Staphylopine and pseudopaline dehydrogenase from bacterial pathogens catalyze reversible reactions and produce stereospecific metallophores

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Running title: Opine dehydrogenase stereospecificity and mechanism

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Keywords: Staphylopine, pseudopaline, opine, metallophore, dehydrogenase, Pseudomonas aeruginosa, Staphylococcus aureus, structural biology, transient kinetics, metal ion sequestration, virulence factor

ABSTRACT
Pseudopaline and staphylopine are opine metallophores biosynthesized by Pseudomonas aeruginosa and Staphylococcus aureus, respectively. The final step in opine metallophore biosynthesis is the condensation of the product of a nicotianamine (NA) synthase reaction (i.e. L-HisNA for pseudopaline and D-HisNA for staphylopine) with an α-keto acid (α-ketoglutarate for pseudopaline and pyruvate for staphylopine), which is performed by an opine dehydrogenase. We hypothesized that the opine dehydrogenase reaction would be reversible only for the opine metallophore product with (R) stereochemistry at carbon C2 of the α-keto acid (prochiral prior to catalysis). A kinetic analysis using stopped-flow spectrometry with (R)- or (S)-staphylopine and kinetic and structural analysis with (R)- and (S)-pseudopaline confirmed catalysis in the reverse direction for only (R)-staphylopine and (R)-pseudopaline, verifying the stereochemistry of these two opine metallophores. Structural analysis at 1.65 – 1.85 Å resolution captured the hydrolysis of (R)-pseudopaline and allowed identification of a binding pocket for the L-histidine moiety of pseudopaline formed through a repositioning of Phe-340 and Tyr-289 during the catalytic cycle. Transient-state kinetic analysis revealed an ordered release of NADP⁺ followed by staphylopine, with staphylopine release being the rate-limiting step in catalysis. Knowledge of the stereochemistry for opine metallophores has implications for future studies involving kinetic analysis, as well as opine metallophore transport, metal coordination, and the generation of chiral amines for pharmaceutical development.

INTRODUCTION
Pseudopaline and staphylopine are metallophores produced by Pseudomonas aeruginosa and Staphylococcus aureus, respectively(1-3). Both metallophores have been shown to chelate multiple transition metal ions that are then returned to the bacterial cell through dedicated transport pathways. This metal ion sequestering mechanism plays an important role in virulence in several infectious diseases(1,4-7). The biosynthesis of these metallophores requires a (S)-adenosyl-L-methionine-dependent aminoalkyltransferase, commonly known as a nicotianamine synthase or NAS (E.C. 2.5.1.43). The NAS links a single aminobutyrate moiety from (S)-adenosyl-L-methionine (SAM) with L-histidine (pseudopaline) or D-histidine (staphylopine; D-histidine is made from L-histidine via a racemase encoded by the staphylopine operon(8)). We have termed this product L- or D-HisNA with the NA referring to nicotianamine. In the second step, an NAD(P)H-dependent oxidoreductase (E.C.
1.5.1.52) known as an opine dehydrogenase (abbreviated PaODH for P. aeruginosa and SaODH for S. aureus) performs a condensation of the HisNA substrate with an \(\alpha\)-keto acid (\(\alpha\)-ketoglutarate, PaODH; pyruvate, SaODH) forming a Schiff base intermediate that is reduced by hydride transfer from NAD(P)H (Figure 1A). The term opine is historical, being derived from the common name octopine, a compound identified in Octopus octopodia muscle tissue in 1927(9). Opines are compounds formed enzymatically by condensation of an amino acid and a \(\alpha\)-keto acid. We have termed the products of these reactions opine metallophores given that they are not true nicotianamines, which incorporate three aminobutyrate moieties from SAM, and because the final enzymatic step forms an opine compound. Pseudopaline and staphylopine are representative of this metallophore class, though many other species possess the biosynthetic machinery to make opine metallophore variants(6).

Although the chemical composition of pseudopaline and staphylopine has been confirmed by both in vitro(1-3) and in vivo(1,3,10) experiments, the stereochemistry formed upon nucleophilic attack at the prochiral carbon of the \(\alpha\)-keto acid has not been determined. Production of either the (R) or (S) diastereomer at this position is possible (Figure 1B). Opine dehydrogenases exist in two structural classes. One class is composed of enzymes with two Rossmann-like fold domains (ex. PDB: 3UH1) that form (S) opines upon reduction of the Schiff base. The other class is composed of enzymes with one Rossmann-like fold domain and one \(\alpha\)-helical domain and produce (R) opines (Figure 1C-E). Indeed, the use of Arthrobacter sp. Strain 1C opine dehydrogenase, which stereospecifically produces the (R) opine \(N^{\alpha}\)-(1-(R)-(carboxyl)ethyl)-(L)-norvaline, by condensing pyruvate and (L)-norvaline(11), is the subject of a U.S. patent(12) proposing the use of this enzyme to generate chiral secondary amines for pharmaceutical development. Full knowledge of opine metallophore stereochemistry is important as it is likely to influence receptor recognition as well as the coordination geometry for metal complexes. This is especially important for pseudopaline, because it incorporates an extra carboxylate ligand from \(\alpha\)-ketoglutarate. As both PaODH and SaODH belong to the (R) opine producing structural class, we have proposed that they produce (R) opine metallophores(13).

