UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacylase (LpxC) is a zinc-dependent enzyme that catalyzes the deacetylation of UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine to form UDP-3-O-(R-hydroxymyristoyl)-glucosamine and acetate. The structural similarity of the active site of LpxC to metalloproteases led to the proposal that LpxC functions via a metalloprotease-like mechanism. The pH dependence of kcat/KM catalyzed by E. coli and A. aeolicus LpxC displays a bell-shaped curve (EcLpxC yields apparent pKₐ values of 6.4 ± 0.1 and 9.1 ± 0.1) demonstrating that at least two ionizations are important for maximal activity. Metal substitution and mutagenesis experiments suggest that the basic limb of the pH profile is due to deprotonation of a zinc-coordinated group such as the zinc-water molecule while the acidic limb of the pH profile is caused by protonation of either Glu78 or His265. Furthermore, the activity decreases and synergy observed for the active site mutants suggest that Glu78 and His265 act as a general acid-base catalyst pair. Crystal structures of LpxC complexed with cacodylate or palmitate demonstrate that both Glu78 and His265 hydrogen bond with the same oxygen atom of the tetrahedral intermediate and the product carboxylate. These structural features suggest that LpxC catalyzes deacylation using Glu78 and His265 as a general acid-base pair and the zinc-bound water as a nucleophile.

Lipopolysaccharide (LPS)1 molecules form the outer-membrane of Gram-negative bacteria and serve to exclude hydrophobic and negatively charged molecules. Lipid A is the hydrophobic portion of LPS that is responsible for anchoring LPS to the membrane and is essential for the viability of Gram-negative bacteria.1 Lipid A is also known as endotoxin and is the immunomodulatory portion of LPS that triggers the immune system in septic shock. As a consequence, inhibition of lipid A biosynthesis is proposed as a strategy for both the development of novel antibiotics and antiendotoxins in the treatment of septic shock.1-5

In E. coli lipid A is synthesized from UDP-N-acetyl glucosamine in a ten step pathway (Figure 1). UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacylase (LpxC) is a zinc-

1 Abbreviations: LPS, lipopolysaccharide; LpxC, UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacylase; UDP-GlcNAc, uridine-5′-diphosphate-N-acetylglucosamine; myrUDP-GlcNAc, UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine; DTT, dithiothreitol; BSA, bovine serum albumin; IPTG, isopropyl D-thiogalactopyranoside; TCEP, tris(carboxyethyl)phosphine; GABC, general acid-general base catalyst; GBC, general base catalyst; GAC, general acid catalyst.; AALpxC, Aquifex aeolicus LpxC; EALpxC, Escherichia coli LpxC.
dependent enzyme that catalyzes the hydrolysis of UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine (myrUDP-GlcNAc) to form UDP-3-O-(R-hydroxy-myristoyl)-glucosamine and acetate. The deacetylation of myrUDP-GlcNAc is the committed step in the biosynthesis of lipid A(7); therefore, this enzyme is a target for the development of inhibitors as antibiotics for the treatment of Gram-negative infections.(2,8-10) A comprehensive understanding of the catalytic mechanism and structure of LpxC will facilitate the development of potent and specific inhibitors of this enzyme.

The enzyme LpxC belongs to a group of enzymes known as the zinc hydrolases. Mononuclear zinc hydrolases can be broadly categorized by two general catalytic mechanisms, one that uses a single bifunctional general acid-base catalyst (GABC) (i.e. metalloproteases) and a second mechanism that uses a GABC pair (i.e. histone deacetylases) to carry out catalysis.(11-14) The structure of A. aeolicus LpxC (AaLpxC) has been solved using x-ray crystallography(15) and NMR spectroscopy(16). This structure (Figure 2) reveals that LpxC contains a unique fold and a novel zinc binding motif, both of which distinguish LpxC from other known zinc hydrolases.(15) One bound zinc ion is an essential cofactor for LpxC catalytic activity (Zn$^{2+}$$_A$), while excess zinc leads to enzyme inhibition(6) by Zn$^{2+}$$_B$ (Figure 2B).(15) The catalytic zinc ion (Zn$^{2+}$$_A$) is coordinated by His79, His238, Asp242, and a solvent molecule, while the inhibitory Zn$^{2+}$$_B$ ion is coordinated by Glu78, His265, a fatty acid and a bridging solvent molecule. Previous mutagenesis experiments suggest that three active site residues (Glu78, Asp246 and His265) are important for catalytic activity, as mutation of these residues to Ala decreases the catalytic activity $>$10$^2$-fold.(17) On the basis of these structural and mutagenesis data, LpxC was proposed to function via a metalloprotease-like mechanism using a single general acid-base catalytic side chain (Glu78) and a zinc-water nucleophile to catalyze deacetylation of its substrate (Figure 3a), as described in detail for metalloproteases.(11-13,15) The Zn$^{2+}$$_A$ ion and His265 provide stabilization of the transition states and oxyanion intermediate prior to protonation of the amine leaving group and collapse of the oxyanion intermediate. In contrast, the deacetylation mechanism for histone deacetylases has been proposed to use a pair of His side chains, one functioning as a general acid and the other as a general base to activate the zinc-water nucleophile.(12,18) The details of the LpxC catalytic mechanism have not yet been fully elucidated. The pH dependence of AaLpxC wild-type and mutants has recently been reported(19) suggesting that Glu78 functions as a general base.

Here, we investigate the catalytic mechanism of LpxC using metal substitution, mutagenesis, pH dependence, and x-ray crystallography. The $k_{cat}/K_M$ for E. coli LpxC (EcLpxC) has a bell-shaped dependence on pH with two pK$_a$'s of $\sim$6.4 and 9.1 similar to values recently reported for AaLpxC.(19) Metal-substitution and mutagenesis experiments suggest that the basic limb of the pH profile represents ionization of a metal-coordinated group, such as the zinc-water molecule. Kinetic evaluation of LpxC mutants and crystal structures of LpxC complexed with cacodylate or palmitate support a mechanism in which Glu78 and His265 function as a general acid-base catalyst pair, wherein the His265 side chain functions as a general acid to protonate the amine leaving group while Glu78 functions as a general base to activate the zinc-water nucleophile.

