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Characterizing the Promiscuity of LigAB, a Lignin Catabolite Degrading Extradiol Dioxygenase from *Sphingomonas paucimobilis* SYK-6

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**Supporting Information**

**ABSTRACT:** LigAB from *Sphingomonas paucimobilis* SYK-6 is the only structurally characterized dioxygenase of the largely uncharacterized superfamily of Type II extradiol dioxygenases (EDO). This enzyme catalyzes the oxidative ring-opening of protocatechuic (3,4-dihydroxybenzoic acid or PCA) in a pathway allowing the degradation of lignin derived aromatic compounds (LDACs). LigAB has also been shown to utilize two other LDACs from the same metabolic pathway as substrates, gallate, and 3-O-methyl gallate; however, *k*<sub>cat</sub>/*K<sub>M</sub> had not been reported for any of these compounds. In order to assess the catalytic efficiency and get insights into the observed promiscuity of this enzyme, steady-state kinetic analyses were performed for LigAB with these and a library of related compounds. The dioxygenation of PCA by LigAB was highly efficient, with a *k*<sub>cat</sub> of 51 s<sup>−1</sup> and a *k*<sub>cat</sub>/*K<sub>M</sub> of 4.26 × 10<sup>6</sup> M<sup>−1</sup>s<sup>−1</sup>. LigAB demonstrated the ability to use a variety of catecholic molecules as substrates beyond the previously identified gallate and 3-O-methyl gallate, including 3,4-dihydroxybenzamide, homoprotocatechuic acid, catechol, and 3,4-dihydroxybenzonitrile. Interestingly, 3,4-dihydroxybenzamide (DHBAm) behaves in a manner similar to that of the preferred benzoic acid substrates, with a *k*<sub>cat</sub>/*K<sub>M</sub> value only ~4-fold lower than that for gallate and ~10-fold higher than that for 3-O-methyl gallate. All of these most active substrates demonstrate mechanistic inactivation of LigAB. Additionally, DHBAm exhibits potent product inhibition that leads to an inactive enzyme, being more highly deactivating at lower substrate concentration, a phenomena that, to our knowledge, has not been reported for another dioxygenase substrate/product pair. These results provide valuable catalytic insight into the reactions catalyzed by LigAB and make it the first Type II EDO that is fully characterized both structurally and kinetically.

The desire to discover and develop renewable alternatives to fossil fuels for the production of energy and as chemical feedstocks has been ever increasing in the past decade, leading to research into the natural ability of microbes to process a wide range of carbon sources. Much attention has been focused on accessing the carbon locked in cellulose, the most abundant natural polymer, while lignin, the second most abundant natural polymer, has not been as extensively investigated. Small natural polymer, while lignin, the second most abundant natural polymer,1 has not been as extensively investigated. Small aromatic molecules produced by the lignin depolymerization process, lignin derived aromatic compounds (LDACs), have been shown to be accessible for use in central metabolism in some bacteria,2–4 thereby unleashing their potential as a possible carbon source for the fermentative production of a wide variety of chemicals. The enzymes capable of oxidative cleavage of the aromatic rings play a key role in the catabolism of these LDACs. Aromatic ring cleaving dioxygenases are generally divided into three classes: intradiol (Class I), extradiol (Class II), and those capable of cleaving aromatics without a diol (proposed Class III).5,6 Extradiol dioxygenases (EDOs) are further divided into types based on superfamily classification: vicinal oxygen chelate (VOC, Type I), cupin (Type III), and a currently undefined superfamily (Type II) hereafter referred to as the protocatechuate dioxygenase (PCAD) superfamily.6,7 EDOs from the VOC and cupin superfamilies have been well characterized by both enzymological and structural methods.8–12

The existence of Type II EDOs, as postulated by protein sequence homology,13,14 has been known for the better part of two decades, and yet, only a handful of these enzymes have been studied beyond sequence alignments, or gene and pathway identification. Ten Type II EDOs have been isolated and characterized in varying degrees either functionally or kinetically (2,3-dihydroxyphenylpropionate 1,2-dioxygenase from *E. coli* (MhpB)14 and *Alcaligenes eutrophus* (MpcI),14,15 protocatechuate 4,5-dioxygenases from *Sphingomonas paucimobilis* SYK-6 (LigAB), *Comamonas testosteroni* Pt-L5,16 and *Comamonas testosteroni* T-2 (PmdAB),17 protocatechuate 2,3-dioxygenase (2,3-PCD) from *Pseudomonas putida* sp. (formerly *Bacillus macerans*),18,19 2-aminophenol 1,6-dioxygenase (1,6-ApDO) from *Pseudomonas pseudocaligenes*,20,21 2'-aminobiphenyl-2,3-diol,1,2-dioxygenase (CarBab) from *Pseudomonas stutzeri*,22,23 the polycyclic arene diole dioxygenase (PhnC) from *Burkholderia sp.*,24 and gallate 2,3-dioxygenase (GDO)25 from *Pseudomonas putida* KT2440). Further, only five other enzymes...
in this superfamily have been functionally identified by molecular biology techniques (homoprotocatechuate 2,3-dioxygenase (HpcB) from *E. coli*), an acid catechol dioxygenase (HppB) from *Rhodococcus globerulus* PWD1, an extradiol dioxygenase EdoD from *Rhodococcus* sp., and protocatechuate 4,5-dioxygenases from *Pseudomonas straminea* (originally ochraceae NGJ1) (ProOab) and *Anthrobacter keyseri* 12B (PcmA). Additionally, the protocatechuate 4,5-dioxygenase LigAB from *Sphingomonas paucimobilis* SYK-6 is the only structurally characterized EDO from this largely undefined superfamily. Until now, full kinetic characterization and mechanistic investigation of Type II EDOs has only been performed for MhpB by Bugg, and 2,3-PCD by Lipscomb, though 2,3-PCD was only identified as a Type II dioxygenase a decade later by Masai. Kinetic analysis beyond specific activity is largely lacking for LigAB and other Type II EDOs.

LigAB was identified as a member of the bacterial LDAC catabolic pathway of *S. paucimobilis* SYK-6, and it catalyzes the extradiol aromatic ring cleavage of protocatechuate by the insertion of O₂ across the C₄–C₅ bond of the aromatic ring to form 4-carboxy-2-hydroxyxymonate-6-semialdehyde, CHMS (Scheme 1). LigAB has been shown to have some promiscuous activity with PCA substrate analogues gallate (GA) and 3-0-methyl gallate (3OMG), two compounds also found in the LDAC catabolic pathway of *S. paucimobilis* SYK-6. This activity has been demonstrated through knockout studies and *in vitro* assays of specific activity; however, the breadth of substrate specificity is previously unknown.

