Active Site Metal Ion in UDP-3-O-((R)-3-Hydroxymyristoyl)-N-acetylglucosamine Deacetylase (LpxC) Switches between Fe(II) and Zn(II) Depending on Cellular Conditions*§

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UDP-3-O-((R)-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase (LpxC) catalyzes the deacetylation of UDP-3-O-((R)-3-hydroxymyristoyl)-N-acetylglucosamine to form UDP-3-O-myristylo-glucosamine and acetate in Gram-negative bacteria. This second, and committed, step in lipid A biosynthesis is a target for antibiotic development. LpxC was previously identified as a mononuclear Zn(II) metalloenzyme; however, LpxC is 6–8-fold more active with the oxygen-sensitive Fe(II) cofactor (Hernick, M., Gattis, S. G., Penner-Hahn, J. E., and Fierke, C. A. (2010) Biochemistry 49, 2246–2255). To analyze the native metal cofactor bound to LpxC, we developed a pulldown method to rapidly purify tagged LpxC under anaerobic conditions. The metal bound to LpxC purified from Escherichia coli grown in minimal medium is mainly Fe(II). However, the ratio of iron/zinc bound to LpxC varies with the metal content of the medium. Furthermore, the iron/zinc ratio bound to native LpxC, determined by activity assays, has a similar dependence on the growth conditions. LpxC has significantly higher affinity for Zn(II) compared with Fe(II) with $K_{m}$ values of 60 ± 20 pm and 110 ± 40 nm, respectively. However, in vivo concentrations of readily exchangeable iron are significantly higher than zinc, suggesting that Fe(II) is the thermodynamically favored metal cofactor for LpxC under cellular conditions. These data indicate that LpxC expressed in E. coli grown in standard medium predominantly exists as the Fe(II)-enzyme. However, the metal cofactor in LpxC can switch between iron and zinc in response to perturbations in available metal ions. This alteration may be important for regulating the LpxC activity upon changes in environmental conditions and may be a general mechanism of regulating the activity of metalloenzymes.

Gram-negative bacteria are important targets in the continuing fight against antibiotic-resistant infections. The development of new antibiotics to treat infections caused by resistant organisms is critically needed and will require novel targets. One potential source of targets in Gram-negative bacteria is the lipid A biosynthetic pathway (Fig. 1A) (1, 2), an essential building block of lipopolysaccharides (LPS) that make up the outer leaflet surrounding the cell wall in Gram-negative bacteria (2–4). In addition to being essential for cell viability, LPS are also referred to as endotoxins (2, 4, 5) and are responsible for immunogenic stimulation in septic shock that can lead to a number of deleterious effects, including severe hypotension, multiple organ dysfunction, and death (5). Consequently, inhibitors of lipid A biosynthesis have the potential to serve as both antibiotics and antiendotoxins (6). The UDP-3-O-((R)-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase (LpxC)3 catalyzes the committed and second overall step in lipid A biosynthesis, the hydrolysis of UDP-3-O-myristylo-N-acetylglucosamine to UDP-3-O-myristylo-glucosamine and acetate (Fig. 1) (7–10). Consequently, inhibitors of LpxC are an active area of drug development (11–14).

LpxC was previously identified as a mononuclear Zn(II) metalloenzyme (Fig. 1B) (15) that also contains a weaker, inhibitory metal ion-binding site. This catalytic metal ion binds and polarizes an inner sphere water molecule that is activated by general base catalysis to serve as the nucleophile for hydrolysis of the acetyl substrate (Fig. 1C) (16, 17). However, recent evidence has shown that LpxC is more active with Fe(II) as its metal ion cofactor compared with Zn(II) (18). This increase in activity may correlate with a change in the ground state metal geometry from 4-coordinate in ZnLpxC to 5-coordinate in the Fe(II)-enzyme (18) that facilitates catalysis by enhancing coordination and polarization of the carbonyl group of the substrate (Fig. 1C). Several enzymes described previously as Zn(II)-dependent enzymes have recently been reclassified as Fe(II) enzymes, including peptide deformylase, S-ribosylhomocysteine, and possibly histone deacetylase 8 (HDAC8) (19–24). Iron is a highly abundant transition metal ion in cells (25, 26); however, the Fe(II) redox state that activates LpxC oxidizes to Fe(III) in air (18). Therefore, it is not yet clear which metal ion activates LpxC in vivo.

