The Active Site of *Escherichia coli* UDP-N-acetylgalactosamine Acyltransferase

CHEMICAL MODIFICATION AND SITE-DIRECTED MUTAGENESIS*

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UDP-N-acetylgalactosamine (UDP-GlcNAc) acyltransferase (LpxA) catalyzes the reversible transfer of an R-3-hydroxyacyl chain from R-3-hydroxyacyl-acyl carrier protein to the glucosamine 3-OH of UDP-GlcNAc in the first step of lipid A biosynthesis. Lipid A is required for the growth and virulence of most Gram-negative bacteria, making its biosynthetic enzymes intriguing targets for the development of new antibacterial agents. LpxA is a member of a large family of left-handed β-helical proteins, many of which are acyl- or acyltransferases. We now demonstrate that histidine-, lysine-, and arginine-specific reagents effectively inhibit LpxA of *Escherichia coli*, whereas serine- and cysteine-specific reagents do not. Using this information in conjunction with multiple sequence alignments, we constructed site-directed alanine substitution mutations of conserved histidine, lysine, and arginine residues. Many of these mutant LpxA enzymes show severely decreased specific activities under standard assay conditions. The decrease in activity corresponds to decreased $k_{cat}/K_m$ values for all the mutants. With the exception of H125A, in which no activity is seen under any assay condition, the decrease in $k_{cat}/K_m$ mainly reflects an increased $K_m$. His125 of *E. coli* LpxA may therefore function as a catalytic residue, possibly as a general base. LpxA does not catalyze measurable UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc hydrolysis or UDP-GlcNAc/UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc exchange, arguing against a ping-pong mechanism with an acyl enzyme intermediate.

UDP-N-acetylgalactosamine (UDP-GlcNAc)$^3$ acyltransferase catalyzes the first step in the biosynthesis of lipid A, the hydrophobic anchor of lipopolysaccharide in Gram-negative bacteria (1–3). This enzyme, the product of the *lpxA* gene (4, 5), transfers an R-3-hydroxyacyl chain from R-3-hydroxyacyl-acyl carrier protein (ACP) to the glucosamine 3-OH of UDP-GlcNAc (Fig. 1) (6–8). The acylation of UDP-GlcNAc is characterized by an unfavorable equilibrium constant (−0.01) (8). Therefore, the second reaction of lipid A biosynthesis, in which the LpxA product UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc is deacetylated (9, 10), is the first irreversible step of the pathway (Fig. 1). The deblocked amino group is then immediately acylated with another R-3-hydroxymyristoyl moiety (6, 11). Mature lipid A is a disaccharide of 2,3-diacylated glucosamine units derived from UDP-2,3-diacylgalactosamine (Fig. 1) (12, 13). *Escherichia coli* lipid A is further phosphorylated at the 1 and 4′ positions and is acylated with laurate and myristate, respectively, at the R-3-hydroxyl groups of the 2′ and 3′ R-3-hydroxacyl chains (Fig. 1) (3, 14, 15).

Lipid A is required for growth of *E. coli* and most other Gram-negative bacteria (16, 17). Lipid A is also necessary for maintaining the integrity of the outer membrane as a barrier to toxic chemicals (18, 19). Furthermore, lipid A is a potent activator of innate immunity in animal systems (3, 20, 21). The study of the enzymes involved in lipid A biosynthesis should therefore prove useful for the development of new anti-infective drugs (22). All enzymes involved in *E. coli* lipid A biosynthesis have now been identified, and their structural genes have been cloned (3, 22–24).

The only enzyme of the pathway for which an x-ray structure is available is LpxA (25). LpxA is a trimer of identical subunits, and it represents the first example of a protein with a left-handed, parallel β-helix as the predominating feature of its secondary structure (25). The 10 coils of the β-helix of *E. coli* LpxA are specified by 24 complete and six incomplete hexapeptide repeats, most of which are contiguous (25). Three repeats (18 amino acid residues) make up one turn of the β-helix. Many other bacterial and eucaryotic proteins contain similar contiguous hexapeptide repeats (26). Three additional hexapeptide repeat proteins have recently been crystallized and shown to contain the same left-handed β-helix structure as seen in LpxA. These are a carbonic anhydrase from *Methanosarcina thermophila* (27), a tetrahydroydrocolinate N-succinyltransferase from *Mycobacterium bovis* BCG (28), and a xenobiotic acetyltransferase from *Pseudomonas aeruginosa* (29). Like LpxA, these enzymes are trimers.

There are no structural clues to the location or mechanism of the LpxA active site, because the LpxA crystal structure was solved in the absence of substrates or inhibitors (25). However, the sequences of more than 15 LpxAs are now available. The amino acid residues conserved across all LpxAs cluster around a deep cleft located between adjacent subunits (Fig. 2), which contains multiple histidines and other basic residues. Because of its symmetry, LpxA has three such clefts (not all visible in Fig. 2). The ACP substrate of LpxA consists of 77 amino acid residues and is very acidic (30, 31). Accordingly, it is plausible that the substrates R-3-hydroxymyristoyl-ACP and UDP-GlcNAc might bind to this basic cleft. Further evidence for the importance of the cleft comes from recent studies of the acyl chain length specificity of *E. coli* LpxA (32). Substitution of glycine 173 (red residue in the shaded area of Fig. 2) with...
methionine switches the acyl chain length selectivity of *E. coli* LpxA from 14 carbons to 10 (32).

