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## Characterization of a Broadly Specific Cadaverine N-hydroxylase Involved in Desferrioxamine B Biosynthesis in *Streptomyces sviceus*

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## RESEARCH ARTICLE

# Characterization of a broadly specific cadaverine *N*-hydroxylase involved in desferrioxamine B biosynthesis in *Streptomyces sviveus*

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## Abstract

*N*-hydroxylating flavin-dependent monooxygenases (FMOs) are involved in the biosynthesis of hydroxamate siderophores, playing a key role in microbial virulence. Herein, we report the first structural and kinetic characterization of a novel alkyl diamine *N*-hydroxylase DesB from *Streptomyces sviveus* (*SsDesB*). This enzyme catalyzes the first committed step in the biosynthesis of desferrioxamine B, a clinical drug used to treat iron overload disorders. X-ray crystal structures of the *SsDesB* holoenzyme with FAD and the ternary complex with bound NADP<sup>+</sup> were solved at 2.86 Å and 2.37 Å resolution, respectively, providing a structural view of the active site environment. *SsDesB* crystallized as a tetramer and the structure of the individual protomers closely resembles the structures of homologous *N*-hydroxylating FMOs from *Erwinia amylovora* (*DfoA*), *Pseudomonas aeruginosa* (*PvdA*), and *Aspergillus fumigatus* (*SidA*). Using NADPH oxidation, oxygen consumption, and product formation assays, kinetic parameters were determined for various substrates with *SsDesB*. *SsDesB* exhibited typical saturation kinetics with substrate inhibition at high concentrations of NAD(P)H as well as cadaverine. The apparent  $k_{cat}$  values for NADPH in steady-state NADPH oxidation and oxygen consumption assays were  $0.28 \pm 0.01 \text{ s}^{-1}$  and  $0.24 \pm 0.01 \text{ s}^{-1}$ , respectively. However, in product formation assays used to measure the rate of *N*-hydroxylation, the apparent  $k_{cat}$  for NADPH ( $0.034 \pm 0.008 \text{ s}^{-1}$ ) was almost 10-fold lower under saturating FAD and cadaverine concentrations, reflecting an uncoupled reaction, and the apparent NADPH  $K_M$  was  $33 \pm 24 \mu\text{M}$ . Under saturating FAD and NADPH concentrations, the apparent  $k_{cat}$  and  $K_M$  for cadaverine in Csaky assays were  $0.048 \pm 0.004 \text{ s}^{-1}$  and  $19 \pm 9 \mu\text{M}$ , respectively. *SsDesB* also *N*-hydroxylated putrescine, spermidine, and L-lysine substrates but not alkyl (di)amines that were branched or had fewer than four methylene units in an alkyl chain. These data demonstrate that *SsDesB* has wider substrate scope compared to other well-studied ornithine

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and lysine *N*-hydroxylases, making it an amenable biocatalyst for the production of desferrioxamine B, derivatives, and other *N*-substituted products.

## Introduction

*N*-hydroxylating monooxygenases (NMOs) play an important role in the biosynthesis of iron-chelators or siderophores. Microorganisms express these flavin-dependent enzymes under iron-limiting conditions to produce siderophores to sequester iron, an essential nutrient required for cell growth and development. Using the electron donor NADPH and molecular oxygen, NMOs catalyze the first committed step in hydroxamate siderophore biosynthesis by oxidizing a primary amine in alkyl amines [1, 2] and amino acids, such as lysine [3] and ornithine [4, 5], to *N*-hydroxylated products. Subsequent acylation of these products form hydroxamates, imparting metal-chelating properties. NMOs are involved in the biosynthesis of a variety of hydroxamate siderophores in many microorganisms, such as *Aspergillus fumigatus* (SidA) [4], *Pseudomonas aeruginosa* (PvdA) [6, 7], *Bordetella bronchiseptica* RB5 (AlcA) [8], and *Streptomyces pilosus* (DesB) [9]. Deleting genes encoding these enzymes in pathogens, such as *A. fumigatus* [10] and *P. aeruginosa* [11], inhibit siderophore production as well as their growth in low iron media. Thus, characterizing NMOs can provide insights into combating pathogen virulence and identifying potential drug targets for the development of antimicrobial agents.

In addition to serving as drug targets against pathogenic microbes, *N*-hydroxylases are indicative of the biosynthesis of hydroxamate siderophores, some of which are used in clinical treatments [12, 13]. The lysine-derived desferrioxamine B (Fig 1) is a tris-hydroxamate siderophore produced by aerobic, Gram-positive soil actinomycetes, such as *S. coelicolor* M145 [12] and *S. pilosus* [14], clinically used for the intravenous treatment of iron poisoning and hemochromatosis [15, 16]. The hydroxamate moiety is the key pharmacophore [17] and also the reason why desferrioxamine B is widely used as a hexadentate chelator in immunoPET and cell tracking [13]. Desferrioxamines are biosynthesized by an operon encoding a lysine decarboxylase (DesA), cadaverine *N*-hydroxylase (DesB), acyl transferase (DesC), and an ATP-dependent nonribosomal peptide synthetase (DesD) [18] (Fig 1). A ferric siderophore lipoprotein receptor (DesE) and a ferric-siderophore hydrolase (DesF) are also found within the gene cluster in various strains of *Streptomyces* (Fig 1) [19, 20]. These genes were first identified in *S. coelicolor* [18] and have since been found in most sequenced *Streptomyces* genomes [20], several species of *Salinispora* [21], as well as plant pathogen *E. amylovora* [2]. While a number of publications have provided insight into the activities of DesA [22], DesB [2], DesC [23], and DesD [23, 24] from *S. pilosus* and *S. coelicolor*, DesA is the only enzyme to be kinetically characterized [22]. Homologs of these biosynthetic enzymes have been identified in several clinical pathogens. For example, there are a number of DesB homologs (36–60% sequence identity) in clinical pathogens, such as *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Yersinia pestis* (S1 Fig in S1 File). Thus, understanding the mechanism of siderophore biosynthetic enzymes, such as DesB, can lead to the identification of new bioactive hydroxamate siderophores and improve the production of other hydroxamates and molecules containing N-O functional groups [25].

We aimed to determine the structure and characterize the kinetics of DesB from *S. sviveus* (SsDesB) to understand the limitations of desferrioxamine biosynthesis and evaluate the substrate scope of alkyl diamine *N*-hydroxylases. Alkyl diamine *N*-hydroxylases, such as the putrescine *N*-hydroxylases GorA from *Gordonia rubripertincta* CWB2 [1] (38% sequence



*pilosus* strain. Steady-state kinetics were performed with putrescine and cadaverine to understand the substrate specificity of SsDesB. We hypothesized that SsDesB would have a broader substrate scope similar to GorA and a comparable three-dimensional structure to other well-studied ornithine (PvdA from *P. aeruginosa*, 31% sequence identity) and alkyl diamine N-hydroxylases (DfoA).

## Materials and methods

### General materials

An *E. coli* codon-optimized, N-terminal hexahistidine-tagged SSEG\_08523 gene encoding SsDesB was synthesized and subcloned into pET28a (+) by GenScript Corp. (Piscataway, NJ). N-hydroxycadaverine was purchased from Enamine Ltd. (Kyiv, Ukraine). All other materials were purchased from Sigma-Aldrich (St. Louis, MO) or ThermoFisher Scientific (Waltham, MA) and used without further purification.

### General methods

Microplate kinetic assays were monitored on an Epoch BioTek microplate spectrophotometer (Winooski, VT). The SsDesB extinction coefficient was determined using an Agilent 8453 diode array UV-vis spectrophotometer (Santa Clara, CA). High resolution electrospray ionization mass spectrometry (ESI-MS) was performed on an Acquity Ultra Performance Liquid Chromatography (UPLC) I-Class System in tandem with a Waters Xevo quadrupole-time of flight (q-TOF) mass spectrometer. Oxygen consumption assays were monitored on an Oxygraph Plus system (Hansatech; Norfolk, UK). DNA sequencing was performed by Eurofins Genomics (Louisville, KY).

### Protein expression of SsDesB with N-terminal hexahistidine tag

The SsDesB gene cloned into pET28a (+) was transformed into *Escherichia coli* BL21 Star (DE3) One Shot® cells (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. Cells were plated on Luria-Bertani (LB)-agar containing 50 µg/mL kanamycin and grown overnight at 37°C. A single colony was used to inoculate LB broth (100 mL) containing 50 µg/mL kanamycin, which was grown overnight at 37°C at 200 rpm. The overnight culture was used to inoculate 1-L of LB broth containing 50 µg/mL kanamycin, which was grown at 30°C at 200 rpm. Cells were induced with 1 mM isopropyl β-D-1 thiogalactopyranoside (IPTG) once an optical density at 600 nm (OD<sub>600</sub>) of 0.6 was reached and grown for four additional hours before being harvested at 4,500 rpm and stored at -80°C.

### Protein purification of SsDesB with N-terminal hexahistidine tag

Cells were lysed twice via the French Press at 20,000 psi after incubation with lysozyme (1 mg/ml) and protease inhibitor cocktail (P8849; Sigma-Aldrich, St. Louis, MO) 50 mM phosphate buffer (pH 8) containing 300 mM sodium chloride and 10 mM imidazole. After centrifugation at 9,100 rpm for 1 hr at 4°C, the supernatant was filtered with a 0.22 µm Millex-GS syringe filter (EMD Millipore; Burlington, MA) and incubated with HisPur™ Ni-NTA resin (ThermoFisher; Waltham, MA). The resin was equilibrated with 50 mM phosphate buffer (pH 8) containing 300 mM sodium chloride and 20 mM imidazole. Protein was eluted in 50 mM phosphate buffer (pH 8) containing 300 mM sodium chloride, 125 mM imidazole, and 2.5% glycerol. Using a 10 K Amicon Ultra centrifugal filter unit (ThermoFisher Scientific; Waltham, MA), protein was buffer-exchanged into 100 mM phosphate buffer containing 1 mM dithiothreitol and 10% glycerol and stored at -80°C.

### Determination of SsDesB extinction coefficient

SsDesB (156  $\mu\text{M}$ ) was heated in triplicate at 95°C for 15 min and clarified by centrifugation at 13,000 rpm for 10 min at room temperature. After the supernatant was removed and reheated again, its absorbance was measured over a range of 200–800 nm. Using Beer's Law ( $A = \epsilon cl$ ) and the extinction coefficient of FAD (11,300  $\text{M}^{-1}\text{cm}^{-1}$ ), the extinction coefficient of DesB was determined, as the concentration of the enzyme should be equivalent to the concentration of free FAD after denaturation. Furthermore, the percent of FAD bound was also determined using Eq (1).

$$\% \text{ of FAD - bound enzyme} = \frac{[\text{Free FAD}]}{[\text{Enzyme}]} \times 100\% \quad (1)$$

### NAD(P)H oxidation assays

Steady-state kinetic parameters for NAD(P)H in oxidation assays were determined by incubating SsDesB (0.7  $\mu\text{M}$ ) with 50  $\mu\text{M}$  FAD, and 10 mM cadaverine at 25°C. Assays were initiated after 5 min with varying concentrations of NAD(P)H (5.5  $\mu\text{M}$ –1 mM), resulting in a total volume of 250  $\mu\text{L}$ . The decrease in absorbance at 340 nm was monitored using an Epoch BioTek microplate spectrophotometer. The initial rate at which the enzyme oxidized NAD(P)H over time was determined using Beer's Law ( $\epsilon_{\text{NAD(P)H}} = 6,220 \text{ M}^{-1}\text{cm}^{-1}$  at 340 nm) and an NAD(P)H standard curve (to determine the quality of cofactor and pathlength of the microplate).

### Oxygen consumption assays

Molecular oxygen consumption consumed by SsDesB was monitored using a Hansatech Oxygraph (Norfolk, England, UK). Steady-state kinetic parameters for substrates were obtained by incubating substrate of various concentrations, 100 mM phosphate buffer, pH 8, SsDesB (7.4  $\mu\text{M}$ ), and 50  $\mu\text{M}$  FAD for 5 min at 25°C with stirring. Reactions were then initiated by the addition of 0.7 mM NADPH (700  $\mu\text{L}$  total volume). Steady-state kinetic parameters for NADPH were obtained by incubating 100 mM phosphate buffer, pH 8, SsDesB (7.4  $\mu\text{M}$ ), 50  $\mu\text{M}$  FAD, and 0.7 mM cadaverine for 5 min incubation with stirring at 25°C. Assays were initiated with various NADPH concentrations (9.1  $\mu\text{M}$ –9.3 mM).

### Detection of hydrogen peroxide

Standard assays (400  $\mu\text{L}$  total volume) consisting of 100 mM phosphate buffer pH 8, 50  $\mu\text{M}$  FAD, 2.12  $\mu\text{M}$  SsDesB, 10 mM cadaverine, and 0.7 mM NADPH were incubated at 25°C. Assays with boiled enzyme as well as no enzyme were also prepared. After 30 s and 72 s, aliquots were removed from assays and the amount of hydrogen peroxide produced was quantified via the QuantiChrom™ peroxide assay kit (BioAssay Systems; Hayward, CA) according to the manufacturer's instructions. A hydrogen peroxide standard curve was used to quantify the amount of hydrogen peroxide produced by SsDesB.

