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LpxI structures reveal how a lipid A precursor is synthesized

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Abstract

Enzymes in lipid metabolism acquire and deliver hydrophobic substrates and products from within lipid bilayers. The structure at 2.55 Å of one isozyme of a constitutive enzyme in lipid A biosynthesis, LpxI from *Caulobacter crescentus*, has a novel fold. Two domains close around a completely sequestered substrate, UDP-2,3-diacylglucosamine, and open to release products either to the neighboring enzyme in a putative multi-enzyme complex, or to the bilayer. Mutation identifies Asp225 as key to Mg²⁺ catalyzed diphosphate hydrolysis. These structures provide snapshots of the enzymatic synthesis of a critical lipid A precursor.

Introduction

Gram-negative bacteria are surrounded by an asymmetric outer membrane in which the inner leaflet is composed primarily of phospholipids, while the outer leaflet contains mainly lipopolysaccharide (LPS) (Fig. 1a). LPS forms a structural barrier that protects Gram-negative bacteria from environmental stressors, including antibiotics^{1,2}. LPS is anchored to the outer membrane via Lipid A, a glucosamine-based saccharolipid. Lipid A biosynthesis is required for Gram-negative species' viability and pathogenesis^{3,4}, and is therefore a promising target against which to develop antimicrobial therapeutics¹. While all steps in the pathway (Fig. 1b) are conserved, the UDP-2,3-diacylglucosamine hydrolysis is carried out by either of two isozymes^{5,6}, by LpxH in 70% of Gram-negative bacteria, and by LpxI in 30% that include *Caulobacter crescentus* and several genera of human pathogens including *Rickettsia*, *Brucella*, and *Leptospira*. We previously characterized LpxI from *Caulobacter crescentus*⁷ (termed CcLpxI) as a peripheral membrane metal-dependent hydrolase that accesses its normally bilayer-resident substrate and produces the same product as LpxH but by a different mechanism. LpxI catalyzes the attack of water on the β-phosphate of UDP-2,3-diacylglucosamine, as opposed to an attack on the α-phosphate that is catalyzed by LpxH⁷. To provide insight into this mechanism, we endeavored to determine the structure of this new class of phosphodiester hydrolase required for lipid A biosynthesis in a subset of Gram-negative bacteria. We hypothesized that these LpxI structures could offer a

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Author contributions

L.M. purified and biochemically characterized the protein, L.M. and J.L. determined and optimized the crystallization conditions, L.M. and J.L. collected the crystallographic data and solved the structures. L.M., J.L., and J.F.-M. refined the structures. L.M., J.F.-M., C.R., and R.S. analyzed and interpreted the structures, L.M. performed and analyzed, with C.R., the LC/MS experiments, L.M. wrote the manuscript, J.F.-M. and R.S. edited the manuscript.

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rare comparison of the substrate and product complexes of an enzyme that acts on saccharolipids, and perhaps reveal a novel and specific lipid-binding domain⁸.

Results

Flexibly linked binding and catalytic domains

The CcLpxI structure (Fig. 2 and Table 1) shows that it incorporates a novel lipid-binding domain attached by a flexible linker to a C-terminal, nucleotide phosphodiester hydrolase. In solution, CcLpxI chromatographs as a dimer (Supplementary Fig. 1a) in which each monomer possesses two domains (Fig. 2). The N-terminal lipid X-binding domain (LXD) (residues 1 through 118) is comprised of a five stranded parallel β -sheet surrounded by two α -helices on one side and three on the other. The LXD is bisected by a lipid-binding cleft formed by β -strands 1–5 on one side, and by α -helices 3–5 on the other (Fig. 2). A linking region (residues 118–144), containing an α -helix, connects the LXD to the C-terminal catalytic domain of CcLpxI (ICD). The N-terminal domain of CcLpxI lacks sequence homology to any other known protein.

The ICD (residues 144–280) is comprised of a five stranded mixed β -sheet bracketed by one α -helix on one side and three on the other (Fig. 2). This domain has structural homology though no sequence homology to a superfamily that includes cytosine deaminase⁹ and various other proteins without conservation of active sites or mechanisms. This motif presumably can be tuned to different enzymatic function.

Among the many LpxI homologues of previously unknown function, the ICD contains 12 absolutely conserved polar residues (Fig. 3), which we probed by mutagenesis for their role in catalysis, some of which we determined to be necessary for CcLpxI activity (Supplementary Table 1). Three of these residues, Gln220, Asp225, and Arg223, are present on a loop between ICD β 3 and α 3 (Fig. 2a). No polar residues are absolutely conserved in the LXD of CcLpxI and its homologues, consistent with non-specific hydrophobic interactions driving the association of the aliphatic chains with LXD.

