Lignostilbene-α,β-dioxygenase (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 (K\text{cat}/K\text{app} = 1.00 ± 0.04 × 10^6 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 inhibited the LsdA-catalyzed cleavage of lignostilbene in a reversible, mixed fashion (K\text{ic} = 6 ± 1 μM, K\text{iu} = 24 ± 4 μM). An X-ray crystal structure of LsdA at 2.3 Å resolution revealed a seven-bladed β-propeller fold with an iron cofactor coordinated by four histidines, in agreement with previous observations on related carotenoid cleavage oxygenases. We noted that residues at the dimer interface are also present in LsdB, another lignostilbene dioxygenase in *S. paucimobilis* TMY1009, rationalizing LsdA and LsdB hom- and heterodimerization in vivo. A structure of an LsdA-phenylazophenol complex identified Phe59, Tyr101, and Lys134 as contacting the 4-hydroxyphenyl moiety of the inhibitor. Phe59 and Tyr101 substitutions with His and Phe, respectively, reduced LsdA activity (K\text{app}) ~ 15- and 10-fold. The K134M variant did not detectably cleave lignostilbene, indicating that Lys134 plays a key catalytic role. This study expands our mechanistic understanding of LsdA and related stilbene-cleaving dioxygenases.

Lignin is a heterogeneous aromatic polymer found in plant cell walls that contributes to the recalcitrance of biomass. It is increasingly recognized that the valorization of lignin is essential to the sustainable biorefining of lignocellulose (1). Deconstructing lignin is thus of great relevance to transforming lignocellulose to biofuels and commodity chemicals (1, 2). The discovery that bacteria are able to at least partially deconstruct lignin has accelerated the study of enzymes and pathways potentially involved in lignin depolymerization and the catabolism of the resulting products (3–5). Lignin-derived aromatic compounds that are efficiently degraded by bacteria include β-aryl ethers, pinoresinol, 2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dicarboxy biphenyl, diaryl propane, and phenylcoumarane (5, 6).

Lignostilbene-α,β-dioxygenase (LSD, EC 1.13.11.43) catalyzes the oxygenolytic fission of lignostilbene (Fig. 1A) to two molecules of vanillin, incorporating both atoms of O2 into the products (7–9). Lignostilbenoids occur in a very limited number of naturally occurring lignins but are prevalent in industrial lignins due to condensation reactions (10, 11). Moreover, lignin-derived stilbenes are proposed to be produced in the bacterial catabolism of diaryl propane and phenylcoumarane (7, 12). Plants produce other stilbenes, such as resveratrol, arachidins, and astringins, as allelochemicals to protect against pathogens and physical damage (13). This variety of stilbenes may explain why some bacterial strains contain multiple LSD homologs (5, 7, 12, 14, 15). The first LSD to be described, LsdA from *Sphingomonas paucimobilis* TMY1009 (TMY1009 hereafter), shares 98% amino acid sequence identity with LSD1 from *Sphingobium* sp. SYK-6 (SYK-6 hereafter), encoded by SLG_37450. LsdA functions as a homodimer and, somewhat unexpectedly, also as a heterodimer with LsdB, a second LSD in TMY1009 with which it shares 70% identity (14). LsdA also shares 49% amino acid sequence identity with the pterostilbene-cleaving CCO1 from *Sphingobium* sp. strain JS1018 (16). Among structurally characterized enzymes, LsdA shares 56% identity with LSD\text{NOV1} from *Novosphingomonas aromaticivorans* NOV1 and 40% with carotenoid oxygenase 1 (CAO1) of *Neurospora crassa* (5, 17–19). Among tested stilbenes, LsdA had the highest

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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; K\text{cat}, competitive inhibition constant; K\text{app}, 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(2-carboxyethyl)phosphine hydrochloride; DCA-S, 3-[(4-hydroxy-3-(4-methoxystyryl)-5-methoxyphenyl)-acrylate; SSRL, Stanford Synchrotron Radiation Lightsource.

This article contains Table S1 and Figs. S1 and S2.
Characterization of LsdA

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 LSD\( \text{NOV1} \) 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-oxy complex to form an \( Fe^{2+} \)-peroxo-substrate cation intermediate. Deprotonation of the hydroxyl moiety is critical in both mechanisms and is assisted by Lys\( ^{\text{136}} \) and Tyr\( ^{\text{101}} \) (LsdA/LSD\( \text{NOV1} \) numbering), two active-site residues conserved among stilbene-cleaving 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 \textit{Escherichia coli} using a pET vector containing \textit{bsd}. 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 \( > 99\% \) apparent homogeneity as judged by SDS-PAGE analysis at yields of \( \sim 10-20 \) mg of purified protein per liter of cell culture. Inductively coupled plasma MS (ICP-MS) analyses revealed that purified LsdA contained \( \sim 1 \) eq of iron per protomer and insignificant amounts of cadmium, 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 eq of iron per LsdA protomer. Preparations of LsdA retained essentially 100% of their activities when exposed to ambient levels of \( O_2 \) for up to 16 h at room temperature.