**EXPERIMENTAL PROCEDURES:**

**Mutagenesis and Protein Expression**

All mutant plasmids were prepared using the Quik-change site-directed mutagenesis kit (Stratagene). The LpxC variants were over-expressed and purified according to published procedures.(6,8,20,21) For expression of less stable LpxC mutants, cells were incubated overnight at 25 °C following addition of isopropyl D-thiogalactopyranoside (IPTG). Additionally, 100 μM IPTG and 200 μM ZnSO$_4$ were added at induction for expression of the D246A EcLpxC mutant.

All purification steps were carried out at room temperature and 4 °C for AaLpxC and EcLpxC, respectively. Following lysis using a microfluidizer, the cell debris was pelleted by centrifugation and the supernatant was loaded onto a DEAE-sepharose column in 25 mM HEPES pH 7, 2 mM dithiothreitol (DTT). Both Aa and EcLpxC were eluted with a linear gradient (0 to
acetyl-glucosamine was prepared from [14C]-UDP-3-O-
triscarboxyethylphosphine (TCEP, 0.5 mM) and
mixtures containing buffer, bovine serum albumin
previously described. The deacetylase activity was measured as
cleaved by LpxC.

contaminating UDP-GlcNAc. Additionally, > 95 % pure as assessed by SDS-PAGE and
were obtained in 90-99% yield, as determined by
tLC analysis verified that
was measured in 20 mM bis-tris propane, pH 7.5,
at seven to nine different concentrations (25 nM to
8 µM) of myrUDP-GlcNAc. The steady state parameters $k_{cat}$, $K_M$ and $k_{cat}/K_M$ were obtained by
fitting the Michaelis-Menten equation to the initial linear velocities measured at various substrate
concentrations.

For the pH dependence experiments, LpxC activity was assayed under $k_{cat}/K_M$
conditions at several concentrations of myrUDP-GlcNAc (50 – 200 nM) to demonstrate a linear
dependence on substrate concentration ([S] < $K_M$)
in the pH range of 5.5 to 10.5. Wild-type and the
mutant LpxC enzymes were linearly dependent on
substrate concentration with the exception of E78A and E78A/H265A at high pH where little
turnover was observed at concentrations below
200 nM, consistent with a $K_M > 200$ nM. The
buffers were either: a combination of 100 mM
acetate/50 mM bis-Tris/50 mM triethanolamine
over the entire pH range or 20 mM MES (pH 5.5-
6.5), 20 mM bis-tris propane (pH 7-9.1) or 20 mM
CAPS (pH 9.8-10.7). Eq. (1) (two ionizations)
was fit to the pH rate profiles. Eq. (2) was fit to
the pH rate profiles for the E78A/H265A mutants,
where $k_1$ is the $k_{cat}/K_M$ at the pH optimum and $k_2$ is
the pH independent value of $k_{cat}/K_M$ at low pH.
LpxC is stable over this pH range under the assay
conditions. The catalytic activity of LpxC
decreases ≤ 2-fold after incubation at the altered
pH for ≤ 5 min followed by measuring the activity at
pH 7.5. For the solvent isotope effect experiments, the initial rates at subsaturating
substrate concentrations in H2O were compared to
the initial rates in ~ 95% D2O. The pD values
obtained for the D$_2$O buffers using the pH meter
readings were corrected by adding 0.4 to these
values.(24)

Eq. (1) \[
\frac{k_{cat}}{K_M} \text{obs} = \frac{k_1}{1 + [H] + [H]^{1/2}}
\]

Eq. (2) \[
\frac{k_{cat}}{K_M} \text{obs} = \frac{k_1 + k_2([H]/[H])}{1 + [H] + [H]^{1/2}}
\]
Crystallography

The C193A/ΔD284-L294 variant of \textit{A. aeolicus} LpxC was used for crystallography experiments as described.\cite{15} Crystallization was achieved by equilibrating a hanging drop containing 3 µL of protein solution (3 mg/mL LpxC, 100 mM HEPES (pH 7.5), 180 mM NaCl, 9-14% PEG3350, and 0.5 mM ZnSO₄) and 3 µL of precipitant buffer [100 mM HEPES (pH 7.5), 180 mM NaCl, 9-14% PEG3350, and 0.5 mM ZnSO₄] over a reservoir containing approximately 1 mL of precipitant buffer. Crystals with maximum dimensions of 0.3 x 0.15 x 0.15 mm³ grew within 3 days and were gradually transferred to a stabilization buffer of 100 mM sodium cacodylate (pH 6.0), 180 mM NaCl, 11-16% PEG 3350, 0.5 mM ZnSO₄, and 1% glycerol. Crystals were flash-cooled in liquid nitrogen following cryoprotection with 22% glycerol and diffracted X-rays to 2.1 Å at Argonne National Laboratory (IMCA-CAT Argonne, IL). Crystals were isomorphous with those prepared at pH 7.0 \cite{15} and belong to space group \textit{P6₁}, with unit cell dimensions \textit{a} = \textit{b} = 101.3 Å, \textit{c} = 122.7 Å. Data were indexed and merged using the program HKL2000.\cite{25} The structure was solved by molecular replacement using the zinc-inhibited enzyme, excluding all zinc ions, solvent and fatty acid molecules, as a search probe for rotation and translation functions calculated with the program AmoRe.\cite{26} It was clear in initial electron density maps that the inhibitory metal ion, Zn²⁺, had dissociated, and that a tetrahedral cacodylate anion was coordinated to the catalytic metal ion, Zn²⁺. In native \textit{AaLpxC}, a fatty acid interpretable as myristate or disordered palmitate occupies the hydrophobic tunnel and coordinates to Zn²⁺; since fatty acids are not included in the crystallization medium, this fatty acid must be a remnant of heterologous expression in \textit{E. coli}. The fatty acid remained bound in the LpxC-cacodylate complex and its carboxylate group was displaced to two alternate conformations as a consequence of Zn²⁺ dissociation. Iterative cycles of refinement and model rebuilding were performed with the programs CNS (27) and O (28), respectively, to improve the structure as monitored by \textit{R} \textit{free}. Data collection and refinement statistics are reported in Table 1.

