2.00 Å, and the structure was solved by molecular replacement (Table 1) (PDB code 5WLY). The core phosphoesterase domain of E. coli LpxH is composed of two 5-stranded β-sheets sandwiched together by α-helices, and this domain overlays very well with the previous LpxH structures (14, 15, 19) (Figs. 1A and 2A). However, the capping helices of E. coli LpxH were displaced from the position observed in previous structures and were largely disordered (Fig. 2A). Residues 122–130 and 162–172 from the capping helices are not visible. Although the unbound form of P. aeruginosa LpxH (PaLpxH) did show increased disorder and an altered conformation where the capping helices bind the product head group and connect to the core domain, the rest of the helices remained packed on top of the active site (Fig. 2D) (15). In E. coli LpxH, the helices are detached from the active site, and only the middle portion (residues 131–161) is visible. This conformation is fortuitously stabilized by crystal contacts (Fig. S1) and is likely transient in solution. However, this structure suggests that the capping helices are much more flexible and disordered in the absence of substrate than was apparent from previous LpxH structures. This structure of E. coli LpxH was the impetus for further experiments to examine the movement of the cap. It is important to note that the capping helices of LpxH4+4, which contains six mutations within the capping helices, or of E. coli LpxH in general may be inherently more flexible than those in H. influenzae or P. aeruginosa LpxH. On the other hand, in previous LpxH crystal structures, the closed conformation was stabilized by binding to lipid X and/or crystal contacts wherein the capping domains were often packed against each other, and this may explain the limited movement observed in the apo PaLpxH structure (14, 15). Because the structures and sequences of LpxH from these species are highly similar (Fig. 2 and Fig. S2), the design and analysis of the following experiments were guided by the hypotheses that the motions

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<th>Parameter</th>
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<th>LpxH4 + 4 SeMet derivative (SAD data)</th>
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Table 1

Diffraction and refinement statistics

The statistics for the highest resolution shell are shown in parentheses. Each data set was collected with a single crystal.
of the capping helices are similar and that the conserved residues play the same roles in these different LpxH variants.

**Hydrogen–deuterium exchange MS (HDX–MS)**

HDX-MS of LpxH4/H11001 showed that the capping domain has a relatively high exchange rate compared with the rest of the protein (Fig. 3, B and C). Residues 146–156, which constitute the first half of the third helix of the capping domain (helix 7), have the highest exchange rate (66.7% in 1 min) of any detectable portion of the protein (Fig. 3). Residues 158–172 have the second highest exchange rate (53.8% for residues 158–169 and 55.9% for residues 170–172 in 1 min) and form the second half of helix 7 and the C-terminal loop of the capping domain. In the LpxH4/H11001 structure, only residues 158–161 were visible from this second region, and residues 159–165 were disordered in the apo PaLpxH structure (Figs. 2 and 3) (15). Residues 84–88, which form a loop and the N terminus of helix 3, have the highest exchange rate outside of the capping domain (47.0% in 1 min) (Fig. 3). Because these residues are largely surface exposed even in closed structures (14, 15), this provides little insight (Fig. 2). Unfortunately, the residues in the long loop between sheet 2 and helix 2, which are covered by the free end of the capping domain in the closed structures but surface-exposed in our structure, were not observed by MS (Figs. 2 and 3). Nonetheless, the high exchange rate of the capping domain, particularly in the 146–156 region that was ordered and attached to the catalytic core in previous structures, supports the hypothesis that this domain is highly dynamic rather than stably attached to the core domain.

**Molecular dynamics simulations show cap flexibility**

PaLpxH was selected for molecular dynamics (MD) simulations because structures of PaLpxH have been determined with and without lipid X bound (15). Moreover, simulations with *E. coli* LpxH would require a large portion of the cap to be computationally built before molecular dynamics could be run; therefore, utilizing PaLpxH structures to minimize the amount of structure that needed to be computationally built gave more reliable starting models for MD simulations. MD simulations were performed on PaLpxH systems with lipid X bound (PDB code 5B49) (LpxH_holo), with lipid X removed (PDB code 5B49) (LpxH_apo_holo), and with the unbound structure (PDB code 5B4C) (LpxH_apo) (15). In our 3-μs simulations of LpxH_holo, lipid X conserved its conformation in the active site. Principal component analysis showed that the cap mostly sampled the conformation observed in the crystal structure and rarely sampled other conformations with the cap slightly more open. In the presence of lipid X, the cap adopted the crystal structure conformation with only small deviations (Fig. 4A). In the absence of a ligand in PaLpxH (LpxH_apo_holo and LpxH_apo), the cap became more flexible, sampling many conformations including an open configuration where residues 123 and 163 were farther apart (Fig. 4A). LpxH_apo displayed an open cap where a large cavity formed near the Mn$^{2+}$ active site that is large enough to accommodate the phosphate and glucosamine moieties of lipid X (Fig. 4B). Overall, MD simulations agreed well with experimental results in that the cap is rather flexible in the absence of lipid X.