In this work, we report the kinetic parameters for anaerobically purified LigAB with both of its physiological substrates, PCA and O₂, as well as an investigation of other substrates containing the catechol scaffold, including GA and 3OMG, with full kinetic characterization of the best substrates. From these analyses, we aim to establish the structural and oxidation potential requirements of substrates for LigAB. Additionally, steady-state kinetic studies are used to establish the pH and metal dependence of LigAB. Furthermore, we demonstrate that anaerobic purification of this enzyme is necessary for optimal stability and kinetic efficiency.

**MATERIALS AND METHODS**

Commerically available reagents and solvents were purchased from Aldrich or Alfa Aesar and used without purification unless otherwise noted. 3-O-Methyl gallate was purchased from ChromaDex. Several substrates were synthesized as previously described: 3,4-dihydroxybenzamide, 1,2-dihydroxy-4-amino-benzene hydrobromide, and methyl-3,4-dihydroxybenzoate. A Varian Mercury 400 MHz NMR was used to collect all ¹H NMR spectra. Absorption spectra were collected with a Varian Cary 100 Bio UV–visible spectrophotometer (Palo Alto, CA). Bradford and Ferene-S assays were conducted in 96-well plate format and absorption values recorded on a Molecular Devices SpectraMax M5 plate reader (Sunnyvale, CA). MAX Efficiency DH5αr and One Shot BL21 Star chemically competent *E. coli* cells were purchased from Life Technologies (Carlsbad, CA). All enzymes used for DNA manipulations were purchased from New England Biolabs (Ipswich, MA). Centrifugation and ultracentrifugation were performed on a DuPont Instruments (Wilmington, DE) Sorvall RC-5B centrifuge and Beckman (Brea, CA) L7-80 Ultracentrifuge with a type 60-Ti rotor, respectively. All cells were lysed using a SIMO-Amino Industry Inc. (Rochester, NY) French press.

**Protein Expression.** Unable to acquire genomic DNA for *Sphingomonas paucimobilis* SYK-6 or the plasmids containing the genes for LigAB, we retrieved the DNA sequence encoding the LigA-LigB gene cluster from the NCBI data bank. A construct for gene synthesis was prepared with insertion of an upstream *Ndel* endonuclease site and additional stop codons followed by a BamHI endonuclease site downstream of the LigA and LigB gene cluster. The modified sequence was synthesized by DNA2.0 (Menlo Park, CA). The synthetic gene sequence was subsequently removed from the provided pJ241 vector by endonuclease cutting with *Ndel* and *BamHI*, ligated into pET-15b (EMD Millipore, Billerica, MA) in the *Ndel* and *BamHI* cloning sites, and transformed by heat shock separately into DH5αr and BL21 competent *E. coli* cells.

His-tagged protein (α subunit N-terminally tagged and β subunit untagged) was expressed from a pET15b plasmid by induction of BL21 competent cells with a final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD₆₀₀ = 0.6 under aerobic conditions at 37 °C. After 20 h, the cell cultures were centrifuged at 5,000 rpm. If not used immediately, cell pellets were stored at –80 °C.

**Aerobic Purification.** Pelleted cells were resuspended in 50 mM Tris (pH 7.0) containing 1:9 glycerol/H₂O with one tablet of Complete-EDTA free protease inhibitor cocktail (Roche, Basel, Switzerland). Resuspended cells were lysed by 3 passes through a French-press at 13,000 psi. The cell lysate was subsequently centrifuged at 25,000 rpm for 45 min in an ultracentrifuge.

The supernatant was applied to a gravity column loaded with HisPur Ni-NTA resin (10 mL) of each fraction were analyzed for purity by SDS–PAGE.

Fractions containing the copurified LigAB α and β subunits were pooled, concentrated using an Amicon concentrator stirred cell with a regenerated cellulose 10 kDa molecular weight cutoff ultrafiltration membrane (EMD Millipore), and buffer exchanged into 50 mM Tris (pH 7.5), 0.5 mM DTT, and 0.5 mM Fe(NH₄)₂(SO₄)₂·6H₂O. Exchanged protein was filtered with a 0.45 μm syringe filter and subsequently flash-purified on a HisPur Ni-NTA resin column (10 mL) and eluted with imidazole at pH 7.5.
frozen in liquid nitrogen and stored at −80 °C. From 6 L of cells, 70 mg of protein was typically obtained and concentrated to ~7 mg/mL.

Anaerobic Purification. Anaerobic purification of LigAB was performed as described above with modification to maintain an oxygen-free environment. After cell lysis, the lysate was transferred to centrifuge tubes, and the head space was flushed with N₂ gas prior to centrifugation at 25,000 rpm for 45 min in an ultracentrifuge. The supernatant was decanted to a serum vial, flushed with N₂ gas, sealed, and transferred into a Vacuum Atmospheres Company (Hawthorne, CA) HE-493/ MO-5 glovebox under N₂ atmosphere, operated at <5 ppm O₂.

All buffers, water, and column resins to be used in the glovebox were prepared as described and thoroughly degassed, to remove O₂, by three cycles of degassing under vacuum followed by N₂ gas bubbling. Degassed buffers were allowed to equilibrate in the glovebox atmosphere overnight prior to use.

The supernatant was applied to a gravity column loaded with HisPur Ni-NTA resin inside the glovebox, and protein was eluted from the column as described above. Fractions (10 mL) were collected for all wash and elution steps. Aliquots (30 μL) of each fraction were removed from the glovebox and analyzed for purity by SDS–PAGE. Fractions containing the copurified LigAB α and β subunits were pooled, concentrated using an Amicon concentrator stirred cell with a regenerated cellulose 10 kDa molecular weight cutoff ultrafiltration membrane (EMD Millipore), and buffer exchanged into 50 mM Tris at pH 8.

Phosphate buffered saline (PBS) was added to the protein solution to a concentration of 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2H₂O, and 2 mM KH₂PO₄ at pH 7.4 followed by bovine derived thrombin (MP Bio) to a concentration of 1 U/mg purified protein to remove the His-tag. His-tag cleaved protein was buffer exchanged into 50 mM Tris (pH 8) to remove excess salt prior to application to a Source-Q ion exchange column (10 mL, GE Healthcare). LigAB was applied to the column with 50 mM Tris (pH 8) and washed with 50 mM Tris (pH 8) and 100 mM NaCl, and eluted with 50 mM Tris (pH 8) and 350 mM NaCl. Fractions (5 mL) were collected for both wash and elution steps. Aliquots (30 μL) of each fraction were removed from the glovebox and analyzed for purity by SDS–PAGE. Those fractions containing LigAB α and β subunits were pooled, concentrated, and buffer exchanged into 50 mM Tris (pH 7.5), 0.5 mM DTT, and 0.5 mM Fe₂(NH₄)₆(SO₄)₂·6H₂O. Exchanged protein was filtered with a 0.45 μm syringe filter and removed from the glovebox in an airtight syringe with the needle sealed by a rubber stopper and subsequently flash frozen in liquid nitrogen and stored at −80 °C. From 6 L of cells, 70 mg of protein was typically obtained and concentrated to ~7 mg/mL.