In a second experiment, crystals were gradually transferred to a stabilization buffer containing 100 mM bis-Tris (pH 6.0), 180 mM NaCl, 11-16% PEG 3350, 0.5 mM ZnSO₄, and 1% glycerol. Following transfer to a 22% glycerol cryoprotectant, crystals were flash-cooled in liquid nitrogen and yielded X-ray diffraction data to 2.7 Å using an R-axis IV++ image plate detector mounted on a Rigaku-200HB rotating anode X-ray generator. Diffraction data were indexed and merged using the program d*Trek.\cite{29} The structure of zinc-inhibited LpxC, excluding all zinc ions, solvent and fatty acid molecules, was used as a search probe in molecular replacement calculations with the program EPMR \cite{30} to phase the initial electron density map. The atomic model was refined and rebuilt using the programs CNS (27) and O (28), respectively. Strict NCS constraints were used during the initial stages of refinement and then relaxed into appropriately weighted restraints as indicated by \textit{R} \textit{free} as refinement progressed. Data collection and refinement statistics are reported in Table 1. Figures 2a, 7a, and 8a were prepared using the program Bobscript.\cite{31}

RESULTS:

Mutations decrease catalytic activity

Previous mutagenesis experiments\cite{17} and the crystal structure of the zinc-inhibited LpxC\cite{15} have suggested that residues Glu78, His265 and Asp246 are important for catalytic activity. Therefore, the steady-state parameters \textit{K} \textit{M}, \textit{k} \textit{cat} and \textit{k} \textit{cat}/\textit{K} \textit{M} were determined for the E78A, H265A, D246A and E78A/H265A \textit{EcLpxC} mutants and compared to the values obtained for WT \textit{EcLpxC} (Table 2). The E78A, D246A and H265A single mutations all decrease \textit{k} \textit{cat}/\textit{K} \textit{M} by 400- to 2200-fold, while the largest decrease in \textit{k} \textit{cat}/\textit{K} \textit{M} was measured for the E78A/H265A double mutant (~1.5 x 10⁴-fold). The decrease in \textit{k} \textit{cat}/\textit{K} \textit{M} values observed for the LpxC mutants is predominantly explained by a decrease in the \textit{k} \textit{cat} values for these enzymes. The smallest decrease in \textit{k} \textit{cat} was observed for the E78A mutant (18-fold), while the largest decrease was observed for the E78A/H265A mutant (1.6 x 10⁴-fold). These mutations have more modest effects on the \textit{K} \textit{M} values, with the E78A mutation causing the largest increase (23-fold). The observed changes in the \textit{K} \textit{M} values rule out the possibility that the activity
in the mutants is due to WT contamination. To further elucidate the functional roles of these residues we measured the pH dependence of catalysis.

**pH dependence of the *LpxC*-catalyzed reaction**

The pH dependence of the *LpxC*-catalyzed reaction for the *Ec* and *Aa*-*LpxC* was determined under subsaturating substrate (*k*~cat~/*K*~M~) conditions to identify ionizations in the free enzyme or substrate that are important for catalytic activity. The *k*~cat~/*K*~M~ values include the rate constants for substrate binding through formation of acetate (first irreversible step).(32) In contrast, the kinetic parameter *k*~cat~ includes the rate constants after formation of the E*S* complex through product dissociation. For enzymes where product release is rate-limiting, as is likely for wild-type *EcLpxC* (Jackman and Fierke, unpublished data), *k*~cat~ provides little information about the rate constant of the chemical step (*k*~chem~).

The pH dependence of *Aa LpxC* was also determined since the structure of *A. aeolicus* LpxC is known, the protein stability is enhanced, and *Aa*-*LpxC* can be readily purified from the background WT *E. coli LpxC*. The pH dependence of *k*~cat~/*K*~M~ for WT *EcLpxC* (Figure 4) affords a bell-shaped curve with two apparent p*K*~a~ values of 6.4 ± 0.1 and 9.1 ± 0.1. The p*K*~a~ values measured for the reaction catalyzed by wild-type *Aa*-*LpxC* are decreased by 0.5 to 1 pH unit (Table 3) and are comparable to literature values (19). These data indicate that at least two ionizations in either LpxC or the substrate are important for catalytic activity.

**Mutations alter the pH dependence of *LpxC***

The pH dependence of *k*~cat~/*K*~M~ for LpxC mutants at Glu78, Asp246 and His265 were examined to determine whether ionization of these groups is responsible for one or more of the ionizations observed in the pH profile (Figure 4, Table 3). In *EcLpxC* the mutation of Glu78, Asp246, or His265 to Ala causes a 300- to 3000-fold decrease in *k*~cat~/*K*~M~ at the pH maximum. The same mutations in *Aa*-*LpxC* decrease the value of *k*~cat~/*K*~M~ at the pH maximum about 10-fold less due mainly to a ~6-fold decrease in the catalytic activity of the WT *Aa*-*LpxC*. All of these single mutations in both *EcLpxC* and *Aa*-*LpxC* retain a bell-shaped pH dependence consistent with two ionizations and, except for E78A *Aa*-*LpxC*, the p*K*~a~ values remain within 0.5 units of the wild-type values. In E78A *Aa*-*LpxC* the value of p*K*~a1~ increases by ~1 pH unit while the value of p*K*~a2~ decreases, with both of these values approaching 7 (Table 3). Taken together, these data suggest that ionization of none of these groups alone is essential for the bell-shaped pH dependence.