We hypothesized that PaODH and SaODH catalyze reversible reactions that specifically produce the (R) opine metallophore diastereomer. The reverse reaction would therefore use only one opine metallophore diastereomer as a substrate. In this study, we measure catalysis in the steady-state for the reverse reactions of PaODH and SaODH using both the (R) and (S) diastereomers of pseudopaline and staphylopine. We also present four X-ray crystal structures of PaODH with ligands bound. PaODH-NADP\(^+\) (1.57 \(\AA\), 0.4 \(\AA\) higher resolution than our previously deposited structure) has NADP\(^+\) bound. PaODH-(S)-Pse(1.64 \(\AA\)) has NADP\(^+\) and (S)-pseudopaline bound. PaODH-(R)-Pse-1hr (1.65 \(\AA\)) was crystallized with NADP\(^+\) and soaked with (R)-pseudopaline, but has density for NADP\(^+\), L-HisNA and partial density for \(\alpha\)-ketoglutarate. PaODH-(R)-Pse-2hr (2.18 \(\AA\)) was crystallized with NADP\(^+\) and soaked with (R)-pseudopaline. This structure only has electron density for the NADP\(^+\) and L-HisNA ligands suggesting catalysis and release of \(\alpha\)-ketoglutarate within the crystal. Together these data define the amino acid residues involved in binding pseudopaline and support the hypothesis that PaODH and SaODH are stereospecific in the formation of the Schiff base intermediate and subsequent hydride transfer resulting in an (R) stereocenter for C2 (Figure 1B) of the respective \(\alpha\)-keto acid. This results in (R)-pseudopaline or (R)-staphylopine products, respectively.

The kinetic mechanism for opine dehydrogenases is best understood for octopine dehydrogenase, an enzyme that was the subject of extensive study by Anna Olomucki and colleagues during the 1970s. They noted a burst phase preceding steady-state turnover at high substrate concentrations that was lost as substrate concentration dropped near the \(K_m\) value. They concluded that hydride transfer was not rate-limiting and that the enzyme most likely proceeded through an ordered-sequential mechanism with NAD(P)H binding first and NADP\(^+\) dissociating last(14,15). SaODH and PaODH have only been examined by steady-state methods that typically mask key steps in the catalytic cycle(16). Here we use stopped-flow spectrometry to conduct transient state kinetic assays of staphylopine dehydrogenase for both the forward and reverse reactions.
observing absorption and fluorescence characteristics of the NADPH dihydronicotinamide ring. Absorption data reveal a reaction that is substantially slower in the steady-state than during the first turnover and fluorescence data suggest that this is due to a slow product release step for the forward reaction. It has recently been proposed that SaODH binds divalent transition metals such as Zn(II), Cu(II), Co(II) and Ni(II) resulting in an inhibition of staphylopine production(17). It was further proposed that SaODH activity is enhanced at low concentrations of Zn(II) or Cu(II) due to preferred catalysis of D-HisNA when coordinated by these two metals. We tested this hypothesis in the transient state and found that the inhibition is due to sequestration of available D-HisNA substrate by metal ion chelation and not due to direct inhibition of the enzyme and also found no evidence of rate enhancement in the presence of Zn(II) or Cu(II).

RESULTS
SaODH and PaODH catalyze reversible reactions – We hypothesized that SaODH and PaODH would catalyze reversible reactions and that both enzymes would exclusively form the (R) product diastereomer, consistent with structurally homologous opine dehydrogenases(18-20). To test this hypothesis, (R)- and (S)-pseudopaline and (R)- and (S)-staphylopine were synthesized as previously described(10,21). SaODH or PaODH were combined with NADP+ and then mixed with varied concentrations of (R)- or (S)-staphylopine (SaODH) or (R)- or (S)-pseudopaline (PaODH) (Figure 2). Substantial inhibition was observed at concentrations above ~25 µM (R)-pseudopaline or (R)-staphylopine (Table S1). NADP+ reduction with (S)-pseudopaline as the varied substrate was, within error, zero at all concentrations (Figure 2B). (S)-staphylopine resulted in limited catalysis with a $k_{cat}$ of 0.02 s$^{-1}$, four-fold above the rate of NADP+ reduction in the absence of opine metallophore in this assay (0.005 s$^{-1}$) (Figure 2B). Reverse reactions initiated with (R)-staphylopine or (R)-pseudopaline showed steady-state turnover with $k_{cat}$ values approximately 60% slower than the previously published forward rates(13). These data indicate that both SaODH and PaODH catalyze reversible reactions consistent with other characterized opine dehydrogenases such as octopine dehydrogenase from Pecten maximus(19).

