Identification of functionally important residues and structural features in a bacterial lignostilbene dioxygenase

Lignostilbene-α,β-dioxygenase A (LsdA) from the bacterium *Sphingomonas paucimobilis* TMY1009 is a nonheme iron oxygenase that catalyzes the cleavage of lignostilbene, a compound arising in lignin transformation, to two vanillin molecules. To examine LsdA’s substrate specificity, we heterologously produced the dimeric enzyme with the help of chaperones. When tested on several substituted stilbenes, LsdA exhibited the greatest specificity for lignostilbene (κcat/Km = 1.00 ± 0.04 × 10⁶ M⁻¹ s⁻¹). These experiments further indicated that the substrate’s 4-hydroxy moiety is required for catalysis and that this moiety cannot be replaced with a methoxy group. Phenylazophenol 4-hydroxy moiety is required for catalysis and that this moiety and X

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2 The abbreviations used are: Lsd, lignostilbene-α,β-dioxygenase; CAO1, carotenoid oxygenase 1; CCO, carotenoid cleavage oxygenase; DMF, dimethylformamide; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; ICP-MS, inductively coupled plasma mass spectrometry; KIC, competitive inhibition constant; KIU, uncompetitive inhibition constant; PDB, Protein Data Bank; RMSD, root mean square deviation; TAPS, 3-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid; TCEP, tris(carboxymethyl)phosphine hydrochloride; DCA-S, 3-(4-hydroxy-3-(4-methoxystyryl)-5-methoxyphenyl)-acrylate; SSRL, Stanford Synchrotron Radiation Lightsource.
substrate specificity \( (k_{cat}/K_M) \) for lignostilbene (14). Moreover, the enzyme appears to act only on trans-stilbenes possessing a 4-hydroxy moiety (20).

LSDs belong to the same protein family as carotenoid cleavage oxygenases (CCOs), which typically catalyze the oxidative cleavage of a double bond in carotenoids (21, 22). These enzymes are characterized by a structural fold comprising a seven-bladed \( \beta \)-propeller (21). The active site occurs at the center of this propeller and contains an \( Fe^{2+} \) coordinated by four histidines (21). In crystal structures of LSDNOV1 and CAO1 in complex with stilbenoid substrates, the organic substrate is bound such that the scissile double bond is in close proximity to the ferrous ion (23, 24).

At least two mechanisms have been proposed for LSDs. In a mechanism proposed by McAndrew et al. (23), the hydroxystilbenoid is activated via the enzyme-catalyzed deprotonation of the 4-hydroxy group, which allows electron delocalization toward an \( Fe^{3+} \)-superoxo electrophile. In an alternate proposal by Sui et al. (24), \( \pi \) electron density from the scissile double bond is redistributed to the iron-oxo complex to form an \( Fe^{2+} \)-peroxo-substrate cation intermediate. Deprotonation of the hydroxyl moiety is critical in both mechanisms and is assisted by Lys136 and Tyr161 (LsdA/LSDNOV1 numbering), two active-site residues conserved among stilbenecleaving oxygenases (23, 24). However, the roles of these residues have not been investigated. Further, it is unclear whether the organic substrate gates the reactivity of the ferrous ion with \( O_2 \) to inhibit the oxidation of the metal ion, as is the case for extradiol dioxygenases (25). For example, EPR analyses have suggested that NO binds to the iron center independent of organic substrate (23). By contrast, crystallographic, X-ray absorption spectroscopy, and Mössbauer spectroscopy data indicate that a number of CCOs do not bind \( O_2 \) in the absence of the organic substrate (24).

Herein, we report the kinetic and structural characterization of LsdA from TMY1009. Steady-state kinetic studies were performed to evaluate the substrate specificity of the enzyme for a variety of stilbenes. The inhibition of the enzyme by a substrate analog was similarly described. Several X-ray crystal structures were determined, including that of LsdA bound to a substrate analog. Conserved active-site residues were substituted to evaluate their roles in substrate specificity and catalysis. The results are discussed with respect to CCOs and bacterial lignin catabolism.