CcLpxI unexpectedly co-purifies and co-crystallizes with its product lipid X

Intending to crystallize “apo” CcLpxI, we were surprised to discover that wild-type CcLpxI co-purified and co-crystallized with its product, lipid X, which is normally present at < 0.2% of total *E. coli* lipid¹⁰. The over-expression of the enzyme unexpectedly caused the accumulation of lipid X, to the level of ~25% of total chloroform soluble *E. coli* lipids (unpublished data, L.M.). This demonstrates that the overexpressed enzyme is active, and suggests that the synthetic pathway to LPS may be regulated to match the throughput of CcLpxI, and restricted in this case by the throughput of the next enzyme LpxB, which uses one molar equivalent of the LpxI substrate, and one equivalent of the LpxI product (see Fig. 1b).

The experimental density maps (2.9 Å) revealed lipid X bound to LXD, but distantly from all residues in the catalytic domain (see Fig. 4a,b,c). The B-factors of the acyl moieties of lipid X are similar to those of the surrounding side chains in the LXD showing that the product is bound with high occupancy. The lipid X binding site is remarkable in that the aliphatic chains are completely buried and surrounded almost entirely by hydrophobic interactions. The glucosamine-1-phosphate moiety is positioned far out into the solvent and the density is consistent with two orientations of lipid X related by a 180° rotation about the long axis (see Fig. 4a,b,c, and Supplementary Fig. 2). The hydrophobic pocket enveloping the acyl chains is precisely complementary to the geometry of a hexose acylated in the equatorial configuration at the 2,3 positions. This may explain the selectivity (as observed for the product here) against binding glycerolphospholipids. The latter can adopt the same

geometry, but with an entropic cost not necessary in the fixed non-reducing hexose ring locked by the 1-phosphate. The binding pocket (Fig. 4c) was calculated to have an internal surface area of $\sim 650 \text{ \AA}^2$, and a volume of $\sim 800 \text{ \AA}^3$ (MetaPocket 2.0¹¹ and CaspT¹²).

To confirm the identity of the molecule that co-purified with CcLpxI, the protein was subjected to a single-phase acidic Bligh-Dyer¹³ extraction, and the organic phase subjected to liquid chromatography run in-line with a time-of-flight electrospray mass spectrometer operating in negative ion mode, as previously described.¹⁴ A singly charged species of exact mass $[M-H]^{-1} 710.42 \text{ amu}$, consistent with pure lipid X, was isolated as a discrete peak eluting from the normal phase column at 22.1 minutes (Supplementary Fig. 3a). Secondary fragmentation yielded components only consistent with lipid X (Supplementary Fig. 3b,c). The total spectrum of the chromatogram did not contain peaks consistent with any other co-purifying lipids, including the common *E. coli* glycerolphospholipids (Supplementary Fig. 3a).

To determine the stoichiometry of lipid X:CcLpxI, we measured the fraction of lipid X bound to CcLpxI by comparing $^{32}\text{PO}_4$ -lipid X on CcLpxI purified from *E. coli* grown in $^{32}\text{PO}_4$, versus total $^{32}\text{PO}_4$ -lipid X, in a manner analogous to that previously described by Metzger and Raetz¹⁵. The total mass of phospholipids was determined from a matched non-radioactive growth. The amount of total lipid X was evaluated by knowing the fraction of total extractable lipids that was lipid X based on thin layer chromatograms. The amount of CcLpxI-bound radiolabeled lipid X was compared with the amount of CcLpxI determined by bicinchoninic acid assay. The stoichiometry of lipid X:protein was estimated to be 0.95:1 (consistent with 1:1 as seen in the structure).