Both iron and zinc exist in high amounts in the cell, with total concentrations around 0.2 mM in Escherichia coli (26). However, the concentrations of the readily exchangeable, or “free,” metal ions are vastly different. Although estimates vary, the best

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3 The abbreviations used are: LpxC, UDP-3-O-((R)-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase; TCEP, tris(2-carboxyethyl)phosphine; ICP-MS, inductively coupled plasma mass spectrometry; LpxC-ZZ, EcLpxC-C63A-Tev-ZZ; NTA, nitrilotriacetic acid; BisTris, 2-(bis[2-hydroxymethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; Bistris propano, 1,3-bis[tris(hydroxymethyl)amino]propano; CDM, Chemically Defined Medium.

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data available for readily exchangeable Fe(II) and Zn(II) come from mammalian cells, where estimates indicate that [Zn(II)_{free}] is picomolar (10–400 pM) (27, 28), and [Fe(II)_{free}] is micromolar (0.2–6 μM) (29–32). The combination of the higher concentration of readily exchangeable Fe(II) and the enhanced reactivity of Fe(II)-bound LpxC (18) suggest that the native cofactor bound to LpxC in E. coli might be Fe(II) rather than Zn(II). To test this hypothesis, we developed rapid purification methods and activity assays to analyze the metal ion status of LpxC in vivo. Using these methods, we demonstrate that under normal growth conditions iron-bound LpxC predominates. Although LpxC binds Zn(II) with much higher affinity than Fe(II), the higher cellular concentration of Fe(II)_{free} contributes to the formation of Fe(II)-bound LpxC in vivo. In summary, these data indicate that Fe(II) is the biologically relevant cofactor for LpxC under common growth conditions, although metal switching occurs in response to metal availability. Furthermore, metal switching may be important for regulating the LpxC activity upon changes in cellular conditions.

MATERIALS AND METHODS

**General Procedures, Protein Expression, and Plasmid Construction**—All solutions were prepared in “metal-free” plasticware, with reagents that did not contain extraneous metal ions and/or were treated with Chelex (Bio-Rad). The metal content of solutions, reagents, and proteins was measured by inductively coupled plasma emission mass spectrometry (ICP-MS; Dr. Ted Huston, University of Michigan). LpxC and LpxC fusion proteins were overexpressed in BL21(DE3) E. coli transformed with pET-derived expression plasmids and purified according to published procedures using DEAE-Sepharose and Reactive Red-120 affinity dye columns (8, 15, 16). The apo-forms and single metal-bound forms of LpxC were prepared by treatment with metal chelators followed by reconstitution with Zn(II) or Fe(II) as described previously (15, 16, 18).

The pEcC63ALpxC expression plasmid encoding E. coli LpxC with the C63A mutation (18) was modified to encode a fusion protein between LpxC and an IgG-binding protein, termed a ZZ tag (33–35), with a tobacco etch virus protease site in the linker region between the two proteins. An NcoI restriction site was introduced at the C-terminal stop codon of LpxC by using the QuikChange mutagenesis kit (Stratagene) with the primer 5'-CCT TCA GCT GTA CTG GCA CCA TGG GGA TCC GAA TTC GAG CTC CG-3' and its reverse complement.

The ZZ tag insert was amplified using PCR from the ZZ region of the commercial vector pEZZ (Amersham Biosciences) with the introduction of XhoI and EcoRI restriction sites as well as a stop codon at the 3' end (forward primer 1, 5'-CGA TGA ACT TGC AAC CCA TGG GAG AAC CTG TAC TTC CAG GGT CTC GAG GAC AAC-3', and reverse primer 1, 5'-TAA AGA ATT CTC AGG TTT CTA GAT TCG CGT CTA CTT TCG G-3'). The DNA fragment was extended using PCR amplification to add a TEV-protease cleavage site and an NcoI restriction site using the forward primer 2, 5'-ATG ACT TGC AAC CCA TGG GAG AAG CTC CTG TCG ATG CTT TTC CAG GGT CTC GAG GAC AAC-3', and the reverse primer 1 (see above). The PCR product and pLpxC plasmid containing the

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**FIGURE 1.** A, role of LpxC in lipid A biosynthesis. B, active site of ZnLpxC derived from Protein Data Bank code 1P42 (49). C, proposed chemical mechanism of LpxC catalysis (16).
new NcoI restriction site were digested with NcoI and EcoRI and ligated together, catalyzed by T4 DNA ligase (New England Biolabs), to create the expression plasmid pEcC63ALpxC-ZZ. The plasmid sequence was verified by the University of Michigan DNA Sequencing Core. The presence of the ZZ tag does not alter the LpxC activity or product affinity. However, the activity of the LpxC-ZZ fusion protein is more sensitive to freeze/thaw cycles than the untagged LpxC (data not shown).