Importantly, both enzymes specifically catalyze the reverse reaction with the (R)-diastereomer of their respective product. This provides definitive evidence that the opine product of the forward reaction is the (R)-opine metallophore.

PaODH binds (S)-pseudopaline in a non-catalytic complex – PaODH crystals were grown in the presence of NADP+ and L-HisNA, (S)-pseudopaline or (R)-pseudopaline. While NADP+ electron density was observed in each case, no substantial additional ligand density was visible in any of the structures, and thus they are not reported here. Co-crystals with 2 mM NADP+ were also grown and soaked for increasing lengths of time with varied concentrations of either substrate or product. Structures co-crystallized with NADP+ in which L-HisNA was soaked (for up to 18 hours) had no density for L-HisNA. However, a structure determined from a crystal grown with NADP+ and soaked for one hour with 10 mM (S)-pseudopaline (PaODH-(S)-Pse) had clear density for both NADP+ and (S)-pseudopaline immediately following molecular replacement. The comparison of this structure to a structure of PaODH grown with 2 mM NADP+ revealed that the two structures are ostensibly identical with a 0.36 Å rmsd for 848 residues (the full length of the dimer). The few differences that are observed are for residues and loops surrounding the active site as described below.

(S)-Pseudopaline binds above the nicotinamide ring of NADP+ (Figure 3). The nicotinamide ring of NADP+ is in the same position in all structures presented herein. The imidazole ring of the L-HisNA moiety of (S)-pseudopaline stacks in between Y243 and F340. F340 rotates down 30° from its position in PaODH-NADP+ to stack above the imidazole ring while the position of Y243 below the imidazole is unchanged. The C2’ carboxylate of the L-histidine moiety of (S)-pseudopaline forms a hydrogen bond with R319 and Y320 originating from the dimerization domain(13). In PaODH-(S)-Pse, Y289 rotates 120° to hydrogen bond the C1 carboxylate. This serves as a “cap” that occludes solvent accessibility to the active site. H219 is proposed to be the catalytic base and is hydrogen bonded to the carboxylate of C5 and to the carboxylate of C2’ in chain B. In chain A, H219 hydrogen bonds only to the C2’ carboxylate and the C5 carboxylate is rotated out of the active site and
hydrogen bonds instead to K168. Each of the residues described above are modeled into complete electron density.

(S)-pseudopaline does not appear to be in a catalytically competent conformation, despite stable binding within the active site. The hydride that is transferred to NADP⁺ during the reverse reaction resides on C2. In the PaODH-(S)-Pse structure C2 is 5.3 Å (chain A) and 7.9 Å (chain B) distant from the nicotinamide hydride. In this reaction, activated water is expected to act as a nucleophile attacking C2 to hydrolyze the Schiff base intermediate, but the (S) configuration at this position results in tight packing between the C1 carboxylate and the imidazole ring providing insufficient space for a water molecule.

PaODH crystals catalyze the hydrolysis of (R)-pseudopaline – To solve structures with (R)-pseudopaline, PaODH crystals were grown in the presence of 2 mM NADP⁺ and soaked in cryoprotectant containing 10 mM (R)-pseudopaline for 15, 45, 60 minutes or 20 mM (R)-pseudopaline for 120 minutes. Each structure was solved and two representative structures are presented here. PaODH-(R)-Pse-1hr was soaked for 1 hour and solved at 1.65 Å and PaODH-(R)-Pse-2hr was soaked for 2 hours and solved at 2.18 Å. For the PaODH-(R)-Pse-1hr structure, complete density is present for the L-HisNA and is positioned identically to the L-HisNA portion of (S)-pseudopaline as described above. Density for α-ketoglutarate is weak, suggesting low occupancy, as seen in the 2mFo-DFc electron density map (Figure 3D). We modeled both (R)-pseudopaline into this density and modeled L-HisNA and α-ketoglutarate separately with the latter model better accounting for the observed density. A Polder map (which eliminates solvent flattening allowing visualization of weaker electron density) generated with L-HisNA and α-ketoglutarate omitted demonstrates this, though C2 and C3 remain poorly fit to the electron density (Figure 3E). In PaODH-(S)-Pse, Y289 is in complete density and is rotated 120° into a “closed” position where it hydrogen bonds with the C2 carboxylate of (S)-pseudopaline. In PaODH-NADP⁺, Y289 is in complete density but is rotated up in an “open” position away from the active site. In the PaODH-(R)-Pse-1hr density is evident for both rotameric positions for Y289.