We now present chemical modification studies to demonstrate that histidine, lysine, and arginine residues are important in LpxA catalysis, whereas serine and cysteine residues are not. We also use site-directed mutagenesis to examine the effects of changing conserved histidine, lysine, and arginine residues. The combined findings support the view that His125 of LpxA is required for catalysis, whereas other conserved basic residues are not. We also use site-directed mutagenesis to examine the effects of changing conserved histidine, lysine, and arginine residues. The combined findings support the view that His125 of LpxA is required for catalysis, whereas other conserved basic residues are not.

![Fig. 1. Role of LpxA in *E. coli* lipid A biosynthesis.](image1)

**FIG. 1.** Role of LpxA in *E. coli* lipid A biosynthesis. The first step in lipid A biosynthesis is catalyzed by LpxA (3). The transfer of the R-3-hydroxymyristoyl moiety from R-3-hydroxymyristoyl-ACP to UDP-GlcNAc is reversible and thermodynamically unfavorable (8). Therefore, the deacetylase encoded by *lpxC* is the first committed step of the pathway and is regulated in response to lipid A content (35). LpxD displays significant sequence homology to LpxA, including conservation of the β-helix and the equivalent of His125 (46). Structures of the other biosynthetic intermediates and the genes encoding the enzymes of the rest of the pathway have been reviewed elsewhere (3, 22).

![Fig. 2. Highly conserved basic amino acid side chains surrounding the proposed LpxA active site cleft.](image2)

**FIG. 2.** Highly conserved basic amino acid side chains surrounding the proposed LpxA active site cleft. The individual monomers of the LpxA homotrimer are shown in yellow, green, and gray (25). Basic residues that are conserved (46) in the LpxAs from 15 diverse Gram-negative organisms (*E. coli, Salmonellatyphimurium, N. meningitidis, P. aeruginosa, Haemophilus influenzae, Yersiniaenterocolitica, Helicobacter pylori, Proteusmirabilis, Aquifexaeolicus, C. vinosum, Rickettsia richettsii, Rickettsiaprowazekii, Brucellabortus, Chlamydia trachomatis, and Synechocystis sp.*) are colored by residue type. His125 is magenta, whereas His144 (just to the left of His125), His122 (immediately behind His125), and His126 (above His125) are colored purple. Lys92 (just below His125) and Arg208 are blue. Gly173 of *E. coli* LpxA, which is conserved as such mainly in 14 carbon-specific LpxAs, is red. Gly173 is buried deeply within the proposed active site cleft, as indicated by the shading. The only known exceptions to the conserved basic residues shown above are H160Y in *N. meningitidis* and K76S in *C. vinosum.*

**EXPERIMENTAL PROCEDURES**

**Materials—**[α-32P]UTP was purchased from NEN Life Science Products. Tryptone, yeast extract, brain heart infusion medium, and agar were from Difco. Antibiotics, glucosamine-1-phosphate, and ACP were products of Sigma. Chloroform, methanol, and acetic acid were from Mallinckrodt. All other chemicals were obtained from Sigma or Mallinckrodt. Silica gel-60 thin layer plates (0.25 mm) were purchased from Merck. Restriction enzymes, Klenow, and T4 DNA ligase were from New England BioLabs or Roche Molecular Biochemicals. Shrimp alkaline phosphatase was from U.S. Biochemical Corp. Primers for mutagenesis were custom-made by Life Technologies, Inc. The LpxC inhibitor L-573,655 (17) was provided by Dr. A. Patchett (Merck Research Laboratories, Rahway, NJ).

**Standard Assay of LpxA Activity in the Forward Direction—**The assay monitors the conversion of [α-32P]UDP-GlcNAc to [α-32P]UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc (7, 8). The standard reaction mixture (10–20 μl) contains 40 mM HEPES, pH 8, 1 mg/ml BSA, 10 μM [α-32P]UDP-GlcNAc (2 × 10^6 cpm/μmol). Substrates were synthesized as described previously (33). The reaction is started by the addition of enzyme (either purified or in a cell extract). The reaction mixture is incubated at 30 °C for 1–10 min. For measuring initial rates, the enzyme concentrations (monomer) are typically 1–10 nM. The reactions are terminated by spotting 2–2.5 ml of the reaction mixture onto a silica thin layer chromatography plate. After the spots are dried, the plates are developed in the solvent chloroform/methanol/water/acetic acid (25:15:4:2, v/v/v/v). The plates are dried and then exposed to imaging screens overnight at room temperature. The plates are visualized, and the extent of the reaction is quantified using a Molecular Dynamics PhosphorImager, operated with ImageQuant software.