**IgG Pulldown Experiments—*E. coli* BL21(DE3) cells transformed with pC63ALpxC-ZZ were grown in Chemically Defined Medium (CDM) (36). Protein expression was induced when the cells reached an *A*$_{600}$ of 0.6 by the addition of 1 mM isopropyl β-d-thiogalactopyranoside along with the addition of either no added metals, 5–20 μM ZnSO$_4$, 5–20 μM ferric ammonium citrate, or both metal supplements, and the cells were incubated overnight at 30 °C. The cells were pelleted by centrifugation, washed once with 5 mM CaCl$_2$, and twice with 10 mM Mops, pH 7. Control experiments demonstrate that 10 mM Ca(II) has no effect on the activity of ZnLpxC or apo-LpxC. The activity of the LpxC-ZZ fusion protein is more sensitive to Ca(II) than the untagged LpxC (data not shown).

The cell pellet was resuspended in 3 ml of pulldown buffer (40 mM Mops, pH 8, 150 mM NaCl, 10 mM TCEP, 0.1% Nonidet P-40), and lysed by incubation with lysozyme (1 mg/ml) at room temperature for 15–30 min. The tubes were sealed with parafilm and removed from the glove box, and the cell lysate was cleared by centrifugation (12,000 rpm, 15–30 min). The cleared lysates were transferred into a glove box; a portion of cleared lysate (200 μl) was set aside for ICP-MS analysis, and the remaining cleared lysate was incubated with 100 μl of IgG-Sepharose beads (Sigma) for 30 min. The IgG-Sepharose beads were washed with 3× 1 ml of pulldown buffer, resuspended in 297 μl of pulldown buffer, and EcC63A LpxC and metal ions were eluted from the IgG beads by either incubation with 3 μl of AcTEV™ protease (10 units/μl, Invitrogen) or 10 mM nitric acid at room temperature overnight. The metals in the eluate were analyzed by ICP-MS. The concentration of EcC63A LpxC in the eluate was determined using the Bradford assay (Sigma) with purified LpxC as the standard. For control experiments, purified apo-LpxC-ZZ (100 μm) was reconstituted with 1 eq of either ZnSO$_4$ or FeCl$_2$ in the anaerobic glove box. The holo-LpxC (9 μM final) was then diluted into cell lysate prepared from BL21(DE3) cells transformed with the pEcC63A plasmid, encoding LpxC without a ZZ tag, and incubated for 30 min at room temperature. The lysate was then applied to IgG-agarose beads and washed as described above, and the bound LpxC-ZZ was removed by incubation in 10 mM nitric acid overnight following by ICP-MS analysis of metal ions in the eluate.

**Native LpxC Deacetylase Activity—Chemically Defined Medium (CDM) (36) (supplemented with 0–20 μM ZnSO$_4$ and/or ferric ammonium citrate) was inoculated with BL21(DE3) cells and grown in a shaker (250 rpm) overnight at 37 °C. The cells were harvested by centrifugation, and the cell pellets resuspended in 20 mM Bistris propane, 10 mM TCEP, pH 7.5. The cells were washed, lyzed, and clarified as described for the pulldown assays above. Metal ion concentrations in the cleared lysate were determined by ICP-MS analysis. The cleared lysate was diluted 10-fold into assay buffer prior to measurement of activity (see below) using 200 nM substrate.