We have modeled Y289 in the “open” position, but display positive electron density (green) from a mFo-DFc map in the “closed” position (Figure 3E). This suggests that rotation of Y289 plays a role in stabilizing substrates during the catalytic cycle and that the “open” position is likely assumed only during binding or release.

Given the stability of the complex observed in PaODH-(S)-Pse, our interpretation of PaODH-(R)-Pse-1hr is that (R)-pseudopaline has been hydrolyzed into L-HisNA and α-ketoglutarate. PaODH-(R)-Pse-2hr provides additional clarity. In this structure, no density is present beyond L-HisNA, providing definitive evidence that the PaODH crystal is catalytically active resulting in a slow hydrolysis of (R)-pseudopaline over 2 hours (Figure 3F). The α-ketoglutarate, which is not well coordinated in the active site and is exposed to solution, is then able to diffuse away leaving only the L-HisNA product. This slow catalysis and/or release of α-ketoglutarate likely contributes to the weak electron density for α-ketoglutarate in the PaODH-(R)-Pse-1hr structure.

Transient state kinetic analysis of SaODH – We analyzed the kinetic mechanism of SaODH using a transient-state kinetic approach. The oxidation of NADPH to NADP⁺ was measured at 340 nm. At 7.8 µM or below for both D-HisNA and pyruvate (Figure 4A and B), oxidation was observed as a single phase. By 31 µM, two phases of NADPH oxidation were clearly apparent; a fast phase with a small amplitude change and a slow phase with a large amplitude change. Experiments with varied D-HisNA were fit globally by numerical integration to model 1 as described in the methods section. Analytical fitting of the data for varied pyruvate from the slow phase to a two exponential decay function, and replotting the rates, illustrates the rate enhancement at low concentrations of pyruvate. This is indicative of a transition from a single turnover to an increasing number of measured turnovers (steady-state turnover at saturating substrate concentrations) as substrate is increased above SaODH concentration (Figure 4, inset).

NADPH oxidation was also observed using diode array detection to scan changes in absorbance during the reaction at wavelengths from 300-500 nm. The 340 nm slice of data from the diode array recapitulated the data measured only at 340 nm.
Thus the fast and slow phases indicate only NADPH oxidation establishing that the absorption of the dihydronicotinamide was not altered by, for example, binding (Figure S1).

NADPH fluoresces with an emission maximum around 450 nm when excited at 340 nm. This signal is both sensitive and known to typically increase when NADPH is shielded from collisional quenching upon binding to an enzyme. We performed transient state fluorescence experiments measuring total emission above 360 nm to examine changes to fluorescence with binding and oxidation of NADPH. An increase in fluorescence was observed when SaODH and NADPH were mixed in the stopped-flow cell at final concentrations of 15 µM SaODH and varied concentrations of NADPH from 2.5 to 80 µM. These data were fit by numerical integration to a one-step equilibrium model, as described in the methods section, giving a forward rate constant, \( k_{+1} = 0.4 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \pm 0.1 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \), a reverse rate constant \( k_{-1} = 27 \pm 5 \text{ s}^{-1} \) for a \( K_{\text{NADPH}} \) of 70 ± 20 µM (Figure 5A). The Fitespace contour indicates good confinement of both rate constants (Figure S2).

Evidence to explain the catalytic processes underlying the data in Figure 4 was obtained by comparing the absorption data with fluorescence signals obtained under the same conditions. The initial increase in fluorescence upon binding of NADPH in the presence of D-HisNA and pyruvate matched the increase in fluorescence upon binding of NADPH in the absence of D-HisNA and pyruvate (Figure 5B – red trace). At approximately 0.01 s the increase in fluorescence slows indicating that hydride transfer has commenced. Rather than a decay to equilibrium a second increase and decrease in fluorescence is observed with an amplitude equivalent to the binding of NADPH in the absence of substrates (Figure 5B – gray trace). This indicates saturation of SaODH by NADPH at equilibrium under the condition of saturating substrate concentration. At progressively lower concentrations of NADPH we observe an incremental loss of the amplitude of this second rise and fall in fluorescence (Figure 5C). For NADPH concentrations below that of the enzyme (15 µM), the reaction is observed as a single decrease following NADPH binding, suggestive of a net single turnover event (Figure 5C – inset). Although the fluorescence data suggests that the reaction slows or even stops with increasing concentration by about 0.3 s, the absorption data demonstrate the greatest amplitude change beginning at this time point (Figure 4A and B).