### Cloning and protein expression of SsDesB for structural studies

The *SsDesB* gene was amplified from *E. coli* codon-optimized DNA obtained from GenScript Corp. (Piscataway, NJ) (SSEG\_08523, pET28a (+)) by polymerase chain reaction (PCR) using the following oligonucleotide primers: 5' -GGC TCG GAG AAC CTG TAC TTC CAG ACC GCG CGT CCG GAG -3' (PE-3112) and 5' -GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT ATT ACA CGC TAA ACT CCT GGA ACG C-3' (PE-3113). The PCR amplicon was then used as the template for a second PCR amplification with primer PE-277 (5' -GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC GGA GAA CCT GTA CTT CCA

G -3') and PE-3113. The amplicon from the second PCR, coding for SsDesB (T2-V425) with an *N*-terminal tobacco etch virus (TEV) recognition site (ENLYFQ/T), was recombined into the entry vector pDONR 221 (Life Technologies; Grand Island, NY) using Gateway cloning technology to generate the entry clone pGL3067, and the nucleotide sequence was confirmed by Sanger sequencing [27]. The modified *SsDesB* gene was then recombined from pGL3067 into pDEST527 to generate the expression vector pGL3070. This plasmid produces an *N*-terminal hexahistidine-tagged SsDesB that can be cleaved by TEV protease to yield the SsDesB enzyme [25]. The protein was expressed in the *E. coli* strain Rosetta2(DE3). Cultures were grown to mid-log phase ( $OD_{600} \sim 0.5$ ) at 37°C in Luria Bertani broth containing 0.2% glucose, 100  $\mu\text{g ml}^{-1}$  ampicillin, and 30  $\mu\text{g ml}^{-1}$  chloramphenicol. Overexpression of the SsDesB fusion protein was induced with 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside for 18–20 h at 18°C. Cells were pelleted by centrifugation and stored at -80°C.

### Protein purification for structural studies

All purification steps were performed at 4–8°C. *E. coli* cell paste (10 g) was resuspended in 200 mL of ice-cold lysis buffer containing 50 mM Tris-HCl pH 7.4, 200 mM NaCl, 25 mM imidazole, and cOmplete, EDTA-free protease-inhibitor cocktail tablets (Roche Diagnostics; Mannheim, Germany). Cells were lysed by passing through an APV-1000 homogenizer (Invensys APV Products, Albertslund, Denmark) at 69 MPa three times, and the lysate was centrifuged for 30 min at 15,500 rpm. The supernatant was filtered by vacuum through a 0.2  $\mu\text{m}$  polyether-sulfone membrane and applied to a 5-mL HisTrap FF column (GE Healthcare, Piscataway, NJ) pre-equilibrated with lysis buffer. The column was washed to baseline with lysis buffer and protein was eluted using a linear gradient from 25 mM to 500 mM imidazole. Fractions containing the His<sub>6</sub>-SsDesB fusion protein were combined and concentrated using an Ultracel 30 kDa ultrafiltration disc (EMD Millipore, Billerica, MA). The concentrated protein was diluted with 50 mM Tris pH 7.4, 200 mM NaCl buffer to achieve an imidazole concentration of 25 mM. The His<sub>6</sub>-SsDesB fusion protein was then digested with 5 mg polyhistidine-tagged TEV protease overnight [27]. The digest was applied to a second HisTrap column (2  $\times$  5 mL) pre-equilibrated with lysis buffer. The deep-yellow colored fractions containing SsDesB were combined and half was concentrated to 12–15 mg/mL in the lysis buffer mentioned above. Aliquots were flash frozen in liquid nitrogen and stored at -80°C for use in structural studies. The protein was judged to be >90% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the molecular weight was confirmed by electrospray ionization mass spectrometry. The remaining portion of protein was incubated overnight at 4°C with 10 mM DTT. The sample was concentrated, as above, and fractionated on a HiPrep 26/60 Sephacryl S-300 HR column (GE Healthcare; Piscataway, NJ) equilibrated with a buffer containing 25 mM Tris-HCl pH 7.4, 150 mM NaCl, and 2 mM tris(2-carboxyethyl)phosphine (TCEP). The peak fractions of recombinant SsDesB were combined and concentrated to 12–15 mg/mL. Aliquots were flash frozen in liquid nitrogen and stored at -80°C for use in biochemical studies. This protein was judged to be >95% pure by SDS-PAGE using a Coomassie stain and its molecular weight was confirmed by electrospray ionization mass spectrometry.

### Product formation assays

Using SsDesB without an *N*-terminal hexahistidine tag, the amount of *N*-hydroxylated product formed in SsDesB assays at 25°C was determined using a modified Csaky iodine oxidation assay [4, 28]. In a 96-well plate, SsDesB (1  $\mu\text{M}$ ) was incubated in 100 mM phosphate buffer, pH 8.0 with 50  $\mu\text{M}$  FAD and varied amounts of substrate (cadaverine or putrescine) for 5 min before reactions were initiated with 0.7 mM NAD(P)H (400  $\mu\text{L}$  total volume). For assays in

which NAD(P)H concentrations were varied (0.031–4 mM), the substrate concentration was 10 mM. Hydroxylamine standards (9.4–300  $\mu$ M) in 100 mM phosphate buffer, pH 8.0, were also prepared. Aliquots of enzyme assays (61.5  $\mu$ L) as well as standards were removed and quenched with perchloric acid (0.03 N). Samples were then centrifuged at 7,000 rpm for 3 min at room temperature before the supernatant (47  $\mu$ L) was removed and added to equal volumes of 10% w/v sodium acetate (47  $\mu$ L) and 0.6% w/v sulfanilic acid in 25% acetic acid (47  $\mu$ L). Samples were then incubated with 0.07% w/v iodine in glacial acetic acid for 15 min with shaking before the addition of 19  $\mu$ L of sodium thiosulfate (0.02 N) and 19  $\mu$ L of 1-naphthylamine (0.6% w/v). After 45 min of shaking at 25°C, 96-well plates were read at 562 nm using an Epoch Biotek microplate spectrophotometer.

### Substrate scope assays

Standard assays (400  $\mu$ L total volume) consisting of 100 mM phosphate buffer pH 8, 50  $\mu$ M FAD, 1  $\mu$ M SsDesB (without an *N*-terminal hexahistidine tag), 10 mM substrate, and 0.7 mM NADPH were incubated at 25°C. The following substrates were assayed: cadaverine, putrescine, L-lysine, 1,3-diaminopropane, spermidine, L-ornithine, *n*-butylamine, and 3-dimethylaminopropylamine.

### Liquid chromatography/mass spectrometry (LC/MS) analysis

Standard assays (400  $\mu$ L) consisting of 100 mM phosphate buffer pH 8, 10 mM substrate, SsDesB (1.0  $\mu$ M, without an *N*-terminal hexahistidine tag) and 0.7 mM NADPH were incubated at 25°C. Assays without substrate were also prepared as a negative control. Aliquots (50  $\mu$ L) were removed after 10 min, quenched with twice the volume of HPLC-grade acetonitrile (ThermoFisher; Waltham, MA), and chilled at -20°C for 10 min. After centrifugation at 12,500 rpm for 3 min at room temperature, the supernatant was incubated with 50  $\mu$ L of 100 mM borate buffer pH 8 followed by the addition of 20  $\mu$ L of 10 mM Fmoc-Cl dissolved in LC/MS grade methanol. After 5 min, samples were incubated with 20  $\mu$ L of 0.1 M 1-adamantylamine in 1:1 acetonitrile: water for an additional 10 min to remove excess Fmoc-Cl. Samples were then analyzed by LC/MS. Using a flow rate of 0.6 mL/min, samples (1  $\mu$ L) were injected onto an Acquity UPLC BEH C18 column (2.1  $\times$  50 mm  $\times$  1.7  $\mu$ m) (Waters; Milford, MA) with an Acquity precolumn filter (2.1 mm  $\times$  0.2  $\mu$ m) attached. The column was warmed to 40°C and UPLC separation was achieved using a 5–100% gradient of acetonitrile: water with 0.1% formic acid over 9 min. ESI-MS in sensitivity mode was used to obtain exact mass measurements of Fmoc-derivatized substrates and products. The following ESI settings were used: 3 kV capillary voltage, 45 V sample cone voltage, 120°C source temperature, 550°C desolvation temperature, 50 L/hr cone gas flow, and 800 L/hr desolvation gas flow. A leucine enkephalin reference (Waters; Milford, MA) was used for the lock spray with a 30 s reference scan frequency, 45 V reference cone voltage, 6 eV collision energy, and a 99.9 dynamic range enhancement setting.

### Kinetic data analysis

Kinetic data were analyzed in Kaleidagraph (Synergy; Reading, PA) and Prism 8 (GraphPad; San Diego, CA). The  $k_{\text{cat}}$  and  $K_{\text{M}}$  of SsDesB with specific substrates were determined by fitting initial rate data to the Michaelis-Menten Eq (2). The substrate inhibition constant ( $K_i$ ) was determined for cadaverine and NAD(P)H (with the exception of oxygen consumption initial rate data with varied NADPH) by fitting the initial rate data to the Haldane Eq (3). All reaction rates were measured such that no more than 10% product formed in enzyme assays. See the S1

[File](#) for fitted data.

$$v = \frac{k_{cat}[S]}{K_M + [S]} \quad (2)$$

$$v = \frac{k_{cat}[S]}{K_M + [S] + \frac{[S]^2}{K_i}} \quad (3)$$

## Crystallization

Purified SsDesB (12 mg/mL in 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 25 mM imidazole) was screened for crystals using several sparse-matrix crystallization screens from Hampton Research, Microlytic, Qiagen, and Molecular Dimensions using a Gryphon crystallization robot (Art Robbins Instruments; Sunnyvale, CA). Optimization screens of the initial crystallization screening hits were carried out using the hanging-drop vapor diffusion method in Easy-Xtal 15-well plates (Qiagen; Germantown, MD). Additional optimization screens were completed using the Hampton Research Additive screen. Crystals of SsDesB for data collection were obtained by mixing 2.5  $\mu$ L of protein solution (12.1 mg/mL) with 2  $\mu$ L well solution (0.1 M Hepes pH 7.5, 0.2 M sodium chloride, 25% w/v polyethylene glycol 3350), and 0.5  $\mu$ L of 0.1 M taurine and sealing over 500  $\mu$ L of well solution. The trays were incubated at 4°C. Yellow, plate-like crystals appeared within 1 week. A single crystal was retrieved from a drop using a Litholoop (Molecular Dimensions; Maumee, OH) and transferred to a 1  $\mu$ L drop of Paratone-N where excess mother liquor was whisked away from the crystal. The crystal was removed with a Litholoop and flash-cooled by plunging it into liquid N<sub>2</sub>.

Crystals of SsDesB in complex with NADP<sup>+</sup> were obtained by incubating 12.1 mg/mL of protein with 1 mM NADP<sup>+</sup> (Sigma-Aldrich, St. Louis, MO) for 2 hrs at 4°C. Precipitated material was removed by centrifugation for 10 min and 2.5  $\mu$ L of the SsDesB:NADP<sup>+</sup> mixture was mixed with 2.0  $\mu$ L of well solution (0.1 M Hepes pH 7.5, 0.2 M sodium chloride, 25% w/v polyethylene glycol 3350) and 0.5  $\mu$ L (0.1 M sarcosine) and sealed over 500  $\mu$ L of well solution. After 1 hr, the drops were streak-seeded with a whisker using previous crystals of SsDesB as a seed source. Yellow, plate-like crystals appeared within 2 days. A single crystal for data collection was retrieved using a Litholoop, transferred to a 1  $\mu$ L drop of Paratone-N, where excess mother-liquor was whisked away from the crystal, and the crystal was flash-cooled by plunging into liquid N<sub>2</sub>.

## Data collection

X-ray diffraction data for crystals of SsDesB and SsDesB complexed with NADP<sup>+</sup> were collected on the 22-ID and 22-BM beamlines, respectively, of the SER-CAT facilities at the Advanced Photon Source, Argonne National Laboratory, Argonne, Illinois. For the SsDesB crystal, 360 images were collected using a wavelength of 1.0000 Å, an oscillation angle of 1.0°, a crystal to detector distance of 300 mm, and an exposure time of 0.25 s. Data were collected from a single crystal of SsDesB complexed with NADP<sup>+</sup> using a wavelength of 1.0000 Å, a crystal-to-detector distance of 300 mm, an oscillation angle of 1.0°, and an exposure time of 20 s. A total of 180 frames of data were collected. All X-ray diffraction images were processed with *HKL-3000* [29].

The 2.37 Å structure of SsDesB in complex with NADP<sup>+</sup> was solved by molecular replacement with the program *PHASER* [30] in the *PHENIX* [31] suite and using the structure of *E. amylovora* DfoA [2] (PDB entry 5o8r, chain A, 53% sequence identity) as a search model after removing all solvent and ligand molecules. Based on the Matthews coefficient of 2.36 Å<sup>3</sup> Da<sup>-1</sup>

and solvent content of 47.9%, a search for 8 molecules in the asymmetric unit was performed [32–34]. Iterative rounds of model-rebuilding were performed manually using *Coot* [35] followed by additional automated model adjustment using the PDBredo server [36]. The PDB coordinate files for the FAD and NADP<sup>+</sup> ligands were prepared using *Molinspiration* (<https://molinspiration.com>), and the ligand restraint files used during refinements were prepared with the program *eLBOW* [37] in *PHENIX*. Refinements were performed with *phenix.refine* [38]. Water molecules were automatically located with *Coot*, manually inspected, and refined with *phenix.refine*.