Results

Purification of LsdA

LsdA of TMY1009 was produced in Escherichia coli using a pET vector containing lsd. Most of the produced protein was insoluble, consistent with a previous account (24). However, the co-production of LsdA in a strain producing the GroEL and GroES chaperones significantly improved the level of soluble LsdA. LsdA was purified to \( \geq 99\% \) apparent homogeneity as judged by SDS-PAGE analysis at yields of \(-10–20 \text{ mg} \) of purified protein per liter of cell culture. Inductively coupled plasma MS (ICP-MS) analyses revealed that purified LsdA contained \(-1 \text{ eq of iron per protomer and insignificant amounts of calcium, cobalt, copper, zinc, manganese, nickel, and lead. Consistent with this result, a colorimetric assay based on Ferene-S yielded a value of } 1.1 \pm 0.2 \text{ eq of iron per LsdA protomer. Preparations of LsdA retained essentially 100\% of their activities when exposed to ambient levels of } O_2 \text{ for up to 16 h at room temperature.}

Substrate specificity

In an oxgenase assay, LsdA was most active at pH 8.5 (Fig. S1). Accordingly, the enzyme was subsequently characterized using air-saturated TAPS \((I = 0.1 \text{ m})\), pH 8.5, at 25 °C. Similar activity was observed using Tris as the buffer. LsdA cleaved 4-hydroxy trans-stilbenes, such as lignostilbene, 4-hydroxystilbene, and resveratrol (Fig. 1B), but not 4-hydroxy-4’-nitro-stilbene. The LsdA-catalyzed cleavage of lignostilbene to vanillin was validated using an HPLC-based assay (Fig. S2). The initial rate of lignostilbene cleavage displayed Michaelis–Menten kinetics (Fig. 2A). As summarized in Table 1, LsdA cleaved the substrates with the following specificity: lignostilbene > 4-hydroxystilbene \( \approx \) resveratrol. LsdA also displayed Michaelis–Menten behavior with respect to \( O_2 \) concentration, with a \( K_{M_{O_2}} \) value of 190 ± 10 \( \mu M \) (Fig. 2B).

In the oxgenase assay, neither stilbene nor 4,4’-dimethoxystilbene was detectably cleaved by LsdA, consistent with their lack of a 4-hydroxy substituent. Other compounds that were not cleaved include phenylazophenol and diethylstilbestrol. The identification of phenylazophenol as a nonhydrolyzable substrate analog that bears a 4-hydroxy substituent prompted us to evaluate its ability to inhibit the LsdA-catalyzed reaction. In steady-state kinetic studies, phenylazophenol inhibited the LsdA-catalyzed cleavage of lignostilbene (\( K_{ic} = 6 \pm 1 \mu M \) and \( K_{iu} = 24 \pm 4 \mu M \); \( K_{ic} \) and \( K_{iu} \) refer to competitive and uncompetitive inhibition constant, respectively) (Fig. 3). Preincubation of LsdA with up to 50 \( \mu M \) phenylazophenol for up to 30 min at room temperature did not significantly affect the enzymatic activity, consistent with reversible inhibition.

Structure of LsdA

LsdA crystallized in space group \( P3_121 \) with two LsdA protomers in the asymmetric unit. The two protomers are related by an approximately 2-fold rotational symmetry (Fig. 4A) and likely represent the dimer that LsdA forms in solution (14). The structure for apo-LsdA, solved to 2.3 Å resolution, was used to solve that of holo-LsdA \((i.e. \text{ bound to } Fe^{2+})\) to 2.6 Å (Table 2). The apo- and holo-LsdA protomers are highly similar in structure, with an average root mean square deviation (RMSD) over all Ca atoms of \( \approx 0.3 \) Å. The structural fold of LsdA is that of a seven-bladed \( \beta \)-propeller, typical of the CCOs (Fig. 4B). Among stilbenoid-cleaving dioxygenases of known structure, LsdA is most similar to the resveratrol-cleaving enzyme LSDNOV1 (Protein Data Bank (PDB) entry 5J53) (23), sharing an RMSD of 1.1 Å over 473 aligned Ca atoms. LSDNOV1 is also a homodimer.