**For assays of** *E. coli* **crude extracts,** 0.2 mg/ml L-573,655 (17) from a 10 mg/ml stock in dimethyl sulfoxide is added to the assay mixtures to inhibit further metabolism of the LpxA reaction product by LpxA. 

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**UDP-GlcNAc + R-3-hydroxymyristoyl-ACP**

- **LpxA**
- **LpxC**
- **Acetate**
- **LpxD**
- **R-3-hydroxymyristoyl-ACP**

**UDP-2,3-diacylglycosamine**

**seven steps**

**Lipid A moiety (Endotoxin)**

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**UDP-GlcNAc + R-3-hydroxyacyl-ACP**

- **LpxA**
- **LpxC**
- **Acetate**
- **LpxD**
- **R-3-hydroxyacyl-ACP**
ent in these extracts (Fig. 1). This is unnecessary for assays of purified LpxA or of *Corynebacterium glutamicum* extracts expressing recombinant *E. coli* LpxA.

**Assay of LpxA in the Reverse Direction**—The assay of LpxA in the reverse direction involves the conversion of UDP-(3-O-acyl-3-deoxy-d-manno-oct-2-ulopyranosyl)-β-d-N-acetylglucosamine (UDP-GlcNAc) to muramyl pentapeptide ([UDP-3-O-5-R-3-hydroxy-3-methylbutyl-D-glucosamine]-L-rhamnomannurate) (UDP-3-O-5-R-3-hydroxyxymyristoyl)-GlCNAC to [α-32P]UDP-GlcNAc (8, 34). The assay mixture (10–20 μl) includes 40 mM HEPES, pH 8, 1 mg/ml BSA, 10 μM acetyl acceptor, 10 μM LpxA (monomer), and 80 μM [α-32P]UDP-3-O-5-R-3-hydroxyxymyristoyl)-GlCNAC (7 × 10^6 cpm/nmol) (35) (provided by Jane E. Jackman, Duke University). ACP, coenzyme A (Sigma), pantetheine (Sigma), N-acetylcycteamine (Aldrich), glutathione (Sigma), and UDP-GPCNAc were tested as acetyl acceptors in parallel reactions (in ethanol) were made each day just prior to an experiment. Exact reactions with LpxA at room temperature were quenched by a 1:50 dilution into 70 μM imidazole (on ice) (at least a 10-fold molar excess over the DEPC). DEPC, ethanol, and imidazole at the concentrations used have no effect on the substrates of the LpxA reaction, and ethanol and imidazole have no effect on LpxA activity at the levels at which they were used. The 70 μM DEPC/imidazole mixtures were further diluted with 1 mg/ml BSA and assayed by the standard method using 40 mM potassium phosphate, pH 7, instead of 40 mM HEPES, pH 8, but this modification has little effect on LpxA activity.

For substrate protection studies, 20 μM purified LpxA was incubated at room temperature for 30 min with various concentrations of pyridoxal 5′-phosphate, phenylglyoxal, or phenylmethanesulfonyl fluoride. These concentrations correspond to about 20 times the ~Km for each substrate. The reactions were quenched by a 1:50 dilution into 40 μM imidazole (a 10-fold molar excess). The mixtures were then further diluted as appropriate into 1 mg/ml BSA and assayed by the standard method with 40 mM potassium phosphate, pH 7, taking into account the residual substrate concentrations carried over from the preincubation.

For NH2OH reactivation studies, 20 μM purified LpxA was incubated with and without 100 μM DEPC for 5 min at room temperature in 40 mM potassium phosphate, pH 7. The reactions were quenched by a 1:50 dilution into 20 μM imidazole. These mixtures were then diluted 1:2 into 40 mM potassium phosphate, pH 7, and 1 mg/ml BSA and were assayed by the standard method with 40 mM potassium phosphate, pH 7.

**Modification of LpxA with Lysine-, Arginine- and Serine-specific Reagents**—In all experiments, 20 μM purified LpxA (monomer) was incubated at room temperature for 30 min with various concentrations of pyridoxal 5′-phosphate, phenylglyoxal, or phenylmethanesulfonyl fluoride. Both reagents were from Sigma, and [α-32P]UDP-3-O-5-R-3-hydroxyxymyristoyl-ACP. These concentrations correspond to about 20 times the ~Km for each substrate. The reactions were quenched by a 1:50 dilution into 40 μM imidazole (a 10-fold molar excess). The mixtures were then further diluted as appropriate into 1 mg/ml BSA and assayed by the standard method with 40 mM potassium phosphate, pH 7.

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A Conserved Histidine Residue Is Required for LpxA Activity

CaCl₂-competent BL21(DE3)/pLysE (Novagen) cells.