Substrate specificity

In an oxygraph assay, LsdA was most active at pH 8.5 (Fig. S1). Accordingly, the enzyme was subsequently characterized using air-saturated TAPS (I = 0.1 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 \( K_M \) value of 190 \( \pm \) 10 \( \mu \)M (Fig. 2B).

In the oxygraph 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 \( P3_1 21 \) 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. 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 \( \sim 0.3 \) 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 LSD\( \text{NOV1} \) (Protein Data Bank (PDB) entry 5J53) (23), sharing an RMSD of 1.1 Å over 473 aligned Ca atoms. LSD\( \text{NOV1} \) 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).

Table 1

| Substrate                | kcatapp (s⁻¹) | Kapp (µM) | Kapp/kcatapp (µM⁻¹) |
|--------------------------|---------------|-----------|---------------------|
| Lignostilbene            | 30 ± 1        | 31 ± 3    | 1000 ± 50           |
| Resveratrol              | 0.63 ± 0.02   | 34 ± 4    | 19 ± 2              |
| 4-Hydroxystilbene        | 0.21 ± 0.01   | 9 ± 1     | 24 ± 3              |
| 4,4′-Dimethoxystilbene   |               |           |                     |

*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 H92521 (Glu20–Leu24). The H11032 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.
Characterization of LsdA

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: Phe^{305}, Tyr^{101}, and Lys^{134}. 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 compared with the WT. Indeed, the K134M variant did not detectably cleave lignostilbene. The Y101F variant had $k_{cat}^{app}$ and $k_{cat}^{app}/K_{M}^{app}$ (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 $\sim 7$ and 3% those of WT LsdA.

**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: Phe^{305}, Tyr^{101}, and Lys^{134}. 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 compared with the WT. Indeed, the K134M variant did not detectably cleave lignostilbene. The Y101F variant had $k_{cat}^{app}$ and $k_{cat}^{app}/K_{M}^{app}$ (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 $\sim 7$ and 3% those of WT 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 LsdA), 20 μM A, 40 μM B, 60 μM C, 80 μM D, 100 μM E, and 120 μM F lignostilbene. The lines represent a best fit of an equation describing mixed inhibition to the data ($K_i = 6 \pm 1 \mu M; K_{cat} = 24 \pm 4 \mu M; K_{M} = 32 \pm 3 \mu M; k_{cat} = 32 \pm 2 s^{-1}$).

**Metal-binding site**

As observed in other CCOs, the LsdA active site harbors a single Fe^{2+} ion that resides at the center of the β-propeller. This metal ion is coordinated in a tetragonal pyramidal fashion by four conserved histidines (His^{197}, His^{218}, His^{282}, and His^{472}) and a solvent molecule (Fig. 5). The average Fe^{2+}–His bond length in the resting state LsdA is $\sim 2.2 \AA$, in agreement with values reported in other CCOs (23, 24). Similar to other CCOs, the sixth metal coordination site (across from His^{282}) is unoccupied and is partly occluded by Thr^{121} (26). Additionally, three of the metal-coordinating histidines interact with conserved acidic residues (Glu^{135}, Glu^{350}, and Glu^{414}) via hydrogen bonds. Finally a cap-like structure on one face of the metal ion is closest to the two azo nitrogen atoms of phenylazophenol and is partly defined by a π–π stacking interaction with the phenolic ring of the inhibitor. Glu^{350}, located within the active-site pocket distal to Tyr^{101}, 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, Ser^{283} and Glu^{353} form hydrogen bonds to one of the hydroxyl groups on resveratrol’s resorcinolic ring. The glutamate is conserved in LsdA (Glu^{350}) 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 (Phe^{305}, Phe^{307}, and Phe^{308}) from the cap. These interactions, together with an interaction with Phe^{395}, help sequester the inhibitor from the solvent. As compared with the ligand-free enzyme, a rotation about C_{β} of the Phe^{305} by $\sim 45^\circ$ 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 Phe^{308} is repositioned by rotations of C_{α} and C_{β} 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 O_{2} 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.

**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 Cα atoms of $\sim 0.3$ 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 Å$^2$ (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 Fe^{2+} ion is closest to the two azo nitrogen atoms of phenylazophenol and is partly defined by a π–π stacking interaction with the phenolic ring of the inhibitor. Glu^{350}, located within the active-site pocket distal to Tyr^{101}, 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, Ser^{283} and Glu^{353} form hydrogen bonds to one of the hydroxyl groups on resveratrol’s resorcinolic ring. The glutamate is conserved in LsdA (Glu^{350}) 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 (Phe^{305}, Phe^{307}, and Phe^{308}) from the cap. These interactions, together with an interaction with Phe^{395}, help sequester the inhibitor from the solvent. As compared with the ligand-free enzyme, a rotation about C_{β} of the Phe^{305} by $\sim 45^\circ$ 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 Phe^{308} is repositioned by rotations of C_{α} and C_{β} 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 O_{2} 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.