The structure of SsDesB at 2.86 Å resolution was solved by molecular replacement with *Phaser* using chain A of the SsDesB-NADP<sup>+</sup> complex structure after removing all solvent and ligand molecules and searching for 8 molecules in the asymmetric unit (2.30 Å<sup>3</sup> Da<sup>-1</sup> and solvent content of 46.5%). Iterative rounds of manual model rebuilding were performed using *Coot* followed by refinement with *phenix.refine*. All structures were validated using the validation tools available in *Coot* and also with the *Molprobtity* server [39]. Refinement statistics and model validation are outlined in Table 4. The coordinates and structure factor files were deposited in the Protein Data Bank under accession codes 6XBB and 6XBC.

## Results

### Protein purification and characterization of FAD cofactor

The purification yielded 30–40 mg of a 51.7 kDa N-terminal hexahistidine tagged protein from 1 L of culture (S4A Fig in S1 File). The UV-visible absorbance spectrum of SsDesB indicated the presence of the FAD cofactor bound to the purified enzyme with characteristic absorbance maxima at 370 nm and 457 nm (S4B Fig in S1 File). The extinction coefficient at 457 nm was 14,478 M<sup>-1</sup>cm<sup>-1</sup> and ~15% of flavin was estimated to copurify with SsDesB.

### NAD(P)H oxidation and coenzyme specificity

Kinetic parameters of SsDesB NAD(P)H oxidation activity was determined by monitoring the decrease in the absorbance of NAD(P)H at 340 nm (Table 1). At a constant concentration of cadaverine (10 mM), SsDesB oxidized NADPH with an apparent  $k_{\text{cat}}$  of  $0.28 \pm 0.01 \text{ s}^{-1}$  and  $k_{\text{cat}}/K_{\text{M}}$  of  $43 \pm 11 \text{ mM}^{-1}\text{s}^{-1}$ . NADH was oxidized with an apparent  $k_{\text{cat}}$  of  $0.38 \pm 0.09 \text{ s}^{-1}$  and  $k_{\text{cat}}/K_{\text{M}}$  of  $3.3 \pm 1.3 \text{ mM}^{-1}\text{s}^{-1}$ , indicating a preference for oxidizing NADPH. There was also evidence of substrate inhibition when assayed with either cadaverine or NAD(P)H at concentrations above 0.7 mM.

### Oxygen consumption assays varying NADPH, cadaverine, and putrescine

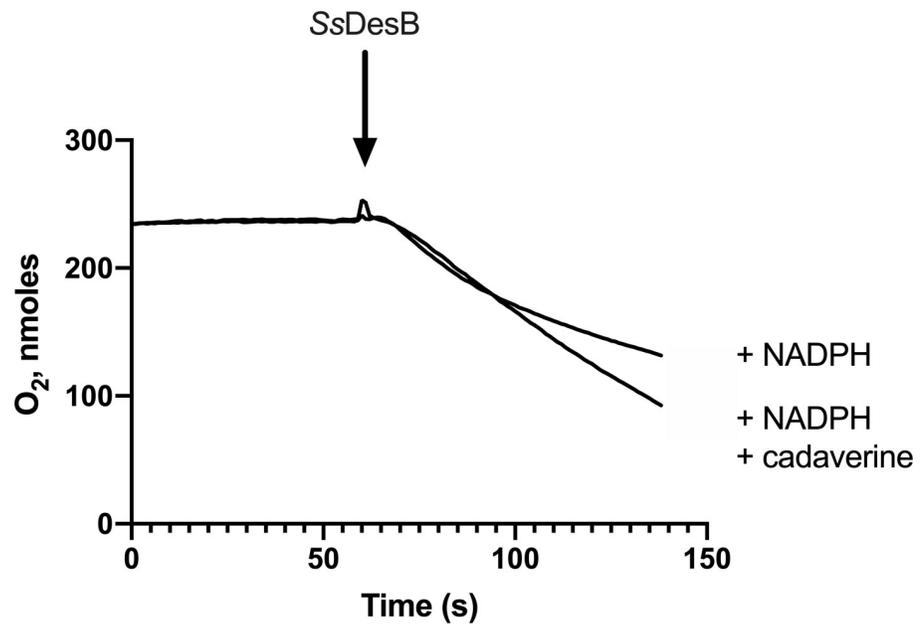
The activity of SsDesB was assessed by monitoring its rate of oxygen consumption with NAD(P)H, FAD, and various substrates. Substrate-independent oxidation was observed as SsDesB

**Table 1. Steady-state kinetic parameters determined in NAD(P)H oxidation assays for different substrates.**

Substrate	$k_{\text{cat}}, \text{s}^{-1}$	$K_{\text{M}}, \mu\text{M}$	$k_{\text{cat}}/K_{\text{M}}, \text{mM}^{-1} \text{s}^{-1}$	$K_{\text{I}}, \text{mM}$
Cadaverine (0.7 mM NADH)	$0.38 \pm 0.07$	$79 \pm 23$	$4.8 \pm 1.6$	$2.2 \pm 1.8$
NADPH (10 mM Cadaverine)	$0.28 \pm 0.01$	$6.4 \pm 1.6$	$43 \pm 11$	
NADH (10 mM Cadaverine)	$0.38 \pm 0.09$	$120 \pm 36$	$3.3 \pm 1.3$	$2.9 \pm 3.4$
Putrescine (0.7 mM NADH)	$0.28 \pm 0.02$	$140 \pm 23$	$2.1 \pm 0.3$	

Initial rates measured with varied concentrations of cadaverine or putrescine (or 10 mM) in NAD(P)H oxidation assays in the presence of SsDesB, 50 μM FAD, and 0.7 mM NAD(P)H (or varied concentrations). All measurements were made in triplicate.

<https://doi.org/10.1371/journal.pone.0248385.t001>



**Fig 2. Oxygen consumption assays.** Oxygraph traces demonstrate high oxidase activity in the absence of substrate. Assays contained 100 mM sodium phosphate buffer, pH 8 with 0.7 mM NADPH and 0.05 mM FAD in the absence or presence of 10 mM cadaverine.

<https://doi.org/10.1371/journal.pone.0248385.g002>

consumed slightly more oxygen (1.5-fold more) with cadaverine (Fig 2). Similar kinetic parameters were determined using the oxygen consumption assay compared to those obtained from NAD(P)H oxidation assays (Table 2). At a constant cadaverine concentration of 10 mM and varying NADPH concentrations, SsDesB consumed molecular oxygen with an apparent  $k_{cat}$  of  $0.24 \pm 0.01 \text{ s}^{-1}$  and  $k_{cat}/K_m$  of  $2.9 \pm 0.4 \text{ mM}^{-1}\text{s}^{-1}$ . These values are somewhat consistent with those determined in NADPH oxidation assays. When the concentration of NADPH was held constant and cadaverine concentrations were varied, SsDesB consumed molecular oxygen with an apparent  $k_{cat}$  of  $0.20 \pm 0.01 \text{ s}^{-1}$  and  $k_{cat}/K_m$  of  $4.3 \pm 0.1 \text{ mM}^{-1}\text{s}^{-1}$ . When putrescine concentrations were varied at constant NADPH concentrations, SsDesB consumed molecular oxygen with a similar apparent  $k_{cat}$  of  $0.29 \pm 0.01 \text{ s}^{-1}$  but lower catalytic efficiency of  $0.62 \pm 0.15 \text{ mM}^{-1}\text{s}^{-1}$ . QuantiChrome assays detected peroxide formation in only assays with enzyme (11  $\mu\text{M}$  in 30 s and 20  $\mu\text{M}$  in 72 s) and none in control assays without enzyme or with boiled enzyme (S5 Fig in S1 File). The presence of peroxide indicated that SsDesB does not use all of its reduced molecular oxygen to form an *N*-hydroxylated product.

### ***N*-hydroxylation activity varying NAD(P)H, cadaverine, and putrescine**

Using SsDesB without a hexahistidine tag at a constant concentration of NADPH, the enzyme *N*-hydroxylated cadaverine with an apparent  $k_{cat}$  of  $0.048 \pm 0.004 \text{ s}^{-1}$  and catalytic efficiency of

**Table 2. Steady-state kinetic parameters determined in oxygen consumption assays for different substrates.**

Substrate	$k_{cat}, \text{s}^{-1}$	$K_m, \mu\text{M}$	$k_{cat}/K_m, \text{mM}^{-1}\text{s}^{-1}$	$K_I, \text{mM}$
Cadaverine(0.7 mM NADPH)	$0.20 \pm 0.01$	$4.7 \pm 1$	$4.3 \pm 0.1$	$37 \pm 4$
NADPH(10 mM Cadaverine)	$0.24 \pm 0.01$	$83 \pm 10$	$2.9 \pm 0.4$	
Putrescine(0.7 mM NADPH)	$0.29 \pm 0.02$	$470 \pm 110$	$0.62 \pm 0.15$	

Initial rates measured with varied concentrations of cadaverine or putrescine (or 10 mM) in the presence of SsDesB, 50  $\mu\text{M}$  FAD, and 0.7 mM NADPH (or varied concentrations). All measurements were made in triplicate.

<https://doi.org/10.1371/journal.pone.0248385.t002>

**Table 3. Steady-state kinetic parameters determined in product formation assays with SsDesB and different substrates.**

Substrate	$k_{\text{cat}}, \text{s}^{-1}$	$K_{\text{m}}, \mu\text{M}$	$k_{\text{cat}}/K_{\text{m}}, \text{mM}^{-1} \text{s}^{-1}$	$K_{\text{I}}, \text{mM}$
Cadaverine	$0.048 \pm 0.004$	$19 \pm 9$	$2.5 \pm 1.2$	$27 \pm 22$
NADPH	$0.034 \pm 0.008$	$33 \pm 24$	$1.0 \pm 0.8$	$1.1 \pm 0.6$
Putrescine	$0.34 \pm 0.006$	$1100 \pm 78$	$0.31 \pm 0.23$	
NADH	$0.094 \pm 0.018$	$130 \pm 49$	$0.71 \pm 0.26$	$1.0 \pm 0.4$

Initial rates measured with varied concentrations of cadaverine or putrescine (or 10 mM) in the presence of SsDesB, 50  $\mu\text{M}$  FAD, and 0.7 mM NAD(P)H (or varied concentrations). All measurements were made in triplicate.

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$2.5 \pm 1.2 \text{ mM}^{-1} \text{ s}^{-1}$  (Table 3). However, the apparent  $k_{\text{cat}}$  value was approximately 6-fold lower than those determined in oxygen consumption and NADPH assays (Fig 3). *N*-hydroxylated product was confirmed by LC/MS in SsDesB assays derivatized with Fmoc-Cl and not in assays without enzyme (Fig 4). At a constant NADPH concentration, SsDesB also *N*-hydroxylated putrescine with a higher apparent  $k_{\text{cat}}$  of  $0.034 \pm 0.006 \text{ s}^{-1}$  but lower catalytic efficiency of  $0.31 \pm 0.23 \text{ mM}^{-1} \text{ s}^{-1}$ , further indicating a preference for *N*-hydroxylating cadaverine over putrescine. The apparent  $K_{\text{M}}$  of cadaverine (31  $\mu\text{M}$ ) with SsDesB was 35-fold lower than that of putrescine (1100  $\mu\text{M}$ ). When the NADH concentration was varied, the apparent  $k_{\text{cat}}$  value was ~2-fold higher than that when NADPH was varied, however the apparent  $K_{\text{M}}$  was ~4-fold larger, indicating a preference for binding NADPH over NADH. At high cadaverine and NAD(P)H concentrations, substrate inhibition was observed. To rule out product inhibition, we assayed SsDesB in the presence of increasing concentrations of *N*-hydroxycadaverine and there was no decrease in enzyme activity (S10 Fig in S1 File). Thus, the initial rate data were fit to Eq 3 when inhibition was observed. Products were confirmed via LC/MS analysis of SsDesB assays with cadaverine detected the generation of new compounds, such as *N*-hydroxycadaverine, after derivatization with Fmoc-Cl that were not present in control assays without enzyme (Fig 4).

### Substrate scope

SsDesB *N*-hydroxylated cadaverine, putrescine, spermidine, and L-lysine substrates (Fig 5). L-ornithine, 1,3-diaminopropane, and 3-dimethylaminopropylamine were not *N*-hydroxylated. The  $m/z$  values of these products are consistent with *N*-hydroxycadaverine ( $[\text{M}+\text{H}]^+$   $m/z$  calcd. for  $\text{C}_{35}\text{H}_{34}\text{N}_2\text{O}_5\text{H}$  563.25; found 562.2563), *N*-hydroxyputrescine ( $[\text{M}+\text{H}]^+$   $m/z$  calcd. for  $\text{C}_{34}\text{H}_{32}\text{N}_2\text{O}_5\text{H}$ : 549.23; found 549.2389), *N*-hydroxylysine ( $[\text{M}+\text{Na}]^+$  calcd. for  $\text{C}_{36}\text{H}_{34}\text{N}_2\text{O}_7\text{Na}$ : 629.23; found 629.2264), and *N*-hydroxyspermidine ( $[\text{M}+\text{H}]^+$   $m/z$  calcd. for  $\text{C}_{37}\text{H}_{39}\text{N}_3\text{O}_5\text{H}$ : 606.29; found 606.2968). See SI for extracted ion chromatograms and high resolution mass spectral data (S11–S15 Figs in S1 File). With the exception of assays with spermidine that yielded two stereoisomers of *N*-hydroxylated products, four stereoisomers of products were detected in all assays (S10–S13 and S15 Figs in S1 File). Assays were also performed with SsDesB containing an *N*-terminal hexahistidine tag was assayed, and no *N*-hydroxylated products were detected in product formation assays containing SsDesB with L-lysine. A slight difference in activity was only observed with L-lysine when it was assayed with the hexahistidine tagged SsDesB, suggesting that the affinity tag interferes with the enzyme *N*-hydroxylating L-lysine.