NADPH fluorescence provides two signals: an emission increase with binding and decrease with oxidation. These signals provide a clear explanation for the absorption data in Figure 4. This is made evident by overlaying absorbance and fluorescence data from each experiment taken with the same concentrations (Figure 5D). During NADPH binding, from 0.001 to 0.02 s, little change in absorbance is observed. The initial drop in fluorescence beginning at 0.02 s correlates in time with the initial fast phase observed by absorption. This fast phase therefore correlates with the completion of one hydride transfer in single turnover consistent with the maximum amplitude of this phase (~15 µM) (Figure 5C – inset). When the substrates are in excess of the enzyme concentration, fluorescence again increases (the signal for NADPH binding). This suggests that NADP+ has been released, clearing the active site for the binding of another NADPH. From this point the catalytic reaction slows substantially suggesting that turnover is contingent on the release of the nascent staphylopine that prevents access of D-HisNA and pyruvate for the next round of catalysis.

We also measured the transient state reaction in the reverse direction, that is the reduction of NADP+ to NADPH followed by the hydrolysis of (R)-staphylopine into D-HisNA and pyruvate (Figure 6). Data from both experiments were fit simultaneously to model 1 (Figure 6). The reverse reaction shows no evidence of delayed release of D-HisNA or pyruvate but proceeds as a single phase. These data were also fit to model 2 which reverses the order of the binding steps (Figure S3). Both simulations fit the data comparably suggesting that binding of NADPH and D-HisNA is independent and random.

Inhibition of SaODH by cobalt(II), nickel(II), zinc(II) and copper(II) – Recently, it was proposed that SaODH is inhibited by the transition metals Co(II), Ni(II), Zn(II) and Cu(II), and that this inhibition plays a role in the regulation of staphylopine biosynthesis(17). The experimental data underpinning this hypothesis were derived from steady-state measurements wherein the authors observed a nearly complete loss of SaODH.
activity as the concentration of each metal exceeded the concentration of substrate. The cause of this inhibition was proposed to be metal binding an SaODH allosteric site. Additionally, in the presence of low concentrations of Zn(II) and Cu(II), a catalytic enhancement was reported. This enhanced rate was proposed to be due to preferential binding and/or catalysis of D-HisNA coordinated by Zn(II) or Cu(II). Our transient state assays provide a sensitive means of exploring these hypotheses. An alternative explanation of the data presented by Hajjar et al. is that all four inhibitory metal ions bind D-HisNA and that while in complex with metal, D-HisNA is not available as a substrate for SaODH.

To test this hypothesis we performed transient-state assays following the absorbance change upon NADPH oxidation for 300 s. Cobalt(II) chloride, nickel(II) chloride, zinc(II) chloride or copper(II) chloride were pre-incubated with D-HisNA or with SaODH prior to initiating the reaction to compare the effect of metal association with substrate versus with enzyme. Data for Co(II) were fit analytically to Eq. 3 or Eq. 4 and by numerical integration to model 1 with the addition of a one-step equilibrium binding term to account for the competition of Co(II) for D-HisNA (Figure 7A, S4A-B, S5A). Analytical fits were not intended to define intrinsic rate constants for reaction steps, but rather to descriptively compare phases of the SaODH reaction between data sets. The fast phase is unchanged in the presence of D-HisNa (100 µM) in excess over Co(II) (25µM) (Figure 7A, 1 – red, blue and green traces). However, when equimolar Co(II) is pre-incubated with D-HisNA, very little turnover is initially observed. This suggests that D-HisNA is unavailable for binding by SaODH (Figure 7A, 1 – black trace). The slow phase traces are initially equivalent when excess unbound D-HisNA is available (Figure 7A, 2 – red, blue and green traces), but in equimolar Co(II), turnover remains very slow. We assume that this slow turnover occurs as a function of the rate of dissociation of the D-HisNA-Co(II) complex (Figure 7A, 3 – black trace). Thus, only when D-HisNA is released by Co(II) is it available as an SaODH substrate and this off rate limits SaODH catalysis. The staphylopine formed is also able to compete for Co(II) which contributes to the off rate by freeing additional D-HisNA. This results in a slow conversion of D-HisNA to staphylopine with an apparent rate constant of 0.02 s⁻¹ (gray trace; Eq. 3 – k₁), greater than 10 times slower than the rate constant used to describe the uninhibited steady-state phase of 0.23 s⁻¹ (red trace; Eq. 4, k₂). By the late steady-state phase, 45 µM D-HisNA has been consumed (Figure 7A, 3 – red, blue and green traces). The free substrate concentration is lower in the presence of Co(II) and the steady-state rate declines toward the apparent off rate for the D-HisNA-cobalt complex. In all cases the reaction proceeds to the same equilibrium because the staphylopine product binds Co(II). Importantly, the inhibition due to the presence of Co(II) is equivalent whether Co(II) is pre-incubated with SaODH (blue trace; k₁ = 0.37 s⁻¹, k₂ = 0.03 s⁻¹) or pre-incubated with D-HisNA (green trace; k₁ = 0.35 s⁻¹, k₂ = 0.03 s⁻¹). Global fitting demonstrates that the data can be simulated using model 1 with only the addition of a one-step equilibrium term that accounts for Co(II) binding of D-HisNA. These data suggest that the inhibition is not due to an association of Co(II) with the enzyme, but instead due to the D-HisNA-Co(II) complex making D-HisNA unavailable as a substrate.