To further examine the origin of the ionizations that alter the activity of LpxC, the pH dependence of the E78A/H265A double mutant was measured in both *EcLpxC* and *Aa*-*LpxC*. These mutants display the largest decreases in *k*~cat~/*K*~M~ (1,700- to 37,000-fold) (Table 3), consistent with the loss of a GABC. Furthermore, the pH dependence of these mutant enzymes is significantly altered. The value of p*K*~a2~ is either increased modestly (*EcLpxC*) or unchanged (*Aa*-*LpxC*). However, at low pH the large decrease in catalytic activity previously described by p*K*~a1~ is not observed (Fig. 4, Table 3). The activity at low pH is relatively independent of pH. Alternatively, the small decrease in catalytic activity observed at low pH can be described by an ionization (p*K*~a3~ = 7.4 ± 0.1) that decreases the catalytic activity modestly (~4-fold). These data demonstrate that an ionization that causes the acidic limb of the pH profile is observed in both the E78A and H265A single mutants but NOT in the E78A/H265A double mutant. One potential explanation of these data is that the catalytic mechanism uses Glu78 and His265 as a GABC pair instead of employing one of these side chains as a single GABC (Figure 3b) and ionization of either residue could lead to the ionization observed as p*K*~a1~ in wild-type and the single mutants.

**p*K*~a2~ reflects ionization of the metal-water ligand**

To ascertain if one of the observed p*K*~a~ values reflects ionization of the metal-bound water molecule, the pH dependence of LpxC substituted with Co(II) or Ni(II) was examined (Table 4). Previous experiments have demonstrated that these metal ions are capable of supporting LpxC activity in lieu of zinc.(6) These data show that p*K*~a2~ shifts upward (Ni(II) > Co(II) > Zn(II)) upon substitution of Zn(II) with Co(II) or Ni(II) for the *E. coli* and *A. aeolicus* enzymes, paralleling the measured acidities of these metal-water ligands.(11,33) Additionally, the value of p*K*~a2~ increases upon substitution of Zn(II) with Co(II) for the H265A...
AaLpxC mutant. The upward shift in the value of \( pK_{a2} \) as the metal-water acidity decreases suggests that \( pK_{a2} \) reflects ionization of a metal-coordinated group, such as water in both the WT and mutant LpxC enzymes.

**Solvent isotope effect**

In both proposed mechanisms (Figure 3), Glu78 functions as a general base catalyst. Solvent isotope effects (\( k_{H2O}/k_{D2O} \)) of 2 to 4 are typically observed for reactions that proceed via GBC. The pH dependence of the solvent isotope effects for WT, E78A, H265A and E78A/H265A E. coli LpxC were measured (Figure 5 and Table 5). A solvent isotope effect (\( V/K_{H2O}/V/K_{D2O} \)) of ~2.1 is calculated for WT EcLpxC by comparing the values at the respective pH maximum (Figure 5), consistent with the transfer of a proton from solvent in the transition state. In the H265A and E78A/H265A mutants a decreased solvent isotope effect (\( V/K_{H2O}/V/K_{D2O} = 1.4 \)) is observed. Unexpectedly, the E78A mutant has an inverse solvent isotope effect of ~0.2 determined from the ratios of the V/K values at the pH maxima. However, in the E78A mutant the value of the solvent isotope effect is difficult to measure accurately since the values of \( pK_{a1} \) and \( pK_{a2} \) are comparable.

**Structure of the LpxC-Cacodylate Complex**

The gradual transfer of LpxC crystals from pH 7.0 to pH 6.0 results in the complete dissociation of the inhibitory zinc ion, \( Zn^{2+}_{B} \), and the electron density map of the LpxC active site reveals that a buffer molecule, the tetrahedral cacodylate anion, coordinates to the catalytic zinc ion, \( Zn^{2+}_{A} \) (Figure 6a). The cacodylate anion coordinates to \( Zn^{2+}_{A} \) in a monodentate fashion (O2- - Zn\(^{2+}\)A separation = 2.1 Å). The O1- - Zn\(^{2+}\)A separation (2.9 Å) is too long for an inner-sphere metal coordination interaction. The O1 atom of cacodylate also accepts hydrogen bonds from the side chains of E78 and H265, while the O2 atom accepts a hydrogen bond from T191 (Fig. 6b). Importantlty, the intermolecular interactions of the cacodylate anion may mimic those of the tetrahedral intermediate and its flanking transition states in LpxC catalysis (Fig. 6c).

A fatty acid molecule interpreted as myristate or disordered palmitate occupied the hydrophobic tunnel and coordinated to \( Zn^{2+}_{B} \) in the active site of the zinc-inhibited enzyme.(15) This fatty acid remains in the hydrophobic tunnel upon cacodylate binding to \( Zn^{2+}_{A} \) and is clearly interpretable as palmitate. Its carboxylate group is displaced to two alternate conformations and does not contact the zinc-bound cacodylate anion. In one conformation the carboxylate hydrogen bonds with His58, in the alternate conformation the carboxylate hydrogen bonds with a water molecule. Neither cacodylate binding nor the pH change triggers any significant conformational changes, and the r.m.s. deviation is 0.2 Å for 267 C\(_\alpha\) atoms between the cacodylate-bound and the zinc-inhibited LpxC structures.

**Structure of the LpxC-Palmitate Complex**

Equilibration of LpxC crystals at pH 6.0 with a non-coordinating buffer, bis-Tris, results in the dissociation of \( Zn^{2+}_{B} \) and the coordination of the palmitate carboxylate to \( Zn^{2+}_{A} \) (Figure 7a). Presumably, the palmitate molecule corresponds to the fatty acid that coordinated to \( Zn^{2+}_{B} \) in the zinc-inhibited enzyme.(15) The electron density map of the LpxC-palmitate complex reveals that palmitate extends from the hydrophobic tunnel and its carboxylate head group makes a nearly symmetric bidentate coordination interaction with \( Zn^{2+}_{A} \) (Figure 7a). Palmitate carboxylate oxygen O1 accepts hydrogen bonds from E78 and H265, and carboxylate oxygen O2 accepts a hydrogen bond from T191 (Figure 7b). These hydrogen bond interactions are comparable to those accommodating the binding of the tetrahedral cacodylate anion (Figure 6). Neither palmitate binding nor the pH change triggers any significant conformational changes, and the r.m.s. deviation is 0.2 Å for 267 C\(_\alpha\) atoms between the palmitate-bound and the zinc-inhibited LpxC structures.