The dimer interface of LsdA has a buried surface area of 1460 Å\(^2\) and contains many polar interactions, including 14 hydrogen bonds and 9 salt bridges, as predicted by PDB-PISA. All but...
one of these interactions are mediated by a linear stretch of residues from the N terminus to Glu31 of each subunit (Fig. 4C), which includes strand H92521 (Glu20–Leu24). The H92521 strands of the two subunits are arranged anti-parallel to each other, with the Glu20 amide from one subunit forming a hydrogen bond with the Asp22 carbonyl of the other. This arrangement creates a 10-stranded anti-parallel H9252 sheet formed by the first propeller blade of each protomer. Other notable interactions involve the side-chain carboxylates of Asp25 and Glu27, which form reciprocal salt bridges with the N terminus (Ala2) and Arg15, respectively, of the two protomers.

Figure 1. A, the LsdA-catalyzed cleavage of lignostilbene. B, compounds used in this study: lignostilbene (1), resveratrol (2), 4-hydroxystilbene (3), 4-hydroxy-4′-nitrostilbene (4), stilbene (5), 4,4′-dimethoxystilbene (6), diethylstilbestrol (7), phenylazophenol (8).

Figure 2. Steady-state kinetic analyses of the LsdA-catalyzed reaction. A, dependence of initial velocity on lignostilbene concentration in air-saturated TAPS (I = 0.1 M, pH 8.5), 25 °C. B, dependence of initial velocity on the O2 concentration in the presence of 125 μM lignostilbene. Red lines, fits of the Michaelis–Menten equation to the data.

Table 1
Apparent steady-state kinetic parameters of LsdA for different substrates
Experiments were performed using TAPS (I = 0.1 M), pH 8.5, at 25 °C. Parameters were calculated using a minimum of 20 data points at various substrate concentrations and were obtained using air-saturated buffer and are thus apparent.

<table>
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<th>Substrate</th>
<th>( k_{cat}^{app} ) s(^{-1})</th>
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<th>( k_{cat}^{app}/K_{M}^{app} ) s(^{-1}) M(^{-1})</th>
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<td>Lignostilbene</td>
<td>30 ± 1</td>
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<td>1000 ± 50</td>
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<td>Resveratrol</td>
<td>63 ± 2</td>
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</tr>
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</table>

\( ^a \) Reported values based on concentration up to 30 μM, above which substrate inhibition was observed.

one of these interactions are mediated by a linear stretch of residues from the N terminus to Glu31 of each subunit (Fig. 4C), which includes strand B1 (Glu20–Leu24). The B1 strands of the two subunits are arranged anti-parallel to each other, with the Glu20 amide from one subunit forming a hydrogen bond with the Asp22 carbonyl of the other. This arrangement creates a 10-stranded anti-parallel β-sheet formed by the first propeller blade of each protomer. Other notable interactions involve the side-chain carboxylates of Asp25 and Glu27, which form reciprocal salt bridges with the N terminus (Ala2) and Arg15, respectively, of the two protomers.
Characterization of LsdA

Figure 3. Dixon plot of the inhibition of LsdA-catalyzed lignostilbene cleavage by phenylazophenol. Experiments were performed using TAPS (pH 8.5), 25 °C, and 10 μM (●), 20 μM (▲), 40 μM (▲), 60 μM (×), 80 μM (□), 100 μM (○), and 120 μM (□) lignostilbene. The lines represent a best fit of an equation describing mixed inhibition to the data (K_u = 6 ± 1 μM; k_cat = 24 ± 4 μM; K_M = 32 ± 3 μM; k_cat = 32 ± 2 s⁻¹).

Metal-binding site

As observed in other CCOs, the LsdA active site harbors a single Fe²⁺ ion that resides at the center of the β-propeller. This metal ion is coordinated in a tetragonal pyramidal fashion by four conserved histidines (His¹⁶⁷, His²¹₈, His²₈₂, and His⁴⁷²) and a solvent molecule (Fig. 5). The average Fe²⁺–His bond length in the resting state LsdA is ~2.2 Å, in agreement with values reported in other CCOs (23, 24). Similar to other CCOs, the sixth metal coordination site (across from His²₈₂) is unoccupied and is partly occluded by Thr¹²¹ (26). Additionally, three of the metal-coordinating histidines interact with conserved acidic residues (Glu¹₃⁵, Glu³₅₀, and Glu⁴¹⁴) via hydrogen bonds. Finally, a cap-like structure on one face of the β-propeller helps shield the metal-binding site from the solvent. This cap is formed by a series of loops that otherwise connect various strands and is structurally similar to that of LSDNOV₁ (RMSD of 3.6 Å over 126 superposed Ca atoms). As with other LSDs, LsdA’s cap lacks the hydrophobic patch found in CCOs that helps localize these enzymes to membranes, where their hydrophobic substrates (carotenoids or isoprenoids) are typically found.