Steady-State Kinetics Assays. The rate of the enzymatic reaction was determined by measuring O₂ consumption using an O₂-sensitive Clark-type electrode with computer integration via an Oxygraph electrode control unit (Hansatech, King’s Lynn, Norfolk, England). Prior to each assay, the electrode was standardized with air-saturated water and water depleted of O₂ by the addition of sodium hydrosulphite as described by the manufacturer. Stock solutions and buffers were prepared fresh daily. Prior to each experiment, 100 μL of LigAB (60 μM) was thawed and buffer exchanged into degassed 50 mM Tris (pH 7.5) containing 1:9 t-butanol/H₂O using a 3 mL Sephadex G-25 desalting gel (GE Healthcare) column in a glovebox flushed with high purity N₂ for 1 h. Exchanged enzyme (3–7 μM) was kept under inert atmosphere (N₂) in a sealed vial on ice for the duration of each experiment.

The effect of pH on the rate of LigAB O₂ consumption was investigated in the pH range of 6–10 at 23 °C. The buffer solutions (50 mM) used to span this pH range were phosphate (pH 6 to 8), Tris (pH 7.5 to 9), BICINE (pH 8 to 9), and CAPSO (pH 9 to 10). The initial rate assays were performed in a final volume of 1 mL of air-saturated 50 mM buffer of desired pH and 250 μM PCA. The reaction was initiated by the addition of 1–2 μL LigAB (3–7 nM final concentration) after the equilibration of all other components for at least 1 min to obtain a constant background O₂ consumption rate of ±0.5 μM/min. Reaction velocities were calculated from the slope of the first 30 s of data after LigAB addition and corrected for background O₂ consumption using 30 s of data immediately prior to LigAB addition. The precise enzyme concentration for each experiment was determined by the Bradford assay (Bio-Rad) after completion. The pKₐ values of effected active site residues were determined by a fit of the reaction velocities as a function of pH to eq 1 (1 acidic pKₐ and 1 basic pKₐ) and eq 2 (2 indistinguishable acidic pKₐ’s and 1 basic pKₐ).

\[
k = \frac{k_{cat}}{1 + \frac{[H^+]^a}{K_{a1}}} + \frac{[H^+]^b}{K_{a2}}
\]

\[
k = \frac{k_{cat}}{1 + \left(\frac{[H^+]^2}{K_{a1}}\right)^2 + \frac{[H^+]^b}{K_{a2}}}
\]

Steady-state kinetic parameters for LigAB with respect to the organic substrate were determined by measuring the rate of O₂ consumption in the presence of varying concentrations of organic substrate (1 μM to 5000 μM). An aqueous stock solution (25 mM) of the desired organic substrate (PCA, GA, or 3OMG) was prepared immediately prior to use in a 10 mL volumetric flask. Stock solutions of 3OMG (25 mM) were prepared in 1:9 DMSO/H₂O. The initial rate assays were performed, as described above, in air-saturated 50 mM Tris (pH 7.5) and initiated by the addition of 1–2 μL of LigAB (3–7 nM). Steady-state kinetic parameters with respect to O₂ were measured in 50 mM Tris (pH 7.5), 1 mM PCA, and 40–450 μM O₂ and initiated by the addition of LigAB (3–7 nM). The buffer was equilibrated prior to each reaction with a fixed mixture of O₂ and N₂ gas using a Cole-Parmer gas proportioner (Vernon Hills, IL), and the reaction chamber was maintained under an atmosphere of the same O₂/N₂ mixture. The precise enzyme concentration for each experiment was determined by the Bradford assay (Bio-Rad) after completion. Kinetic parameters were determined by a least-squares fitting of the Michaelis–Menten equation to the data using KaleidaGraph (Synergy).

Substrate Induced Enzyme Inactivation. The partition ratios and rates of enzyme inactivation (j_pp (max)) in the presence of PCA, GA, 3OMG, and DHBA am were calculated using methods previously described for mechanism based inactivation of dioxygenases. The apparent rate of inactivation (j_pp (max)) in air-saturated buffer during catalytic turnover was determined from the curvature of O₂ consumption progress curves of reactions performed at varying substrate concentrations using anaerobically purified enzyme. The rate constant of inactivation at a given substrate concentration (j_i) was determined by fitting individual progress curves to eq 3 using KaleidaGraph. In this equation, P₀ is the concentration of the product formed at
time $t$, $P_{\text{comp}}$ is the concentration of the product formed at reaction completion, and $P_i$ is the concentration of the product at the start of the reaction.

$$P_i = P_{\text{comp}}(1 - e^{-t}) + P_i$$

(3)

The apparent rate of inactivation ($j_{\text{app}}$) was subsequently determined by a fit of eq 4 to the values of $j_i$ as a function of substrate concentration, for substrates showing a substrate concentration dependent inactivation. For substrates not showing a concentration dependence of the rate of inactivation, $j_{\text{app}}$ was calculated as the average of the $j_i$ values over the range of substrate concentrations.

$$j_i = \frac{k_{\text{cat}} [S]}{K_{\text{app}} + [S]}$$

(4)

The partition ratio was then calculated using the $k_{\text{cat}}$ determined from the steady-state kinetics assays and the $j_{\text{app}}$ value determined above using eq 5.

$$\text{partition ratio} = \frac{\text{substrate consumed}}{\text{enzyme inactivated}} = \frac{k_{\text{cat}}}{j_{\text{app}}^{\text{act}}}$$

(5)

**Metal Dependence.** The influence of different divalent metals on PCA dioxygenase activity was tested via a method adapted from Diaz, et al.\textsuperscript{25} Anaerobically purified enzyme was buffer exchanged into degassed 100 mM Tris (pH 7.5) containing 1:9 t-butanol/H₂O and subsequently incubated under N₂ in a sealed vial with 2 mM 2,2′-dipyridyl and/or 2,2′-bipyridine, bipy) at 4 °C for 1 and 24 h. Apo-enzyme reactivation was attempted by the incubation of the apoenzyme solution in a 2 mM solution of the desired divalent metal salt (FeSO₄, CuSO₄, CoSO₄, MnSO₄·H₂O, MgSO₄·7H₂O, NiSO₄, or ZnSO₄) under anaerobic conditions for 1 h and/or 24 h at 4 °C. Activities of untreated enzyme (no bipy added after 1 and 24 h), apoenzyme samples, and samples incubated with metal salts were determined by O₂ consumption assays of 250 μM PCA in 50 mM Tris (pH 7.5). The concentration of enzyme in the buffer exchanged stock was determined by the Bradford assay, and the concentration of enzyme in bipy and metal salt treated samples was calculated by calculating the dilution of the buffer exchanged stock.