Cloning Mutant lpxA Genes into pGK1—pET23c-based plasmids containing wild type and mutant lpxA genes were digested with NdeI and BamHI to excise the lpxA gene. This fragment was treated with Klenow fragment and then ligated into a BamHI-digested and Klenow fragment containing pGK1 (pShuttleUmu/bacterium/E. coli shuttle vector) (37). The plasmid with wild type lpxA is pTO8 (32). Other plasmids are named for the mutated gene that they carry prefaced by pGK, e.g. pGK-H122A. Plasmids were introduced into C. glutamicum R163 by electroporation (32, 44). Competent cells were made by growing a 500-ml culture of C. glutamicum in LB broth at 30 °C (225 rpm) to A₆₀₀ = 0.2. Cells were washed twice with 25 ml of water and resuspended in 2.5 ml of the same. Electroporation was done under the following conditions: 50 µl of cells and 1 µl (1 µg) of DNA in a 0.2-cm cuvette (Bio-Rad) at 2.5 kV, 25 microfarad, and 200 Ω. Cells were allowed to recover for 2 h at 30 °C in 1 ml of brain heart infusion (BHI) medium and then plated appropriately to yield approximately 100 colonies at 30 °C on BHI plates (15 g/liter agar) supplemented with 50 µg/ml rifampicin and 5 µg/ml chloramphenicol.

Western Blotting—C. glutamicum extracts (1.5 µg) and purified LpxA (50 ng) were analyzed by SDS-polyacrylamide gel electrophoresis (15%). Proteins were transferred to a polyvinylidene difluoride (Bio-Rad) membrane by the following procedure. A piece of extra thick filter paper (Bio-Rad) was wetted in 1 × transfer buffer (25 mm Tris base and 200 mm glycine containing 20% methanol). On top of this was placed the polyvinylidene difluoride membrane that had been prewetted in methanol, followed by water and then by 1 × transfer buffer containing 20% methanol. The gel was placed on top of the membrane and covered with a piece of extra thick filter paper wetted in 1 × transfer buffer containing 0.05% SDS. The transfer was done at 20 V for 40 min with a Bio-Rad Trans-Blot SD semi-dry transfer apparatus. After transfer, the polyvinylidene difluoride membrane was blocked overnight at room temperature with blocking buffer (PBS containing 0.2% Tween-20) (Sigma) and 5% Krogger nonfat dried milk; PBS consists of 1.45 g of Na₂HPO₄, 0.2 g of KH₂PO₄, 0.2 g of KCl, and 8.0 g of NaCl/liter at pH 7.4). The next day the membrane was washed twice quickly with 25 ml of PBS containing 0.2% Tween-20 and then incubated for 1 h at room temperature with 20 ml of blocking buffer containing primary antibody (provided by Dr. Garry Dotson). The primary antibody is polyclonal rabbit antibody generated against purified His-tagged LpxA (45). This antibody was partially purified using a Ni²⁺ column charged with His-tagged LpxA and then a protein A column (45). The primary antibody was used at a 1:10,000 dilution from a 1 mg/ml stock. The membrane was then washed three times for 5 min with 30 ml of blocking buffer. The membrane was washed twice quickly with 25 ml of PBS containing 0.2% Tween-20 and then incubated for 1 h at room temperature with 20 ml of blocking buffer containing secondary antibody. The secondary antibody is donkey anti-rabbit immunoglobulin conjugated with horse-radish peroxidase (Amersham Pharmacia Biotech). The secondary antibody was used at a 1:20,000 dilution from a 0.5 mg/ml stock. The membrane was washed as described above and then washed three more times with 30 ml of PBS containing 0.2% Tween-20. Detection was done using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) according to the manufacturer’s directions.

Determination of Kinetic Parameters—Initial velocities were measured with the standard assay in the forward direction using 100 µM R-3-OH-myristoyl-ACP (wild type LpxA K_m/ACP = 1 µM) and varying concentrations of UDP-GlcNAc. Purified wild type LpxA (3.6 nm monomer), as well as extracts of C. glutamicum cells (1–6 mg/ml) carrying plasmids that express wild type or mutant lpxA genes, were assayed at concentrations appropriate for following the initial rate. Because of the unfavorable equilibrium constant of the LpxA reaction (8), initial velocities could only be measured for 100 µM UDP-GlcNAc. For purified wild type LpxA and extracts containing wild type, K₇6A, or K₇8R LpxA, steady-state kinetic parameters were determined by fitting the initial velocities to the Michaelis-Menten equation by nonlinear regression using the Kaleidograph (Synergy Software) curve-fitting program. For extracts containing H122A, H144A, or H160A LpxAs, the initial velocities increased linearly with UDP-GlcNAc concentrations up to 10 mM. In these cases, k_cat/K_m values were determined by fitting initial velocities to a line using the Kaleidograph program, and lower limits for k_cat and K_m were then extrapolated.

The concentration of LpxA in C. glutamicum extracts was estimated in the following manner. The specific activity of purified wild type LpxA under standard conditions is 265,000 pmol/min/mg, and the specific activity under standard conditions in C. glutamicum extracts containing wild type LpxA is 347 pmol/min/mg. Dividing these two values gives the conversion factor of 784 mg extract/mg LpxA. Because expression

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![Figure 3. Time course of inactivation of E. coli LpxA by DEPC.](image)

**FIG. 3.** Time course of inactivation of E. coli LpxA by DEPC. Purified LpxA (20 µM monomer) was preincubated at room temperature without (○) or with 40 µM (△), 80 µM (■), 160 µM (●), or 320 µM DEPC (■). At the indicated times, a portion of the preincubation mixture was quenched with imidazole, diluted, and assayed for remaining LpxA activity. Levels are very similar in all the C. glutamicum extracts for wild type and mutant LpxA, as judged by Western blotting (not shown), this conversion factor was used to estimate the k_cat and K_m values for all the LpxAs expressed in the C. glutamicum system.