**The role of loop (residues 80–83) in cap flexibility**

In our MD simulations of apo LpxH starting from the lipid X-bound conformation (LpxH_apo_holo), we observed that the cap generally maintained its positioning as observed in the PaLpxH crystal structures. However, as a notable deviation...
from the crystal structure, we found that residues 80–83, which formed a flexible loop underneath the cap, move upward to and protrude into the cap. In particular, Phe\textsubscript{82} and Leu\textsubscript{83} side chains interact with the cap residues via hydrophobic interactions. To further investigate the role of the loop, we simulated a F82GL83G double mutant of PaLpxH (LpxH\textsubscript{mut}). Principle component analysis of the LpxH\textsubscript{mut} showed that the mutations caused the cap to collapse on itself, adopting a closed conformation (Fig. 4). Based on this, the loop may have a role in regulating the opening or closing of the cap and thus the exposure of the active site for ligand binding.

We then studied the root mean square deviation (RMSD) of the loop for individual systems to elucidate the dynamics of the loop. We aligned the trajectories against two reference conformations (loop-down and loop-up conformations) to identify which conformation the loop adopts over time. The loop-down conformation has the loop further away from the cap as in the crystal structure, whereas the loop-up conformation has the loop protruded inside the cap as seen in simulations (Fig. 4C). In both LpxH\textsubscript{mut} and LpxH\textsubscript{holo} systems, the loop adopted the down conformation away from the cap, whereas the loop in the LpxH\textsubscript{apo_apo} and LpxH\textsubscript{apo_holo} systems sampled both the up and down conformations and conformations in-between (Fig. 4C).

To test whether the loop-up and loop-down conformations of the loop correlate with the open and closed states of the cap, we calculated the distance between the cap and the loop and the distance between residues 123 and 163, the two residues at the opposite ends of the cap (Fig. 4B). These two residues provide a good measure of the cap’s open and closed conformations. As
seen in Fig. 4D, the data indicated a negative correlation between the distance between residues 123 and 163 and the distance between the loop and the cap; thus, the loop-up conformation correlates with the open conformation. Taken together, our results suggest a mechanism where the loop protrudes into the cap to keep it open, forming a solvent-exposed cavity for ligand binding. Therefore, mutation of residues 82 and 83 (F82G and L83G) would be expected to decrease activity by inhibiting substrate binding. To test this hypothesis, these conserved residues were mutated to Gly in the soluble E. coli LpxH forming LpxH4/GG, and its activity was tested in vitro as described below.

**Activity of E. coli LpxH mutants**

Activity assays showed that the solubilized E. coli LpxH crystallized in this study was catalytically competent for hydrolyzing UDP-DAG to lipid X (Fig. 5 and Table 2). In fact, the crystallized form showed significantly more activity than the WT enzyme with a C-terminal mCherry fusion (LpxH–mCherry) (Table 2). These results indicate that none of the eight mutated residues were important for activity on detergent-solubilized substrate. The reason for increased activity is unknown but may simply reflect improved solubility: less aggregated protein is present. Alternatively, the six mutations in the capping helices may increase the flexibility of the cap and thus facilitate cap opening. The LpxH3/H110014-W136H mutant, wherein Phe141 was restored and Trp136 was mutated as an alternate solubilizing mutation, has similar activity to LpxH4/H110014. In H. influenzae LpxH (HiLpxH), the corresponding Phe142 contacts the amide-linked acyl chain of lipid X, whereas the residue corresponding to Trp136 is surface exposed in all LpxH structures (Fig. 6) (14, 15).
The similar solubility and activity of these variants is more consistent with the hypothesis that increased activity reflects improved solubility of the LpxH variants rather than effects on substrate binding. However, mutating Ile47 and Arg149 to Cys (LpxH4̶H11001̶4-CC) decreased activity significantly placing it on par with LpxH–mCherry (Table 2): Ile47 forms part of the hydrophobic pocket that binds the hydrocarbon tails of the substrate, and Arg149 forms a salt bridge with Asp50 in structures where the cap is positioned on top of the active site, not displaced as in the LpxH4̶H11001̶4 structure (Fig. 6). The loss of this salt bridge that stabilizes the position of the cap may explain the decreased activity of LpxH4̶H11001̶4-CC. As described above, molecular dynamics simulations suggested that residues Phe82 and Leu83 are important for stabilizing an open cap conformation in which the active site is accessible to substrate. When both of these residues were mutated to Gly (LpxH4+4-GG), activity was decreased significantly below that of LpxH–mCherry consistent with the effect predicted by molecular dynamics simulations (Table 2). However, these mutations could also decrease substrate binding efficiency by decreasing the hydrophobic contacts available to the amide-linked acyl chain of lipid X. In addition, the melting temperature (Tm) of LpxH4+4-GG was decreased by 2.6 °C relative to LpxH4+4, indicating that these mutations partially compromise protein stability (Table S1). However, the Tm remained well above the temperature (21 °C) at which activity assays were run. Finally, because even the most active LpxH variant tested here had a specific activity 12500-fold lower than that measured for WT HiLpxH (16), it is important to note that our reaction conditions were not identical. In particular, we determined specific activities with reactions run at 21 °C versus 30 °C used previously (16).