**DISCUSSION:**

*Mutations significantly decrease k\(_{cat}\) and k\(_{cat}/K_M\)*

The large decreases in the \( k_{cat} \) and \( k_{cat}/K_M \) values for the E78A, D246A, H265A and E78A/H265A mutants further demonstrate the importance of these side chains in the catalytic mechanism. Preliminary transient kinetic data (Jackman and Fierke, unpublished data) suggest that for WT EcLpxC the kinetic parameter \( k_{cat} \) is limited by a rate constant for a step that occurs
after the rate constant for deacetylation ($k_{chem}$). However, in these LpxC mutants the deacetylation rate constant is decreased, so that $k_{cat}$ is presumed to measure $k_{chem}$. Therefore, the decreases in $k_{cat}$ are a lower limit for the effects these mutations have on $k_{chem}$. The large diminution in $k_{cat}$ (18- to 950-fold) observed for mutations at E78, D246 and H265 demonstrate the catalytic importance of these residues in LpxC. The E78A/H265A double mutation has partially additive effects on both $k_{cat}$ and $k_{cat}/K_M$ compared to the E78A and H265A single mutations.

**pH dependence of the LpxC-catalyzed reaction**

The pH dependence of the LpxC-catalyzed reaction for the *E. coli* and *A. aeolicus* enzymes indicates that at least two ionizations are important for catalytic activity (Figure 4). The pH dependence can be reasonably described by two ionizations (one protonation and one deprotonation) that each decrease the catalytic activity by more than 100-fold. The pH dependence under $k_{cat}/K_M$ conditions reflects ionizations in both the unbound enzyme and substrate.(35) However, mutagenesis and metal-substitution experiments alter both of the $pK_a$ values (Tables 3 and 4) suggesting that ionization of the substrate does not cause either of the primary $pK_a$ values. The simplest explanation of the pH dependence data is that the acidic limb represents an ionization whose deprotonation is important for activity and the basic limb of the pH profile represents an ionization wherein protonation is important for activity.

$pK_{a2}$ reflects metal-water ionization

The molecular origins of the ionizations in the pH rate profile were investigated through metal substitution and mutagenesis studies. The correlation between the value of $pK_{a2}$ for metal-substituted LpxC (Tables 4) with the Lewis acidity of the substituted metal ions(11,33) in WT and the AaH265A mutant suggests that $pK_{a2}$ represents ionization of a metal-coordinated group, most likely the water molecule. We propose that one role of zinc in the reaction is to lower the $pK_a$ of the ligated water molecule which serves as the nucleophile in the LpxC-catalyzed reaction. However, ionization of the metal-water to metal-hydroxide would be predicted to increase the rate constant for nucleophilic attack of the metal-solvent ligand on the carbonyl carbon of the substrate, similar to the reaction catalyzed by carbonic anhydrase. The simplest explanation for the observed decrease in activity following ionization of the metal-water molecule would be that the proton on this water molecule is required for protonation of the amine leaving group to facilitate breakdown of the tetrahedral intermediate to form products (Fig. 3).

$pK_{a1}$ may reflect ionization of E78

Two ionizations are observed for wild-type LpxC and each of the H265A, D246A and E78A mutants. However, the acidic limb of the pH profile essentially disappears in the E78A/H265A double mutant suggesting that ionization of either E78 or H265 is observed in $pK_{a1}$. Although the data do not clearly distinguish between these two groups, several pieces of evidence argue that ionization of Glu78 is the origin of $pK_{a1}$ in WT, D246A and H265A LpxC enzymes. First, no changes are observed in the measured $pK_a$ values for the H265A and D246A mutations (Table 3) indicating that ionization of neither of these side chains is observed in the pH rate profile. In contrast, the E78A mutation significantly increases the value of $pK_{a1}$ in *AaLpxC*. Second, the crystal structures (Figs. 6 and 7) indicate that E78 donates a hydrogen bond to the zinc-bound carboxylate oxygen of palmitate and to O1 of cacodylate, suggesting that E78 is protonated at pH 6, consistent with a $pK_{a1}$ of 6.4. Third, the $pK_a$ of H265 in *AaLpxC* has been measured by pH titration of the chemical shift in the NMR spectrum as 7.6, significantly higher than the measured values of $pK_{a1}$. However, the retention of two ionizations in the pH rate profile of the E78A mutant suggests that ionization of a different group, such as H265, is observed in the E78A mutant. The positioning of both His265 and Glu78 within hydrogen bond distance of O1 of the bound cacodylate and palmitate suggests that either group could function as a GBC to activate the zinc-water nucleophile.

A third ionization that modulates activity 4-fold is unmasked in the E78A/H265A mutant. This $pK_a$ could reflect ionization of a number of different groups, including the diphosphate in the substrate (UDPGlcNAc $pK_a$'s ~2 and 7.5, unpublished data), and could also alter the shape of the pH rate profile of WT LpxC.
The magnitude of the decreases in $k_{cat}/K_M$ at the pH maximum for the E78A and H265A mutations (Table 2) is on the low side for deletion of a side chain that functions as a GABC (10$^2$- to 10$^4$-fold); however, the 10$^3$-10$^4$-fold lower activity of the E78A/H265A double mutant is more consistent with the loss of a GABC.(37,38) Furthermore, the magnitude of the decrease in the maximal $k_{cat}/K_M$ for the E78A/H265A mutant is ~10-fold smaller than predicted if the effects of the single mutations are completely additive. In the absence of structural perturbations, partially additive effects can be explained by either a cooperative interaction of the mutated residues to facilitate the same rate-limiting step or independent interactions of the mutated residues to facilitate consecutive non-rate-limiting steps.(39,40) The partially additive effects of the E78A and H265A mutants suggest that both groups might stabilize a common intermediate; for example by functioning as a general acid-base pair in catalysis (Fig. 3b).