**RESULTS**

**Inactivation of LpxA by DEPC**—Incubation of purified LpxA with DEPC, a histidine reagent, causes a rapid loss of enzymatic activity (Fig. 3). LpxA is stable under the preincubation conditions in the absence of DEPC. The loss of activity depends upon the time of preincubation and the concentration of DEPC, and inactivation is complete in 10 min with only a small (4-fold) molar excess of DEPC over LpxA monomer.

To determine whether the inactivation of LpxA results from modification of an active site residue, we looked for substrate protection against DEPC inactivation. UDP-GlcNAc has no effect, but R-3-hydroxymyristoyl-ACP largely protects LpxA from inactivation by DEPC (data not shown). ACP itself contains one histidine residue, and consequently care was taken to ensure that ACP was not simply scavenging the DEPC reagent. The incubation included a 2-fold molar excess of acyl-ACP over LpxA monomer, and a 10-fold molar excess of DEPC. Therefore, even if the histidine of ACP reacted with the DEPC, there would still be more than enough DEPC (an 8-fold excess) to cause full inactivation of LpxA. Protection by acyl-ACP suggests that the modified residue accounting for the inactivation is in the E. coli ACP active site or at least in the cleft proposed to dock ACP (Fig. 2), which contains four conserved histidine residues.

**Reactivation of DEPC-inactivated LpxA by NH₂OH**—In some cases, DEPC has been shown to react with side chains other than histidine (38). The small amount of DEPC needed for the inactivation of LpxA and the rapid rate of the inactivation (Fig. 3) argue that side chains other than histidine are not being protected. In addition, the inactivation by DEPC can be reversed by incubation with NH₂OH (not shown). NH₂OH reverses DEPC modification of histidine and tyrosine residues but not of lysine or cysteine side chains (38). The reaction of DEPC with tyrosine residues on LpxA can be ruled out, given that no change in the absorbance of LpxA at 280 nm occurs upon reaction with DEPC (not shown). Thus, the inactivation of LpxA by DEPC can be attributed solely to the modification of histidine residue(s).
A Conserved Histidine Residue Is Required for LpxA Activity

Correlation between the Number of Histidine Residues Modified and the Loss of LpxA Activity—When DEPC reacts with a histidine residue, a carbethoxyhistidine moiety is formed that absorbs light at 240 nm (ε = 3200 M⁻¹ cm⁻¹) (38). We used this phenomenon to calculate the number of histidine residues modified per LpxA monomer during the inactivation process. Throughout the incubation of LpxA with DEPC, portions of the reaction mixture were removed, quenched with imidazole, and assayed for LpxA activity. The correlation between the number of histidine residues modified and the loss of catalytic activity is shown in Fig. 4. When an average of one histidine residue/monomer is modified, only 20% of the initial activity remains. Because LpxA contains 12 histidines/monomer, it is reasonable that some monomers would have acquired more than one modified histidine residue, whereas 20% might have no modified histidines. These results suggest that one modified histidine/monomer leads to complete inactivation of LpxA. This would correspond to three modified histidines/trimer, consistent with the idea of three functional active sites/trimer.

Effects of Other Chemical Modification Reagents on LpxA Activity—We next examined LpxA inactivation by pyridoxal 5′-phosphate/sodium borohydride, phenylglyoxal, and phenylmethanesulfonyl fluoride, lysine-, arginine-, and serine-specific reagents, respectively (40). Low millimolar concentrations of both pyridoxal 5′-phosphate/sodium borohydride and phenylglyoxal are capable of inhibiting LpxA completely (not shown). Phenylmethanesulfonyl fluoride has little effect on LpxA activity (not shown). In addition, no time-dependent inactivation of LpxA is seen using the cysteine reagent, methyl methane thiosulfonate (not shown). These findings suggest that lysine and arginine residue(s) might play important roles in LpxA substrate binding and/or catalysis, whereas serine and cysteine residues probably do not, consistent with the fact that there are no conserved serine or cysteine residues in diverse LpxAs.

Site-directed Mutagenesis of E. coli LpxA—The sequences of LpxA proteins from 15 Gram-negative bacteria were found by BLAST searching of the nonredundant protein data base (46). All LpxAs have conserved, solvent-exposed histidine residues at positions corresponding to 122, 125, and 144 in the E. coli LpxA sequence (Fig. 2). The histidine at position 160 (Fig. 2) is conserved in all LpxAs except for the enzyme from Neisseria meningitidis (47), in which that position is a phenylalanine. Lysine 76 (Fig. 2) is conserved across all LpxAs except for the enzyme from Chromatium vinosum, in which it is a serine. Arginine 204 (Fig. 2) is conserved in all LpxA homologues identified to date. Accordingly, each of these residues was mutated to alanine, and the mutant LpxAs were overexpressed under T7 promoter control in the E. coli strain BL21(DE3)/pLysE. All the mutants expressed comparable levels of LpxA protein, as judged by analysis of membrane-free supernatants by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining (not shown).