Inhibition

Nayar et al. (20) identified an inhibitor of E. coli LpxH via genetic screening. Thus, we tested the ability of this inhibitor (3) (Fig. 7) to inhibit LpxH in vitro. Luminescence-based assays

Figure 4. Flexibility of the capping helices. A, density map of the first principal component is shown. PC1 motion is also shown in Video S1. B, the open and closed forms of the cap found in MD simulations are shown. Cap residues (residues 121–168) are colored in blue, whereas the rest of the protein is shown in silver surface. Mn2+ ions are shown in pink. The loop containing Phe82 and Leu83 is colored orange, and the residues are colored with green carbons and a yellow surface. C, a box plot of the RMSD calculation aligned against the loop-up and loop-down conformations. The loop-up and loop-down protein conformations in ribbon diagram are shown at the bottom on the right and left, respectively. The cap and the loop are highlighted in blue and orange, respectively, and Phe82 and Leu83 are shown with cyan carbons. D, heat map showing correlation of the cap opening with the cap–loop distance. The cap opening distance is measured between residues 123 and 163, whereas the cap–loop distance is determined by the distance between the center of mass of the cap and the center of mass of the loop. Individual points represent frequency of conformations observed during the time course of MD simulations. Lighter colors represent greater point density.
Figure 5. Inhibition of LpxH. Reactions (10 μl) were run at 30 °C with 62 μM UDP-DAG, 10 nm LpxH, 0.1 mM Tris-HCl pH 8.0, 0.1% Triton X-100, 10% DMSO, and 1 mg/ml BSA at various concentrations of the inhibitor (3) (Fig. 7) (shown in micromolar). The reactions were quenched by spotting on HPTLC silica gel 60 plates, which were run with 25/15/4/2 chloroform/methanol/water/acetic acid mobile phase and charred with 20% sulfuric acid in ethanol and a heat gun. Reactions prepared with UDP-DAG but no enzyme act as a negative control and UDP-DAG standard. Independently purified lipid X product is also included as a standard. LpxH–mCherry reactions were run for 30 min. The reaction proceeded to completion in the absence of inhibitor but is slightly inhibited by 1 μM inhibitor. At 2–8 μM inhibitor, the reaction proceeded to ~50% completion, and it is mostly inhibited at 10 μM and above. These plates were not repeated. LpxH + 4 reactions were run for 20 min. No inhibition was observed up to 1 mM inhibitor. This plate was replicated twice. LpxH + 4-W136H reactions were run for 40 min. A small amount of UDP-DAG remains at 100 μM inhibitor, suggesting weak inhibition at high inhibitor concentrations. This plate was replicated thrice. Analysis of band intensities is presented in Fig. S6, and the full plates are shown in Fig. S7.

Table 2
LpxH specific activities
The reactions were run in triplicate at ambient temperature (21 °C) with 100 μM UDP-DAG and 5–20 nm LpxH (in 0.5 M NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM DTT, and 2.5 mM MnCl2), 0.1 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, and 1 mg/ml BSA. Inhibitor (3) was dissolved in DMSO, resulting in a final concentration of 10% DMSO in these reactions. Activity was measured with a UMP/CMP-Glo kit (Promega), which quantifies UMP concentration with a luciferase-coupled assay. Specific activities with standard errors and confidence intervals were calculated by linear regression analysis of three time points within the linear range of the reactions in GraphPad Prism v7.03.

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<tr>
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<th>95% confidence interval</th>
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<tr>
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<tr>
<td>3 + 4-W136H</td>
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<tr>
<td>4 + 4-CC</td>
<td>17.94 ± 2.81</td>
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</tbody>
</table>