### Structural analysis of SsDesB

Crystal structures of SsDesB were obtained for the FAD-bound holoenzyme and the ternary complex with bound FAD and  $\text{NADP}^+$  at 2.86 Å and 2.37 Å, respectively. Data collection and

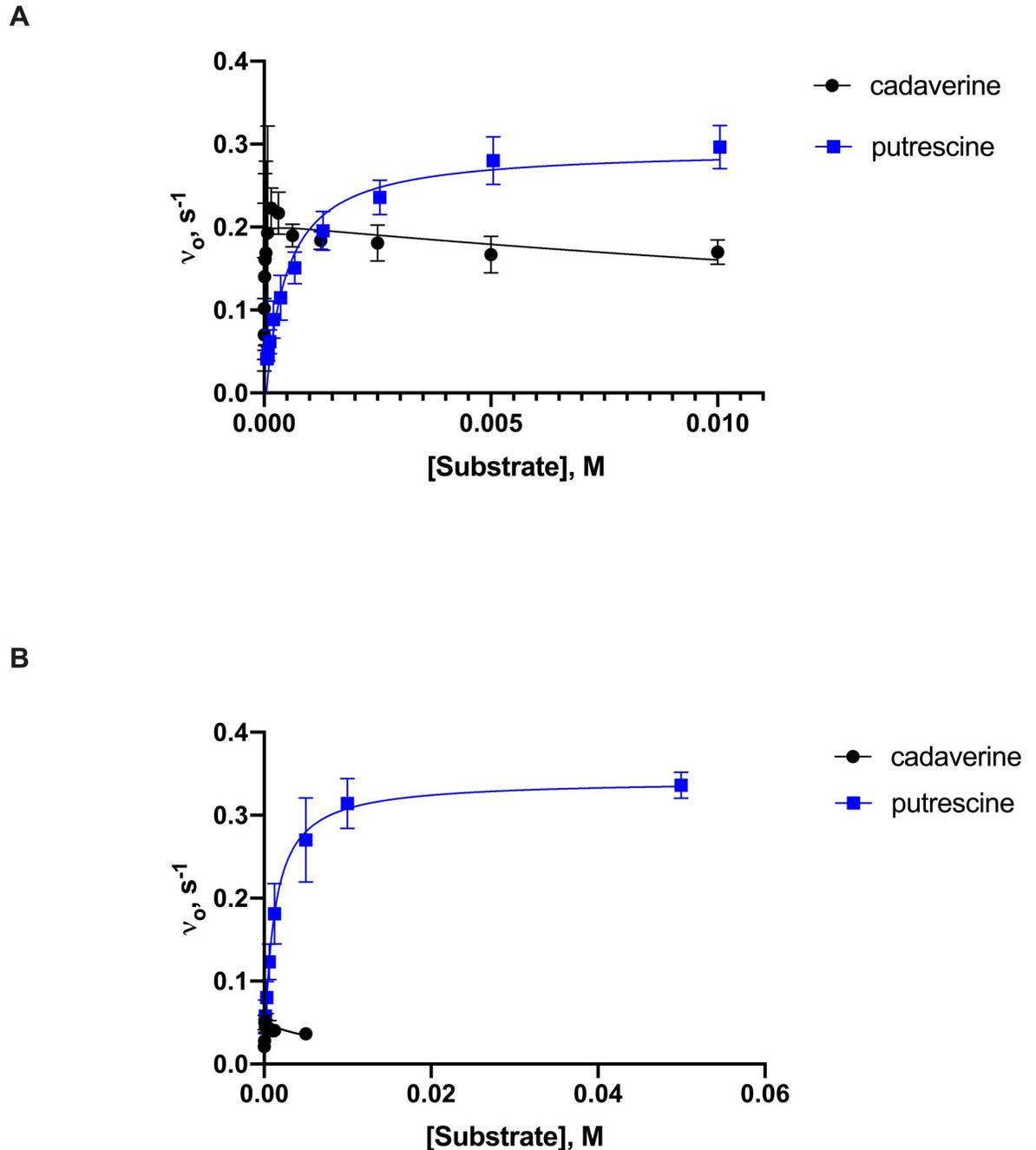
**Table 4. Crystallographic data collection and refinement statistics.**

<b>Data collection statistics:</b>		
	<b>SsDesB-FAD</b>	<b>SsDesB-FAD-NADP<sup>+</sup></b>
Program used:	<i>HKL3000</i>	<i>HKL3000</i>
Beamline	APS, SER-CAT, 22-ID	APS, SER-CAT, 22-BM
Wavelength (Å)	1.0000	1.0000
Space group	<i>P2<sub>1</sub></i>	<i>P2<sub>1</sub></i>
<i>a, b, c</i> (Å)	83.05, 151.17, 141.40	84.13, 153.43, 141.31
$\alpha, \beta, \gamma$ (°)	90, 91.58, 90	90, 92.44, 90
Resolution range*	50–2.86 (2.90–2.86)	50.0–2.37 (2.40–2.37)
Number of unique reflections measured	78957 (3917)	139487 (6942)
Completeness (%)	98.7 (98.5)	96.3 (96.3)
Redundancy	3.2 (3.3)	3.7 (3.7)
Mean <i>I</i> / $\sigma$ ( <i>I</i> )	15.4 (2.2)	14.2 (2.0)
<i>R<sub>sym</sub></i>	0.066 (0.451)	0.089 (0.692)
<i>R<sub>merge</sub></i>	0.043 (0.355)	0.059 (0.580)
<i>R<sub>pim</sub></i>	0.042 (0.288)	0.053 (0.418)
CC ½	0.996 (0.860)	0.996 (0.729)
<b>Refinement statistics</b>		
Program used:	phenix.refine	phenix.refine
Resolution range (Å)	39.1–2.86	37.02–2.37
Number of reflections used in refinement	73546 (3617)	136497 (6786)
R/ <i>R</i> <sub>free</sub>	0.199/0.258	0.189/0.240
No. of atoms		
Protein, chain A,B,C,D,E,F,G,H	3340/3340/3326/3326/3340/3333/3553/3340	3353/3340/3340/3346/3346/3340/3340/3370
FAD, chain A,B,C,D,E,F,G,H	53, all chains	53, all chains
NADP <sup>+</sup>	-	48, all chains
Water	-	1201
Average B factor (Å <sup>2</sup> )		
Protein, chain A,B,C,D,E,F,G,H	43.5/51.2/52.3/55.4/41.0/49.3/47.4/42.1	30.9/32.8/34.4/39.2/33.7/39.8/37.0/38.3
FAD, chain A,B,C,D,E,F,G,H	41.2/50.6/57.5/62.2/38.2/56.0/49.4/38.9	29.2/30.6/33.9/33.1/29.4/38.3/33.2/35.4
NADP <sup>+</sup>	-	31.5/33.4/35.6/38.3/36.2/41.0/38.5/38.8
Water	-	40.9
Root-mean-square- deviation from ideal		
Bond length (Å)	0.003	0.002
Bond angle (°)	0.6	0.5
<b>Ramachandran plot</b>		
Favored (%)	94.4	95.7
Allowed (%)	5.1	4.1
Outliers (%)	0.5	0.2
<b>Molprobrity analysis</b>		
All atoms contact clash score	5.54 (100 <sup>th</sup> percentile)	3.1 (100 <sup>th</sup> percentile)
Molprobrity score	1.66 (100 <sup>th</sup> percentile)	1.4 (100 <sup>th</sup> percentile)
PDB accession code	6XBC	6XBB

\*Values in parenthesis are for the highest resolution shell

<https://doi.org/10.1371/journal.pone.0248385.t004>

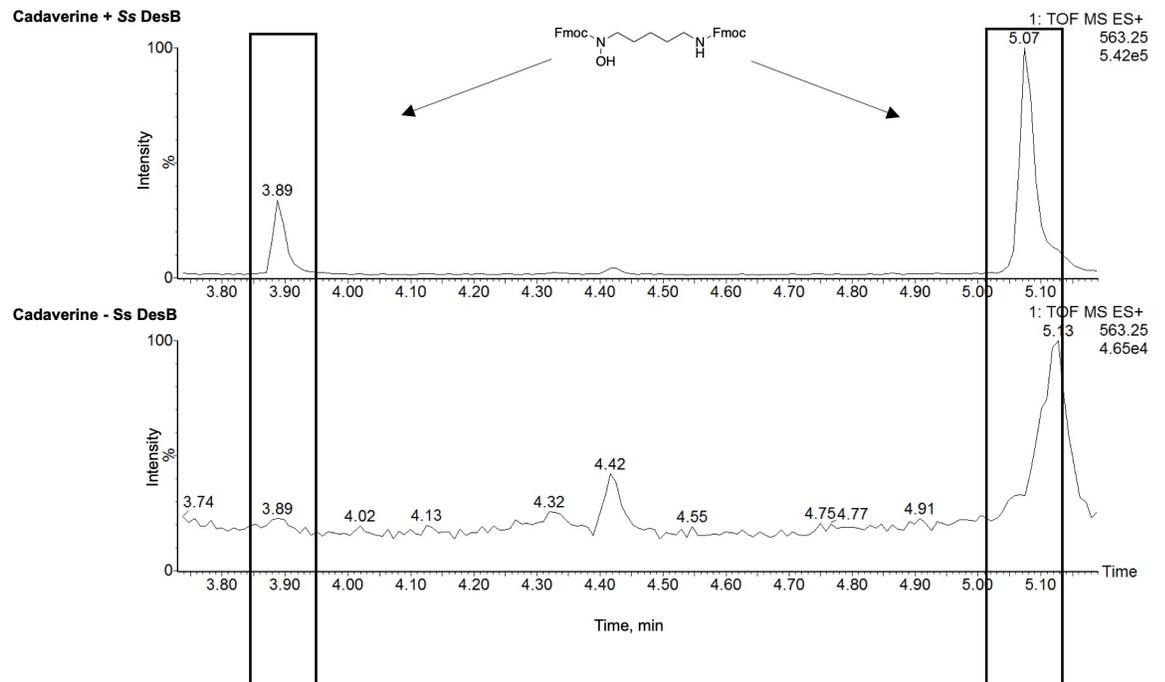
processing statistics are presented in Table 4. SsDesB crystallized as a homotetramer with each protomer consisting of two dinucleotide-binding Rossmann-fold domains [40]: an FAD-binding domain (red) and an NADPH-binding domain (blue) (Fig 6). In both structures, each



**Fig 3. Representative steady-state kinetics of SsDesB in oxygen consumption and product formation assays.** A. Initial rate kinetic data obtained with cadaverine and putrescine in oxygen consumption assays. Kinetic data for putrescine assayed with SsDesB, 0.05 mM FAD, and 0.7 mM NADPH. These data were obtained in triplicate and fit to the Haldane substrate inhibition (cadaverine) and Michaelis-Menten equations (putrescine), respectively. B. Initial rate data for cadaverine and putrescine assayed with SsDesB, 0.05 mM FAD, and 0.7 mM NADH in product formation assays. These data were obtained in triplicate and fit to the Haldane substrate inhibition (cadaverine) and Michaelis-Menten equations (putrescine), respectively.

<https://doi.org/10.1371/journal.pone.0248385.g003>

molecule in the asymmetric unit contained bound FAD and in the SsDesB-FAD-NADP<sup>+</sup> complex, a bound NADP<sup>+</sup> molecule was clearly resolved in each protomer. Based on a query of the Protein Data Bank (PDB) using PDBefold [41, 42], the overall structure of the SsDesB resembles those of previously determined crystal structures of the flavin-dependent monooxygenases



**Fig 4. LC/MS detection of *N*-hydroxycadaverine ( $m/z$  563.25).** LC/MS select ion chromatograms of Fmoc-derivatized *N*-hydroxycadaverine in assays with and without SsDesB. Diastereomers are observed.