The LpxC activity in the cleared lysates was measured either immediately in the glove box or after 2.5 h of incubation in air. The ratio of iron and zinc bound to LpxC was estimated from the activity measured either anaerobically (A$_a$), reflecting the activity of both ZnLpxC and FeLpxC, or after 2.5 h of incubation in air (A$_e$), reflecting the activity due to ZnLpxC, assuming that all of the FeLpxC forms ZnLpxC under these conditions (18). Equation 1 was used to describe the activity at A$_a$ where $S_{Zn}$ and $S_{Fe}$ are the specific activity of Zn- and FeLpxC, respectively, whereas $E_{Zn}$ and $E_{Fe}$ are the concentration of Zn- and FeLpxC, respectively. Using the ~6-fold higher activity of FeLpxC relative to ZnLpxC ($E_{Fe}/E_{Zn} = 6$) (18), $A_a$ was solved in terms of the zinc enzyme and $E_{tot}$ (Equation 2). Using the relationship $A_{2.5} = S_{Zn}E_{tot}$, the $E_{Fe}/E_{tot}$ ratio in the anaerobic sample was calculated (Equation 3), and the $E_{Fe}/E_{Zn}$ ratio was obtained by conservation of mass. To demonstrate that the native activity observed was specifically due to LpxC activity, control assays were performed with the LpxC-specific hydroxamate inhibitor L-161,240 (18).

$$A_a = S_{Zn}E_{Zn} + S_{Fe}E_{Fe} \quad \text{(Eq. 1)}$$

$$\frac{A_a}{E_{tot}} = 6S_{Zn} - S_{Zn}E_{Fe} \quad \text{(Eq. 2)}$$

$$\frac{E_{Zn}}{E_{tot}} = \frac{E_{Fe}}{S_{Fe}} \quad \text{(Eq. 3)}$$

**LpxC Assay—**LpxC activity was measured as described previously (16, 37, 38). For native activity measurements, assays were performed in 20 mM Bistris propane, 10 mM TCEP, pH 7.5, bovine serum albumin (BSA, fatty acid-free, 1 mg/ml), and [3H]UDP-3-O-(3-hydroxymaryristoyl)-N-acetyl-glucosamine (200 nm). Mixtures were pre-equilibrated at 30 °C, and the reactions were initiated by the addition of cell lysate. LpxC assays were also carried out in Fe(II) buffers for affinity determinations, as described below. After incubation for various times, the reactions were quenched by the addition of sodium hydroxide, which also cleaves the myristate substituent for ease of separation. Substrate and product were separated on PEI-cellulose TLC plates (0.1 μm guanidinium HCl) and quantified by scintillation counting. Initial rates of product formation (<20% reaction) were determined from these data.

**In Vitro Metal Ion Affinity and Exchange—**To remove contaminating metal ions, ultrafiltration devices (Microcon molecular weight cutoff of 30,000) were incubated with 10 mM Hepes, pH 7.5, 500 μM EDTA, 100 mM dipicolinic acid for 30 min (500 μl), followed by 3× 500-μl washes with ultrapure water. To examine metal exchange *in vitro*, 10 μM LpxC C63A with either bound Zn(II) or Fe(II) was incubated in an anaerobic chamber with various concentrations of ZnSO$_4$ or FeCl$_2$ (0–300 μM) for ≥15 min in 20 mM Bistris propane, pH 7.5, 10 mM TCEP. The free and bound metal ions were separated by centrifugation (3000 × g, 4 min) in an ultrafiltration device, and the metals in an equal volume of filtrate and retentate were then analyzed by ICP-MS.

For Zn(II) affinity measurements, apo-LpxC C63A (1–200 μM) was incubated in a metal ion and pH buffer containing 1 mM nitritotriacetic acid (NTA), 5 mM Mops, pH 7, 2.5 μM dipic-
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RESULTS

LpxC Purified from E. coli Contains Mainly Bound Fe(II)—LpxC purified from E. coli was previously shown to contain bound Zn(II); however, this protein was purified under aerobic conditions with 2 mM dithiothreitol as the reducing agent (15), which is not sufficient to prevent oxidation of bound Fe(II) (18). Subsequent experiments demonstrated that under anaerobic conditions Fe(II)-bound LpxC is 6–8-fold more active than ZnLpxC (18). The absorbance spectrum of Fe(II)-bound LpxC has a broad peak with a maximum at 420 nm (ε_{420} = 1080 M^{-1} cm^{-1}) (supplemental Fig. S1), similar to the spectra of other low spin non-heme Fe(II) complexes (41, 42) and consistent with the extended x-ray absorption fine structure data suggesting pentacoordinate metal geometry (18).