gave an IC50 of 1.2 ± 0.2 μM for LpxH–mCherry (Table 2 and Fig. S3). TLC–based assays also showed inhibition of LpxH–mCherry by 3, giving incomplete conversion of UDP–DAG to lipid X (Fig. 5); in TLC-based assays, the IC50 appeared to be low micromolar (~5 μM) (Fig. 5). On the other hand, the crystallized form of E. coli LpxH was not inhibited by 3 (Fig. 5). This result is likely due in part to the F141H mutation because E. coli LpxH-F141L was previously identified as a resistant mutant (20). However, when Phe141 was reintroduced in LpxH3 + 4-W136H, inhibition was still weak at inhibitor concentrations as high as 1 mM (Fig. 5). Thus, Leu142, Leu146, and/or Phe147 are likely also involved in inhibitor binding, possibly by influencing the position of Phe141. Influence on the position of Phe141 may also explain the resistance of the LpxH-R149H mutant: the corresponding Arg packs against Phe142 in the HiLpxH structure (PDB code 5K8K) (Fig. 6). The G48D and L84R resistance mutations likely function by introducing a charged residue into the hydrophobic pocket thus blocking inhibitor binding. The location of these mutations strongly suggest the inhibitor acts by competitively binding to LpxH and blocking binding of the hydrocarbon tails of the substrate (20) (Fig. 6). When the inhibitor was docked into the HiLpxH active site with AutoDock Vina (21), it tended to occupy the same space as the amide-linked acyl chain (Fig. 6D). In one compelling model, the inhibitor is engaged with the Phe142 (F141 in E. coli LpxH) by π-stacking, which could explain the resistance conferred by the otherwise conservative F141L mutation (Fig. 6D) (20). The HiLpxH structure was taken to be the best candidate for molecular docking because PaLpxH already contains Leu141.

Conclusions
In this work, we have provided multiple lines of evidence that the substrate-binding cap of LpxH is more dynamic than was apparent from previously published crystal structures alone (14, 15). Molecular dynamics simulations showed how this dynamic nature of the cap can allow for facile substrate binding and product release between the helices of the cap. Further-
more, these simulations identified two residues that may act as a wedge to promote cap opening to allow substrate entry, and mutation of these residues was found to decrease LpxH activity as predicted. Finally, we quantified the potency for a previously identified LpxH inhibitor and modeled a plausible binding mode consistent with identified resistance mutations. The greater understanding of the structure and dynamics of the LpxH substrate-binding pocket provided by this research will
aid in the development of more effective inhibitors and antibi-
otic leads targeting this step in the synthesis of Gram-negative endotoxin.

**Experimental procedures**

**Cloning, site-directed mutagenesis, and expression**

*E. coli* lpxH was PCR-amplified from DH5α cells (NEB) and inserted into the NdeI (NEB) and XhoI (NEB) sites of pET24a (+) (Novagen). Expression of WT LpxH was poor. To improve expression, LpxH was solubilized by the mutation of predicted surface-exposed hydrophobic residues. These residues were predicted from a homology model generated by the Phyre2 server with a distantly related hydrolase, Rv0805 (PDB code 3IB7, 17% identity) (22, 23). Several mutations were tested, and F141H, L142S, L146S, and F147H were identified as the best mutations for improving solubility and expression. However, this protein failed to crystallize. To improve crystallization, surface entropy reduction mutations (E14A, E15A, K161T, and E162A) were introduced that were identified by the SER server (24). This eight-residue mutant (LpxH4 +4) was used for the remainder of experiments except where noted otherwise. A second eight-residue mutant (LpxH3 +4-W136H) was also generated in which Trp136 was mutated to His instead of Phe to test the effect of the F141H mutation on inhibitor binding as discussed below. In addition, I47C and R149C were added to the LpxH4 +4 mutant (LpxH4 +4-CC) in an attempt to introduce a disulfide bond that could lock down the capping domain; however, only the presence of intermolecular disul-
fides was observed by SDS-PAGE (data not shown). Finally, F82G and L83G were added to LpxH4 +4 (LpxH4 +4-GG) to test the effect on activity as suggested by molecular dynamics simulations (discussed below).

LpxH was expressed in BL21 DE3 cells (Lucigen) grown in Miller’s Luria Broth (RPI) (40 mg/liter kanamycin (Teknova)) at 37 °C to an optical density at 600 nm (OD600) of 0.8 and induced at 18 °C with 1 mM isopropyl β-D-1-thiogalactopyranoside (GoldBio) overnight. The cells were pelleted at 4,540 g (4 °C, 25 min) (Beckman Avanti J-25 I, JA-25.50). The lysate was batch-bound for 1 h at 4 °C with 6 ml of pre-equilibrated HisPur nickel–nitrilotriacetic acid resin (Thermo) with the addition of 10 mM imidazole (Chem-Impex Intl’l). A gravity-flow column was used to collect the resin, which was then washed with 50 ml of equilibration buffer (0.5 M NaCl, 50 mM Tris–HCL, pH 7.4, and 5 mM β-ME) with 25 mM imidazole and 15 ml of equilibration buffer with 40 mM imidazole. LpxH was eluted in 20 ml of equilibration buffer with 250 mM imidazole. Elutant was dialyzed against 500 ml of 0.5 M NaCl, 20 mM Tris-HC1, pH 7.4, 5 mM DTT (GoldBio), and 5 mM MnCl2 at 4 °C in 3.5-kDa cutoff tubing (Spectra/Por) overnight. Dialyzed elutant was concentrated in a 10-kDa cutoff centrifugal filter (Millipore), and concentrated protein was run on HiLoad 26/60 Seperdex 200 column (GE Healthcare) in 0.5 M NaCl, 20 mM Tris–HCL, pH 7.4, 5 mM DTT, and 2.5 mM MnCl2 at 4 °C. Peak fractions corre-
sponding to monomeric LpxH were concentrated to 14.2 mg/ml, as measured by A280 (ε = 28,085 M−1 cm−1) (Nanodrop 8000 Thermo), flash frozen in liquid N2, and stored at −80 °C. The selenomethionine (Sem) derivative of LpxH was produced by the Met synthesis inhibition method with 50–75 mg/10 ml Sem (Chem-Impex International) (25). The Sem derivative was purified as for the native protein except the concentrations of DTT and β-ME were increased to 10 mM.