A single point mutant of CcLpxI co-purifies with its substrate

Since CcLpxI co-purified with its product lipid X, we sought to produce a catalytically deficient point-mutant wherein the substrate UDP-2,3-diacylglucosamine was bound to the enzyme, but not hydrolyzed. We therefore made separate mutations to alanine at each of a selected set of seven polar residues in the ICD that are absolutely conserved among CcLpxI orthologues (Fig. 3), and expressed these constructs in *E. coli* (Supplementary Table 1, and Supplementary Figs. 4,5). The chloroform-soluble fractions from acidic Bligh-Dyer extractions of crude lysates from these cells were analyzed by electrospray time-of-flight mass spectrometry (Supplementary Fig. 6). In *E. coli* in which mutant CcLpxI-D225A was expressed, we observed the accumulation of doubly charged and singly charged species consistent with the substrate UDP-2,3-diacylglucosamine (Supplementary Fig. 6i,j). The MS/MS fragmentation pattern for the singly charged species isolated from purified CcLpxI-D225A established that it was UDP-2,3-diacylglucosamine (Supplementary Fig. 3d,e,f). No species of UDP-2,3-diacylglucosamine were observed in *E. coli* expressing empty vector (Supplementary Fig. 6b,c), wild-type CcLpxI (Supplementary Fig. 6e,f), or the other CcLpxI point mutants.

CcLpxI-D225A was purified to near-homogeneity as with CcLpxI (Online Methods). We confirmed that CcLpxI-D225A co-purified with UDP-2,3-diacylglucosamine by directly spotting protein from size exclusion chromatographic fractions on a thin layer chromatography plate, and visualizing the lipid by charring with H_2SO_4 (Supplementary Fig. 1). We performed LC MS/MS on the single-phase acidic Bligh-Dyer extracted purified CcLpxI-D225A as described above for CcLpxI. A singly charged species having a mass consistent with UDP-2,3-diacylglucosamine (exact mass/charge ratio of $[M-2H]^{-2}$ of 507.73, or $[M-H]^{-1}$ of 1016.47) was isolated in a discrete peak eluting from the normal phase column at 21.2 minutes (Supplementary Fig. 3d). Secondary fragmentation yielded ions characteristic of UDP-2,3-diacylglucosamine (Supplementary Fig. 3e,f). The total spectrum of the LC run did not contain peaks consistent with other co-purifying lipids,

including common *E. coli* glycerolphospholipids (Supplementary Fig. 3d). Therefore Asp225 is a key catalytic residue and D225A still binds its substrate without hydrolyzing it.

The structure of CcLpxI-D225A reveals that the substrate-binding pocket is comprised of two domains

Purified CcLpxI-D225A was crystallized in different conditions and in a different space group than CcLpxI (Table 1). The structure was therefore determined independently by single-wavelength anomalous diffraction to 3.0 Å resolution from Se methionine derivatized crystals. A low-resolution structural model was built into this density and used to phase a 2.55 Å native data set. Strong density indicated that UDP-2,3-diacetylglucosamine substrate spanned both the LXD and the ICD; with LXD enveloping the acyl chains of the lipid X moiety, and ICD engaging in well defined hydrophilic interactions with the head-group (Fig. 4d,e,f). The glucosamine moiety of the substrate is in strained chair conformation (Fig. 4e). The LXD and ICD domains are hinged closed around the substrate (Fig. 4f). As a result of domain closure, the binding pocket has a greatly increased internal surface area of ~ 1500 Å², and volume of ~ 2500 Å³ relative to 650 Å², 800 Å³ in the product liganded structure. The B-factors of the UDP-2,3-diacetylglucosamine atoms are similar to those of the surrounding side chains (Fig. 5a)

Evidence for the CcLpxI dimerization interface

The molecular weight of CcLpxI is 29.4 kDa. The apparent solution molecular masses of purified CcLpxI-D225A and CcLpxI were estimated by sedimentation equilibrium as described in the Methods. For CcLpxI, two separate measurements yielded apparent molecular masses of 58.9 and 56.9 kDa, indicating that the protein is a dimer in solution. For CcLpxI-D225A, measurements yielded 63.7 and 57.0 kDa. Elution volumes on calibrated size exclusion columns are also consistent with both CcLpxI and LpxI-D225A being dimers. Both CcLpxI (space group I4₁22) and CcLpxI-D225A (P6₁22) share a common interface across a crystallographic two fold axis (Fig. 5b,c). The interface in CcLpxI has a buried surface area of 2*1150 Å² versus 2*1225 Å² in CcLpxI-D225A. Comparison of interactions between monomers using the program PISA¹⁶ predicted this to be a stable interface. E185 and R193, the only conserved polar residues outside the substrate-binding cavity lie in the interface. They form an intramolecular buried salt bridge, and coordinate an intermolecular pi-stacking of both R193 amidinium groups in the interface (see Fig. 5a inset). When Glu185 was mutated to alanine CcLpxI-E185A and expressed in *E. coli*, the UDP-2-3-diacetylglucosamine hydrolase activity of the crude lysate was reduced ~ 400-fold relative to wild-type CcLpxI (Supplementary Table 1, and Supplementary Fig. 5), and it was proteolyzed *in vivo* (Supplementary Fig. 4), suggesting a role for this interaction in stabilizing the dimer interface. The R193A mutant of CcLpxI had a wild-type level of UDP-2,3-diacetylglucosamine activity (Supplementary Table 1 and Supplementary Fig. 5), and thus its role in putative interface stabilization is unclear.