To clarify the identity of the biologically relevant metal ion cofactor, we purified LpxC under anaerobic conditions to re-examine the identity of the native metal cofactor. For all of these experiments, we used a previously characterized LpxC variant, EcC63A (18), that has high catalytic activity but reduced affinity for metal ions at the inhibitory site. This protein was fused to a cleavable C-terminal ZZ tag (33–35) (EcLpxCc63A-Tev-ZZ or LpxC-ZZ) to facilitate rapid purification of LpxC from crude cell lysates with minimal metal contamination. LpxC-ZZ was induced in BL21(DE3) cells grown in minimal medium containing varied metal supplements and rapidly isolated by incubation with IgG beads followed by elution with either nitric acid or TEV protease. The concentration of metals and protein in the column eluate were determined by ICP-MS and Bradford assays, respectively. This method minimizes contamination of LpxC by adventitious metal ions. Control experiments using cells containing LpxC without the ZZ tag demonstrate that little or no (<0.1 μM) protein or metal ions are observed in the eluate. In general, LpxC-ZZ co-purifies with mainly Fe(II) and some Zn(II) (Table 1); no other divalent metal ions (e.g. cobalt, nickel, and copper) are observed at greater than trace levels (<3–5%). A substoichiometric metal ratio is also observed under all conditions, suggesting either that LpxC-ZZ is not fully saturated with metals under physiological conditions or metal ions dissociate during the purification steps. This latter possibility is further examined in control experiments described below. Calculations based on metal affinities (see under “Discussion”) also rationalize the substoichiometric metal bound to LpxC under some conditions. LpxC-ZZ purified from cells grown in minimal (CDM) medium with or without iron supplementation contains mainly bound iron (Table 1); however, supplementation of the medium with zinc increases the iron/zinc ratio bound to LpxC-ZZ. In fact, the iron/zinc ratio bound to LpxC-ZZ varies with the (iron/zinc)_{total} ratio in the cell lysate in a roughly linear fashion (Fig. 2A), suggesting that LpxC co-purifies with the most abundant metal ion in the cells. The same trend is observed when the cellular concentration of LpxC is decreased (Fig. 2A), indicating that the metal content is not an artifact of protein overexpression.

One possible explanation for the correlation between the metals bound to LpxC-ZZ and the metal content of the cell lysate is that the metals bound to LpxC equilibrate with the cell lysate after cell lysis. To examine this possibility, LpxC-ZZ reconstituted with either stoichiometric Zn(II) or Fe(II) was incubated with cell lysates, purified using IgG beads, and the bound metal content determined by ICP-MS. These experiments demonstrate that the metal ion bound to LpxC-ZZ does not significantly dissociate (stoichiometry >0.7 Me(II)/LpxC) or equilibrate with metals in the cell lysate during the pulldown procedure because the iron/zinc ratio depends on the identity of the original metal ion bound to LpxC (Fig. 2B). Consequently, the pulldown experiments measure the bound metal content of LpxC-ZZ expressed in E. coli when cells are lysed.

### TABLE 1

| Growth medium | Lysate Fe/Zn ratio | Fe/LpxC/E_{max} | ZnLpxC/E_{max} |
|---------------|--------------------|----------------|---------------|
| CDM          | 3.2                | 0.41 ± 0.11   | 0.08 ± 0.01   |
| Fe(II)       | 22                 | 0.59 ± 0.14   | 0.04 ± 0.01   |
| Zn(II)       | 0.34               | 0.10 ± 0.07   | 0.25 ± 0.07   |
| Fe and Zn(II)| 2.9                | 0.42 ± 0.10   | 0.10 ± 0.06   |

* Data represent the average and S.D. from two replicate experiments. Data for LpxC isolated from cells grown in varied metal concentrations are shown in Fig. 2A. ICP-MS data have S.E. of ≤5%.

Chemically defined medium was without metal supplements or supplemented with 20 μM ZnSO_{4}, 20 μM ferric ammonium citrate, or both.
and indicate that LpxC incorporates the most available metal ion in a manner reflective of the cellular iron/zinc ratio.