**Differential scanning fluorimetry**

Differential scanning fluorimetry was utilized to identify additives that would stabilize LpxH for crystallization. Thermal shift assays were performed in a CFX96 real-time system C1000 Touch Thermal Cycler (Bio-Rad). Samples (25 μl) were pre-
pared in a 96-well plate with a final concentration of 0.484 mg/ml protein, 80X Sypro Orange (Life Technologies) (1.6% DMSO), and 2-fold diluted Solubility and Stability Screen (HR2–072 Hampton Research). The temperature was increased from 20 to 95 °C at 0.5 °C per cycle with a 30 s cycle. Sucrose (1 μl) and 10 μM GSH/GSSG GSH were identified as strong stabilizing agents.

Differential scanning fluorimetry was also utilized to identify low-salt conditions appropriate for HDX–MS of LpxH4 +4 and to compare the stability of LpxH4 +4 and LpxH4 +4-

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**Figure 7. Synthesis of LpxH inhibitor.** 1-5-([4-(3-trifluoromethyl)phenyl]piperazin-1-yl) sulfonamido indolin-1-yl) ethan-1-one (3) was synthesized from piperazine derivative (2), 1-acetylindoline-5-sulfonamide chloride (1), and trieth-
ylamine dissolved in dichloromethane. The reaction was performed at ambient temperature for 2 h with stirring. Following purification, the final yield was 79% at ≥95% purity.

**Figure 6. Inhibitor binding and cap mobility.** Residues that have been shown to be important for inhibitor binding are shown. G48D, L84F, F141L, and R149H were found to provide E. coli with resistance to the inhibitor by Nayar et al. (20). In this study, LpxH4 +4, which contains F141H, was also found to be resistant, but reintroduction of Phe in LpxH3 +4-W136H was not sufficient to allow micromolar inhibition, suggesting some contribution to resistance by one or more of L142S, L146S, and F147H. A, HilpxH (PDB code 58BK) (14) has residue numbering +1 that of E. coli LpxH and PalpxH. Arg103 is forming a ion pair with Asp1 (3.5 Å). The amide-linked acyl chain of lipid X is interacting with Phe42. B, PalpxH (PDB code 58G9) (15) has Leu at 141 instead of Phe, suggesting that P. aeruginosa has a natural resistance to the inhibitor. Furthermore, Leu44 appears to allow the amide-linked acyl chain to assume a conformation different from that observed in HilpxH. Arg44 is forming an ion pair with Asp42 (2.8 and 2.9 Å). C, the positions of these residues in the LpxH structure solved in this study. Displacement of the cap has broken the ion pair between Arg42 and Asp42. D, docking of the inhibitor (tan carbon) into the HilpxH structure with AutoDock Vina (21) shows that it may occupy the same space as the amide-linked acyl chain of lipid X (gray carbons). In the docking model selected (4th ranked of 9), the inhibitor is engaged in a π-stacking interaction with Phe42, which may explain the specificity for Phe over Leu at this position. An overlay of the top four docking models is shown in Fig. S8. E, another view of the docking model in D, with mesh surface rendering, lipid X removed, and the inhibitor rendered in space-filling model. F, the 2D structure of the inhibitor, which was rendered in ChemBioDraw Ultra 14.0.
F82GL83G, which was identified by molecular dynamics simulations as discussed below, in size-exclusion buffer. These experiments were performed as above except that the final concentration of LpxH was 5 μM, and sample volume was 40 μl.