**Product Determination.** LigAB reaction products were identified by two different methods. The reaction product of the PCA starting material was generated under O₂ saturated conditions in 1 mL of 50 mM phosphate buffer (pH 7.5), 2 mM PCA, and incubation with ∼10 μg (50 μL of a 45 μM stock) of anaerobically purified LigAB added portion-wise over 1 h. The reaction was monitored by ¹H NMR for the disappearance of the PCA aromatic chemical shifts, with 3-(trimethylsilyl)-propionic-2,2,3,3-d₄ acid sodium salt (DSS, Aldrich) as a reference. Additionally, the product, CHMS, was converted to 2,4-lutidinic acid as previously described by the addition of 200 μL of concentrated NH₄OH.\textsuperscript{46–48} After stirring at room temperature for 2 h, the ¹H NMR spectrum was recorded. All aqueous ¹H NMR spectra were recorded in 1:9 D₂O/H₂O with water suppression. The UV–vis spectra of the CHMS reaction product were recorded under reaction (pH 7.5), acidic (pH 1), and alkaline (pH 14) conditions by the addition of 4 M HCl or 4 M NaOH, respectively. The UV–vis spectrum of the product formed by the reaction of CHMS with ammonium hydroxide was also recorded.

**Substrate Promiscuity Assessment.** A wide range of potential substrates were screened using methods analogous to those previously described, using anaerobically purified LigAB and 5 mM compound. Reactions were performed in 1 mL of air-saturated 50 mM Tris (pH 7.5) with 5 mM substrate at 25 °C and monitored by oxygen consumption. LigAB was added to initiate the reaction as previously described. In addition to stock solutions of PCA and gallate (25 mM in H₂O), and 3OMG (25 mM in 1:9 DMSO/H₂O), stock solutions of the organic substrates used for alternative substrate determination were prepared as 25 mM solutions in H₂O (3,4-dihydroxybenzaldehyde, 2,3,4-trihydroxybenzoic acid, 2,3-dihydroxybenzoic acid, homoprotocatechuic acid, 4-hydroxybenzoic acid, 3,4-dihydroxybenzamide, 1,2-dihydroxy-4-aminobenzene, 4-nitrocatechol, catechol, 4-methyl catechol, 3-methoxycatechol, 2-aminoophenol, and 3,4-dihydroxybenzonitrile), 1:9 DMSO/H₂O (benzoic acid, vanillic acid, syringic acid, 2-iodobenzoic acid, vanillin, 3,4-dimethoxy benzaldehyde, syringaldehyde, 3-ethoxy-4-hydroxy benzaldehyde, methyl-3,4-dihydroxybenzoate, 2-methoxy-4-methylenephon, 3-methoxyphenol, and veratrol), or as 12.5 mM solutions in 1:4 DMSO/H₂O (caffeic acid and p-coumaric acid). In an effort to see activity from potentially poor substrates, varying concentrations of LigAB (3–350 nM) were added to assays of the organic substrates. Assays with an addition of anaerobic enzyme greater than 2 μL exhibited O₂ dilution effects upon addition. The dilution effect was corrected by subtraction of the progress curve for the addition of an equivalent volume of degassed 50 mM Tris and 1:9 t-butanol/H₂O buffer containing no enzyme. Kinetic parameters were determined for substrates identified to have significant reactivity. Substrates found to have low levels of O₂ consumption activity are reported as a percentage of the activity of LigAB with 5 mM PCA.

The oxidation potentials of a series of PCA analogues where the C1 substituent was varied were calculated using Gaussian 09 (Wallingford, CT).\textsuperscript{49–51} Initial structures were minimized by a molecular mechanics procedure, starting from several different initial geometries to ensure the location of the global minimum, followed by complete geometry optimization at the density functional B3LYP/6-31+g(d)-PCM level. Because solvation is especially important with charged species, solvation energies of all species were computed by the commonly used polarized continuum method of Tomasi.\textsuperscript{51} The computed free energies (in kcal/mol) of each parent anion and the corresponding neutral species formed by the removal of an electron from the parent were computed. The difference (ΔG₃₀₀) between the two energies is a measure of the ease of electron removal from the anion. ΔG₃₀₀ was then converted to the absolute oxidation potential (independent of reference electrode) of the substance through the relationship ΔG₃₀₀ = −r(ΔG₃₀₀), in which Faraday (r, the proportionality constant between electrochemical and chemical free energies) = 23.06 kcal/volt. Absolute potentials have been shown to be highly correlated with experimentally oxidation potentials in other systems.\textsuperscript{52}

**Inhibition Assessment.** All molecules that exhibited no oxygen consumption upon incubation with LigAB were examined for their ability to bind to and inhibit the dioxygenation of PCA by LigAB. Stock solutions of all molecules were prepared as previously described. Reactions were performed in 1 mL of air-saturated 50 mM Tris (pH 7.5) with 500 μM PCA and 1 mM potential inhibitor at 25 °C, and monitored by oxygen consumption; LigAB was added to initiate the reaction (3–7 nM). Inhibition was determined by comparison of the rates of inhibitor containing assays to the
rates obtained from assays performed directly before and after where only 500 μM PCA (and no potential inhibitor) was present.

**pH Effect on Iron Binding.** Thawed anaerobically purified enzyme was buffer exchanged using Sephadex G-25 desalting gel as described above into degassed 50 mM Tris (pH 7.5) and 1:9 DMSO/H2O to remove excess iron from storage. A 30 μL aliquot of each enzyme sample was removed for use in a Bradford assay to determine the enzyme concentration. Desalted enzyme was subsequently buffer exchanged under ambient conditions into a 50 mM buffer of pH 6 (phosphate), 7.5 (phosphate), and 10 (CAPSO) using Amicon Ultra 0.5 mL centrifugal filters with a 10 kDa molecular weight cutoff (Millipore). After buffer exchanging, the protein samples were diluted to their original volume with water, and a Ferene-S assay adapted for the 96-well plate format was performed, as described by Capyk, et al.53 to determine the iron content of all samples. The iron concentration was divided by the concentration of enzyme determined from the Bradford assay to give the relative iron−enzyme ratios.

**RESULTS AND DISCUSSION**

LigAB has been the subject of several studies since it was first reported as a protocatechuate 4,5-dioxygenase in 1990 by Noda, et al.54 The crystal structure, the first for this superfamily, and reaction kinetics (specific activity) of LigAB have been previously reported and are often cited by those investigating dioxygenase enzymes believed to be members of the PCAD superfamily. However, in these previous studies, LigAB was expressed and purified aerobically.37−39 Our attempts to mimic these purification conditions did indeed lead to the production of active enzyme; however, experiments to confirm the kinetic parameters of the enzyme revealed inconsistent initial rates at a given organic substrate concentration as well as loss of activity.
Incubation of aerobically purified enzyme with reductants, such as dithiothreitol or ascorbate, failed to recover or enhance the activity. The adoption of the anaerobic protein purification techniques described here and by others has produced pure copurified LigAB with greater than 10-fold higher activity over that of the aerobically purified enzyme and lower loss of activity over time after thawing from storage. Additionally, no loss of activity has been observed in the anaerobically purified enzyme stored at ~80 °C over a period of 6 months.