**Native Activity Is Oxygen-sensitive and Dependent on Iron/Zinc Concentration**—We have previously shown that LpxC activity in crude cell lysates from cells grown in LB medium is redox-sensitive, consistent with the presence of Fe(II)-LpxC (18). However, under anaerobic conditions, the activity of LpxC in cell lysates is stable for >30 min, demonstrating that the metal ions do not rapidly dissociate or exchange. To further examine the in vivo metal content of LpxC, we measured the redox sensitivity of the native LpxC activity of E. coli cell lysates (no overexpression of LpxC) prepared from BL21(DE3) cells grown in minimal medium supplemented with iron and/or zinc. The cells were lysed in an anaerobic chamber, and the LpxC activity was assayed immediately after lysis (reflecting the activity of both ZnLpxC and FeLpxC) as well as after sufficient incubation in air (2.5 h) to allow the Fe(II) cofactor bound to LpxC to oxidize and be replaced by Zn(II) (18). These data demonstrate that the ratio of Fe(II)-LpxC to Zn(II) (see “Materials and Methods”) bound to natively expressed LpxC also depends on the (iron/zinc)total ratio in the cell lysate, comparable with the activity measured in iron-supplemented cell lysates is stable for 4 days (20 mM metal supplementation). These experiments demonstrate that the Fe(II) cofactor bound to LpxC readily exchanges with excess Zn(II) (Fig. 4B), although little exchange is observed when Zn(II)-LpxC is incubated with excess Fe(II). These data are consistent with the higher affinity of LpxC for zinc compared with iron, and the data can be modeled by competitive binding equations (Equations 4 and 5 and Figs. 4A and 4B) using the measured metal affinities. These calculations demonstrate that at the concentrations used for the in vitro exchange experiments, ZnLpxC is the predominant form at equilibrium.

\[
\frac{E \cdot Fe}{E_{tot}} = \frac{1}{1 + \frac{K_{D}^{Fe}}{Fe_{free}}(1 + \frac{Zn_{free}}{K_{D}^{Zn}})} \quad (Eq. 4)
\]

\[
\frac{E \cdot Zn}{E_{tot}} = \frac{1}{1 + \frac{K_{D}^{Zn}}{Zn_{free}}(1 + \frac{Fe_{free}}{K_{D}^{Fe}})} \quad (Eq. 5)
\]

The rate constants for dissociation of LpxC-bound metal ions were determined from the time-dependent decrease in
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activity upon dilution of the enzyme into EDTA and are not dependent on the concentration of chelator. These measurements reveal that the rate constant for dissociation ($k_{\text{off}}$) of Zn(II) and Fe(II) from LpxC are 0.032 ± 0.003 and 0.067 ± 0.004 min$^{-1}$, respectively (Fig. 4C). The similarity in the dissociation rate constants for Fe(II) and Zn(II) is unexpected given the significantly higher affinity of LpxC for Zn(II). Therefore, the discrimination between the two metal ions is observed mainly in the apparent association rate constants that are estimated from the values of $K_D$ and $k_{\text{ass}}$ as $\sim 9 \times 10^4$ (zinc) and $1 \times 10^4$ M$^{-1}$ s$^{-1}$ (iron), assuming a one-step binding mechanism. Addition of palmitate to ZnLpxC slows the dissociation rate constant by $\sim$1.3-fold to a half-time of 30 min at 25 °C suggesting that metal exchange in cell lysates could be inhibited by ligand binding. Although LpxC activity in cell lysates is stable under anaerobic conditions, addition of 1 mM EDTA to the lysate causes a 2-fold decrease in activity in 30 min, a time scale that is in the same range as the metal dissociation constants measured in vitro. These data suggest that EDTA decreases inhibition of metal exchange in lysates. Additionally, these data demonstrate that metals bound to LpxC can dissociate readily in vitro, disfavoring the possibility that in vivo metal selectivity is determined solely by a kinetic trap.

**DISCUSSION**

LpxC Is Activated by Either Fe(II) or Zn(II) in Cells—LpxC purified under aerobic conditions contains bound Zn(II) (15). However, LpxC is activated in vitro by a number of divalent metal ions, including zinc, iron, nickel, and cobalt (15, 18) and Fe(II)-LpxC is 6–8-fold more active than Zn(II)-LpxC (Table 2) (18). Substitution of Fe(II) for Zn(II) also enhances the affinity of LpxC for fatty acids by $\sim$6-fold and the affinity for hydroxamate inhibitors by $\sim$2-fold (18). X-ray absorption experiments demonstrate an increase in the ground state metal geometry from 4-coordinate in ZnLpxC to 5-coordinate in the Fe(II)-enzyme (18). These data suggest that enzyme function may correlate with coordination number; a 5-coordinate metal ion may enhance coordination of both water and substrate to lower the pK$_a$ value of the metal-bound water and to polarize the carbonyl group of the substrate to enhance catalysis. Despite the higher activity of Fe(II)-LpxC under in vitro conditions, the metal cofactor that activates LpxC under cellular conditions was unclear.