Crystallization and X-ray diffraction

LpxH4+4 (7.1 mg/ml) with 20 mM reduced GSH (Calbiochem) was crystallized in 1.5-μl hanging drops (2:1 protein to well solution) over 0.1 M Tris-HCl, pH 8.2, 70 – 80 mM magnesium formate (Fluka), and 1–5% 2-propanol (Fisher) at 19 °C. Plate-shaped crystals grew within 2 days. The crystals were cryo-protected with 0.25 M NaCl, 10 mM Tris-HCl, pH 7.4, 2.5 mM DTT, 1.25 mM MnCl₂, 50 mM Tris-HCl, pH 8.2, 0.5% 2-propanol, 35 mM magnesium formate, 10 mM reduced GSH, and 30% 2-methyl-2,4-pentanediol (ACROS Organics). The crystals were shot at the Advanced Photon Source (Argonne National Laboratory, Lemont, IL) on Beamline 24-ID-E at 0.979 Å and 100 K. The best crystals diffracted to 2.0 Å (Table 1). The Sem derivative was crystallized under the same conditions as the native protein with the addition of 5 mM tris(2-carboxyethyl)phosphine-NaOH (Soltect Ventures), pH 6.6, to the well solution. The Sem derivative formed plate-shaped crystals, which were cryo-protected with the same solution as the native crystals. The best Sem derivative crystal diffracted to 2.63 Å at the APS on Beamline 24-ID-E at 0.979 Å and 100 K (Table 1). The diffraction data were indexed, integrated, and scaled in the beamline’s Rapid Automated Processing of Data software (XDS (26) and CCP4 (27)).

Model building

The structure of E. coli LpxH was solved in the P2₁2₁2₁ space group by molecular replacement in PHENIX Phaser with a structure of P. aeruginosa LpxH (PDB code 5B4A) (47% identity), which contained a mutation that eliminated Mn²⁺ binding but was still bound to lipid X, as the search model (15). Most of the amino acids, 198, and one Mn²⁺ ion were built automatically by PHENIX (28). The rest of the structure was built manually in Coot with refinement and automated ligand placement performed in PHENIX (28, 29). Residues 122–130 and 162–172 and the C-terminal His tag are disordered and not visible in the electron density map. The structure was refined with Ramachandran statistics of 94.86% favored, 4.67% allowed, and 0.47% outliers. The Sem derivative data were used to create an anomalous difference map that confirmed the positions of the selenium atoms in the structure (Fig. S4).

Hydrogen–deuterium exchange MS

LpxH was prepared at 20 μM for hydrogen–deuterium exchange in 0.1 M NaCl and 10 mM Tris-HCl, pH 8.2. Protein was diluted 10-fold with D₂O at 10 °C (100 μl final). Exchange was quenched with an equal volume of quench buffer (1.25% formic acid, 1.5 mM guanidine HCl) at 0 s, 10 s, 1 min, 3 min, 5 min, 10 min, 20 min, 30 min, 60 min, and 120 min, and 100 μl was injected on a Waters ACQUITY HDX LC system. The LC system was maintained at 0 °C except a separated chamber for the pepsin digestion (10 °C). The samples were digested on a Prozyme pepsin cartridge (2.1 mm × 3.0 mm, Applied Biosystems), trapped, and desalted on a Waters ACQUITY BEH C18 VanGuard pre-column (130 Å, 1.7 μm, 2.1 mm × 5 mm) for 3 min with a flow rate of 100 μl/min buffer A (0.1% formic acid in water). The resulting peptic peptides were resolved on a Waters ACQUITY UPLC BEH C18 column (1.7 μm, 1.0 × 100 mm) with a linear gradient ranging from 5% to 60% acetonitrile with 0.1% formic acid in 6 min at 40 μl/min, which was coupled to a Waters Synapt G2 HDMS q-TOF mass spectrometer. Mass spectra were collected in positive ion MS² mode. Nondeuterated peptides were identified in PLGS 3.0 (Waters), and deuterated peptides were identified and quantified in DynamX 3.0 (Waters) with correction for deuterium incorporation during proteolysis as previously described (30). Hydrogen exchange was measured on 61 peptic peptides, which cover 87.1% primary sequence of LpxH with an average redundancy of 3.51 (Fig. S4).

Molecular dynamics simulations

We performed triplicate MD simulations for four systems: LpxH with lipid X in the binding site (LpxHboom), apo LpxH starting from the holo crystal structure (LpxHapo_holo), LpxH F82G/L83G mutant (LpxHmut), and apo LpxH starting from the apo crystal structure (LpxHapo_apo). The crystal structures of LpxH from P. aeruginosa we used as starting structures are PDB code 5B49 (with bound lipid X) for the first three systems and PDB code 5B4C (with no ligand bound) for the last system (15). We used Gaussian09 (31) to generate RESP HF 6–31G* charges and the Antechamber module of the Amber14 suite (32) to parametrize Lipid X with the Generalized Amber Force Field (33). The systems were built with Amber ff14SB force field (34) in a TIP3P (35) water box. Chloride ions were added to neutralize the positively charged systems. Histidine protonation states were determined by the PROPKA program (36) implemented in the NBCR PDB2PQR webserver (37).