**pH Effects on Rate.** Despite exhaustive searching, a previous determination of the pH rate maximum of LigAB was not found. The pH rate maximum of a related enzyme, gallate 3,4-dioxygenase (DesB), from the same LDAC degradation pathway was determined, by oxygen consumption, to be 8.5 in the presence of gallate. Kinetic parameters (specific activity) were previously determined for LigAB as well as DesB and DesZ (3-O-methylgallic acid 3,4-dioxygenase) using the pH optimum conditions for DesB. Here, LigAB was found to have a pH rate maximum of 7.5 in phosphate buffer for the consumption of O₂ in the presence of PCA (Figure 2A) when fit to eq 2. This pH rate maximum is consistent with pH values found for other Type II EDOs, including PCA and homoprotocatechuete (HPCA) dioxygenases. The pKₐ values determined from the fit of eq 2 suggest that there are two residues with a pKₐ of 6.3 and a third residue with a pKₐ of 9.7. The slope of the basic arm, 1.8, from the plot of log(κᵢᵥ) as a function of pH and fit to eq 2 (Figure 2B) also supports the presence of two residues with acidic pKₐ values, and the slope of the basic arm, 0.6, supports the presence of one residue with a basic pKₐ.

In addition to the pH rate effect, changes in buffering agents also influenced the reaction rate, as can be seen in the pH region of 7.5 to 9 (Figure 2A). The effect was most evident at pH 7.5 where the initial rate of reactions performed in phosphate buffer displayed a small rate enhancement (20%) over the initial rate of reactions performed in Tris buffer. The small size and the diol like structure of Tris may contribute to the ability of Tris to possibly bind and occupy the LigAB active site, thus causing a mild inhibitory effect. Despite the rate enhancement in phosphate buffer, assays of LigAB activity were performed in Tris buffer to remain consistent with previous studies, and the pH was adjusted to 7.5 corresponding to the pH rate maximum found here.

LigAB contains two active site histidine residues, His127β and His195β, which based upon homology to mechanistically characterized Type I and Type III EDOs are believed to aid in substrate alignment and play a role as a catalytic base and catalytic acid, respectively (Figure 3). His127β is likely one of the two active site residues that becomes protonated and leads to decreased activity at lower pH. The second residue to be protonated may be one of the iron chelating residues, most likely Glu242β, although His12β and His61β are also candidates, leading to a loss of iron from the active site and subsequently a decrease in activity (see below for a discussion of metal dissociation experiments). The loss of activity at high pH corresponds to a single deprotonation event. Although His195β has been suggested to play a role in substrate alignment in the active site, it is possible that this residue plays an additional role as a catalytic acid, based upon homology to the mechanisms of other dioxygenases. Additional explanations for the decrease in activity at high pH corresponding to the relatively high pKₐ of 9.7 could include oxidation of the active site iron or deprotonation of pKₐ, depressed Ser269β. Deprotonation of Ser269β would create an unfavorable charge–charge interaction with the carboxylic acid of PCA, preventing proper alignment in the active site. Further mechanistic analysis, including mutagenesis of these residues, will be performed to test mechanistic assignments.

**Metal Dependence.** In metalloenzymes, the relative promiscuity of active site ligands to bind a variety of different metal ions of the same oxidation state makes it necessary to carefully determine which metal(s) are able to promote catalysis. In a variety of enzyme superfamilies, like the amidohydrolase superfamily, the number of metal ions necessary for catalysis and also the identity of the metal ion(s) can be an important diagnostic for membership in an enzyme subtype, which can be informative when trying to determine the reaction catalyzed. Since nonheme iron extradiol dioxygenases have been observed to be catalytically active with a variety of metal ions, most commonly Fe²⁺, but also with other divalent metals such as Mn²⁺, Co²⁺, Cu²⁺, Zn²⁺, or Ni²⁺, we sought to determine the iron metal dependency of LigAB. Since many nonheme iron dioxygenases are expressed and purified aerobically, and subsequently doped with an iron(II) salt (or other divalent metal ions) to reconstitute the enzyme activity, LigAB was grown and purified in the absence of any exogenous metal. Despite the absence of any metals in the growth media or lysis buffer, the enzyme copurified with metal under both aerobic and anaerobic conditions, in a catalytically active state.

In order to obtain apo enzyme, LigAB was incubated alternately with EDTA and bipy to remove the liganded metal. Incubation for 1 h with either bipy or EDTA, as described by Diaz, was insufficient to completely eliminate LigAB activity suggesting that LigAB has a more tightly bound and/or less accessible iron center that other dioxygenases (LigAB maintained 65% activity upon incubation with EDTA and 50% activity upon incubation with bipy, after 1 h of incubation at pH 7.5). In the case of LigAB, complete loss of activity was only achieved by incubation of the enzyme with 2 mM bipy for 24 h. Incubation of LigAB with EDTA (1 or 2 mM) for the same time frame yielded the enzyme, which maintained 35%
Fe remaining bound to LigAB at this pH is likely in the Fe(III) state, which has been observed with the addition of pH-dependent UV-visible spectral changes of CHMS (pH 7.5, dashed line; pH 2, dotted line; pH 14, dashed-dotted line) and the spectrum of 2,4-lutidinic acid (solid) formed upon the addition of NH₄OH.

Iron Dissociation. To investigate the potential contribution of Fe(III) dissociation to the decreases in rate observed in the pH rate profile, the iron content of enzyme exchanged and incubated at pH 6.5, 7.5, and 10 (Table 3). At pH 0.2, as compared with the exchange rates observed at pH 6.6.66

The product of the dioxygenation of PCA by LigAB, 4-carboxy-2-hydroxymuconate-6-semialdehyde (CHMS), was found to have an iron to enzyme ratio of 1:1 Fe/PCA (Table 3) at pH 7.5. The reduction of Fe(III) to Fe(II) was observed with the addition of pH-dependent UV-visible spectral changes of CHMS (pH 7.5, dashed line; pH 2, dotted line; pH 14, dashed-dotted line) and the spectrum of 2,4-lutidinic acid (solid) formed upon the addition of NH₄OH.

Product Identification. The product of the dioxygenation of PCA by LigAB, 4-carboxy-2-hydroxymuconate-6-semialdehyde (CHMS), was found to have an iron to enzyme ratio of 1:1 Fe/PCA (Table 3) at pH 7.5. The reduction of Fe(III) to Fe(II) was observed with the addition of pH-dependent UV-visible spectral changes of CHMS (pH 7.5, dashed line; pH 2, dotted line; pH 14, dashed-dotted line) and the spectrum of 2,4-lutidinic acid (solid) formed upon the addition of NH₄OH.