<https://doi.org/10.1371/journal.pone.0248385.g004>

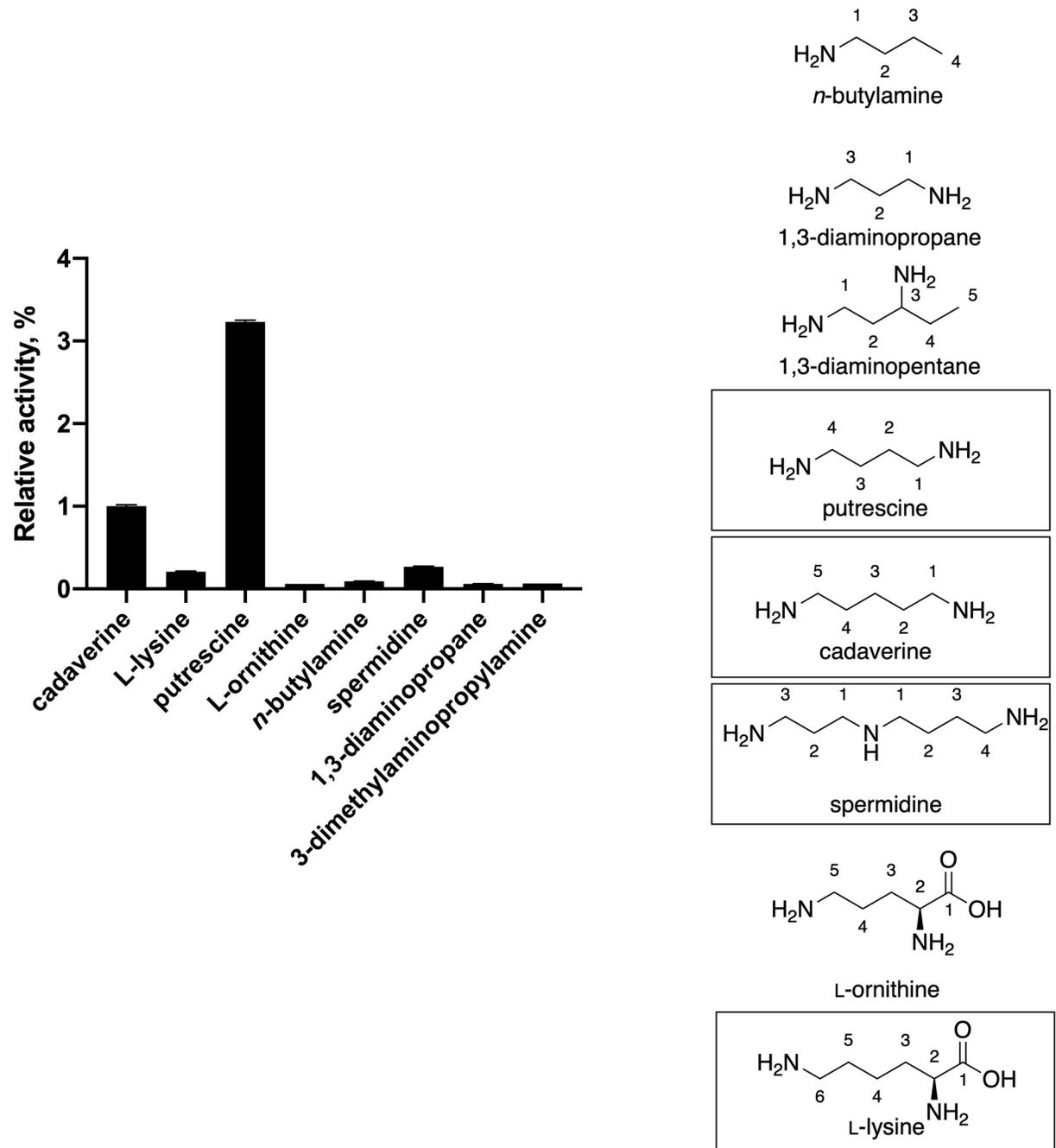
*E. amylovora* DfoA (PDB entry 5O8P, r.m.s.d. 0.85 Å over 407 aligned residues) [43], the ornithine hydroxylase KtzI from *Kutzneria* sp. 744 (PDB entry 4TM1, r.m.s.d. 1.74 Å over 364 aligned residues) [5], the *P. aeruginosa* ornithine hydroxylase PvdA (PDB entry 3S5W, r.m.s.d. 1.96 over 361 aligned residues) [44], and the *A. fumigatus* ornithine hydroxylase, SidA, (PDB entry 5CKU, r.m.s.d. 2.08 Å over 362 aligned residues) (Fig 7 and S1 Table in S1 File).

### FAD-bound enzyme

In the SsDesB-FAD complex structure, the FAD molecule is bound in an elongated conformation with the fully planar isoalloxazine ring positioned at the interface of the two dinucleotide-binding domains and a large area of the molecule, particularly the adenine dinucleotide portion, is exposed to solvent. During purification, after passage through the gel filtration column, fractions containing SsDesB lost the deep yellow color, suggesting that FAD was lost during the process. For our crystallization efforts, we used concentrated protein eluted from an IMAC column in which the aliquots retained a deep yellow color. However, similar crystallization hits could also be obtained after adding exogenous FAD to the protein aliquots after passage through a size exclusion column and then concentrating the protein. Loss of FAD during purification was also reported with other homologs [3]. In the holoenzyme, FAD is held in the active site pocket primarily by hydrogen bonding interactions involving S43, E42, K44, H50, P390, and W49 along the flavin adenine nucleotide backbone while residues Q61 and L392 contribute direct hydrogen bonds to the planar isoalloxazine ring primarily via the backbone amide nitrogen atoms (Fig 8).

### FAD and NADP<sup>+</sup>-bound enzyme

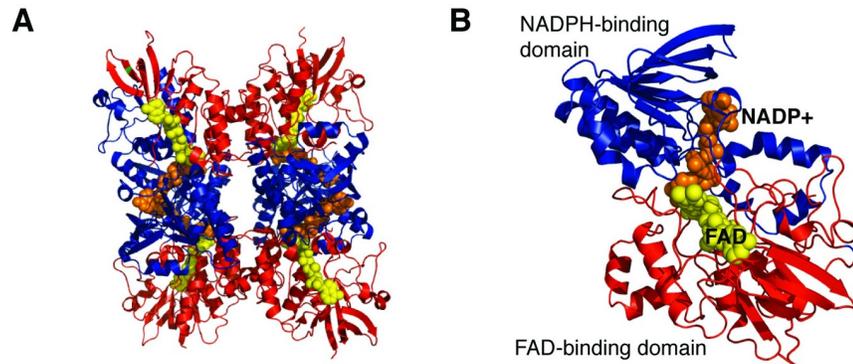
There are no significant global structural changes observed upon binding of the NADP<sup>+</sup> molecule (r.m.s.d. = 0.26 over 2741 aligned atoms). The higher resolution 2.37 Å structure of the



**Fig 5. Relative SsDesB activity with different substrates.** Initial rates measured with 10 mM L-lysine, cadaverine, spermidine, putrescine, *n*-butylamine, L-ornithine, 1,3-diaminopropane, and 3-dimethylaminopropylamine in product formation assays in the presence of SsDesB, 50  $\mu$ M FAD, and 0.7 mM NADPH. All initial rates are relative to that of cadaverine. *n*-Butylamine, L-ornithine, 1,3-diaminopropane, and 3-dimethylaminopropylamine did not yield any Fmoc-derivatized *N*-hydroxylated products by LC/MS. All assays were performed in triplicate and molecular structures are shown with numbered carbon chains.

<https://doi.org/10.1371/journal.pone.0248385.g005>

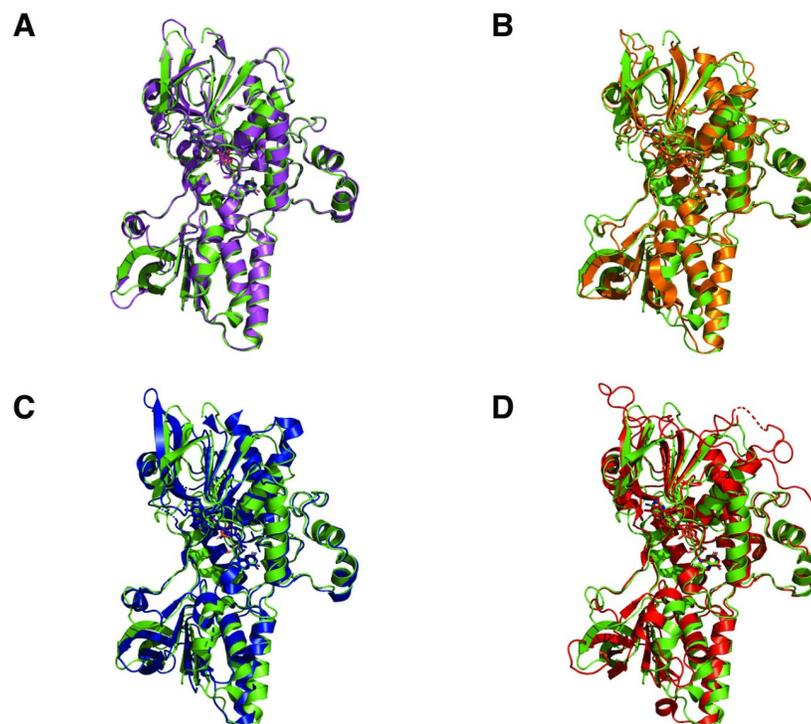
ternary complex shows that the  $\text{NADP}^+$  cofactor is held in the active site pocket in an elongated manner via several direct hydrogen bonds to SsDesB and a network of water-mediated hydrogen bonding bridges between SsDesB residues and  $\text{NADP}^+$  (Fig 9). The electron density is well-defined for both the FAD and  $\text{NADP}^+$  cofactors, including the nicotinamide ring, in all



**Fig 6. Overall structure of tetrameric SsDesB in complex with NADP<sup>+</sup>.** NAD<sup>+</sup> and FAD-bound SsDesB (PDB code: 6XBB). A) Tetrameric structure of SsDesB complexed with NADP<sup>+</sup> illustrating the FAD-binding domain (red-ribbons) and the NADPH-binding domain (blue-ribbons). The bound NADP<sup>+</sup> molecule is shown in orange spheres and the FAD molecule in yellow spheres. B) A view of the SsDesB protomer.

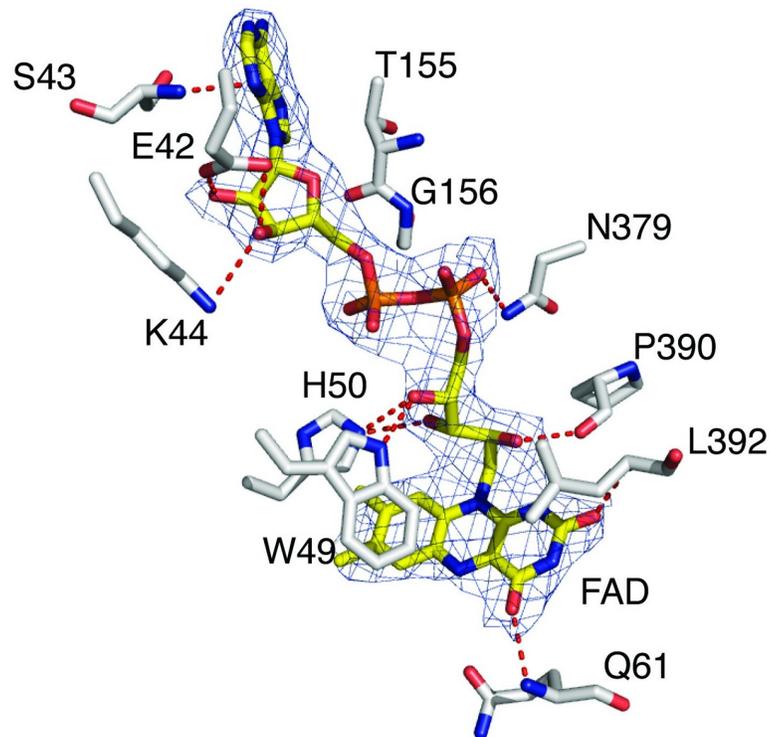
<https://doi.org/10.1371/journal.pone.0248385.g006>

8 protomers in the asymmetric unit. The nicotinamide ring is held in position by hydrogen bonding interactions between the nicotinamide N7N atom and main chain carbonyl oxygen atom of H59 and the N5 atom of the FAD isoalloxazine ring. The nicotinamide O7N atom participates in water-mediated hydrogen bonding interactions via water 679 to the E202 side chain and H50 main chain carbonyl oxygen atom. The side chain of Q61 also contributes to stabilizing interactions via a hydrogen bond between the side chain OE1 oxygen atom and the



**Fig 7. Structural comparison of SsDesB and homologs.** Superimposed crystal structures of chain A of SsDesB (PDB code: 6XBC) with A) *E. amylovora* DfoA (PDB code: 5O8P), B) *Kutzneria* sp. 744 ornithine hydroxylase, Ktzl, (PDB code: 4TM1), C) *P. aeruginosa* ornithine hydroxylase, PvdA, (PDB code: 3S5W), and D) *A. fumigatus* ornithine hydroxylase, SidA, (PDB code: 5CKU).

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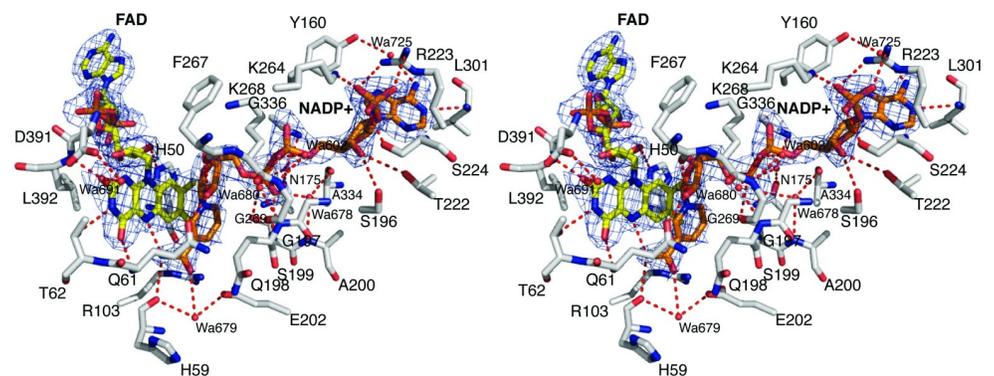


**Fig 8. Active site environment of SsDesB holoenzyme.** View of the active site residues (carbon atoms in gray, nitrogen atoms in blue, and oxygen atoms in red) mediating FAD (carbon atoms in yellow, and phosphate atoms in orange) binding in the FAD-bound SsDesB structure (PDB code: 6XBC). The fit of the FAD molecule to the final  $2F_o - F_c$  electron density map (blue mesh, 2.86 Å resolution, contoured at  $1\sigma$  level) is shown.