Structure of the LsdA-phenylazophenol complex

To further explore substrate binding in LsdA, the enzyme was co-crystallized with phenylazophenol. Co-crystals of LsdA-phenyl-azophenol were yellow, similar to that of the inhibitor. Two protomers constitute the asymmetric unit, as in the inhibitor-free structures, and the complex was refined to 3.0 Å (Table 2). The structure of LsdA in the complex is virtually indistinguishable from that of the inhibitor-free enzyme: comparison between the two yielded an RMSD over all Ca atoms of ~0.3 Å. Inspection of an omit difference density map revealed positive density consistent with the presence of an inhibitor molecule adjacent to the metal at each active site (Fig. 6A). The active site of the LsdA-phenylazophenol complex has a lower metal occupancy, as indicated by a weaker electron density associated with the metal ion as compared with the inhibitor-free structure.

Phenylazophenol was modeled at full occupancy with an average B-factor of 76.7 Å² (Fig. 6B). However, the resolution of the structure precluded defining the binding orientation on the basis of density fitting alone. Instead, the binding orientation was derived from the polarity of surrounding amino acid residues and by comparison with the structures of enzyme–ligand complexes of homologous enzymes (Fig. 6C) (23, 24). In the model, the Fe²⁺ ion is closest to the two azo nitrogen atoms of phenylazophenol (~3–4 Å). The 4-hydroxy moiety of the phenylazophenol forms hydrogen bonds with each of Tyr¹⁰¹ and Lys¹³⁴ (Fig. 6B). In addition, Phe³⁹ forms a π–π stacking interaction with the phenolic ring of the inhibitor. Glu³⁵₀ located within the active-site pocket distal to Tyr¹⁰¹, does not contact the inhibitor, which lacks substituents on the nonphenolic ring. These four residues are conserved throughout stilbene-cleaving dioxygenases and have been previously identified in binary complexes of LSDNOV₁ and CAO1, respectively, with resveratrol (23, 24). In LSDNOV₁, Ser²₈₃ and Glu³₅₃ form hydrogen bonds to one of the hydroxyl groups on resveratrol’s resorcinolic ring. The glutamate is conserved in LsdA (Glu³⁵₀) and CAO1, but the serine is replaced by glycine in both LsdA and CAO1. In LsdA, the bound phenylazophenol is covered by a constellation of phenylalanine residues (Phe³⁰⁵, Phe³⁰⁷, and Phe³⁰⁸) from the cap. These interactions, together with an interaction with Phe³⁹, help sequester the inhibitor from the solvent. As compared with the ligand-free enzyme, a rotation about X₁ of the Phe³⁰⁵ by ~45° coupled with small changes in conformation in the main chain repositions the phenyl ring to accommodate binding of phenylazophenol. In addition, the phenyl ring of Phe³⁰⁸ is repositioned by rotations of X₁ and X₂ by 70 and 115°, respectively, to form the ligand-binding pocket in the LsdA-phenylazophenol complex. Finally, no density corresponding to a metal-bound solvent or O₂ species was observed in the LsdA-phenylazophenol complex, in contrast to what was reported in the CAO1-resveratrol and LSDNOV₁-resveratrol complexes, perhaps due to the low resolution.

Active-site variants

To evaluate the roles of key residues in substrate recognition and catalysis, we substituted each of three residues of LsdA identified to interact with the 4-hydroxy moiety of the phenylazophenol: Phe³⁰⁵, Tyr¹⁰¹, and Lys¹³⁴. These residues were substituted with histidine, phenylalanine, and methionine, respectively. The variants were purified in similar yields as the WT LsdA. Further, they all contained a full complement of iron. As summarized in Table 3, all of the variants had significantly less activity as compared with the WT. Indeed, the K₁₃⁴M variant did not detectably cleave lignostilbene. The Y101F variant had k_cat/app K_M(app) and k_cat/K_M(app) (the superscript “app” refers to apparent parameters) values that were 10 and 20% those of the WT, whereas the corresponding values for the F59H variant were ~7 and 3% those of WT LsdA.
Discussion