The domains of the CcLpxI-D225A-substrate complex swing open on forming the CcLpxI-product complex

While LXD and ICD are closed in the substrate-bound D225A complex, they are opened by ~70° and rotated by ~80° in the product-bound complex (Fig. 5b,c). The domains are essentially unchanged between states (rmsd 0.6 Å for Cα of the LXD; rmsd 0.46 Å for Cα of the ICD on superposition). The linking loop in the CcLpxI-D225A-substrate complex becomes a helix in the CcLpxI-product complex (Fig. 2 and Fig. 5b,c).

While the observed conformational differences between CcLpxI-D225A and CcLpxI may represent states along a true catalytic pathway, we cannot exclude that these conformations are artifacts of crystal packing. To probe the solution conformations, we therefore

determined their sedimentation velocities. The sedimentation coefficient of CcLpxI-D225A was 3.85 ± 0.09 svedbergs ($n = 5$) while that of CcLpxI was 3.56 ± 0.11 svedbergs ($n = 5$), consistent with the more elongated shape of the product complex. Additional evidence for the substrate complex having a smaller hydrodynamic radius than the product complex is the increase in elution volume on a calibrated size exclusion column (Supplementary Fig. 1a).

Discussion

An interfacial lipid hydrolase

LpxI is a UDP-2,3-diacylglucosamine hydrolase present in many Gram-negative bacteria. In these species, it plays an essential role in the biosynthesis of lipid A (Fig. 1), the hydrophobic anchor of lipopolysaccharide (LPS) in several pathogenic genera including *Rickettsia*, *Brucella*, and *Leptospira*^{1,2}. Here we report the first structure of any UDP-2,3-diacyl glucosamine hydrolase, which represents a new family of phosphodiester hydrolases. As it is required for growth and viability of these and other lethal pathogens, and as it has no apparent mammalian homologue, LpxI is a potentially attractive enzyme target for antimicrobials. LpxI is a divalent metal ion-dependent hydrolase that catalyzes the attack of water on the β -phosphate of UDP-2,3-diacylglucosamine, an integral membrane lipid substrate and product⁷ (Fig. 1b).

Crystals of CcLpxI were obtained in the presence of lipid X, which serendipitously co-purified stoichiometrically with CcLpxI. The structure, determined at 2.9 Å resolution, consists of two domains: an N-terminal lipid X binding domain (LXD), and a C-terminal LpxI catalytic domain (ICD) (Fig. 2 and Fig. 5). This is a new arrangement of domains; each domain has a fold similar to distantly related protein domains having unrelated functions, but the combination of these in one protein is novel. The hydrophobic binding pocket is strikingly similar to that observed in Sec14¹⁷ and in various PH-domain (lipid binding domain)-fused proteins^{8,18}. Relatively few residues are positioned such that they might interact with the head group of lipid X. For this reason, the mode of lipid X binding to CcLpxI is reminiscent of how exogenous lipids are bound by many members of the CD1 family of receptors^{19,20}, or of how lipid A binds to human Toll-like receptor 4^{21,22}, albeit without participation of the 1-phosphate. In this regard, LpxI differs from the structure of product-bound LpxA, the first enzyme of the lipid A biosynthetic pathway, which has high specificity for its ligand's hydrophilic head-group^{23,24}. To date, fewer than twenty families of lipid binding domains have been described^{25,26}; the LXD of CcLpxI represents a new specific lipid-binding domain.

Thermodynamic coupling at the dimer interface

The presence of two absolutely conserved LpxI residues at the dimer interface (Fig. 5) supports the idea that it is an obligate dimer of catalytic subunits. The substrate-bound form has 150 Å² larger buried surface area than the product bound dimer, and has several more hydrogen bonds and salt bridges. This suggests that the cost of introducing strain (Fig. 4e,f) into the glucosamine moiety upon enzyme closure can be balanced in part by the dimer interface to favor substrate binding and product release when substrate is available, in this conformationally coupled mechanism.