The effect of Ni(II), Zn(II) and Cu(II) were also evaluated and the data provide similar conclusions (Figure 7B-D, S5B-D). In no case was the reaction rate observed to be faster, regardless of preincubation of the metal ion with enzyme or with substrate. In every case, the presence of metal resulted in either equivalent rates or in inhibition. Ni(II) binds D-HisNA with sufficient affinity that equimolar Ni(II):D-HisNA resulted in no turnover on a 5 min time scale (Figure 7B, S5B). Reducing Ni(II) concentrations left free D-HisNA that was converted into staphylopine. The reactions did not proceed to the equilibrium observed in the absence of metal suggesting that staphylopine was unable to free D-HisNA from Ni(II). When Ni(II) was preincubated with SaODH more staphylopine was made than when Ni(II) was preincubated with D-HisNA. This suggests a slow rate of association between Ni(II) and D-HisNA that allows significant catalysis to occur prior to formation of the Ni(II):D-HisNA coordination complex.

When Zn(II) was preincubated with SaODH the initial rate was faster than for preincubation with D-HisNA, but both rates were slower than in the absence of Zn(II) (Figure 7C, S5C). This again suggests a slow rate of association between Zn(II) and D-HisNA allowing significant
catalysis prior to complex formation. When equimolar Zn(II) was preincubated with D-HisNA the reaction was slowed approximately 10-fold. The results with Cu(II) are similar to those of Zn(II). No rate enhancement was observed. Equimolar Cu(II) preincubated with D-HisNA nearly halted the reaction with only one turnover every 7.5 minutes (Figure 7D, S5D). In no case is there evidence of metal-dependent allosterism or of a rate enhancement due to preferential catalysis of a metal:D-HisNA complex.

DISCUSSION

Staphylopine was first synthesized by Toronto Chemical Company for Arnoux et al. (1) and the total synthesis was later published by Lei et al. (21) In both cases the (S)-staphylopine diastereomer was made, but evidence for the production of the (S) stereocenter by the opine dehydrogenase has been absent from the literature. The stereochemistry of the opine metallophore products may affect receptor recognition, efficiency of transport and metal coordination making full stereochemical determination an important goal. Structural evidence has also been lacking for substrate and opine metallophore product ligands. As a result, the amino acid residues involved in binding and catalysis have only been hypothesized previously (13). Steady-state kinetic methods have been employed to study substrate specificity and $k_{cat}$ in the opine dehydrogenases, but transient-state methods have not been used to examine underlying catalytic mechanisms. Finally, a recent report suggested allosteric enhancement and inhibition for Zn(II) and Cu(II) under different conditions and allosteric inhibition by Co(II) and Ni(II)(17). The data presented herein address each of the above areas of opine dehydrogenase research.

PaODH and SaODH, as well as octopine dehydrogenase (PDB:1BG6), have been previously observed to crystallize with open active sites (13,22). The holoenzyme structures of PaODH-(S)-Pse and PaODH-(R)-Pse are only slightly different. In monomer A and B of PaODH-(S)-Pse the distance between C4 of the nicotinamide ring and C2 of (S)-pseudopaline is too distant for hydride transfer at 5.3 Å and 7.9 Å respectively. The PaODH-(R)-Pse structures are similar, but the more open monomer B has weaker electron density for L-HisNA and we chose to leave this density unmodeled. These higher resolution structures do provide additional insight into the question of active site dynamics during the catalytic cycle. The stacking arrangement of F340 and the rotation of Y289 into a closed position eliminates solvent accessibility within the L-HisNA binding pocket (Figure S6). E123 hydrogen bonds with the hydroxyls of the nicotinamide ribose sugar and is found on α-helix N, as described previously (13) (Figure 3B). αN is a core helix of the catalytic domain and E123 acts from the catalytic domain to stabilize NADP+ in the Rossmann-like fold domain. The position of E123, so near to NADP+ suggests that αN does not move to position the substrate for hydride transfer. Rather, it may be the long loop from αM to β13 that transiently closes the active site. This loop contains F340 which is repositioned upon L-HisNA binding. Thus, the dihydronicotinamide ring may approach the Schiff base only transiently to affect hydride transfer.