**Solvent isotope effect**

The solvent isotope effect of 2 ($k_{cat}/K_M$) that is observed for WT LpxC is consistent with the rate-limiting transfer of a solvent proton in the transition state, as indicated by a general base catalysis mechanism.(34) The most striking result from these experiments is the inverse solvent isotope effect observed for the E78A mutant. One possible explanation for this result is that the values of the $pK_a$’s are inverted in E78A EcLpxC such that a small fraction of the total enzyme is active at any pH. The addition of D$_2$O could alter the values of the $pK_a$’s in the E78A mutant so that a higher fraction of the active enzyme is observed in D$_2$O and therefore an inverse solvent isotope effect is obtained. However, the inverse isotope effect could also be explained by a change in mechanism for the E78A mutant to a reaction that proceeds via GAC, such as rate-limiting acid-catalyzed breakdown of the tetrahedral intermediate, or a reaction that involves use of a metal-chelated water.(34,41)

**LpxC-cacodylate and -palmitate complexes support a general acid-base pair mechanism**

Both Glu78 and His265 hydrogen bond with the O1 atoms of zinc-bound cacodylate (Fig. 6) and palmitate (Fig. 7), suggesting that both residues could simultaneously interact with a zinc-bound solvent nucleophile. Notably, palmitate can be considered as a “bi-product analogue”- i.e., it contains structural features present in both products of LpxC catalysis: a carboxylate group like that of acetate, and a fatty acid side chain like that of myrUDP-GlcNAc. Therefore, the intermolecular interactions of palmitate, and especially those of the palmitate carboxylate, are relevant for understanding product binding in the LpxC active site. The close contact between E78 and the zinc-bound carboxylate oxygen of palmitate likely implies that E78 is predominantly protonated at pH 6.0 to accommodate this interaction, consistent with the $pK_a$ value of 6.4 in the acidic limb of the pH rate profile assigned to E78. It is possible that a similar interaction accommodates the binding of the acetate product in the final step of the hydrolysis reaction. However, the ionization of E78 to the negatively charged carboxylate is required to regenerate the active form of the general base, so the resultant electrostatic repulsion between the negatively charged carboxylate group of E78 and zinc-bound palmitate may facilitate product release.

Combining these structural data with the pH rate profiles leads to the proposal that $pK_{a1}$ reflects ionization of Glu78 in WT and H265A LpxC, which functions as a general base to activate the zinc-water nucleophile. However, in E78A LpxC the side chain of His265 replaces Glu78 as the GBC, albeit with reduced efficiency, and ionization of this group is observed in the pH rate profile as $pK_{a1}$. Although it is possible for the apparent $pK_a$ values for the acidic and basic groups to be switched(35), the observed values for $pK_{a1}$ and $pK_{a2}$ are reasonable for ionization of an active site Glu and zinc-bound water, respectively.

**Overall mechanism**

On the basis of these data we propose that Glu78 and His265 function as a GABC pair and that the ionizations observed for WT LpxC represent the ionization of Glu78 ($pK_{a1}$) and the zinc-bound water molecule ($pK_{a2}$). The assignment of these $pK_a$ values to Glu78 and the zinc-bound water molecule is consistent with those reported in a recent study; however, the mechanisms arrived at based on these data are different.(19) We propose that Glu78 and His265 are poised to function as a GABC pair in the LpxC-catalyzed reaction via the mechanism shown...
in Figure 3b, with zinc and Thr191 providing stabilization of the transition states and oxyanion intermediate. This mechanism is consistent with the structure of the bound tetrahedral cacodylate anion (Figure 6) which mimics the structure of the oxyanionic intermediate and its flanking transition states; the hydrogen bond interactions of cacodylate with both E78 and H265 demonstrate that both of these side chains are positioned to function as general acid-base catalysts. The finding that the E78A and H265A mutations have partially additive effects can also be explained by this mechanism, since these residues are functioning cooperatively in the chemical step. One of the major differences between the mechanisms in Figure 3 is the role of His265. This residue is structurally/electronically precluded from fulfilling both of the proposed roles as an electrostatic catalyst (Fig. 3a) and general acid catalyst (Fig. 3b). We propose that the mechanism wherein His265 functions as a GAC is preferable to the metalloprotease-like mechanism where His265 provides stabilization of the transition states and oxyanion intermediate for the following reasons. The His265 is part of a His/Asp charge-relay and normally these charge-relays mediate proton-transfer reactions.\(^\text{42,43}\) Although the distances from both E78 and H265 to the CH\(_3\) of the cacodylate complex (equivalent to leaving group amine in the product) are longer than expected for a proton donor (>4 Å), H265 is closer. Moreover, His265 can make a closer approach to the leaving group by rotating about side chain torsion angle \(\chi_1\), which would weaken or break the hydrogen bond with D246. This would also lower the pK\(_a\) of H265 and facilitate proton transfer to the leaving amino group and collapse of the tetrahedral intermediate. Participation of T191 in the reaction via electrostatic stabilization of the oxyanion intermediate and flanking transition states is supported by the structures of the LpxC-cacodylate and -palmitate complexes (Fig. 6 and 7) where hydrogen bonds between T191 and ligand O2 (corresponding to the carbonyl oxygen atom of the substrate) atoms are observed. In summary, these data support the proposal that the LpxC-catalyzed reaction proceeds via a general acid-base pair mechanism.

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FIGURE LEGENDS:

**Figure 1.** The biosynthesis of Lipid A.

**Figure 2.** (a) Structure of zinc-inhibited LpxC (PDB accession code 1P42) (15); A = catalytic zinc ion, B = inhibitory zinc ion. (b) Metal coordination interactions in the binuclear zinc cluster of zinc-inhibited LpxC.

**Figure 3.** The proposed mechanisms for LpxC using either (A) a single bifunctional GABC or (B) a GABC pair.

**Figure 4.** The pH dependence of the (●) WT, (○) H265A, (□) E78A and (◆) E78A/H265A EcLpxC-catalyzed reactions. The values for $k_{cat}/K_M$ were measured at 30 °C using subsaturating concentrations of myrUDP-GlcNAc (50 - 200 nM), as described in the Materials and Methods section. The $pK_a$ values listed in Table 3 were determined by fitting an equation including two ionizations (Eq. 1) or two ionizations where one $pK_a$ decreases the activity modestly (Eq. 2, dashed line) to the data.