Interfacial catalytic mechanisms

We propose a catalytic model in which the LXD and ICD each form halves of an inter-domain active site (Fig. 6). The mode of LpxI catalysis requires Asp225, Mg⁺², and catalytic water(s) not yet seen in the D225A structure. The fact that we purified substrate (in the mutant) and the lipid-product complexes from whole cells suggests that the default state *in vivo* is lipid-product bound (without UMP bound), and that available substrate must

contribute to product release (Fig. 6). The driving force for the LXD and ICD domain rearrangement could be hydrolysis and release of the soluble UMP product. Another possible driving force is the strained conformation of the substrate's chair form glucosamine ring (Fig. 4e,f). Substrate binding and product release must be effected either by adjacent enzymes in a hypothetical multimeric lipid A synthome, such as LpxB (Fig. 1b), or by the lipid bilayer.

Glutamates and aspartates play diverse roles in phosphodiester cleavage. We have shown that Asp225 is required for LpxI-catalyzed hydrolysis. However, even within a family of hydrolases, such as the nudix proteins^{27–31}, these “catalytic bases” can have different functions, including the direct deprotonation of the catalytic water, the orientation of a catalytically essential water shell, the liganding of divalent metals which in turn coordinate catalytic water and/or the substrate's phosphodiester moiety. Although detailed mechanistic hypotheses cannot be developed based on the present data, it is clear that CcLpxI operates differently than most other nucleotide pyrophosphatases. While such phosphatases perform catalysis by a variety of mechanisms and with sundry ensembles of active-site residues, most appear to do so without major macro-molecular rearrangements. CcLpxI differs in evoking large conformational dynamic trajectories. Asp225's role in LpxI could be probed further by mutating Asp225 to Cys, then reacting the resulting D225C mutant with iodoacetate, restoring a carboxyl. Asp225's role may be directly catalytic, or may affect substrate binding.

To our knowledge, CcLpxI is the only example of a nucleotide pyrophosphatase that remains bound to its non-nucleotide product following catalysis. This unusual property may reflect the cost of solvating lipid X binding site/lipid X on removal of the protein from the bilayer environment (Fig. 6). We therefore speculate that with LpxI substrate binding drives the release of lipid X. In this regard, LpxI may more closely resemble two-domain glycosyl-transferases, wherein acceptor nucleotide-sugar hydrolysis appears to drive domain movements and/or product release³².

An interesting possibility is that LpxI, under chromosomal expression control, may normally form a complex with the next enzyme in the pathway, LpxB (Fig. 1b), in its cognate species. The lipid A disaccharide synthase LpxB, like LpxI, acts on lipid substrates located in the inner leaflet of the inner membrane of Gram-negative bacteria. Given the low steady-state levels of UDP-2,3-diacylglucosamine and lipid X in bacteria^{10,33,34}, the hypothesis that LpxI (or its analogue LpxH in other species) transfers one molecule of lipid X for fusion with one of UDP-2,3-diacylglucosamine on LpxB as part of a bacterial lipid A synthesis enzyme complex seems plausible in the Raetz pathway. The present study provides a basis for future biochemical and biophysical characterization of this unique family of metal-dependent UDP-diacylglucosamine hydrolases, and constitutes a starting point for structure-based inhibitor identification and antimicrobial development, perhaps focusing on uridine analogues. The structure opens the horizon for understanding interfacial catalysis of lipids at the membrane surface.

Online Methods

Strain Construction, Protein Purification, and Activity Assay

Briefly, CcLpxI and CcLpxI-D225A were expressed from a high-copy, Isopropyl β -D-1-thiogalactopyranoside-inducible plasmid conferring N-terminal Tobacco Etch Virus protease cleavable 10-histidine tags. Expression was accomplished using a T7 expression system in *E. coli* strain C41(DE3)³⁵. Cells were lysed by mechanical disruption, and tagged CcLpxI or CcLpxI-D225A were purified by immobilized metal exchange chromatography, followed by anion-exchange chromatography, cleavage of the polyhistidine tag using Tobacco Etch

Virus protease, reverse immobilized metal exchange chromatography, and finally size exclusion chromatography.