<https://doi.org/10.1371/journal.pone.0248385.g008>

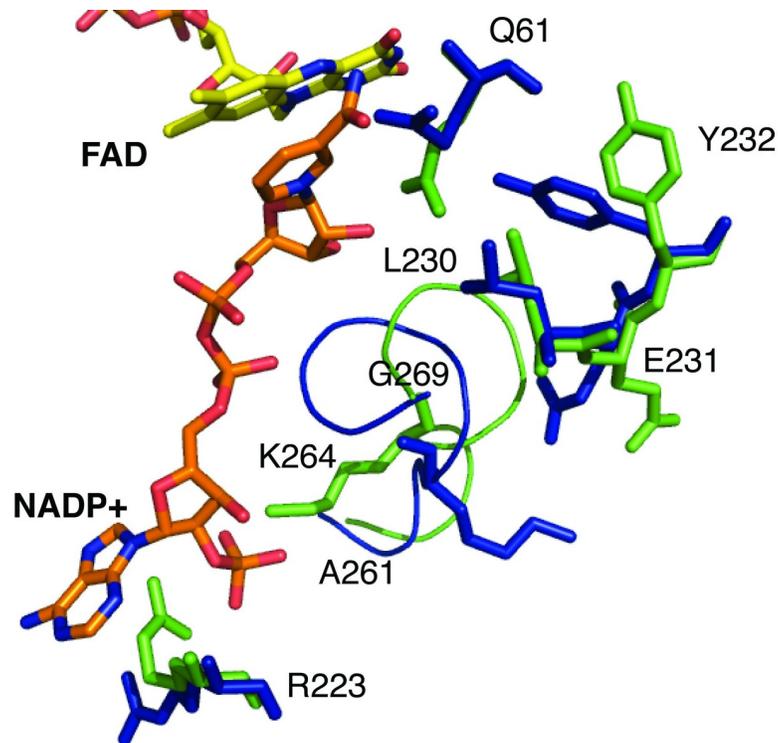
O2D ribose oxygen atom. The phosphate moiety of NADP<sup>+</sup> is held in place by a salt bridge between the O2X oxygen and the side chain of K264, and the R232 side chain to the O2X and O1X oxygen atoms. In addition, there is a hydrogen bond between the O3X oxygen and the side chain of S224.

In comparison to the holoenzyme structure, upon NADP<sup>+</sup> binding, several structural adjustments are observed among the residues surrounding the NADP<sup>+</sup> binding pocket. At the



**Fig 9. Active site environment of SsDesB-NADP<sup>+</sup> bound structure.** Stereo-view of the active site of the SsDesB-NADP<sup>+</sup> bound structure (PDB code: 6XBB). The fit of the FAD molecule (carbon atoms in yellow, nitrogen atoms in blue, oxygen atoms in red, and phosphate atoms in orange) and the NADP<sup>+</sup> molecule (carbon atoms in orange) to the final  $2F_o - F_c$  electron density map is shown (blue mesh, 2.37 Å resolution, contoured at  $1\sigma$  level).

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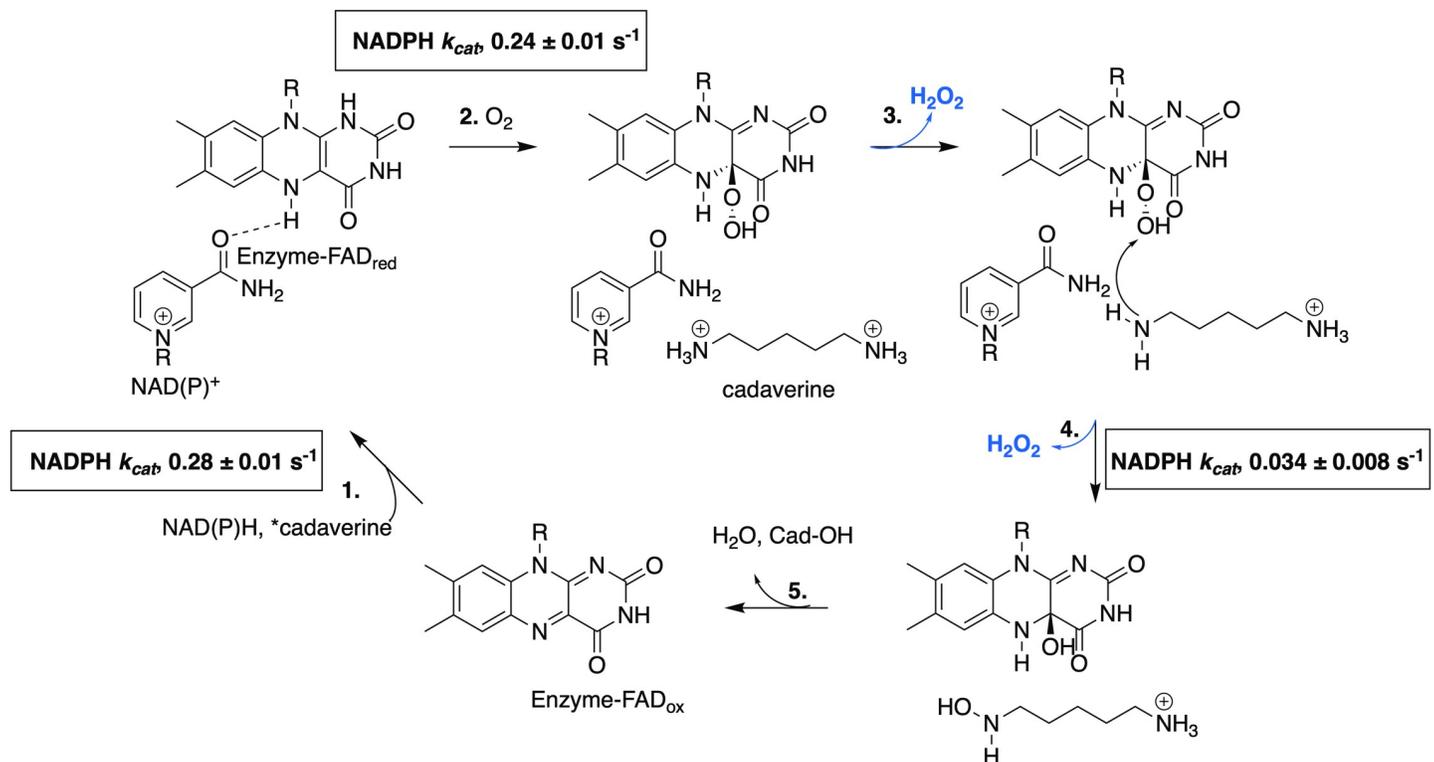
**Fig 10. Structural changes upon NADP<sup>+</sup> binding.** View of the superimposed active sites of the SsDesB holoenzyme (blue; PDB code: 6XBC) and SsDesB-NADP<sup>+</sup> bound (green; PDB code: 6XBB) structures highlighting the positional shift of residues in the active site upon NADP<sup>+</sup> binding.

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interface of the FAD isoalloxazine ring and NADP<sup>+</sup> nicotinamide ring, the side chain of Q61 shifts away from the FAD isoalloxazine to accommodate the binding of the nicotinamide ring of NADP<sup>+</sup>, which in turn, induces a shift in the rotamer positions of the nearby L230, E231, and Y232 side chains (Fig 10). Additionally, the D391 side chain is now engaged in a water-mediated hydrogen bond bridge between the side chain OD2 oxygen atom and the isoalloxazine ring O2 atom via water 691 (Fig 9). The side chain of T62 is also hydrogen bonded to the isoalloxazine ring N3 nitrogen atom (Fig 9). A shift in the conformation of the loop consisting of residues 261–269 is observed which avails space for NADP<sup>+</sup> binding and also properly positions the side chain of K264 to engage in an ionic interaction with the phosphate moiety of NADP<sup>+</sup>. The side orientation of R223 also shifts and is now properly positioned for an ionic interaction with the NADP<sup>+</sup> phosphate and is also stacked against the terminal adenine moiety.

## Discussion

Alkyl diamine *N*-hydroxylases are class B flavin monooxygenases that catalyze the *N*-hydroxylation of diamines using NADH or NADPH as their redox partners. These NMOs play an essential role in committing diamines to the biosynthesis of hydroxamate siderophores, which are used to sequester iron for microorganisms, including several human pathogens. Furthermore, they are used in the industrial fermentation of pharmaceuticals, such as desferrioxamine B, and are currently being explored as biocatalysts, as they accept complex substrates and introduce the unique N-O functionality in a single step, which can be further derivatized [45]. However, they are not as well-studied as the L-lysine and L-ornithine NMOs, particularly



**Fig 11. General mechanism for Class B flavin-dependent monooxygenases.** The NADPH oxidation, oxygen consumption, and *N*-hydroxylation steps are represented by steps 1, 2, and 4, respectively. The loss of peroxide is indicated in blue and the apparent  $k_{cat}$  values for steady-state kinetic assays with varied NADPH concentrations are shown. \*, the substrate is either added before or after the reduction of molecular oxygen.

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those involved in the biosynthesis of nocobactin in *N. farcinica* [3] and ferrichrome in *A. fumigatus* [4], respectively. Here we report the structural and kinetic characterization of SsDesB, a cadaverine *N*-hydroxylase from *S. sviveus*.

The general mechanism proposed for class B flavin-dependent monooxygenases (Fig 11) commences with an oxidized FAD being reduced by the C4-pro-R position of NAD(P)H nicotinamide to the N5 position of the flavin isoalloxazine ring to produce FADH<sup>-</sup> (FAD<sub>red</sub>). Then molecular oxygen adds to FAD<sub>red</sub> to form an activated C4a-(hydro)peroxyflavin intermediate that undergoes nucleophilic attack by the primary amine of the bound substrate, which could bind before or after the reduction of molecular oxygen. NADP<sup>+</sup> typically remains bound to prevent the quenching of the C4a-(hydro)peroxyflavin species [46, 47]. Once the hydroxylated product is formed, the oxidized FAD is regenerated by the loss of water and the products dissociate, completing the catalytic cycle. Based on the kinetic analysis of the NADPH oxidation, O<sub>2</sub> consumption, and *N*-hydroxylation steps, SsDesB appears to have a mechanism consistent with the proposed mechanism for a Class B flavin-dependent monooxygenase. However, Fig 2 shows that SsDesB consumes oxygen when incubated with NADPH in the absence of substrate, suggesting that the substrate may not be required to be bound to SsDesB for FAD to be reduced (step 1 in Fig 11), which is not uncommon for *N*-hydroxylases [46, 48].

While the apparent  $k_{cat}$  values for the initial NADPH oxidation and oxygen consumption steps for SsDesB were of the same order of magnitude, there was a ten-fold difference in the apparent  $k_{cat}$  values of those steps to that of the *N*-hydroxylation step. The significant decrease in the apparent  $k_{cat}$  among those three steps indicates the uncoupling of the reaction. The detection of peroxide in SsDesB assays with cadaverine further supports this notion, as not all

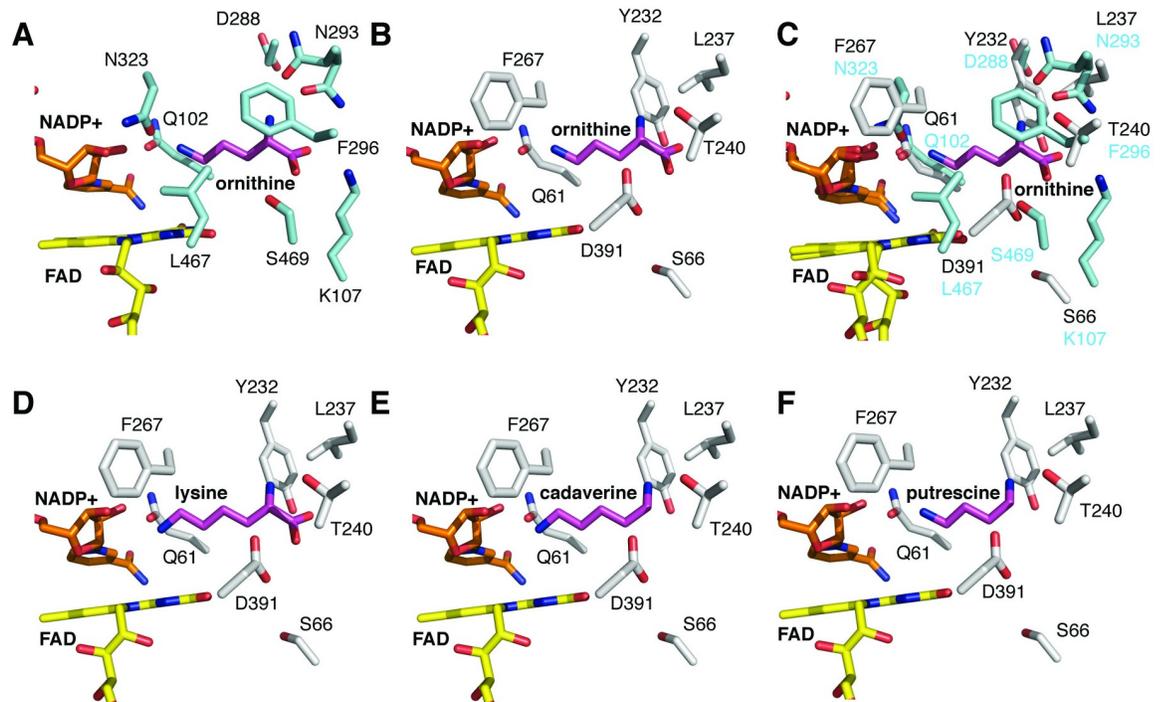
molecular oxygen is converted into *N*-hydroxylated product. Other *N*-hydroxylases have been reported to catalyze uncoupled reactions, such as NbtG (*Nocardia farcinica*) [49], MbsG (*Mycobacterium smegmatis*) [49], PvdA [48], and GorA [1].