The relative activities of the membrane-free supernatants containing these LpxA mutants as compared with membrane-free supernatant containing overexpressed wild type LpxA are as follows (Table I). H122A, H144A, and K76A LpxAs have 2–6% wild type specific activity under standard assay conditions in extracts made from IPTG induced cells, whereas H160A and H125A have less than 1% of the wild type activity (Table I). Mutation of the conserved arginine at position 204 (Fig. 2) to alanine likewise displayed <1% wild type activity.² Each mutant shows increased protein levels upon induction with IPTG as judged by gel electrophoresis (not shown), and all but H125A and H160A show corresponding 5–10-fold induction of LpxA activity compared with extracts of uninduced cells (Table I). The observation that increased levels of H125A protein do not lead to increased activity suggests that H125A LpxA is completely inactive. Indeed, the activity of H125A extracts is in the range of the pET23c vector control (Table I).

The next set of mutant LpxAs that we constructed included H122N, H125N, H144N, H160F, and K76R (Table I). Asparagine can sometimes take the place of histidine in hydrogen bond formation. The H160F mutant was made to test whether the N. meningitidis residue could take the place of His¹⁶⁰ in E. coli LpxA, and the K76R mutant was made to determine whether a positive charge at position 76 was sufficient to support activity. K76R LpxA has 5-fold more activity than K76A LpxA, but none of the other mutants have significantly more activity than the original alanine mutants (Table I). Like H125A, the extracts of strains expressing H125N do not show increased LpxA activity upon induction of these mutant proteins with IPTG (Table I), nor was activity restored by the addition of imidazole to H125A LpxA, or a H125G mutant (not shown).

The mutant enzymes H122A, H125A, H144A, H160A, K76A, and K76R were purified as described under “Experimental Procedures.” Each mutant LpxA eluted from each column at the same point in the NaCl gradients as did wild type LpxA. Analytical ultracentrifugation was used to demonstrate that each of these mutant enzymes formed trimers in aqueous solution, just like the wild type (data not shown). These results taken together suggest that the global protein folds of the mutant LpxAs are similar to that of the wild type. The loss of activity associated with each of the mutations (Table I) is therefore likely to be due to the loss of a residue that is important for catalysis and/or substrate binding rather than to a major structural change. However, the possibility of small local conformational changes associated with these point mutations cannot yet be excluded.

Expression of E. coli LpxA Mutants in C. glutamicum R163—When LpxA mutants are expressed in E. coli, the background of wild type LpxA activity made from the chromosomal copy of the essential lpxA gene complicates the kinetic analysis. Because the mutant LpxAs all purify exactly like the wild type enzyme, even the “purified” mutant enzymes are contaminated with

² C. Sweet and C. R. H. Raetz, unpublished results.
residual wild type LpxA. To avoid this problem, we expressed H122A, H125A, H144A, H160A, K76A, and K76R LpxAs in a Gram-positive host, C. glutamicum (37). Gram-positive bacteria do not contain any of the lpx genes, because they do not make lipid A (48). C. glutamicum has previously been shown to be a good system for expressing and assaying LpxA point mutants (32).

Mutated lpxA genes were removed from the pET23c vector and cloned into the C. glutamicum/E. coli shuttle vector, pGK1 (37). These plasmids were then used to express the mutant LpxAs in C. glutamicum. Overexpression of the enzymes in these extracts is 2 orders of magnitude lower than in the T7 promoter-driven E. coli system, and the LpxA protein band cannot be visualized by Coomassie staining after SDS-polyacrylamide gel electrophoresis of C. glutamicum crude extracts (not shown). Therefore, a Western blot with an anti-LpxA antibody was used to demonstrate that all mutant LpxAs were expressed to similar levels (data not shown).

The relative specific activities under standard assay conditions of the LpxA mutants compared with the wild type, when expressed in C. glutamicum extracts, are shown in Table II. With no LpxA activity background from the host cells, it is clear that H125A is completely inactive. The other mutants have similar relative specific activities as observed in the E. coli extracts (Table I).

**Kinetic Analysis of LpxA Point Mutants**—The initial kinetic analysis of the LpxA point mutants was carried out using the C. glutamicum extracts, because they have no wild type LpxA background activity, and the mutants all seem to behave similarly in the C. glutamicum system as in E. coli extracts (Tables I and II). In addition, the wild type $k_{cat}$ and $K_m$ values saturated levels of acyl-ACP (100 $\mu$M) (8) are the same for purified LpxA and wild type LpxA expressed in C. glutamicum extracts (Table III).