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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: Phe^{305}, Tyr^{101}, and Lys^{134}. 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 compared with the WT. Indeed, the K134M variant did not detectably cleave lignostilbene. The Y101F variant had $k_{cat}^{app}$ and $k_{cat}^{app}/K_{M}^{app}$ (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 $\sim 7$ 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 4-hydroxy moiety.

Table 2
X-ray diffraction data collection and refinement statistics for LsdA structures

|                  | Apo-LsdA | Holo-LsdA | LsdA-phenylazophenol |
|------------------|----------|-----------|----------------------|
| **Data collection** |          |           |                      |
| Resolution range (Å) | 37.08–2.30 (2.38–2.30) | 60.92–2.60 (2.69–2.60) | 61.00–3.00 (3.11–3.00) |
| Space group       | P3_21    | P3_21     | P3_21               |
| Unit cell dimensions (Å) | a = b = 181.3, c = 94.9 | a = b = 181.3, c = 95.1 | a = b = 181.9, c = 96.4 |
| Unique reflections | 79,776 (7876) | 56,663 (5634) | 34,286 (3443) |
| Completeness (%)  | 99.9 (99.4) | 99.8 (99.6) | 92.0 (93.6) |
| Redundancy        | 13.5 (13.2) | 9.2 (9.9)  | 2.0 (2.0)           |
| Average I/σI     | 20.3 (2.1) | 15.4 (2.2) | 9.9 (2.5)           |
| Rmerge           | 0.146 (1.353) | 0.048 (0.337) | 0.053 (0.259) |
| CC1/2            | 0.998 (0.731) | 0.996 (0.733) | 0.990 (0.875) |
| Wilson B-factor (Å^2) | 35.8 | 39.0 | 55.7 |
| Anisotropy       | 0.356 | 0.115 | 0.637 |
| **Data refinement** |          |           |                      |
| Rwork (Rfree)    | 0.180 (0.203) | 0.187 (0.215) | 0.213 (0.241) |
| Protein residues | 964 | 944 | 962 |
| No. of water molecules | 547 | 351 | 2 |
| RMSD bond length (Å) | 0.003 | 0.003 | 0.002 |
| RMSD bond angles (degrees) | 0.62 | 0.61 | 0.59 |
| Average B-value (Å^2) | 40.9 | 43.0 | 56.0 |
| Ramachandran plot (%) | Most favored regions 97.6 | 96.3 | 94.9 |
| | Disallowed regions 0.0 | 0.2 | 0.2 |
| | PDB code 6OJR | 6OJW | 6OJT |

* Data collection values in parentheses represent the data for the highest resolution shell.

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 Fe^2+ 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.
4-hydroxyl group in each of the LSDNOV1-resveratrol, CAO1-resveratrol, and LSDa-phenylazophenol complexes (23, 24).

The inhibition of LSDa by phenylazophenol is consistent with previous studies in which LSDa was unable to cleave stilbenoids with substitutions at either C6 or C9 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 postulated that the electronically optimal binding pocket (≤3.8 Å) are indicated using dashed lines.

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 (≤3.8 Å) are indicated using dashed lines.

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.

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 proteomes 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 TMY1009, 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 TMY1009 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 DiamondTM system to a resistance of at least 18 megaohms. Lignostilbene was a gift from Prof. Victor Snieckus and Dr. Timothy E. Hurst (Queens 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-isofrom of LSD in TMY1009, was synthesized by back-translating the protein’s amino acid sequence.
was resuspended in the cell resuspension buffer supplemented with 30 mg/liter kanamycin, 30 mg/liter chloroform, at which time the medium was further supplemented with 0.8 M (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, 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.

### 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 chloroamphenicol, 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. (NH₄)₂SO₄ was added to the cleared lysate to a final concentration of 1.2 M, and the supernatant was removed by centrifugation. The protein pellet was resuspended in the cell resuspension buffer supplemented with 0.8 M (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.

### 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).

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**Table 3**

| Enzyme | \( K_{\text{cat}}^{\text{app}} \) | \( K_{\text{M}}^{\text{app}} \) | \( K_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}} \) |
|---|---|---|---|
| WT | 30 ± 1 | 31 ± 3 | 1000 ± 50 |
| F59H | 2.0 ± 0.1 | 72 ± 5 | 28 ± 2 |
| Y101F | 3.1 ± 0.1 | 15 ± 1 | 210 ± 20 |
| K134M | ND | ND | ND |

* 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.
Characterization of LsdA

Steady-state kinetics

Kinetic assays were performed by monitoring the consumption of O2 using a Clark-type polarographic O2 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 stilbenes 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 O2-depleted water via the addition of sodium hydroxysulfite. 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 O2 were evaluated using 125 μM lignostilbene and initial concentrations of O2 from 16 to 600 μM. The initial O2 concentrations were achieved by equilibrating the reaction mixture with humidified mixtures of O2 and N2 gasses. Final O2 levels were normalized to the ambient O2 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 P321 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 P321 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 LSDNOV1 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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