The open character of the active site suggests that NADPH and HisNA can bind PaODH and SaODH without any dependency on order. Our kinetic data support this observation. Indeed, it is possible to reverse the first two steps of model 1 (D-HisNA and NADPH binding steps) and achieve similar fits for both the forward and reverse transient state reactions for SaODH. Once NADPH and HisNA are associated with the enzyme, the α-keto acid enters and condensation occurs forming the Schiff base intermediate. This is followed rapidly by a transient “closure” event resulting in hydride transfer. Product release is rate-limiting with NADP+ released first followed by slow release of the metallophore product (Scheme 1).

We have defined the residues that stabilize the L-HisNA moiety of (R)-pseudopaline. In all the structures solved for this manuscript, the electron density for the C1” carboxylate and the imidazole ring of L-HisNA are the most well-defined features in the substrate or product electron density maps. This suggests the importance of these contact points in the recognition and binding of L-HisNA and raises the question of whether D-HisNA is bound by staphylopine dehydrogenase using the same residues. In PaODH, R319 and Y320 hydrogen bond to the C1” carboxylate and F340 and Y243 stack above and below the imidazole group. SaODH has an rmsd of 2.2 Å over 427/432 Ca residues in a structural comparison with PaODH. The resulting overlay demonstrates that SaODH...
conserves all four of these residues in the same position, but to accommodate the D-HisNA diastereomer using the same contact points would require the secondary amine to be positioned at an angle 120° from the L-HisNA secondary amine. This is certainly possible within the large active site cavity. Interestingly, we have noted previously (13) that SaODH is also able to accept L-HisNA as a substrate with a \( k_{cat}/K_m \) only 3-fold lower than for D-HisNA. PaODH is specific for L-HisNA.

Recently, Arnoux and colleagues published two structures of SaODH that were modelled with (S)-staphylopine (PDB:6H3F) and D-HisNA (PDB:6H3D) bound, respectively (17). The difficulty with this interpretation is that the electron density modeled with (S)-staphylopine is limited to three disconnected areas (Figure 8A). Most strikingly, the C1 carboxylate of pyruvate is modeled in a position within hydrogen bonding distance of NADP+ . It seems more probable that this density is a water molecule or an ion. In the second structure density in the shape of H-dhistidine is present, but the aminobutyrate moiety for D-HisNA is not present (Figure 8A), as noted by the authors. Additionally, the H-dhistidine moiety is positioned very differently between the two structures with its carboxylate group in opposite orientations. An alignment of PaODH-(R)-Pse-1hr and SaODH with staphylopine bound (PDB:6H3F), as described above, suggests that the position of H-dhistidine as co-crystallized with SaODH is likely correct as it hydrogen bonds with R319 in a manner similar to PaODH. The electron density presented as representing (S)-staphylopine most likely represents solvent molecules rather than the metallophore product (Figure 8A and B).

In the same paper, data are presented to demonstrate allosteric inhibition of SaODH in the presence of Co(II), Ni(II), Zn(II) and Cu(II). Our data indicate inhibition by each of these metals, but not due to an association of the metal with the enzyme. Not surprisingly, D-HisNA binds Co(II), Ni(II), Zn(II) and Cu(II) effectively, as the SaODH reaction adds only one additional ligand group, a carboxylate from pyruvate, during the biosynthesis of staphylopine. When bound by metal, the D-HisNA substrate moieties that would be recognized by the SaODH active site are instead coordinated by the metal. The metal:HisNA coordination complex results in a very different geometry than the elongated L-HisNA pose seen in our structures of PaODH. It is, therefore, unlikely that the metal:D-HisNA complex is a substrate for SaODH as was hypothesized by Hajjar et. al. Rather, the metal:D-HisNA coordination complex reduces the freely available D-HisNA pool and depresses the observed activity of SaODH as a function of diminished substrate concentration. The authors also report on a structural basis for metal binding to SaODH based on three X-ray crystal structures. These crystals were soaked in either 5 mM Mn(II), Zn(II) or Ni(II). We note, however, that these data are not archived in the PDB so further analysis of these structures is not possible.

Our data definitively show that SaODH and PaODH catalyze reversible reactions specific for the (R) diastereomer of the respective opine products, staphylopine and pseudopaline. To generate the (R) diastereomer and not the (S), the ODH must position the \( \alpha \)-keto acid for nucleophilic attack on the re face of the \( sp^2 \)-hybridized carbon C2. PaODH-(R)-Pse-1hr is valuable as it provides evidence for catalysis within the PaODH protein crystal, but the position of \( \alpha \)-ketoglutarate during the catalytic cycle is defined by weak electron density representing low occupancy. PaODH-(R)-Pse-2hr captures the completion of catalysis prior to L-HisNA release for the reverse reaction. Thus, both our kinetic and structural data support the conclusion that SaODH and PaODH produce metallophores with (R) stereochemistry at C2.