The $K_{m}$UDP-GlcNAc, $k_{cat}$, and $k_{cat}/K_{m}$ values for the LpxA point mutants are shown in Table III. The $k_{cat}/K_{m}$ values for the LpxA mutants are lower than wild type in all cases. This decrease is due to an increase in $K_{m}$ values for all of the mutants. In addition, H160A LpxA probably has a lower $k_{cat}$ than wild type. However, H125A shows no activity under any assay condition, and therefore no kinetic parameters can be measured. These results suggest that Lys$^{76}$, His$^{122}$, His$^{144}$, and possibly His$^{160}$ play roles in substrate binding and that His$^{225}$ may be directly involved in catalysis.

### Table I

**Specific activities of wild type and mutant LpxAs expressed in E. coli extracts**

Standard LpxA assays at pH 8 were performed with membrane-free supernatants prepared from BL21(DE3)/pLysE cells carrying pET23c-based plasmids containing wild type or mutant lpxA genes.

| Plasmid         | Specific activity (uninduced) pmol/min/mg | Specific activity (induced) pmol/min/mg | Relative activity (%) |
|-----------------|------------------------------------------|----------------------------------------|----------------------|
| **Experiment I** |                                          |                                        |                      |
| pET23c (vector) | 49                                       | 62                                     |                      |
| pTO1 (wild type)| 5210                                     | 38500                                  | 100                  |
| pTO-H122A       | 435                                      | 2360                                   | 6.1                  |
| pTO-H125A       | 181                                      | 117                                    | 0.50                 |
| pTO-H144A       | 196                                      | 1370                                   | 3.5                  |
| pTO-H160A       | 123                                      | 222                                    | 0.58                 |
| pTO-K76A        | 164                                      | 1080                                   | 2.8                  |
| pTO-K76R        | 513                                      | 5050                                   | 13                   |
| **Experiment II** |                                         |                                        |                      |
| pET23c (vector) | 113                                      | 151                                    |                      |
| pTO1 (wild type)| 17000                                    | 55000                                  | 100                  |
| pTO-H122N       | 182                                      | 816                                    | 2.1                  |
| pTO-H125N       | 203                                      | 201                                    | 0.52                 |
| pTO-H144N       | 464                                      | 7000                                   | 18                   |
| pTO-H160F       | 499                                      | 516                                    | 1.1                  |

### Table II

**Specific activity of wild type and mutant LpxAs expressed in C. glutamicum extracts**

Standard LpxA assays at pH 8 were performed with extracts prepared from C. glutamicum cells carrying pGK1-based plasmids containing wild type or mutant lpxA genes.

| Plasmid | Specific activity pmol/min/mg | Relative activity % |
|---------|------------------------------|---------------------|
| pGK1 (vector) | <0.01* | <0.003 |
| pTO8 (wild type) | 347 | 100 |
| pGK-H122A | 27 | 7.7 |
| pGK-H125A | <0.01 | <0.003 |
| pGK-H144A | 22 | 6.3 |
| pGK-H160A | 2.4 | 0.69 |
| pGK-K76A | 27 | 7.7 |
| pGK-K76R | 33 | 9.5 |

* 0.01 pmol/min/mg is the lower limit of detection for this assay.
In the C-terminal α-helical region of LpxA (25). All these residues cluster around a deep cleft situated between the monomers of the LpxA trimer (Fig. 2). Another residue in the same area, glycine 173 (Fig. 2, red), is involved in determining the acyl-chain length selectivity of LpxA (32). We presume that LpxA has three identical active sites, consistent with the locations of the active sites in other β-helix proteins (27–29). The active site of M. thermophila carbonic anhydrase is defined by the location of its catalytic zinc atom (27). The locations of the active sites of the P. aeruginosa xenobiotic acetyltransferase (29) and of the B. bovis tetrahydrodipicolinate N-succinyltransferase (28, 51) are based on x-ray structures of co-crystals with substrate or inhibitor, respectively.

What roles might the conserved residues of the putative LpxA active site play in catalysis? To answer this question, we investigated LpxA mutants in which each residue was mutated to alanine (Tables I and II). The H125A LpxA mutant is completely inactive as shown by activity assays of the enzyme expressed in the Gram-positive host C. glutamicum (Table II). However, the other mutant LpxAs do possess low levels of activity. The absolute requirement for His125 suggests a catalytic role, such as that of a general base in a direct transfer mechanism (Fig. 6), whereas the other residues may be involved in substrate binding.

We further investigated these LpxA mutants by measuring $K_{m,\text{UDP-GlcNAc}}$ and $k_{cat}$ and $k_{cat}/K_{m,\text{UDP-GlcNAc}}$ values for the enzymes expressed in C. glutamicum. We did not measure $K_{m,\text{acyl-ACP}}$ values because it is not technically feasible with the current assay. The wild type $K_{m,\text{acyl-ACP}}$ is $2 \mu M$ (8). The wild type $K_{m,\text{UDP-GlcNAc}}$ value is 3 orders of magnitude higher (1 mM), and the mutant LpxAs have $K_{m,\text{UDP-GlcNAc}}$ values that are another order of magnitude higher ($>10$ mM) (Table III). The equilibrium constant for the LpxA reaction is 0.01 (8). With these constraints, it is impossible to measure the conversion of [3-32P]UDP-GlcNAc to [3-32P]UDP-3-(R-3-hydroxymyristoyl)-GlcNAc at very high UDP-GlcNAc and very low acyl-ACP concentrations. Determination of accurate $K_{m,\text{acyl-ACP}}$ values for the LpxA mutants will have to await the development of a new LpxA assay involving radioactive acyl-ACP as the donor and unlabeled UDP-GlcNAc as the acceptor.