All MD simulations were performed using the Amber GPU workflow (38). We first relaxed each system with multistep miniminations followed by four consecutive restrained MD simulations. The first 2,000 minimization steps constrained all heavy atoms. The second 2,000 minimization steps constrained only the protein heavy atoms and ions. The third 2,000-step minimization was similar to the second minimization but with restraints on the Mn²⁺ ions removed. The fourth 10,000-step minimization held only the protein backbone atoms constrained. The final 20,000 steps minimized all atoms without any constraints. After minimizations, the systems were slowly heated from 0 to 310 K while keeping a positional restraint of all heavy atoms for 250 ps. Then the systems were subjected to three consecutive 250-ps restrained MD simulations consecutively releasing the initial 3 kcal/(mol. Å²) restraint on heavy atoms to 2 and 1 kcal/(mol. Å²). Finally, three independent copies of 1-μs production MD simulations with 2-fs time steps were performed at 310 K and 1 atm for all four systems, resulting in 12 μs of MD simulations in total. The MD simulations were stable as shown in RMSD plots (Fig. S5).

Principle component analysis and distance analysis

Principle component analysis was performed on all the systems using Amber cpptraj (39). The motion of the cap (residues 121–168) and an interacting loop (residues 80 – 83) was captured in a covariance matrix and diagonalized to find the eigen-
values, and the conformation variation of each system was shown by projecting onto the eigenvalues. Distances were calculated using VMD (40). R was used for plotting.

**Chemical synthesis of LpxH inhibitor**

The general procedures were as follows. All commercial chemicals were used as supplied unless otherwise indicated. Flash chromatography was performed on a Teledyne CombiFlash RF-200 with RediSep columns (silica) and indicated mobile phase. All moisture-sensitive reactions were performed under an inert atmosphere of ultrapure argon with oven-dried glassware. $^1$H was recorded on a Varian 600 MHz. Mass data were acquired on an Agilent 1100 LC/MSD TOF mass spectrometer (G1969A). Analysis of sample purity was performed on a Varian Preparat SD-1 HPLC system with a Phenomenex Gemini, 5-µm C18 column (250 mm × 4.6 mm). HPLC conditions: solvent A = H$_2$O, solvent B = acetonitrile; flow rate = 1.0 ml/min; compounds were eluted with a gradient of 5% acetonitrile/H$_2$O for 0–5 min and to 95% acetonitrile/H$_2$O from 5 to 30 min followed by 100% acetonitrile from 35 to 40 min. Purity was determined by total absorbance at 254 nm. Compound has a purity of ≥95%.

Synthesis of 1-(5-((4-((3-(trifluoromethyl) phenyl) piperazin-1-yl) sulfonyl) indolin-1-yl) ethan-1-one (3) (Fig. 7): To a solution of piperazine derivative (2) (100 mg, 0.43 mmol, 1.0 eq) in dichloromethane (5 ml) at 0 °C, 1-acetyllindoline-5-sulfonyl chloride (1) (0.12 g, 0.48 mmol, 1.1 eq) and triethylamine (0.08 ml, 0.56 mmol, 1.3 eq) was added dropwise, and the reaction mixture was stirred for 2 h at room temperature. The reaction was diluted with dichloromethane (10 ml) and quenched with sodium bicarbonate (10 ml). The organic layer was separated, and the aqueous layer was extracted with dichloromethane (2 × 10 ml). The combined organic layers were washed with brine, dried over sodium sulfate, and evaporated in vacuo to yield the desired crude product. Purification of the crude using CombiFlash with a gradient of 0–2% methanol in dichloromethane yielded the title compound as colorless crystals (0.16 g, 79%). $^1$H NMR (600 MHz, DMSO-$d_6$) δ 8.21 (d, $J = 8.2$ Hz, 1H), 7.59 (d, $J = 11.0$ Hz, 2H), 7.40 (t, $J = 7.9$ Hz, 1H), 7.19 (d, $J = 8.4$ Hz, 1H), 7.15 (s, 1H), 7.09 (d, $J = 7.5$ Hz, 1H), 4.17 (t, $J = 8.4$ Hz, 2H), 3.32 (s, 3H), 3.23 (t, $J = 8.4$ Hz, 2H), 2.99 (s, 4H), 2.19 (s, 3H). HRMS-ESI (+)/m/z calculated for C$_{21}$H$_{25}$F$_2$N$_2$O$_5$S, 454.1412 [M+H]$^+$; found: 454.1406.

**Purification of LpxH–mCherry**

To test the inhibition of WT LpxH, a C-terminal fusion with mCherry was designed. The LpxH and mCherry genes were fused together with a KpnI (NEB) site followed by three consecutive Gly codons. The gene fusion was ligated into the BamHI (NEB) and XhoI sites of pET24. This fusion allowed for the purification of enough LpxH–mCherry for enzymatic assays. The nickel–nitrilotriacetic acid portion of the purification protocol was the same as for the native soluble form of LpxH except that β-ME was decreased to 1 mM, Tris-HCl, pH 7.4, was replaced with HEPES-NaOH pH 8.0 (Fisher), and 10% glycerol (Fisher) was added to the buffer. The eluant (15 ml) was dialyzed against 500 ml of 5% glycerol, 0.5 M NaCl, 50 mM HEPES-NaOH, pH 8.0, 5 mM MnCl$_2$, and 1 mM DTT at 4 °C in 3.5-kDa cutoff tubing overnight. The dialyzed eluant was concentrated in a 30-kDa cutoff centrifugal filter (Millipore), and concentrated protein was run on a Superdex 200 Increase 10/300 GL column in 5% glycerol, 0.5 M NaCl, 50 mM HEPES-NaOH, pH 8.0, 5 mM β-ME, and 2.5 mM MnCl$_2$ at 4 °C. The most pure fractions were combined, and the concentration was measured as 0.413 mg/ml (7.52 µM) as measured by $A_{280}$ ($ε = 62,340$ M$^{-1}$ cm$^{-1}$).