Unlike many amino acid *N*-hydroxylases with  $K_M$  values ranging from 0.3 to 4 mM, the apparent  $K_M$  values for cadaverine, NADPH, and NADH were significantly lower, approaching the limit of detection of the multistep product formation assay. The apparent  $k_{cat}$  of SsDesB with cadaverine was at least 10-fold less than the published  $k_{cat}$  values of other amino acid *N*-hydroxylases [50]. SsDesB also exhibited higher apparent  $k_{cat}$  values with putrescine, which were the same order of magnitude in product formation and oxygen consumption assays, suggesting the putrescine substrate stabilizes the C4a-(hydro)peroxyflavin intermediate. However, cadaverine is the preferred substrate based on its higher apparent catalytic efficiency. We are aware that the apparent  $K_M$  values for cadaverine and putrescine in product formation assays were slightly higher than those determined in the other assays, which is likely due to greater error associated with the multistep product formation assay.

The difference in the magnitudes of the apparent  $K_M$  values for cadaverine and putrescine, which only differ by one methylene group, likely reflects the physiological relevance of these polyamines in *S. sviveus*. For example, in *S. putrefaciens*, desferrioxamine B is synthesized once putrescine has been depleted [51]. The more than 20-fold difference between the apparent  $K_M$  values of cadaverine and putrescine with SsDesB could represent a way to manage iron acquisition in *S. sviveus*. The *S. sviveus* genome contains genes to transport putrescine into the cell; thus, putrescine could be used as a SsDesB substrate to produce *N*-hydroxyputrescine and other putrescine-derived hydroxamate siderophores under certain growth conditions.

Based on the *N*-hydroxylation of cadaverine and putrescine, SsDesB has broader substrate and cofactor specificity than other class B flavin-dependent monooxygenases supporting our initial hypothesis. SsDesB exhibited similar activity to GorA [1] and PubA [26], accepting both NADPH and NADH cofactors as well as multiple diamine substrates. However, a higher apparent catalytic efficiency was determined with NADPH, indicating a preference for NADPH over NADH. A preference for NADPH was also reported for GorA, in which the relative amount of hydroxylated product made with NADPH was significantly higher than with NADH, which produced too little product for quantification in assays containing 150  $\mu$ M of either cofactor. Similar to GorA, SsDesB also exhibits higher activity with putrescine followed by cadaverine [1]. However, the authors did not report steady-state kinetic data over a wide enough range of cadaverine concentrations to confirm that putrescine is the preferred GorA substrate [1]. While SsDesB exhibits higher activity with putrescine at concentrations above its apparent  $K_M$ , cadaverine is the preferred substrate based on its low micromolar  $K_M$ , increasing its apparent catalytic efficiency.

Molecules containing alkyl chains with less than four methylene groups or branched alkyl amines were not *N*-hydroxylated by SsDesB. Spermidine and L-lysine were accepted to a lesser degree, respectively, indicating that SsDesB can tolerate longer alkyl diamines as well as a carboxyl group. LC/MS detected at least four stereoisomers of these *N*-hydroxylated products with the exception of spermidine, which produced two stereoisomers likely due to the *N*-hydroxylation of the longer alkyl chains, as the amines with 3-carbon alkyl chains were not *N*-hydroxylated. The spermidine substrate is more readily *N*-hydroxylated than L-lysine (Fig 5) and may form more stable hydrogen bonding interactions with its additional nitrogen atoms. Interestingly, assays with SsDesB containing an *N*-terminal hexahistidine tag did not *N*-hydroxylate lysine, whereas SsDesB did without a hexahistidine tag, suggesting that additional residues near the *N*-terminus prevent the *N*-hydroxylation of amino acid substrates (S16 Fig in S1 File).



**Fig 12. Structural analysis of substrate specificity.** A. Active site structure of SidA bound to NADP<sup>+</sup> and ornithine (PDB code: 4b63). B. Model of ornithine binding to the active site of SsDesB obtained by superimposing the coordinates of SsDesB (PDB code: 6XBB) onto the coordinates of SidA bound to ornithine (PDB code: 4b63). C. Comparison of the active site environment between SsDesB and SidA. D. Modeling of lysine binding to SsDesB. E. Modeling of cadaverine binding to SsDesB. F. Modeling of putrescine binding to SsDesB.

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The structural basis for SsDesB *N*-hydroxylation was explored by comparing the structure of SsDesB bound to NADP<sup>+</sup> with experimental crystal structures of the SidA homolog with bound substrate. Similar to other NMO structures [5], SsDesB is a homotetramer with its active site within a subunit at the interface of three domains, including Rossmann-type FAD and NAD(P)H binding domains (Fig 6). The SsDesB active site appears to be more solvent exposed, which may contribute to its uncoupled mechanism as a lower amount of FAD copurified with the enzyme compared to that of other *N*-hydroxylases (75% flavin in NbtG [3] and 50–60% flavin in SidA [4]) in which FAD is buried and inaccessible by solvent. While SsDesB accepts a broad range of substrates, such as cadaverine and putrescine, it does not *N*-hydroxylate L-ornithine. To gain structural insight into the substrate specificity of SsDesB, we superimposed the coordinates of the SsDesB-NADP<sup>+</sup> complex onto its SidA homolog, which accepts L-ornithine as a substrate, crystallized with NADP<sup>+</sup> and L-ornithine (PDB code: 4b63) [52] (Fig 12). Fig 12A shows that SidA binds L-ornithine through several hydrogen bonds involving N323, N293, S469, and K107 residues (these residues are conserved in the PvdA and KtzI homologs); however, SsDesB lacks the equivalent residues (Fig 12B and 12C). In particular, SsDesB lacks the equivalent K107 residue, which would hydrogen bond to the carboxylate moiety of L-ornithine and contribute a stabilizing positive charge, anchoring the substrate. Additionally, SsDesB contains a nonpolar L237 residue (N293 in SidA), eliminating the possibility of hydrogen bonding interactions at this position with the carboxylate oxygen and  $\delta$ -carbon primary amine of L-ornithine. In SidA, N323 hydrogen bonds with the  $\alpha$ -carbon primary amine; however, SsDesB contains a nonpolar F267 residue in the equivalent position (Fig 12C). Furthermore, a clashing negative charge provided by SsDesB D391 (L467 in SidA, L404 in KtzI and L408 in PvdA) would likely repel the negative charge of the L-ornithine

carboxylate group. These highlighted structural features provide insight into why SsDesB does not accept L-ornithine as a substrate.

Given that SsDesB accepts L-lysine, cadaverine, and putrescine as substrates, we modeled the position of these substrates into the SsDesB active site by superimposing the SsDesB coordinates onto available crystal structures from homologs with bound substrates (Fig 12). The position of L-lysine in the SsDesB active site was modeled by overlaying the SsDesB coordinates onto those of the SidA-NADP<sup>+</sup>-L-lysine complex structure (Fig 12D, PDB code: 4b64) and the position of L-ornithine was based on the superimposed coordinates with the SidA-NADP<sup>+</sup>-L-ornithine structure (Fig 12B, PDB code: 4b63), while the position of cadaverine (Fig 12E) and putrescine (Fig 12F) was modeled based on the removal of atoms from the L-lysine substrate in PDB entry 4b64 and L-ornithine substrate in PDB entry 4b63, respectively. While exact hydrogen bonding interactions are difficult to predict based on modeling alone, we can suggest that given proper positioning of cadaverine, the side chain of D391 may hydrogen bond with the  $\epsilon$ -carbon amine and Q61 is within hydrogen bonding distance to the  $\alpha$ -carbon amine (Fig 12). Similar interactions could possibly be observed with putrescine, which is one methylene shorter than cadaverine, given proper positioning of the substrate. However, given putrescine's lower apparent  $K_M$  with SsDesB, these interactions may be weaker due to the shorter chain length. Although we observed low activity with L-lysine, we could not fully rationalize the binding of L-lysine based on our modeling studies. If the side chain of D391 moved away from the carboxylate moiety of L-lysine, then the nearby T240 side chain could hydrogen bond with L-lysine. However, experimental X-ray crystal structures and additional site-directed mutagenesis studies are needed to fully characterize the structural basis for the binding of SsDesB substrates and identify key catalytic residues.

We also examined the X-ray crystal structure to understand the preference of SsDesB for NADPH over NADH. The ornithine N-hydroxylase from *P. aeruginosa*, PvdA [5, 48], only uses NADPH as the electron donor during the oxidation of substrates. In the 1.9 Å resolution crystal structure of PvdA with bound NADP<sup>+</sup> and ornithine (PDB code: 3S5w), there is no electron density available to sufficiently model the nicotinamide ring, suggesting that flexibility may be required for catalysis [5]. However, in SsDesB, the electron density for the nicotinamide ring of NADP<sup>+</sup> is fully resolved in all molecules in the asymmetric unit. The specificity of PvdA for NADPH is determined by two residues, R240 and S210 [5]. R240 forms two hydrogen bonds with the 2'-phosphate on the adenine ribose and the S286 residue also forms a hydrogen bond to the phosphate. In contrast, SsDesB has an R223 residue, which is equivalent to R240 in PvdA, but lacks the equivalent serine residue (S3 Fig in S1 File). Instead, a positively charged lysine, K268, is positioned away from the 2'-phosphate, which may reduce the specificity of SsDesB for NADPH (Fig 9).

## Conclusions

The present study is the first kinetic and structural characterization of SsDesB, providing insight into the biosynthesis of desferrioxamine and derivatives. The kinetic and structural data revealed the broader substrate scope of alkyl amine N-hydroxylases compared to the more selective ornithine and lysine N-hydroxylases. Furthermore, an uncoupled mechanism was observed when SsDesB was assayed with cadaverine and not with putrescine. Understanding the structural and catalytic basis for activity can lead to the development of biocatalysts and inhibitors that target homologs in human pathogens. Future studies include the kinetic and structural characterization of mutated SsDesB residues to identify key catalytic residues, understand why the enzyme-catalyzed reaction is uncoupled with cadaverine, and expand the

SsDesB substrate scope to produce structurally diverse hydroxamate siderophores and *N*-hydroxylated molecules.

## Supporting information

### S1 File.

(DOCX)

### S1 Raw images.

(PDF)

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**Funding acquisition:** Lesley-Ann Giddings.

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**Visualization:** George T. Lountos.

**Writing – original draft:** Lesley-Ann Giddings, George T. Lountos.

**Writing – review & editing:** Lesley-Ann Giddings, George T. Lountos.

## References

1. Esuola CO, Babalola OO, Heine T, Schwabe R, Schlömann M, Tischler D. Identification and characterization of a FAD-dependent putrescine N-hydroxylase (*GorA*) from *Gordonia rubripertincta* CWB2. *J Mol Catal.* 2016; 134:378–89.
2. Salomone-Stagni M, Bartho JD, Polsinelli I, Bellini D, Walsh MA, Demitri N, et al. A complete structural characterization of the desferrioxamine E biosynthetic pathway from the fire blight pathogen *Erwinia amylovora*. *J Struct Biol.* 2018; 202(3):236–49. <https://doi.org/10.1016/j.jsb.2018.02.002> PMID: 29428557