The substrate specificity studies of LsdA are consistent with previous reports that the enzyme cleaves only 4-hydroxystilbenes. More particularly, it had previously been determined that LsdA does not cleave 2-hydroxy, 3-hydroxy, or 4-methoxy stilbenes (20). Different LSD homologs have different substrate specificities, as exemplified by the isoforms of LSDTMY1009 (14). Moreover, there are conflicting reports on whether LSDNOV1 can cleave rhapontigenin and rhaponticin, both of which are 4-methoxy stilbenes (19, 23). Nevertheless, most characterized LSDs appear to require the 4-hydroxy moiety for activity (8, 23, 27). This is consistent with the conserved active-site lysyl and tyrosyl residues in LSDs and the strikingly similar manner in which they interact with the.

Table 2

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Figure 4. Crystal structure of LsdA dimer and protomer. A, ribbon and surface representation of the dimeric assembly of LsdA. The different protomers of the asymmetric unit and presumed dimer are yellow and teal, respectively. Bound Fe2+ ions are shown as black spheres. B, ribbon diagram of LsdA protomer, colored using a gradient from the N (blue) to C (red) termini. C, stick representation of residues at the dimer interface. Carbon atoms and secondary structure elements of the two subunits are yellow and teal, respectively. Intersubunit salt bridges and hydrogen-bonding interactions (≤4.0 Å) are indicated using dashed lines.

Table 2

X-ray diffraction data collection and refinement statistics for LsdA structures

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Characterization of LsdA

4-hydroxyl group in each of the LSD

Figure 5. The metal-binding site of LsdA. Shown is a stick representation of LsdA in the resting state (PDB code 6OJW). The Fe²⁺ ion and solvent species are represented as orange and red spheres, respectively. Ligand bonds, as well as polar and hydrogen-bonding interactions (≤3.0 Å) are indicated using dashed lines.

The inhibition of LsdA by phenylazophenol is consistent with previous studies in which LsdA was unable to cleave stilbenoids with substitutions at either C₃ or C₆ of the vinyl group (28, 29). Further, both N-benzylideneaniline and N-benzylamine potently inhibit LsdA (29). Similarly, CAO1 did not cleave fluoro-resveratrol (24). Phenylazophenol behaved as a mixed inhibitor, which suggests the presence of multiple binding sites. Whereas only a single binding site was observed in the LsdA-phenylazophenol structure, electron density consistent with multiple ligands was observed in a Co²⁺-substituted CAO1-resveratrol complex (24).

Although LsdA cleaves lignostilbene quite efficiently, the enzyme’s physiological substrate remains unknown. Inspection of the substrate-binding site of LsdA revealed several pockets adjacent to the phenyl rings of the inhibitor, suggesting that the enzyme’s physiological substrate has multiple substitutions along the aromatic groups. Interestingly, the stilbenoid 3-(4-hydroxy-3-(4-hydroxy-3-methoxystyryl)-5-methoxyphenyl)acrylate (DCA-S) has been identified as a metabolite in the catabolism of dehydrodiconiferyl alcohol in SYK-6 (12). It is presumed that DCA-S is cleaved by at least one of the eight LSD homologs harbored by SYK-6 (5). Nevertheless, LsdA’s specific activity for DCA-S was 2 orders of magnitude lower than for lignostilbene (7).

In the two proposed reaction mechanisms of LSDs, conserved Lys¹³⁴ and Tyr¹⁰¹ have been postulated to assist in the deprotonation of the 4-hydroxyl (23, 24). Our data indicate that although both residues hydrogen-bond with the substrate’s hydroxyl, only Lys¹³⁴ is essential for catalysis. Although the high pKₐ of lysine’s ε-amino group (~10) makes it ill-suited to function as a base catalyst, it functions as such in several dehydrogenases (31–33). In these enzymes, the catalytic lysine does not act directly as the base catalyst, but instead promotes the deprotonation of the catalytic tyrosine by lowering the pKₐ of the latter’s phenolic hydroxyl group via electrostatic polarizaton (34). Our data are consistent with the catalytic lysine of LSDs acting directly on the substrate.