All CcLpxI assay conditions described in this work were identical to those previously described⁷. Briefly, each 25 μ l reaction contained the following components: 100 μ M UDP-2,3-diacetylglucosamine, 0.5 mg/ml bovine serum albumin (BSA), 0.05% w/v Triton X-100, 20 mM HEPES pH 8.0, and 2 mM $MgCl_2$, and 1000 dpm/ μ l β -³²PO₄ labeled UDP-2,3-diacetylglucosamine. All assays were conducted at 30°C, and quenched by spotting portions of the reaction onto silica TLC plates, which were developed, visualized, and quantified as previously described⁷.

Crystallization and data collection for CcLpxI and CcLpxI-D225A

Native and Se-Met substituted CcLpxI were crystallized and cryo-protected in identical conditions. Mixed drops of CcLpxI (20–30 mg/ml) and well solution were set up in a ratio of 7:3 v/v in sitting-drop vapor-diffusion chambers. The well solution consisted of 100 mM MES pH 5.7, 3–7% w/v PEG 6000, and 2.5% v/v glycerol. Crystals, with typical dimensions of $\sim 200 \times 200 \times 400$ μ m, formed at 4°C in between 1 and 3 days, and continued to grow for 5–7 days. The crystals were rectangular pyramids and bi-pyramids. This crystallization condition was robust, and was reproducible with reagents from different suppliers.

Crystals of CcLpxI were fragile, and disintegrated when touched with nylon loops or other crystal-harvesting implements. Therefore, CcLpxI was cryo-protected *in situ* by gradually exchanging the sitting-drop buffer with glycerol-containing cryo-protection solutions, until a final concentration of 40% v/v glycerol was achieved. During cryo-protection, the dimensions of the crystals decreased slightly, as expected for a vitrification process. In concentrations of glycerol greater than 30% v/v, the CcLpxI crystals could be manipulated without damaging them. While other cryo-protectants were tested, including PEG400, xylitol, MPD, and ethylene glycol, screening revealed that optimal X-ray diffraction could be obtained by cryo-protection with buffers containing 35–40% v/v glycerol, and supplemented with $MgCl_2$ to 2 mM.

Se-substituted CcLpxI-D225A crystals were grown at 20°C, by hanging-drop vapor diffusion. The well solution consisted of 100 mM Tris pH 7.5–9.0, 180–200 mM NaCl, 1.3–1.6 M $(NH_4)_2SO_4$, and 6% v/v glycerol. Large crystals, in the form of hexagonal prisms and spears, often exceeded 500 μ m in length. Crystals appeared in 1–2 days, and continued to grow for another 3–5 days. This crystallization condition was robust, and was reproducible.

Native CcLpxI-D225A were grown at 20°C, by hanging-drop vapor diffusion. The well solution consisted of 1.6–2.2 M NaCl, and 8–14% v/v PEG6000. Large crystals, in the form of hexagonal prisms and spears, often exceeded 500 μ m in length. Crystals appeared in 1–2 days, and continued to grow for another 3–5 days. This crystallization condition was robust, and was reproducible with reagents from different suppliers.

In contrast to CcLpxI, CcLpxI-D225A crystals were not very fragile, and could be easily harvested using nylon loops. Se-methionine CcLpxI-D225A crystals were cryoprotected using well solution substituted with glycerol to $\sim 20\%$ w/v, while native CcLpxI-D225A crystals were cryoprotected in their well solution supplemented with PEG400 in steps of 5% w/v until a concentration of 25% w/v PEG400 was obtained. Cryoprotection with glycerol and ethylene glycol yielded poorer diffraction than did PEG400.

Diffraction data were collected at the Lawrence Berkeley National Laboratory Advanced Light Source, Beamline 8.3.1, under a -170°C nitrogen cryo-stream. As no homologues existed with which to phase CcLpxI, SAD datasets were collected on Se-Met derivatized

crystals at the Se absorption peak. The highest resolution Se-Met crystals diffracted to 2.9 Å. Phases were determined by SAD. For CcLpxI-D225A, SAD and MAD datasets were collected on Se-Met derivatized crystals at the Se absorption peak, near, and far edges. The highest resolution Se-Met dataset diffracted to 3.0 Å. Phases were determined by MAD. Native CcLpxI-D225A datasets were collected to 2.55 Å, and the unrefined structure was used to determine the phases for the native dataset. Data processing was performed using either HKL2000³⁶ or ELVES³⁷. Phasing by molecular replacement was performed using Phenix³⁸. Building was done using Coot³⁹, while refinement was accomplished using Coot, Phenix, and CCP4⁴⁰. The MolProbity^{41–44} server was employed for structural validation and improvement.