**Figure 5.** Solvent isotope effect of *E. coli* WT LpxC in (●) H2O and (○) 95% D2O and E78A in (■) H2O and (□) 95% D2O. The values for $V/K$ were measured at 30 °C using subsaturating concentrations of myrUDP-GlcNAc (50 - 200 nM), as described in the Materials and Methods section. The $pK_a$ values listed in Table 5 were determined by fitting an equation including two ionizations (Eq. 1) to these data.

**Figure 6.** (a) Omit electron density map of cacodylate bound to the catalytic zinc ion, Zn2+ (contoured at 4σ). Atoms are color-coded as follows: C = yellow, O = red, N = blue, S = green, As = black; zinc appears as a gray sphere. Zinc coordination interactions are designated by green dotted lines, and hydrogen bonds are indicated by black dotted lines. (b) Hydrogen bond and metal coordination interactions in the LpxC-cacodylate complex. Distances are indicated in Ångstroms. (c) Proposed scheme for hydrogen bond and metal coordination interactions of the tetrahedral intermediate in catalysis, by analogy with (b).

**Figure 7.** (a) Omit electron density map of the LpxC-palmitate complex (contoured at 4σ). Atoms are color-coded as follows: C = yellow, O = red, N = blue; zinc appears as a gray sphere. (b) Hydrogen bond and metal coordination interactions in the LpxC-palmitate complex. Distances are indicated in Ångstroms.
TABLES:

**Table 1:** Data Collection and Refinement Statistics

| Complex                  | LpxC-cacodylate complex | LpxC-palmitate complex |
|--------------------------|-------------------------|------------------------|
| Resolution               | 30-2.1                  | 50-2.7                 |
| Reflections              | 257853/                 | 114604/                |
| (measured/unique)        | 41614                   | 19665                  |
| Completeness             | 100/                    | 98.2/                  |
| (%)                      | 100                     | 100                    |
| $R_{\text{merge}}$       | 0.054/                  | 0.115/                 |
| ($I/\sigma$)             | 0.359                   | 0.335                  |
| Protein atoms            | 4298                    | 4298                   |
| Solvent atoms            | 318                     | 117                    |
| Glycerol molecules       | 1                       | N/A                    |
| Chloride ions            | 3                       | 3                      |
| Sulfate ions             | 2                       | N/A                    |
| Metal ions               | 5                       | 5                      |
| Ligand atoms             | 46                      | 36                     |
| Reflections              | 39481/                  | 17605/                 |
| used in refinement       | 2088                    | 1919                   |
| $R/R_{\text{free}}$      | 0.182/                  | 0.201/                 |
| r.m.s. deviations        |                         |                        |
| bonds (Å)                | 0.005                   | 0.006                  |
| Angles                   | 1.2                     | 1.2                    |
| proper dihedral          | 23.5                    | 23.3                   |
| improper dihedral        | 0.7                     | 0.8                    |

$R_{\text{merge}} = \frac{\sum |I_j - \langle I_j \rangle|}{\sum I_j}$, where $I_j$ is the observed intensity for reflection $j$ and $\langle I_j \rangle$ is the average intensity calculated for reflection $j$ from replicate data. $R$ is per asymmetric unit. $R = \frac{\sum |F_o| - |F_c|}{\sum |F_o|}$, where $R$ and $R_{\text{free}}$ are calculated by using the working and test reflection sets, respectively.
Table 2. Steady-state kinetic parameters for *E. coli* LpxC mutants

| *E. coli* LpxC<sup>a,b</sup> | $k_{\text{cat}}$ (min<sup>-1</sup>) | $K_M$ (µM) | $k_{\text{cat}}/K_M$ (µM<sup>-1</sup>min<sup>-1</sup>) | Fold-decrease ($k_{\text{cat}}/K_M$) |
|-------------------------------|-----------------|----------|-----------------|--------------------------|
| WT – Zn<sup>2+</sup>          | 90 ± 2          | 0.19 ± 0.01 | 460 ± 10       | --                      |
| WT – Co<sup>2+</sup>          | 27 ± 2          | 0.47 ± 0.08 | 65 ± 0.6        | 7                       |
| E78A                          | 5.0 ± 0.4       | 4.3 ± 0.6  | 1.16 ± 0.09     | 400                     |
| H265A                         | 0.30 ± 0.05     | 1.4 ± 0.5  | 0.21 ± 0.04     | 2190                    |
| D246A                         | 0.094 ± 0.009   | 0.25 ± 0.06 | 0.38 ± 0.06     | 1210                    |
| E78A/H265A                    | 0.058 ± 0.003   | 1.9 ± 0.2  | 0.031 ± 0.002   | 14,800                  |

<sup>a</sup> The metal-substituted enzymes were prepared with a stoichiometry of 1:1 as described in the Materials and Methods section.

<sup>b</sup> The initial rate for LpxC-catalyzed deacetylase activity was determined at 30 °C (20 mM bis-tris propane, pH 7.5, 1 mg/mL BSA, 0.5 mM TCEP) with myrUDPGlcNAc as the substrate. The kinetic parameters were obtained from the initial velocities, as described in the Materials and Methods section.
Table 3. The pH dependence of $k_{cat}/K_M$ catalyzed by *E. coli* and *A. aquifex* LpxC mutants$^a$