In the presence of oxygen, the Fe(II) cofactor bound to LpxC is oxidized to Fe(III), which dissociates and is replaced by Zn(II)

**TABLE 2**

| Kinetics and thermodynamic parameters of LpxC-C63A | $K_D$ (M$^{-1}$) | $k_{\text{ass}}$ (min$^{-1}$) | $k_{\text{off}}$ (s$^{-1}$) |
|---|---|---|---|
| Zn(II)-C63A LpxC | 60 ± 20 | 170 ± 34 |
| Fe(II)-C63A LpxC | 112 ± 43 | 990 ± 130 |
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(18). Therefore, to examine the in vivo metal content of LpxC, we developed a rapid pulldown experiment using an IgG-binding ZZ tag that could be carried out under anaerobic conditions. Subsequent determination of the bound metal by ICP-MS demonstrates that although the majority of bound metal to LpxC purified from cells grown in minimal medium is iron, the enzyme also contains bound zinc. In fact, the ratio of iron/zinc bound to LpxC-ZZ is linearly dependent on the iron/zinc ratio in the cell lysate (Fig. 2A). Control experiments (Fig. 2B) demonstrate that the measured LpxC metal content reflects the metals bound to LpxC in vivo when the cells are lysed. Furthermore, the iron/zinc ratio bound to natively expressed LpxC correlates with the iron/zinc ratio obtained from the pulldown assays (Fig. 2A), demonstrating that the observed metal variation in LpxC is not an artifact of protein overexpression. These data demonstrate that LpxC in E. coli binds and is activated by the “most available” metal ion. Therefore, although LpxC normally functions using Fe(II) as the co-factor, the metal cofactor switches to Zn(II) to adapt to environmental conditions and/or to regulate activity.

Metal Selectivity Is Determined by Association Kinetics and Thermodynamics—In contrast to the enhanced activity of Fe(II)-LpxC compared with Zn(II)-LpxC, the affinity of LpxC for Fe(II) is 1000-fold weaker than Zn(II) (K_D ~ 100 nM and 60 pm, respectively) (Fig. 4A). This differential affinity is consistent with the increased Lewis acidity and greater ligand stabilization energy predicted by the Irving-Williams series of metal stability constants (44, 45).

Unexpectedly, the rate constants for dissociation of Zn(II) and Fe(II) from the LpxC-metal complex are comparable (0.032 and 0.067 min⁻¹, respectively (Fig. 4C)) despite the large difference in binding affinity. This result indicates that metal selectivity occurs in the apparent association rate constant, estimated as 10⁷ and 10⁶ M⁻¹ s⁻¹ for Zn(II) and Fe(II), respectively, and implies that association of Fe(II) is not a diffusion-controlled step. Similar metal association kinetics have previously been observed for human carbonic anhydrase II where differential association rate constants for Zn(II) and Cu(II) (10⁴–10⁵ and 10⁸ M⁻¹ s⁻¹, respectively) have been observed (46, 47). Analysis of the zinc binding kinetics of human carbonic anhydrase II mutants suggests a two-step mechanism as follows: diffusion-controlled zinc association to form a complex with two protein ligands followed by a slower slower step that includes coordination of the third protein ligand (48). A similar two-step mechanism could explain the kinetics for Fe(II) binding to LpxC.

Given the relatively facile dissociation of the active site metal under in vitro conditions (t½ = 10–25 min), retention of the in vivo metal ion during purification of LpxC could have been difficult. However, little metal exchange or dissociation was observed in cell lysates (Fig. 2B) indicating that metal exchange/dissociation from LpxC is inhibited in lysates. One possible mechanism for this inhibition is the formation of a ternary LpxC–metal-ligand complex that decreases the metal dissociation rate constant. For example, a second metal ion or a fatty acid, such as palmitate (Fig. 4C), may bind to LpxC to inhibit metal exchange/dissociation (18, 37, 49).