The Fe²⁺ cofactor of LSDs and other CCOs is relatively resistant to oxidative inactivation. This is in contrast to another class of nonheme Fe²⁺ dioxygenases, the extradiol dioxygenases, which are inactivated upon exposure to O₂ as well as during catalytic turnover (25). Whereas LSDs may use a gating mechanism similar to the extradiol dioxygenases to prevent the binding and activation of O₂ in the absence of organic substrate, this protective measure is unlikely to involve structural changes to modulate the enzyme’s affinity toward O₂. Substrate binding in CCO did not affect the protein structure or significantly change the electronic state of the metal (24). Indeed, the active-site metal appears to contribute minimally toward substrate binding and overall protein structure. However, ligand binding may have a steric or electronic effect in the formation of the ternary complex.

The residues that mediate dimerization in LsdA are conserved in LsdB. These include all of the residues involved in the predicted 14 hydrogen bonds and nine salt bridges between the two protomers of the LsdA dimer (Fig. 4C). The high degree of conservation between LsdA and LsdB rationalizes how LsdA and LsdB form homo- and heterodimers in vivo (14). Further, these residues are conserved in CAO1, consistent with its dimeric structure. LsdA and LsdB share >95% amino acid sequence identity with LSD1 and LSD2 (SLG_36640), respectively, from SYK-6. The conserved residues include all of those at the dimer interface. This suggests that the occurrence of heterodimeric LSDs is not uncommon to TMY10009, although the physiological significance of these heterodimers is unclear. The relatively small size of the dimer interface suggests that subunits might readily swap in solution. Finally, it is noted that these interfacial residues are not required for dimerization, as the third LSD of TMY10009 is dimeric despite lacking these residues (15).

In conclusion, this study presents a more in-depth look into the first characterized LSD and establishes the relative importance of Lys¹³⁴ for catalysis. Further work is required to establish the physiological role of the LSDs, particularly in bacteria containing multiple homologs. To this end, we are investigating the various LSDs of SYK-6.

Experimental procedures

Chemicals and reagents

All reagents were of analytical grade unless otherwise noted. Restriction enzymes and the Phusion PCR system used for cloning were from New England Biolabs. Water for buffers was purified using a Barnstead Nanopure Diamond™ system to a resistance of at least 18 megaohms. Lignostilbene was a gift from Prof. Victor Snieckus and Dr. Timothy E. Hurst (Queen’s University, Kingston, Ontario, Canada). All other stilbenes were commercially sourced.

DNA manipulation

DNA was purified, manipulated, and propagated using standard procedures (35). The lsdA gene (locus tag: 1917171A), which encodes for the α'-isoform of LSD in TMY1009, was synthesized by back-translating the protein’s amino acid sequence
supernatant was removed by centrifugation. The protein pellet was collected from 2 liters of culture were suspended in 20 ml of 20 mM HEPPS, 2 mM DTT, 0.5 mM (NH4)2Fe(SO4)2, pH 8.0 and lysed using codon optimized for expression in E. coli (GenScript USA Inc.). The gene was subcloned into pET41LsdA (Novagen) using PCR-based mutagenesis using a pair of overlapping primers. The nucleotide sequences of key constructs were confirmed by sequencing. The oligonucleotides used in this study are listed in Table S1.