Lipid extraction and mass spectrometry

Co-purified lipids were extracted from 0.2 mg samples of protein (or crude *E. coli* cell lysate), purified by the method above, with a 5.8 ml acidic two-phase Bligh-Dyer system¹³. The extraction was performed as previously described¹⁵. When indicated, portions of the lipid extracts were spotted on silica thin layer chromatography plates, separated, and imaged as previously described¹⁵.

LC-MS analysis of total, single-phase Bligh-Dyer extracted protein or *E. coli* crude lysate samples, using a normal phase column run with subsequent monitoring by ESI-MS/MS in the negative mode, was performed as previously described⁴⁵. A 20 µl sample of each 7.6 ml single-phase acidic Bligh-Dyer extraction (0.25% of the total single-phase volume) was loaded onto a ~600 µl normal phase column (2.5% of bed volume). ESI-MS was performed by a QSTAR XL time-of-flight mass spectrometer coupled to an LC system. Data were analyzed with the aid of the Analyst QS software package.

Analytical ultracentrifugation

Sedimentation equilibrium experiments were used to estimate the molecular weights of purified CcLpxI and CcLpxI-D225A. Samples were prepared by diluting concentrated protein stocks into their storage buffer (20 mM HEPES pH 8.0, 200 mM NaCl). For each construct, concentrations of 1, 2, and 3 mg/ml were each centrifuged at 8,060, 18,150, and 201,600 × *g*, in a Beckman An-60Ti rotor, using a Beckman Optima XL-A analytical ultracentrifuge. Centrifugation was performed at 20°C. Samples were paired with reference cells containing buffer identical to that with which they were diluted. Absorbance was monitored at 240 nm. The program SEDNTERP⁴⁶ was used to calculate the buffer density ($\rho = 1.0088$), and the amino acid sequence of CcLpxI was used to calculate its partial specific volume ($v_{\text{bar}} = 0.7371$). Best-fit buoyant molecular weight (MW^b) was estimated using the XL-A XLI-1 software suite, from which molecular weight was calculated using the equation: $MW = MW^b / (1 - v_{\text{bar}}\rho)$ ^{46,47}. Sedimentation velocity experiments were used to determine the sedimentation coefficients of CcLpxI and CcLpxI-D225A. The proteins were diluted, as above, to 0.7, 1.4, 2.1, 2.6, and 3.1 mg/ml. These samples, paired with reference cells containing the protein buffer, were centrifuged at 2900 × *g* in a Beckman An-60Ti rotor using a Beckman Optima XL-A analytical ultracentrifuge. For CcLpxI, absorbance was monitored at 245 nm, while CcLpxI-D225A was monitored at both 245 and 260 nm. Data were processed using ProteoLab XL-A software, and weight-average sedimentation coefficients (*s*) were determined by second-moment integration of the sedimentation absorbance profiles between the meniscus and solution plateaus⁴⁸.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Lipid A is an essential membrane component of Gram-negative bacteria
a) A cross-section of a generic Gram-negative bacterial cell envelope. Lipid A comprises the hydrophobic membrane anchor of lipopolysaccharide (LPS). LPS is the major structural component of the outer leaflet of the outer membrane. **b)** A summary of the constitutive lipid A biosynthesis pathway in a generic LpxI-containing Gram-negative bacterium. The glucosamine saccharides of the lipid A precursors are drawn in cyan, while lipid A biosynthetic enzymes are shown in purple or blue. LpxI (or LpxH) and LpxB perform catalysis at the cytoplasmic interface of the inner membrane. Kdo₂-Lipid A is the minimum structure required for growth and viability in most Gram-negative bacteria.

Figure 2. Topology and overall fold of CcLpxI and CcLpxI-D225A in complex with lipid X and UDP-2,3-diacylglucosamine, respectively

a) CcLpxI is represented by a topology diagram. Flat arrows are β -strands and cylinders are α -helices. Structural elements colored in blue belong to the “Lipid X Binding Domain” (LXD), while the “LpxI Catalytic Domain” (ICD) is colored yellow-orange. The domains are joined by an α -helical linking region, which is colored dark purple. **b,c)** These panels show ribbon diagrams of the X-ray crystal structures of CcLpxI and CcLpxI-D225A, respectively, colored as in (a). Ligands are rendered in teal sticks, with oxygen colored red, nitrogen colored blue, and phosphorous colored dark orange.