Despite the higher affinity of LpxC for Zn(II), the in vivo data paradoxically indicate that Fe(II) is the predominant metal bound to LpxC in E. coli (Figs. 2 and 3 and Table 1). The mechanisms for determination of the iron/zinc metal selectivity of proteins in a cell are currently unclear. Metal selectivity has previously been proposed to depend on metal availability and/or be regulated by metallochaperones, although no zinc-specific metallochaperones have yet been identified (50, 51). As described in detail below, the observed in vivo metal content of LpxC can be rationalized solely from the metal affinity and concentration. Although the total cellular iron and zinc concentrations are comparable, at ~0.2 mM in most systems (26), the best estimates for the free or “readily exchangeable” cellular concentrations of Fe(II) and Zn(II) are vastly different. The best measurements, carried out mainly in mammalian cells, indicate that the concentration of Zn(II) is 10–400 µM, while the concentration of Fe(II) is 0.2–6 µM or higher (27–32). Therefore, the readily exchangeable Fe(II) concentration is predicted to be higher than the K_D Fe for LpxC, although the readily exchangeable zinc concentration is near the K_D Zn, rationalizing the predominance of Fe(II)-LpxC in vivo.

Using purely thermodynamic considerations, the percentage of iron- and zinc-bound LpxC can be calculated from the metal affinities and concentrations using a competitive model for LpxC metal binding (Equations 4 and 5). These calculations reveal that if metal binding to LpxC is under thermodynamic control, the balance of iron- to ZnLpxC is readily altered by changes in the free metal concentrations (Fig. 4D). For example, at the highest Fe_free and lowest Zn_free concentrations reported (6 µM and 10 pm, respectively), LpxC is calculated to contain 98% iron, 0.3% zinc, and 1.7% apoenzyme (iron/zinc ratio = 330). At the opposite extreme, 0.2 µM Fe_free and 400 pm Zn_free, LpxC is calculated to bind 19% iron and 70% zinc with 11% apoenzyme (iron/zinc ratio = 0.27). The measured values for Fe-, Zn-, and apo-LpxC in cells grown in CDM (Table 1) can be recapitulated using readily exchangeable cellular concentrations of Zn(II) and Fe(II) of 10 µM and 0.1 µM, respectively. These calculations indicate that the observed metal content of LpxC, including the substoichiometric metal content, can be explained solely using a thermodynamic model. Furthermore, these calculations demonstrate that modest perturbations in the cellular metal ion pool could influence the metal content of LpxC, as observed in response to alterations in the metal content of the media. Therefore, LpxC is well suited for metal switching in vivo. Importantly, this model for LpxC metal ion specificity demonstrates that thermodynamic considerations are sufficient to explain the observed behavior of LpxC in vivo. However, this correlation does not rule out the involvement of chaperones or other regulatory elements in determining the in vivo metal selectivity of LpxC.

Function of Metal Switching in LpxC—These data demonstrate that LpxC is the newest member of a growing class of non-heme Fe(II) hydrolases that were previously thought to be zinc enzymes, such as peptide deformylase, S-ribosylhomocysteinease, and possibly HDAC8 (19–24, 52, 53). However, LpxC should not be considered an Fe(II)-specific metalloenzyme but rather a Me(II)-dependent enzyme that is generally activated by Fe(II) but is subject to switching to
Zn(II)-LpxC in response to altered cellular conditions, such as variations in metal availability in the medium (Fig. 2A). Other environmental triggers, such as infection in a host or conditions of oxidative stress, may also perturb cellular metal ion homeostasis, and this alteration could be reflected in the active site metal ion bound to LpxC. Iron/zinc metal switching is likely to provide growth advantages under conditions where iron availability is limited, such as those encountered in infection (54–56). Metal switching may also be a novel regulatory mechanism, whereby the activity and possibly stability of LpxC can be finely tuned by changes in intracellular metal ion concentrations. Furthermore, it is possible that many prokaryotic metal-dependent hydrolases similarly switch the active site metal between Fe(II) and Zn(II) in response to changes in cellular metal homeostasis, as suggested by the enzymes previously identified as zinc- or iron-dependent hydrolases (21, 24, 57–60). This newly proposed mechanism to regulate the activity of LpxC and possibly other metalloenzymes has important implications for understanding metalloenzyme homeostasis as well as future drug design for LpxC and other antibiotic targets.

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