3. Binda C, Robinson RM, Martin del Campo JS, Keul ND, Rodriguez PJ, Robinson HH, et al. An unprecedented NADPH domain conformation in lysine monooxygenase NbtG provides insights into uncoupling of oxygen consumption from substrate hydroxylation. *J Biol Chem*. 2015; 290(20):12676–88. <https://doi.org/10.1074/jbc.M114.629485> PMID: 25802330
4. Chocklett SW, Sobrado P. *Aspergillus fumigatus* SidA Is a highly specific ornithine hydroxylase with bound flavin cofactor. *Biochemistry*. 2010; 49(31):6777–83. <https://doi.org/10.1021/bi100291n> PMID: 20614882
5. Olucha J, Lamb AL. Mechanistic and structural studies of the N-hydroxylating flavoprotein monooxygenases. *Bioorg Chem*. 2011; 39(5):171–7. <https://doi.org/10.1016/j.bioorg.2011.07.006> PMID: 21871647
6. Meneely KM, Lamb AL. Biochemical characterization of a flavin adenine dinucleotide-dependent monooxygenase, ornithine hydroxylase from *Pseudomonas aeruginosa*, suggests a novel reaction mechanism. *Biochemistry*. 2007; 46(42):11930–7. <https://doi.org/10.1021/bi700932q> PMID: 17900176
7. Ge L, Seah SYK. Heterologous expression, purification, and characterization of an L-ornithine N5-hydroxylase involved in pyoverdine siderophore biosynthesis in *Pseudomonas aeruginosa*. *J Bacteriol*. 2006; 188(20):7205–10. <https://doi.org/10.1128/JB.00949-06> PMID: 17015659
8. Kang HY, Brickman TJ, Beaumont FC, Armstrong SK. Identification and characterization of iron-regulated *Bordetella pertussis* alcaligin siderophore biosynthesis genes. *J Bacteriol*. 1996; 178(16):4877–84. <https://doi.org/10.1128/jb.178.16.4877-4884.1996> PMID: 8759851
9. Schupp T, Toupet C, Divers M. Cloning and expression of two genes of *Streptomyces pilosus* involved in the biosynthesis of the siderophore desferrioxamine B. *Gene*. 1988; 64(2):179–88. [https://doi.org/10.1016/0378-1119\(88\)90333-2](https://doi.org/10.1016/0378-1119(88)90333-2) PMID: 2841191
10. Hissen AH, Wan AN, Warwas ML, Pinto LJ, Moore MM. The *Aspergillus fumigatus* siderophore biosynthetic gene *sidA*, encoding L-ornithine N5-oxygenase, is required for virulence. *Infect Immun*. 2005; 73(9):5493–503. <https://doi.org/10.1128/IAI.73.9.5493-5503.2005> PMID: 16113265
11. Minandri F, Imperi F, Frangipani E, Bonchi C, Visaggio D, Facchini M, et al. Role of iron uptake systems in *Pseudomonas aeruginosa* virulence and airway infection. *Infect Immun*. 2016; 84(8):2324–35. <https://doi.org/10.1128/IAI.00098-16> PMID: 27271740
12. Mobarra N, Shanaki M, Ehteram H, Nasiri H, Sahmani M, Saeidi M, et al. A review on iron chelators in treatment of iron overload syndromes. *Int J Hematol Oncol Stem Cell Res*. 2016; 10(4):239–47. PMID: 27928480
13. Petrik M, Zhai C, Haas H, Decristoforo C. Siderophores for molecular imaging applications. *Clin Transl Imaging*. 2017; 5(1):15–27. <https://doi.org/10.1007/s40336-016-0211-x> PMID: 28138436
14. Schupp T, Waldmeier U, Divers M. Biosynthesis of desferrioxamine B in *Streptomyces pilosus*: evidence for the involvement of lysine decarboxylase. *FEMS Microbiol Lett*. 1987; 42(2–3):135–9.
15. Cao A, Galanello R. Beta-thalassemia. *Genet Med*. 2010; 12(2):61–76. <https://doi.org/10.1097/GIM.0b013e3181c6d68ed> PMID: 20098328
16. Codd R, Richardson-Sanchez T, Telfer TJ, Gotsbacher MP. Advances in the chemical biology of desferrioxamine B. *ACS Chem Biol*. 2018; 13(1):11–25. <https://doi.org/10.1021/acscchembio.7b00851> PMID: 29182270
17. Bertrand S, Hélesbeux J-J, Larcher G, Duval O. Hydroxamate, a key pharmacophore exhibiting a wide range of biological activities. *Mini Rev Med Chem*. 2013; 13(9):1311–26. <https://doi.org/10.2174/13895575113139990007> PMID: 23701657
18. Barona-Gómez F, Wong U, Giannakopoulos AE, Derrick PJ, Challis GL. Identification of a cluster of genes that directs desferrioxamine biosynthesis in *Streptomyces coelicolor* M145. *J Am Chem Soc*. 2004; 126(50):16282–3. <https://doi.org/10.1021/ja045774k> PMID: 15600304
19. Patel P, Song L, Challis GL. Distinct extracytoplasmic siderophore binding proteins recognize ferrioxamines and ferricoelichelin in *Streptomyces coelicolor* A3(2). *Biochemistry*. 2010; 49(37):8033–42. <https://doi.org/10.1021/bi100451k> PMID: 20704181
20. Barona-Gómez F, Lautru S, Francou F-X, Leblond P, Pernodet J-L, Challis GL. Multiple biosynthetic and uptake systems mediate siderophore-dependent iron acquisition in *Streptomyces coelicolor* A3(2) and *Streptomyces ambifaciens* ATCC 23877. *Microbiology*. 2006; 152(11):3355–66.
21. Nett M, Ikeda H, Moore BS. Genomic basis for natural product biosynthetic diversity in the actinomycetes. *Nat Prod Rep*. 2009; 26(11):1362–84. <https://doi.org/10.1039/b817069j> PMID: 19844637
22. Burrell M, Hanfrey CC, Kinch LN, Elliott KA, Michael AJ. Evolution of a novel lysine decarboxylase in siderophore biosynthesis. *Mol Microbiol*. 2012; 86(2):485–99. <https://doi.org/10.1111/j.1365-2958.2012.08208.x> PMID: 22906379

23. Telfer TJ, Gotsbacher MP, Soe CZ, Codd R. Mixing up the pieces of the desferrioxamine B jigsaw defines the biosynthetic sequence catalyzed by DesD. *ACS Chem Biol*. 2016; 11(5):1452–62. <https://doi.org/10.1021/acscchembio.6b00056> PMID: 27004785
24. Ronan JL, Kadi N, McMahon SA, Naismith JH, Alkhalaf LM, Challis GL. Desferrioxamine biosynthesis: diverse hydroxamate assembly by substrate-tolerant acyl transferase DesC. *Philos Trans R Soc Lond B Biol Sci*. 2018; 373(1748). <https://doi.org/10.1098/rstb.2017.0068> PMID: 29685972
25. Mindt M, Walter T, Kugler P, Wendisch VF. Microbial engineering for production of *N*-functionalized amino acids and amines. *Biotechnol J*. 2020; 15:1900451. <https://doi.org/10.1002/biot.201900451> PMID: 32170807
26. Li B, Lowe-Power T, Kurihara S, Gonzales S, Naidoo J, MacMillan JB, et al. Functional identification of putrescine *C*- and *N*-hydroxylases. *ACS Chem Biol*. 2016; 11(10):2782–9. <https://doi.org/10.1021/acscchembio.6b00629> PMID: 27541336
27. Raran-Kurussi S, Cherry S, Zhang D, Waugh DS. Removal of affinity tags with TEV protease. In: Burgess-Brown NA, editor. *Heterologous gene expression in E coli: methods and protocols*. New York, NY: Springer New York; 2017. p. 221–30.
28. Csaky T. On the estimation of bound hydroxylamine in biological materials. *Acta Chem Scand*. 1948:450–4.
29. Minor W, Cymborowski M, Otwinowski Z, Chruszcz M. HKL-3000: the integration of data reduction and structure solution—from diffraction images to an initial model in minutes. *Acta Crystallogr D*. 2006; 62(8):859–66.
30. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. *J Appl Crystallogr*. 2007; 40(4):658–74. <https://doi.org/10.1107/S0021889807021206> PMID: 19461840
31. Zwart PH, Afonine PV, Grosse-Kunstleve RW, Hung L-W, Ioerger TR, McCoy AJ, et al. Automated structure solution with the PHENIX suite. In: Kobe B, Guss M, Huber T, editors. *Structural proteomics: high-throughput methods*. Totowa, NJ: Humana Press; 2008. p. 419–35.
32. Matthews BW. Solvent content of protein crystals. *J Mol Biol*. 1968; 33(2):491–7. [https://doi.org/10.1016/0022-2836\(68\)90205-2](https://doi.org/10.1016/0022-2836(68)90205-2) PMID: 5700707
33. Kantardjiev KA, Rupp B. Matthews coefficient probabilities: improved estimates for unit cell contents of proteins, DNA, and protein–nucleic acid complex crystals. *Protein Sci*. 2003; 12(9):1865–71. <https://doi.org/10.1110/ps.0350503> PMID: 12930986
34. Weichenberger CX, Rupp B. Ten years of probabilistic estimates of biocrystal solvent content: new insights via nonparametric kernel density estimate. *Acta Crystallogr D*. 2014; 70(6):1579–88. <https://doi.org/10.1107/S1399004714005550> PMID: 24914969
35. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. *Acta Crystallogr D*. 2010; 66(4):486–501. <https://doi.org/10.1107/S0907444910007493> PMID: 20383002
36. Joosten RP, Long F, Murshudov GN, Perrakis A. The PDB\_REDO server for macromolecular structure model optimization. *IUCrJ*. 2014; 1(4):213–20. <https://doi.org/10.1107/S2052252514009324> PMID: 25075342
37. Moriarty NW, Grosse-Kunstleve RW, Adams PD. electronic Ligand Builder and Optimization Workbench (eLBOW): a tool for ligand coordinate and restraint generation. *Acta Crystallogr D*. 2009; 65(10):1074–80. <https://doi.org/10.1107/S0907444909029436> PMID: 19770504
38. Afonine PV, Grosse-Kunstleve RW, Echols N, Headd JJ, Moriarty NW, Mustyakimov M, et al. Towards automated crystallographic structure refinement with phenix.refine. *Acta Crystallogr D*. 2012; 68(4):352–67. <https://doi.org/10.1107/S0907444912001308> PMID: 22505256
39. Williams CJ, Headd JJ, Moriarty NW, Prisant MG, Videau LL, Deis LN, et al. MolProbity: more and better reference data for improved all-atom structure validation. *Protein Sci*. 2018; 27(1):293–315. <https://doi.org/10.1002/pro.3330> PMID: 29067766
40. Rao ST, Rossmann MG. Comparison of super-secondary structures in proteins. *J Mol Biol*. 1973; 76(2):241–56. [https://doi.org/10.1016/0022-2836\(73\)90388-4](https://doi.org/10.1016/0022-2836(73)90388-4) PMID: 4737475
41. Krissinel E, Henrick K. Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta Crystallogr D*. 2004; 60(12 Part 1):2256–68.
42. Krissinel E. On the relationship between sequence and structure similarities in proteomics. *Bioinformatics*. 2007; 23(6):717–23. <https://doi.org/10.1093/bioinformatics/btm006> PMID: 17242029
43. Setser JW, Heemstra JR, Walsh CT, Drennan CL. Crystallographic evidence of drastic conformational changes in the active site of a flavin-dependent *N*-hydroxylase. *Biochemistry*. 2014; 53(38):6063–77. <https://doi.org/10.1021/bi500655q> PMID: 25184411

44. Robinson R, Qureshi IA, Klancher CA, Rodriguez PJ, Tanner JJ, Sobrado P. Contribution to catalysis of ornithine binding residues in ornithine *N*5-monooxygenase. *Arch Biochem Biophys*. 2015; 585:25–31. <https://doi.org/10.1016/j.abb.2015.09.008> PMID: 26375201
45. Mügge C, Heine T, Baraibar AG, van Berkel WJH, Paul CE, Tischler D. Flavin-dependent N-hydroxylating enzymes: distribution and application. *App Microbiol and Biotechnol*. 2020; 104(15):6481–99. <https://doi.org/10.1007/s00253-020-10705-w> PMID: 32504128
46. Palfey BA, McDonald CA. Control of catalysis in flavin-dependent monooxygenases. *Arch Biochem Biophys*. 2010; 493(1):26–36. <https://doi.org/10.1016/j.abb.2009.11.028> PMID: 19944667
47. van Berkel WJH, Kamerbeek NM, Fraaije MW. Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. *J Biotechnol*. 2006; 124(4):670–89. <https://doi.org/10.1016/j.jbiotec.2006.03.044> PMID: 16712999
48. Meneely KM, Barr EW, Bollinger JM, Lamb AL. Kinetic mechanism of ornithine hydroxylase (PvdA) from *Pseudomonas aeruginosa*: substrate triggering of O<sub>2</sub> addition but not flavin reduction. *Biochemistry*. 2009; 48(20):4371–6. <https://doi.org/10.1021/bi900442z> PMID: 19368334
49. Robinson R, Sobrado P. Substrate binding modulates the activity of *Mycobacterium smegmatis* G, a flavin-dependent monooxygenase involved in the biosynthesis of hydroxamate-containing siderophores. *Biochemistry*. 2011; 50(39):8489–96. <https://doi.org/10.1021/bi200933h> PMID: 21870809
50. Neumann CS, Jiang W, Heemstra JR Jr., Gontang EA, Kolter R, Walsh CT. Biosynthesis of piperazic acid via *N*5-hydroxy-ornithine in *Kutzneria* spp. 744. *ChemBioChem*. 2012; 13(7):972–6. <https://doi.org/10.1002/cbic.201200054> PMID: 22522643
51. Soe CZ, Pakchung AAH, Codd R. Directing the biosynthesis of putrebactin or desferrioxamine B in *Shewanella putrefaciens* through the upstream inhibition of ornithine decarboxylase. *Chem Biodivers*. 2012; 9(9):1880–90. <https://doi.org/10.1002/cbdv.201200014> PMID: 22976977
52. Franceschini S, Fedkenheuer M, Vogelaar NJ, Robinson HH, Sobrado P, Mattevi A. Structural insight into the mechanism of oxygen activation and substrate selectivity of flavin-dependent *N*-Hydroxylating monooxygenases. *Biochemistry*. 2012; 51(36):7043–5. <https://doi.org/10.1021/bi301072w> PMID: 22928747