**Protein production and purification**

LsdA was produced heterologously using E. coli BL-21 λDE3 containing pET41LsdA and pGro7 (Takara Bio Inc.). Freshly transformed cells were grown at 37 °C in lysogeny broth supplemented with 30 mg/liter kanamycin, 30 mg/liter chloramphenicol, and 1 mg/ml L-arabinose to an A600 of ~0.7. Expression of LsdA was induced with 1 mM isopropyl-β-D-thiogalactopyranoside, at which time the medium was further supplemented with 0.5 mM FeCl3, and the cells were incubated at 16 °C for an additional 16 h. Cells were harvested by centrifugation and stored at −80 °C until further processing. Cells collected from 2 liters of culture were suspended in 20 ml of 20 mM HEPPS, 2 mM DTT, 0.5 mM (NH4)2Fe(SO4)2, pH 8.0, and lysed at 4 °C using an EmulsiFlex-C5 homogenizer (Avestin). Cellular debris was removed by centrifugation. The supernatant was loaded onto a Source 15 phenyl column and eluted with a linear gradient from 0.2 to 1M (NH4)2SO4, and the precipitate was removed by centrifugation and filtration at 0.45 μm. Subsequent purification steps were performed anaerobically by manipulating the sample inside a glovebox (Labmaster Model 100, Mbraun). Chromatography was performed using an AKTA Purifier interfaced to the glovebox with buffers and fraction collection inside the glovebox. Buffers used for purification were sparged with N2 before being placed in the glovebox for equilibration overnight. The supernatant was loaded onto a Source 15 phenyl column and eluted with a linear gradient from 0.8 to 0 M (NH4)2SO4 in 120 ml of 20 mM HEPPS, 2 mM DTT, 0.5 mM (NH4)2Fe(SO4)2, pH 8.0 (AKTA Purifier, GE Healthcare). Fractions containing LsdA, as determined through SDS-PAGE, were pooled and dialyzed into 20 mM HEPPS, 2 mM DTT, pH 8.0. LsdA was purified further using a MonoQ 10/100 GL column (GE Healthcare). The protein was eluted with a linear gradient from 0.2 to 1 M NaCl in 120 ml of 20 mM HEPPS, 2 mM DTT, pH 8.0. Fractions containing LsdA were pooled, dialyzed into 20 mM HEPPS, 80 mM NaCl, 2 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), pH 8.0, concentrated to ~10 mg/ml, flash-frozen as beads in liquid N2, and stored at −80 °C until further use. The variants were purified similarly. Apo-LsdA was purified using a similar protocol except that the purification was performed aerobically and neither the media nor the buffers were supplemented with iron.

### Table 3

**Apparent steady-state kinetic parameters of LsdA and select variants for lignostilbene**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{app}^{cat}$</th>
<th>$k_{app}^{cat}$</th>
<th>$k_{app}^{cat}/k_{app}^{cat}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>$30 ± 1$</td>
<td>$31 ± 3$</td>
<td>$1000 ± 50$</td>
</tr>
<tr>
<td>F59H</td>
<td>$20 ± 0.1$</td>
<td>$72 ± 5$</td>
<td>$28 ± 2$</td>
</tr>
<tr>
<td>Y101F</td>
<td>$3.1 ± 0.1$</td>
<td>$15 ± 1$</td>
<td>$210 ± 20$</td>
</tr>
<tr>
<td>K134M</td>
<td>ND†</td>
<td>ND†</td>
<td>ND†</td>
</tr>
</tbody>
</table>

| Experiments were performed using air-saturated TAPS (f = 0.1 m), pH 8.5, at 25 °C. Parameters were calculated using a minimum of 20 data points at various lignostilbene concentrations. These parameters were obtained using air-saturated buffer and are thus apparent. |

† ND, not detected.

**Protein analytical methods**

Protein purity was evaluated using SDS-polyacrylamide gel stained with Coomassie Blue according to established procedures (35). Protein concentration was determined using a micro-BCA™ protein assay kit (Pierce) using BSA as a standard. Iron concentrations were determined colorimetrically using the Ferene-S assay and ferric chloride solution as a standard (36). ICP-MS was performed using a NexION 300d mass spectrometer (PerkinElmer Life Sciences) calibrated using IV-Stock-4 synthetic standard (Inorganic Ventures). To liberate metal ions, the protein samples were treated with concentrated HNO3 and H2O2 as described previously (37).
Characterization of LsdA