| LpxC Mutant  | LpxC Source | $pK_{a1}$  | $pK_{a2}$  | $k_{cat}/K_M$ pH maximum ($\mu$M$^{-1}$ min$^{-1}$) | Fold-decrease$^b$ |
|--------------|-------------|------------|------------|-------------------------------------------------|------------------|
| WT           | *Ec*        | 6.2 ± 0.1  | 9.2 ± 0.1  | 371 ± 18                                         | 1                |
|              | *Aa*        | 5.8 ± 0.2  | 7.9 ± 0.3  | 65 ± 11                                          | 1                |
| E78A         | *Ec*        | 6.7 ± 0.1  | 8.7 ± 0.1  | 1.3 ± 0.06                                       | 285              |
|              | *Aa*        | 7.0 ± 0.1  | 7.0 ± 0.1  | 1.6 ± 0.3                                        | 41               |
| H265A        | *Ec*        | 6.4 ± 0.1  | 8.6 ± 0.1  | 0.28 ± 0.02                                      | 1325             |
|              | *Aa*        | 6.0 ± 0.1  | 8.2 ± 0.2  | 0.39 ± 0.04                                      | 167              |
| D246A        | *Ec*        | 6.0 ± 0.2  | 9.0 ± 0.2  | 0.12 ± 0.01                                      | 3090             |
|              | *Aa*        | 5.5 ± 0.1  | 8.3 ± 0.1  | 0.47 ± 0.02                                      | 138              |
| E78A/H265A   | *Ec*        | —$^d$      | 10.2 ± 0.1 | 0.01 ± 0.001                                     | 37,000           |
|              | *Aa*        | —$^d$      | 8.4 ± 0.2  | 0.039 ± 0.001                                    | 1,700            |

$^a$ The $k_{cat}/K_M$ values for deacetylation of myrUDPGlcNAc catalyzed by *EcLpxC* and *AaLpxC* mutants were determined at 30 °C and 60°C, respectively, as a function of pH. The pH of the buffers was measured at the reaction temperature. The pH dependence of the $k_{cat}/K_M$ values was used to determine the $pK_a$ values as described in the Materials and Methods section.

$^b$ Calculated from the ratio of $(k_{cat}/K_M)^{WT-Zn}/(k_{cat}/K_M)^{mutant}$.

$^c$ *Ec* and *Aa* denote *E. coli* and *A. aquifex* LpxC, respectively.

$^d$ A second $pK_a$ with a value of ~7.4 ± 0.1 that decreases the catalytic activity ~4-fold at low pH was also observed for this mutant.
Table 4. Active site metal alters the pH dependence of $k_{\text{cat}}/K_M$ catalyzed by *E. coli* and *A. aquifex* LpxC

| LpxC Mutant | LpxC Source | Active Site Metal Ion$^b$ | $pK_{a1}$ | $pK_{a2}$ | $k_{\text{cat}}/K_M$ pH maximum ($\mu$M$^{-1}$ min$^{-1}$) |
|-------------|-------------|--------------------------|----------|----------|--------------------------------------------------|
| WT          | *Ec*        | Zn(II)                   | 6.4 ± 0.1| 9.1 ± 0.1| 370 ± 20                                         |
|             | *Ec*        | Co(II)                   | 6.3 ± 0.2| 10.3 ± 0.2| 37 ± 3                                           |
| WT          | *Aa*        | Zn(II)                   | 5.8 ± 0.2| 7.9 ± 0.3| 65 ± 11                                          |
|             | *Aa*        | Co(II)                   | <6       | 8.4 ± 0.1| 47 ± 1                                           |
|             | *Aa*        | Ni(II)                   | <6       | 8.8 ± 0.3| 68 ± 8                                           |
| H265A       | *Aa*        | Zn(II)                   | 6.0 ± 0.1| 8.2 ± 0.2| 0.39 ± 0.04                                     |
|             | *Aa*        | Co(II)                   | 7.1 ± 0.2| 9.4 ± 0.4| 0.44 ± 0.06                                     |

$^a$The $k_{\text{cat}}/K_M$ values for deacetylation of myrUDPGlcNAc catalyzed by *Ec*LpxC and *Aa*LpxC were determined at 30 °C and 60°C, respectively, as a function of pH. The pH of the buffers was measured at the reaction temperature. The pH dependence of the $k_{\text{cat}}/K_M$ values was used to determine the $pK_a$ values as described in the Materials and Methods section.

$^b$The single catalytic Zn(II) cofactor was removed by incubation with dipicolinic acid and replaced by incubation with Co(II) or Ni(II), as described in the Materials and Methods section.

$^c$*Ec* and *Aa* denote *E. coli* and *A. aquifex* LpxC, respectively.
Table 5. Solvent isotope effects on *E. coli* LpxC<sup>a</sup>

| *E. coli* LpxC | pK<sub>a1</sub> | pK<sub>a2</sub> | V<sub>H2O</sub>/V<sub>D2O</sub> |
|----------------|---------------|---------------|-----------------|
| WT (H<sub>2</sub>O) | 6.4 ± 0.1 | 9.1 ± 0.1 | 2.1 |
| (D<sub>2</sub>O) | 7.4 ± 0.1 | 9.6 ± 0.1 | |
| E78A (H<sub>2</sub>O) | 6.2 ± 0.3 | 8.5 ± 0.3 | ~0.2 |
| (D<sub>2</sub>O) | 7.2 ± 0.6 | 7.9 ± 0.5 | |
| H265A (H<sub>2</sub>O) | 6.0 ± 0.2 | 9.3 ± 0.2 | 1.2 |
| (D<sub>2</sub>O) | 7.0 ± 0.2 | 9.8 ± 0.3 | |
| E78A/H265A (H<sub>2</sub>O) | -- | 9.1 ± 0.2 | 1.4 |
| (D<sub>2</sub>O) | -- | 9.1 ± 0.3 | |

<sup>a</sup> The LpxC activity was determined using myrUDPGlcNAc as the substrate. The pH dependence of k<sub>cat</sub>/K<sub>M</sub> was fit using equations (1) or (2) to calculate the pK<sub>a</sub> values, as described in the Materials and Methods section.

FIGURES

Figure 1:
Figure 2:

a

b

Fatty Acid

Thr191

His238

His265

Glu78

Asp242

Asp246
Figure 3:

(A) R = myrUDP-Glc

(B) R = myrUDP-Glc
Figure 6:
Figure 7:

a

b

[Diagram showing molecular structure with labeled atoms and distances: Asp246, His265, Glu78, O, N, and Zn].
UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase functions through a general acid-base catalyst pair mechanism
Marcy Hernick, Heather A. Gennadios, Douglas A. Whittington, Kristin M. Rusche, David W. Christianson and Carol A. Fierke

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