Figure 4. UV-visible spectra of LigAB catalysis with PCA. (A) Spectra of PCA (solid line) and CHMS at reaction completion (dashed line) at pH 7.5. (B) pH dependent UV-visible spectral changes of CHMS (pH 7.5, dashed line; pH 2, dotted line; pH 14, dashed-dotted line) and the spectrum of 2,4-lutidinic acid (solid) formed upon the addition of NH₄OH.

Biochemistry
hyde (CHMS), was identified by UV-vis spectroscopy and 1H NMR, aided by a secondary conversion of CHMS to 2,4-lutidinic acid. Since CHMS is involved in an extensive equilibrium of structural isomeric forms, its isolation is difficult. The UV-vis spectra of CHMS under multiple pH conditions have been previously reported, and the spectra recorded here (Figure 4A) are consistent with previous data showing a $\lambda_{\text{max}}$ of 298 nm and a second $\lambda_{\text{max}}$ of 410 nm at pH 7.5. Additionally, the spectra of PCA at pH 7.5 is reported here. The spectral changes observed upon reducing the pH to 1 or increasing the pH to 14 are also consistent with previous observations of CHMS (Figure 4B). The conversion of CHMS to 2,4-lutidinic acid also provides a diagnostic UV-vis spectral change that is consistent with the conversion to this product. In addition to the UV-vis spectral changes, the reaction was monitored by 1H NMR spectroscopy (Figure S1, Supporting Information). The disappearance of PCA can be clearly observed on the NMR time scale; however, peaks corresponding to product formation were not observed in the 1H NMR spectrum of the reaction at completion due to the equilibrium of multiple product isomers. Addition of NH₄OH to convert CHMS to 2,4-lutidinic acid results in the appearance of three aromatic proton signals consistent with a spectrum of 2,4-lutidinic acid from the Sigma-Aldrich spectral library.71

**Steady-State Kinetics.** Although Masai and co-workers previously established the relative rates of reaction for LigAB with PCA, GA, and 3OMG, these experiments were conducted under less than optimal conditions with aerobically purified enzyme and a pH optimum determined for a different, yet related, dioxygenase.39 Also, the kinetic parameters of $k_{\text{cat}}$ and $k_{\text{cat}}/K_{\text{m}}$ were not determined. Results of the steady-state kinetics for LigAB with PCA, gallate, and 3OMG obtained in this study are shown in Figure 5. The steady-state kinetic parameters are listed in Table 1. The values of $K_{\text{cat}}^{\text{app}}$ for these substrates are consistent with those previously determined; however, here we have found that 3OMG binds much less tightly than previously observed: 2319 ± 316 μM compared to 937 ± 18 μM, respectively. The values of $k_{\text{cat}}^{\text{app}}$ determined here for these substrates demonstrate a similar relative ratio of activities to the previously determined specific activity with PCA having the highest turnover, followed by GA, and then 3OMG. The CS-substituent effect on reactivity and substrate specificity is quite apparent from this series, likely due to the steric clash with Phe103α (Figure 3); however, not all steric and electronic contributions to substrate specificity are evident from these data. Significantly, as described above, the LigAB purified anaerobically in this study is greater than 10-fold more active than the enzyme expressed from the same source but purified aerobically. While the previously reported results for LigAB are significant and inspired our work with this enzyme, anaerobic purification techniques allow for a more accurate characterization of LigAB.

The steady-state kinetic parameters determined for LigAB with the native substrate, PCA, as compared to other dioxygenases with their native substrates are listed in Table S1, Supporting Information. Dioxygenases of all types (I-VO, II-PCAD, and III-cupin) are quite proficient enzymes, with $k_{\text{cat}}/K_{\text{m}}$ values ranging from $10^5$ to $10^8$ M$^{-1}$s$^{-1}$.30,31,36,62,69,70 and LigAB (4.3 × 10$^6$ M$^{-1}$s$^{-1}$) fits well within this trend. Additionally, the kinetic parameters of LigAB coincide well with those determined for 2,3-PCD, MhpB, and 4,5-PCD (C. testosteroni Pt-L5), the only other Type II dioxygenases with a complete set of steady-state kinetic parameters determined.16,18,33 The $K_{\text{m}}$ for LigAB (162 ± 16 μM), determined from Figure 6, was also found to be in the range of those determined for other extradiol dioxygenases (Table S1, Supporting Information) and is nearly identical to that determined for the Type II dioxygenase 2,3-PCD (142 ± 14 μM).

**Substrate Specificity and Inhibition.** Intradiol and extradiol dioxygenases have long been known to exist for the preferential cleavage of a wide variety of substrates. Substrate classes include catechols, protocatechuates, gentisates, 2-aminophenols, quinones, aromatic amino acids, and even halogenated aromatics.7 Dioxygenase specificities range from the highly specific to the quite promiscuous. LigAB has been shown here and in previous studies to be a promiscuous enzyme capable of efficient catalytic turnover of several substrates in the LDAC catabolic pathway of S. paucimobilis. (PCA, GA, and 3OMG). The promiscuity of LigAB with GA and 3OMG contrasts with another Type II dioxygenase, gallocate dioxygenase (GDO) from *Pseudomonas putida* KT2440 (39% sequence identity to LigB). GDO was found to have no oxygen consumption activity with PCA and is believed to be a gallocate specific dioxygenase.25 Additionally, several PCA 4,5-dioxygenases (4,5-PCD) have been identified for which substrate specificity, substrate uptake by live cells, and the inhibition effect of other substrates have been studied; however, reaction kinetics remain largely unstudied for these 4,5-PCDs17,29,46,48,61,64,78–80 with the exception of the 4,5-PCD from *Comamonas testosteroni* Pt-L5 (formerly *Pseudomonas testosteroni* Pt-L5) (Table S1, Supporting Information).16 Despite the evidence of promiscuity in LigAB, the range of accepted substrates was never explored beyond the PCA analogues identified from within its pathway. Here, we have tested multiple classes of substrates analogous to the basic catechol scaffold with substitutions primarily at C1, C5, and functional modification of the catecholic hydroxyl groups (Table 2 and Figure S2, Supporting Information). In addition to the three known substrates, LigAB was demonstrated to

![Figure 5. Steady-state kinetics of LigAB with PCA and substrate analogues: (red circle) PCA, (blue square) gallate, (green diamond) 3OMG, (orange triangle) DHBAm. The dependence of the initial rates on the organic substrate concentration was determined in air-saturated buffer (250–275 μM O₂, pH 7.5, 25 °C), and the curves are fit to the Michaelis–Menten equation.](image-url)
Menten equation to the data. The activity of LigAB with 500 mM PCA.

observed for DHBAm; however, the behavior is inconsistent with eq 4 due to highly inactivating product inhibition.

concentrations of gallate.

average of the

μ concentration was measured at high substrate concentration (1000 μM PCA, pH 7.5, 25 °C).

dependence of the initial rates on the O₂ concentration was measured at high substrate concentration (1000 μM PCA and 1 mM inhibitor in air-saturated 50 mM Tris bu... 373 μM PCA, pH 7.5, 25 °C. Calculated as a percentage of the activity of LigAB with 5 μM PCA.