Steady-state kinetics

Kinetic assays were performed by monitoring the consumption of O\textsubscript{2} using a Clark-type polarographic O\textsubscript{2} electrode OXYG1 (Hansatech) connected to a circulating water bath. Assays were performed in 1 ml of air-saturated 40 mM TAPS (I = 0.1 M, pH 8.5) at 25 °C and initiated by adding the stilbene. Stock solutions of the stilbene were made in dimethylformamide (DMF). The final concentration of DMF in the assay solutions was <0.5% (v/v). Reaction velocities were corrected for the background reading prior to substrate addition. The electrode was calibrated daily according to the manufacturer’s instructions using air-saturated water and O\textsubscript{2}-depleted water via the addition of sodium hydrosulfite. Stock solutions were prepared fresh daily. Steady-state kinetic parameters were evaluated by fitting the Michaelis–Menten equation to the data using the least-squares fitting of LEONORA (38). The effect of pH on the rate of the LsdA-catalyzed reaction was evaluated using I = 0.1 M solutions of citrate (pH 6.0), HEPPS (pH 7.0–9.0), and carbonate (pH 9.6 and 10.3). The apparent steady-state kinetic parameters for O\textsubscript{2} were evaluated using 125 μM lignostilbene and initial concentrations of O\textsubscript{2} from 16 to 600 μM. The initial O\textsubscript{2} concentrations were achieved by equilibrating the reaction mixture with humidified mixtures of O\textsubscript{2} and N\textsubscript{2} gasses. Final O\textsubscript{2} levels were normalized to the ambient O\textsubscript{2} level prior to the adjustment. The electrode was equilibrated with air-saturated buffer between runs. The inhibition of LsdA by phenylazophenol was evaluated by monitoring the initial velocity using varying concentration of lignostilbene and the inhibitor. The inhibition constants were determined by fitting an equation describing mixed inhibition to the data using LEONORA (38).

Protein structure determination

Crystals of apo-LsdA were grown aerobically by sitting drop at room temperature in a 1:1 mixture of ~10 mg/ml apo-LsdA in 20 mM HEPPS, 80 mM NaCl, 2 mM TCEP, pH 8.0, with reservoir solution containing 0.2 M tripotassium citrate and ~20% PEG 3350 (v/v). Crystals were briefly soaked in reservoir buffer supplemented with ~30% glycerol (v/v) for cryoprotection and flash-frozen in liquid nitrogen. Diffraction data were collected at the SSRL on beamline 7-1. Data were processed and integrated using Mosflm and CCP4 AIMLESS (44, 45). Holo-LsdA crystallized in the space group P\textsubscript{3}\textsubscript{2}1 with two molecules in the asymmetric unit. The structure was solved using molecular replacement with LsdA protomer coordinates from the solved apo-structure (described above) as a search model in the program PhaserMR from the Phenix package (40, 41). The refined structure has residues 2–481 modeled for chain A. Chain B has residues 2–481 modeled with two gaps spanning residues 306–315 and 381–386 that were not modeled due to poor electron density. The model also contains two iron molecules, one magnesium ion, three glycerols, and 351 water molecules.

A crystal structure of LsdA-phenylazo-phenol was obtained by co-crystallizing the enzyme and inhibitor. The crystals were prepared aerobically by sitting drop at room temperature using a 1:1 ratio of ~10 mg/ml LsdA in protein buffer and reservoir buffer containing 0.2 M sodium fluoride and ~17% PEG 3350 (v/v) supplemented with ~1 mM of phenylazophenol in DMF. Crystals were flash-frozen in liquid nitrogen. Diffraction data were collected at the Canadian Light Source on beamline 08ID-1 and data were processed and integrated using Mosflm and CCP4 AIMLESS (44, 45). The crystal was of space group P\textsubscript{3}\textsubscript{2}1 with two molecules in the asymmetric unit. The structure was also solved using molecular replacement with LsdA protomer coordinates from the solved apo-structure as a search model in PhaserMR from Phenix (40, 41). The refined structure has residues 2–482 modeled for each protomer, but there was poor electron density for residues 309–315 and 380–386 in both protomers. A single solvent molecule was modeled in each active site.

Data collection and refinement statistics for all three structures are summarized in Table 2. The program MolProbity was used for structure validation including calculation of the fit to a Ramachandran plot (46). The coordinates and observed structure factor amplitudes have been deposited in the PDB under the accession codes 6OJR, 6OJW, and 6OJT for apo-LsdA, holo-LsdA, and LsdA-phenylazophenol, respectively. Structure figures were generated in PyMOL (PyMOL Molecular Graphics System, version 1.8, Schrödinger, LLC, New York). RMSD calculations between different LsdA structures were performed using the least-squared superposition tool of Coot (4). RMSD calculations between different LsdA and LSD\textsubscript{NOV}1 structures were performed using DALI (30).

Author contributions—E. K. and L. D. E. designed experiments and analyzed data. E. K. conducted most of the experiments. A. K. N. L. assisted in the kinetic characterization. E. K., M. M. V., M. J. K., and M. E. P. M. performed the structural refinement. E. K. and L. D. E. wrote the paper with input from M. M. V. and M. E. P. M.
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References


Characterization of LsdA