Figure 3. Absolutely conserved polar residues mapped to the structure of CcLpxI-D225A
a) CcLpxI-D225A, in grey cartoon rendering of the putative CcLpxI-D225A dimer with a semi-transparent grey surface shown on one monomer of the dimer, with its substrate, UDP-2,3-diacylglucosamine, shown as yellow sticks, absolutely conserved polar residues thought to be involved in substrate binding and/or catalysis are shown as teal sticks, and absolutely conserved polar residues residing at the hypothetical dimerization interface are shown as magenta sticks. The C-β of Ala225 is shown as a purple stick, and is labeled D225A to indicate its mutation from Asp225 in this structure. **b)** Absolutely conserved polar residues within the wild-type Cc-LpxI sequence (boxed, colored residues). Residues thought to be involved in substrate binding and/or catalysis are colored in teal, residues hypothesized to be involved in dimerization are shown in magenta, and Asp225 is shown in purple. An asterisk under a residue indicates that alanine-scanning mutagenesis was performed on that residue (see Supplementary Table 1).

Figure 4. Ligand binding in CcLpxI and CcLpxI-D225A

a), A schematic model of the lipid X binding site in CcLpxI. **b,c)**, Two views of lipid X in the binding pocket of CcLpxI. Lipid X was refined in two overlapping, partially occupied orientations (see Supplementary Fig. 2); for clarity, only the major orientation is shown here. **d-f)**, CcLpxI-D225A in complex with UDP-2,3-diacylglucosamine. Coloring matches that in Fig. 2. In (c) and (f), grey shading indicates the ligand binding pockets.

Figure 5. CcLpxI dimerization

a), the CcLpxI-D225A dimer colored by B-factor (with red corresponding to high B-factor and blue to low B-factor), with the inset panel showing absolutely conserved Glu185/Arg193 dyad positioned at the interface. **b,c)**, Dimers of substrate-bound CcLpxI-D225A and product-bound CcLpxI, respectively, colored as in Fig. 2.

Figure 6. A model for CcLpxI-mediated catalysis

a–f) One possible model of LpxI catalysis at the membrane interface. Lipid X and UDP-2,3-diacylglucosamine are shown as cartoons: teal circles with attached lines represent 2,3-diacylglucosamine moieties, red circles are phosphates, and white boxes denote uridine. The LpxI cartoon shows a dimer, and is colored as in Fig. 2. **a)** CcLpxI searches the membrane interface for its substrate. **b)** The enzyme binds to its substrate. **c)** Withdrawal of substrate from the bilayer and domain movement result in the release of product into the membrane. **d)** LpxI hinged shut, with the UDP-2,3-diacylglucosamine positioned for catalysis; this state may be represented by the CcLpxI-D225A structure. **e)** Phosphodiester hydrolysis and/or UMP release may drive opening of the active site, returning LpxI to its open, product-liganded state in **(f)**. While this schematic shows simultaneous action of monomers, CcLpxI catalysis may be cooperative.

Table 1

Data collection and refinement statistics

	Native CcLpxI-D225A	SeMet CcLpxI-D225A	SeMet CcLpxI
Data collection			
Space group	P6 ₁ 22	P6 ₁ 22	I4 ₁ 22
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	107.30, 107.30, 81.29	112.95, 112.95, 81.35	74.29, 74.29, 364.64
<i>α</i> , <i>β</i> , <i>γ</i> (°)	90, 90, 120	90, 90, 120	90, 90, 90
		<i>Peak</i>	<i>Peak</i>
Wavelength	1.11	0.9795	0.9796
Resolution (Å)	61.2 – 2.55	50.0 – 3.0	45.5 – 2.9
<i>R</i> _{sym} or <i>R</i> _{merge}	12.7 (99.1)	7.4 (59.2)	9.0 (63.4)
<i>I</i> / <i>σI</i>	32.0 (3.3)	28.0 (2.8)	17.8 (3.2)
Completeness (%)	98.1 (95.0)	98.3 (98.3)	99.7 (99.0)
Redundancy			
Refinement			
Resolution (Å)	40 – 2.55		45.5 – 2.9
No. reflections	9603		11,932
<i>R</i> _{work} / <i>R</i> _{free}	22.8/28.5		21.6/27.2
No. atoms			
Protein	1977		2044
Ligand/ion	68		49
Water	19		7
<i>B</i> -factors			
Protein	81.4		94.1
Ligand/ion	60.4		112.2
Water	64.4		74.9
R.m.s. deviations			
Bond lengths (Å)	0.009		0.008
Bond angles (°)	1.46		1.31