Figure 6. Steady-state kinetics of LigAB with varying O₂ concentrations. Dependence of the initial rates on the O₂ concentration was measured at high substrate concentration (1000 μM PCA, pH 7.5, 25 °C), and the curve is a fit of the Michaelis–Menten equation to the data.

utilize four new substrates: 3,4-dihydroxybenzamide (DHBAm), homoprotocatechuate (HPCA), catechol, and 3,4-dihydroxybenzonitrile. DHBAm was the most active of the newly identified substrates, displaying only a small fraction of the activity (less than 1%) as seen for LigAB with PCA (Table 2). Interestingly, a number of molecules were identified that were not substrates but showed competitive inhibition of the LigAB reaction with PCA, including 4-nitrocatechol and 4-methylcatechol (Table 2). All of these molecules are analogues of PCA, where the C1 substituent (using the PCA numbering scheme) is varied. To explain these observations, we initially performed calculations...inhibitor in air-saturated 50 mM Tris buffer at 25 °C and pH 7.5. Calculated as a percentage of the activity of LigAB with 500 μM PCA with no inhibitor present.

Table 1. LigAB Steady-State Kinetic Parameters Measured with PCA and Analogous Substrates in Air-Saturated Buffer

<table>
<thead>
<tr>
<th>substrate</th>
<th>k₉⁰⁰ₐ (μM)</th>
<th>k₉⁰⁰ₜ (s⁻¹)</th>
<th>k₉⁰⁰/K₉⁰⁰ (M⁻¹ s⁻¹)</th>
<th>partition ratio⁸⁺</th>
<th>k₉⁰⁰/K₉⁰⁰ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCA</td>
<td>51 ± 4</td>
<td>216 ± 3</td>
<td>4.26 × 10⁷</td>
<td>~18000</td>
<td>0.012³</td>
</tr>
<tr>
<td>gallate</td>
<td>441 ± 55</td>
<td>53 ± 2</td>
<td>1.21 × 10⁷</td>
<td>~2500</td>
<td>0.021²</td>
</tr>
<tr>
<td>3OMG</td>
<td>2319 ± 316</td>
<td>7.3 ± 0.5</td>
<td>3.13 × 10⁷</td>
<td>~5600</td>
<td>0.0013⁴</td>
</tr>
<tr>
<td>DHBAm</td>
<td>3263 ± 327</td>
<td>104 ± 5</td>
<td>3.20 × 10⁷</td>
<td>~3200</td>
<td>0.033¹</td>
</tr>
</tbody>
</table>

⁸Estimated from the j₉⁰⁰ and k₉⁰⁰ (eq 5). Calculated from a fit of eq 4 to jₙ values determined from the fitting of progress curves (eq 3) at different concentrations of gallate. Concentration dependent inactivation was not observed for PCA or 3OMG. j₉⁰⁰ was calculated as an average of the jₙ values determined from the fitting of eq 3 to progress curves at different substrate concentrations. Concentration dependent inactivation was observed for DHBAm; however, the behavior is inconsistent with eq 4 due to highly inactivating product inhibition. k₉⁰⁰ was calculated from an average of the jₙ values determined from the fitting of eq 3 to progress curves of reactions with 1000–5000 μM DHBAm.

Table 2. Library of Alternative Substrates: Observed Activity, Inhibition, and Calculated Oxidation Potentials for Analogues of PCA

<table>
<thead>
<tr>
<th>substrate</th>
<th>activity observed</th>
<th>% activity⁶⁺</th>
<th>% inhibition⁶⁺</th>
<th>E°substrate oxidation (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(HPCA) Homoprotocatechuate</td>
<td>Y</td>
<td>0.2</td>
<td>28</td>
<td>5.40</td>
</tr>
<tr>
<td>4-methylcatechol</td>
<td>N</td>
<td></td>
<td>5.71</td>
<td></td>
</tr>
<tr>
<td>PCA</td>
<td>Y</td>
<td>100</td>
<td>5.82</td>
<td></td>
</tr>
<tr>
<td>catechol</td>
<td>Y</td>
<td>0.17</td>
<td>5.88</td>
<td></td>
</tr>
<tr>
<td>3,4-dihydroxybenzamide</td>
<td>Y</td>
<td>33</td>
<td>6.11</td>
<td></td>
</tr>
<tr>
<td>3,4-dihydroxybenzaldehyde</td>
<td>N</td>
<td></td>
<td>6.19</td>
<td></td>
</tr>
<tr>
<td>3,4-dihydroxybenzonitrile</td>
<td>Y</td>
<td>0.04</td>
<td>6.30</td>
<td></td>
</tr>
<tr>
<td>4-nitrocatechol</td>
<td>N</td>
<td>31</td>
<td>6.46</td>
<td></td>
</tr>
<tr>
<td>gallate</td>
<td>Y</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-O-methylgallate</td>
<td>Y</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,4-trihydroxybenzoic acid</td>
<td>N</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3-dihydroxybenzoic acid</td>
<td>N</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-methoxycatechol</td>
<td>N</td>
<td>26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

⁶Measured with 5 mM substrate in air-saturated 50 mM Tris buffer at 25 °C and pH 7.5. Calculated as a percentage of the activity of LigAB with 5 mM PCA. Measured with 500 μM PCA and 1 mM inhibitor in air-saturated 50 mM Tris buffer at 25 °C and pH 7.5. Calculated as a percentage of the activity of LigAB with 500 μM PCA with no inhibitor present.
was correlated with the catalytic efficiency, using Gaussian 09 (Table 2). From the data, it is clear that a range of organic substrate oxidation potentials (5.40–6.30 eV) is tolerated for accepted substrates; however, the incorporation of O₂ is most facile when the oxidation potential of the organic substrate is 5.82–6.11 eV, as for PCA and 3,4-dihydroxybenzamide. 4-Methyl catechol and catechol, with oxidation potentials of 5.71 and 5.88 eV, respectively, are close to or within the facile range, yet oxygen consumption is not seen or is very low. Some molecules on the fringe of the oxidation potential range such as HPCA (5.40 eV) and 3,4-dihydroxybenzonitrile (6.30 eV) are tolerated as substrates by LigAB, but the activity is severely diminished. 4-Nitrocatechol with the highest oxidation potential of the C1 PCA analogues is not a substrate but is rather an inhibitor, suggesting that compounds that have a higher potential than 6.30 eV are not electronically accessible by LigAB.