**Activity assays**

For TLC-based assays, LpxH variants were prepared at 100 nM in 0.5 M NaCl, 20 mM Tris, pH 7.5, and 2.5 mM MnCl$_2$. The reactions (10 µl) were prepared with 62 µM UDP–DAG (as measured by $A_{260}ε = 9.9$ µM$^{-1}$ cm$^{-1}$), 10 nM LpxH or 1 µl of dilution buffer, 100 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, and 1 mg/ml BSA. UDP–DAG and lipid X were prepared as reported previously (41). The reactions were run at 30 °C and were quenched by spotting onto an HPTLC Silica gel 60 plate (EMD Millipore). The spots were dried with a Dual-Temp heat gun (Genesis) set on low (300 °C). The plate was run with a 25/15/4/2 chloroform/methanol/water/acetic acid mobile phase. After running, the plate was allowed to dry for 1 h. Then the plate was developed by spraying with 20% sulfuric acid in ethanol and charring with the heat gun set on high (538 °C) until the bands appeared. Reactions to test the effect of the LpxH inhibitor contained 10% DMSO (TCI) and were run for the amount of time required for the uninhibited enzyme to reach completion (Table S2) as determined above.

Although TLC-based assays have the benefit of directly showing changes in the substrate and product of interest, absolute quantification requires concurrently run standard curves (42, 43). To determine specific activities for more quantitative comparisons of LpxH variants, UMP concentration was quantified with a UMP/CMP-Glo glycosyltransferase assay kit (Promega) as described by the manufacturer. The reactions (10 µl) were prepared as above except that enzyme dilution buffer included 5 mM DTT, reaction LpxH concentration was 5 nM (20 mM for LpxH4-4GG), and UDP–DAG concentration was 0.1 mM. The reactions were run at ambient temperature (21 °C) in a white 384-well plate (Greiner Bio-One 781074). The reactions were quenched with 10 µl of UMP/CMP detection reagent (Promega), and the plate was shaken 30 s at 1440 rpm and 1-mm amplitude in a Spark 10M plate reader (Tecan). The luciferase reaction was incubated at ambient temperature 1 h, and then the total luminescence of each well was measured in a luminometer developed by Fluorescence Innovations (Minneapolis, MN). Concurrently run UMP standard wells allowed luminescence of LpxH reaction wells to be converted to UMP concentration. All reactions and standards were run in triplicate. To determine specific activities, reactions were quenched at three time points within the early, linear product accumulation range of the LpxH reactions, and linear regression was performed in GraphPad Prism v7.03. To determine the IC$_{50}$ of the LpxH inhibitor, specific activities of LpxH–mCherry (5 nM) were determined as above with 10% DMSO and 2–50 µM inhibitor. These specific activities with standard errors were fit to a three-variable dose–response inhibition curve in GraphPad Prism v7.03.
Structural dynamics of LpxH capping helices

\[ \text{SA} = \frac{\text{SA}_{\text{max}} - \text{SA}_{\text{min}}}{1 + 10^{(\text{logIC}_{50} - \text{logIC}_{50})}} \]  \hspace{1cm} \text{(Eq. 1)}

For the purpose of curve fitting, an inhibitor concentration of 0.1 nm was paired with the uninhibited (10% DMSO) LpxH–mCherry specific activity.

Molecular docking

The LpxH inhibitor 1-(5-((4-(3-(trifluoromethyl) phenyl)piperazin-1-yl) sulfonyl) indolin-1-yl) ethan-1-one (3) (Fig. 7) (20) was docked into the HiLpxH (PDB code 5K8K) structure (14) with AutoDock Vina (21). The protein and ligand structures were prepared for docking in AutoDockTools from the MGLTools 1.5.6 suite (44, 45). The active site was defined by a box centered at coordinates (0.2, 16.529, 13.923) with x, y, and z lengths of 24, 18, and 36 Å. Exhaustiveness was set to 10; 9 structures were output.

Data availability

The coordinates and structure factors of the crystal structure reported in this article are available at the Protein Data Bank under accession number 5WLY. All other data presented are available within this article or its supplementary information or from the corresponding authors by request.


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References

Structural dynamics of LpxH capping helices