We measured the $K_{m,\text{UDP-GlcNAc}}$ of purified wild type LpxA to be 1 mM, an order of magnitude higher than that reported previously (8), and two-fold higher than that reported for wild type LpxA in cell extracts (7). The substrate preparations used in the present study are of higher quality than those used in the earlier investigations. In addition, the use of a Phosphor-Imager in our work has greatly facilitated the collection of a much larger number of accurate data points. For these reasons
we are confident that our $K_{m, \text{UDP-GlcNAc}}$ values are correct.

Mutation of Lys$^{76}$, His$^{122}$, His$^{144}$, or His$^{160}$ to alanine results in $k_{\text{cat}}/K_{m, \text{UDP-GlcNAc}}$ values that are lower than that for wild type LpxA (Table II). This reflects a higher $K_{m, \text{UDP-GlcNAc}}$ in all cases, suggesting that the mutated residues are involved in UDP-GlcNAc binding and/or formation of the ternary complex of UDP-GlcNAc, acyl-ACP, and LpxA. In particular, K76A has a $K_{m, \text{UDP-GlcNAc}}$ over an order of magnitude higher than wild type (Table III). The value is two-fold lower when Lys$^{76}$ is replaced with arginine. A positive charge at position 76 (Fig. 2) could be involved in binding the phosphates of UDP-GlcNAc or an acidic side chain of the acyl-ACP. In addition to a higher $K_{m, \text{UDP-GlcNAc}}$, H160A LpxA has a lower $k_{\text{cat}}$ than wild type LpxA (Table III). His$^{160}$ may therefore play a more direct role in catalysis and/or substrate binding. More detailed kinetic analyses in conjunction with crystal structures of LpxA in complex with its substrates will be needed to define more precisely the roles of these histidine and lysine residues.

We propose that LpxA is not likely to utilize a ping-pong mechanism with an acyl enzyme intermediate, because LpxA does not remove the acyl chain from UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc in the absence of a thiol-containing acyl acceptor (Fig. 5). The absence of either a conserved serine or cysteine residue and the inability of serine- or cysteine-specific reagents to inactivate LpxA render any mechanism involving an acyl-enzyme intermediate on serine or cysteine extremely unlikely. However, we cannot exclude the possibility of a mechanism in which binding of both the acyl-donor and the acylacceptor is required for the formation of an acyl-enzyme intermediate in the ternary complex on a residue other than serine or cysteine, perhaps on His$^{125}$. Nevertheless, a more likely role for His$^{125}$ would be as a general base, abstracting the hydrogen from the 3-OH of UDP-GlcNAc and preparing that substrate for direct nucleophilic attack of the acyl chain carbonyl group of the acyl-ACP (Fig. 6). Interestingly, LpxD (Fig. 1) displays significant sequence homology to LpxA (11) in that it also contains an extensive $\beta$-helix domain, and it appears to have conserved the equivalent of the LpxA His$^{125}$ residue (46).

A better studied precedent for the mechanism shown in Fig. 6 is that proposed for the type III chloramphenicol acetyltransferase (49, 50), in which co-crystals of the enzyme with chloramphenicol, affinity labeling, site-directed mutagenesis, and kinetic studies all strongly support the model. However, the chloramphenicol acetyltransferase does differ from LpxA in that it can catalyze two distinct acetylations of its substrate.

In the process of studying the reverse LpxA reaction, we discovered that, in addition to ACP, pantetheine, coenzyme A, and DTT function as weak acyl-acceptors (Fig. 5). Although this finding contradicts earlier reports that R-3-hydroxymyristoyl-CoA is not a substrate in the forward direction (7, 8), LpxA activity with pantetheine, CoA, or DTT in the reverse direction is 5 orders of magnitude slower than with ACP and is therefore unlikely to have any physiological significance. Given the technical constraints of the LpxA assay in the forward direction, it would be extremely difficult to detect acyltransferase activity with such poor substrate analogs.
The LpxA assay in the reverse direction (Fig. 5) offers a very sensitive probe for certain low activity substrates. The sensitivity of the reverse assay might therefore be useful for additional kinetic and thermodynamic studies of LpxA. For instance, a comparison of the LpxA equilibrium constants for coenzyme A versus ACP, approached from the product side (as in Fig. 5), might provide some insights into the physical basis for the unfavorable equilibrium constant observed for the LpxA reaction with its natural substrates. The acyl chain length specificity in the reverse direction, as well as the binding constants for each of the LpxA substrates and products, might also prove to be very informative.

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