Since a clear correlation between catalytic efficiency and oxidation potential for the library of C1 analogues of PCA could not be established for all of the compounds in this series, we then evaluated the likely binding interactions of the various molecules with the LigAB active site residues. We noted that the two worst substrates pyrocatechol and 3,4-dihydroxybenzonitrile as well as one of the inhibitors 4-methyl catechol would all be unable to have favorable hydrogen bonding interactions with the NH₂ group of Asn270β, which is predicted to hydrogen bond to the carboxylic anion of PCA (Figure 3). Sterics could also explain the relatively low catalytic efficiency of LigAB with HPCA (a C1 substrate analogue which moves the carboxylate further into the pocket by the addition of a methylene) and the absence of catalysis or inhibition with methyl-3,4-dihydroxybenzoate and caffeic acid, which are likely just too big to fit into the binding site, perhaps due to steric conflict with Thr271β (Figure 3). Conversely, the inhibitory nature of 4-nitrocatechol, which is isosteric with PCA and could accept hydrogen bonds from the NH₂ group of Asn270β, is likely dominated by electronic effects. In other systems, it has been observed that substrate/inhibitor scope is generally smaller than that observed for LigAB. In the 4,5-PCD from C. testosteroni, sulfonylcatechol (a C1 sulfonyl substitution of the PCA carboxylic acid) was the only known substrate analogue with a C1 modification to be turned over. Additionally, catechol (C1-H) and 4-methyl catechol (C1-Me) were observed to be inhibitors of the 4,5-PCD of Pseudomonas sp. but nonsubstrates of the 4,5-PCD of C. testosteroni.

Interestingly, 3,4-dihydroxybenzaldehyde was neither a substrate nor an inhibitor of LigAB despite the structural similarity to PCA and its seemingly acceptable oxidation potential. The inability to turnover 3,4-dihydroxybenzaldehyde has been observed in 4,5-PCDs from Pseudomonas sp. and C. testosteroni, however, in these instances 3,4-dihydroxybenzaldehyde does act as an inhibitor. Examination of other aldehyde containing compounds including vanillin revealed that none of these compounds were substrates or inhibitors for LigAB despite their structural similarity to viable LigAB substrates. Knowing that vanillin is the substrate of the enzyme two ahead of LigAB in the S. paucimobilis LDAC catabolic pathway, we hypothesize that perhaps LigAB has evolved to preclude the binding of aldehydes in the active site.

Taken together, the results of the library suggest that there are both structural and electronic requirements which are necessary for organic substrate activity with LigAB. The first requirement is an electron withdrawing group in the C1 position such that the oxidation potential of the substrate is 5.40–6.30 eV (~5.8 eV being the sweet spot). Second, a 3,4 or a 4,5 diol is required. Functional modification of hydroxyls in these positions, such that hydroxyls ortho to one another are not present, eliminates activity. For example, 3OMG remains a viable substrate because the 4,5-diol motif is conserved, yet vanillate and syringate, which have a methylether rather than a hydroxyl ortho to the C4-hydroxyl, displayed no activity. Mutagenesis of various active site residues will be pursued to test for possible gain of function for the various substrates that are believed to be electronically allowed but sterically disfavored.

**Mechanism Based Inactivation in Steady-State Kinetics.** As has been previously reported for other dioxygenase enzymes, LigAB is susceptible to inactivation during catalytic turnover under air-saturated conditions. While inactivation of a Type II dioxygenase has not previously been reported, the rates of inactivation for LigAB (Table 1) are similar to those reported for the well characterized Type I dioxygenases 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD) and 2,6-dichlorohydroquinone 1,2-dioxygenase (PcpA). Inactivation was seen with each of the four most preferred substrates (PCA, GA, 3OMG, and DHBAm); however, only gallate showed substrate concentration dependent inactivation rates that could be fit to eq 4, resulting in a k_{app} of 0.021 s⁻¹. Inactivation during the turnover of PCA (0.012 s⁻¹) and 3OMG (0.0013 s⁻¹) was independent of substrate concentration within the experimental concentration range. While there has been some debate over how mechanism based inactivation occurs, the strongest evidence suggests that the active site Fe(II) becomes irreversibly oxidized to Fe(III) due to the release of superoxide. Superoxide release can occur before or after organic substrate binding, though it tends to occur more slowly in the free enzyme due to a lower affinity (higher K_mox) for oxygen in the absence of substrate as was observed for DHBD. Additionally, while inactivation tends to be more pronounced in the presence of poor substrates, inactivation has also been observed in the presence of the preferred substrate(s). While the data presented here does not strictly follow this trend (Table 1), the rate of inactivation for the three most preferred substrates (PCA, gallate, and DHBAm) is consistent with this observation with PCA being the least inactivating and DHBAm being the most inactivating of these substrates. 3OMG, despite being the poorest of the preferred substrates, displayed the least inactivation. The increased stericity of 3OMG likely prevent this substrate from properly binding; however, once bound in a geometry suitable for catalysis, 3OMG may be more active toward C=O bond formation with superoxide thus preventing premature release and slowing the oxidation of the LigAB active site iron.

**CONCLUSIONS**

Steady-state kinetic studies have demonstrated that LigAB is highly specific for catecholic substrates with an electron...
While LigAB was previously known to have promiscuous activity, this promiscuity has now been expanded beyond molecules of the LDAC degradation pathway of *S. paucimobilis* SYK-6. Analysis of the structure and oxidation potentials of the accepted substrates suggests that LigAB is highly specific for diol containing substrates with an oxidation potential between 5.40 and 6.30 eV. Additionally, steric and hydrogen bonding interactions by active site residues (such as Phe103α, Thr271β, or Ser269β) likely impart some of LigAB’s substrate specificity, modulating the catalysis of accepted substrates and preventing some substrate-like molecules that meet these parameters from being acceptable substrates. Mutagenesis of LigAB will likely reveal the relative importance of these residues in controlling substrate specificity. LigAB, like other dioxygenases, is subject to mechanistic inactivation, although not all substrates show a concentration dependence on the rate of inactivation. In the case of the newly discovered substrate DHBAm, dead-end product inhibition dominates the mechanisms of inactivation. While inhibition of LigAB is not directly applicable to our research goals, a number of competitive inhibitors were also identified in this study. These results could provide insight toward identifying lead compounds in a system where dioxygenase inhibition is desired. Furthermore, the pH rate studies suggest this enzyme would have either two catalytic bases or that Fe(II) coordination is highly pH sensitive. Further studies suggest this enzyme would have either two catalytic intermediates or that Fe(II) coordination is highly pH sensitive. Further mechanistic analyses, including mutagenesis, might shed light on these results and allow comparisons to mechanisms proposed for dioxygenases from other enzyme superfamilies. In total, these results make LigAB the first Type II dioxygenase to be fully characterized both structurally and kinetically and solidify its position as the de novo member of the Type II dioxygenase superfamily.

## ASSOCIATED CONTENT

* Supporting Information

Steady-state kinetic parameters for Type I, II, and III extradiol dioxygenases, NMR characterization of CHMS, and UV–vis spectra of the PCA LigAB reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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## REFERENCES