Interestingly, it was recently shown that when 100 \( \mu \)M (S)-pseudopaline is added to wild type \textit{P. aeruginosa} PAO1 grown on Vogel-Bonner minimal media with 50 \( \mu \)M of added EDTA the intracellular concentrations of Zn(II), Fe(II), Co(II) and Ni(II) are increased (10). 100 \( \mu \)M (R)-pseudopaline did not increase metal uptake in comparison to wild type \textit{P. aeruginosa} PAO1 grown in the absence of any added metallophore. The authors also demonstrated the uptake of a pseudopaline derivative into \textit{P. aeruginosa} PAO1 cells via the conjugation of a fluorescence probe detected by fluorescence microscopy. These intriguing data could suggest that the presence of 100 \( \mu \)M (R)-pseudopaline inhibits additional (R)-pseudopaline biosynthesis while (S)-pseudopaline is treated as an exogenous metallophore and does not directly affect regulation. The \( \Delta G^\circ \) for the SaODH reaction is -1.9 \( \pm \) 0.5 kJ/mol (calculated from the product formation measured in five different transient state absorbance experiments).
Thus, production of staphylopine is highly dependent on the maintenance of low staphylopine concentrations in the cytosol. Assuming PaODH operates similarly, adding exogenous (R)-pseudopaline to the media could directly slow the native production of pseudopaline and lead to lower levels of metal uptake in comparison to (S)-pseudopaline supplemented media. Nevertheless, the observation that (S)-pseudopaline both binds to PaODH (structures herein) and that it is taken up into *P. aeruginosa* PAO1, even with a conjugated fluorophore attached(10), has exciting implications for the possible development of an inhibitor for PaODH or for the delivery of an analog conjugated to an antibiotic.

**CONCLUSIONS**

SaODH and PaODH catalyze reversible reactions generating (forward biosynthetic reaction) or consuming (reverse reaction) the (R) diastereomer of staphylopine (SaODH) or pseudopaline (PaODH). L-HisNA binding is directed primarily by the histidine moiety in PaODH with a similar binding contacts likely in SaODH due to high structural similarity. The kinetic mechanism has random association of D-HisNA and NADPH followed by the alpha-keto acid substrate. Product release is strictly ordered by slow staphylopine release and this process was found to be rate-limiting for SaODH. Metal inhibition by Co(II), Ni(II), Zn(II) and Cu(II) is due to competition for substrate rather than direct inhibition of the enzyme.

**EXPERIMENTAL PROCEDURES**

*Protein expression and purification –* SaODH and PaODH were purified as previously described(2) with the following modification. For the purification of PaODH, 1 mM octyl β-D-glucopyranoside (BOG) was added to the buffers for both the nickel affinity and size exclusion chromatography purification steps. 1 mM dithiothreitol (DTT) was added to the buffer for the size exclusion chromatography step. Protein concentration was determined by absorbance at 280 nm using the method of Pace *et. al*(23). Previously, we used the Bradford assay to determine concentration for PaODH and SaODH, but global fitting of single turnover reactions revealed these values to be artificially high. Absorbance at 280 nm gives a concentration lower than by Bradford assay by a factor of 6.7 for both enzymes. Multiplying our previously published *k*<sub>cat</sub> values(13) by this factor gives corrected *k*<sub>cat</sub> and *k*<sub>cat</sub>/*K*<sub>m</sub> values.

*Reagents and reaction buffer –* Sodium pyruvate, NADPH and NADP<sup>+</sup> were obtained from Sigma. Purified SaODH was stored at a concentration of 50 µM in a buffer containing 50 mM potassium phosphate pH 8.0, 100 mM sodium citrate and 20% glycerol. All transient state and steady state experiments were performed using rapid mixing with a Hi-Tech stopped-flow spectrometer (TgK Scientific). In all subsequent descriptions, we report only the final concentrations in the stopped-flow cell after mixing. SaODH was first diluted to 30 µM using reaction buffer (50 mM Tris pH 8.0) giving a concentration of 12% glycerol. Upon mixing in the stopped-flow cell, the final concentration of glycerol was diluted to 6%.

*Steady-state kinetics of the reverse reaction –* Initial rates were measured by observing NADP<sup>+</sup> reduction as an increase in absorbance at 340 nm using a stopped-flow spectrometer at 24 °C. The reaction buffer was 50 mM Tris pH 8.0 (SaODH) or 50 mM potassium phosphate pH 8.0 (PaODH). 300 nM ODH and 224 µM NADP<sup>+</sup> were combined in one syringe and mixed 1:1 with a second syringe containing varied concentrations of either (R)- or (S)-staphylopine (*S. aureus*) or (R)- or (S)-pseudopaline (*P. aeruginosa*). To determine kinetic parameters, plots of initial rates were fit to the Michaelis-Menten equation modified to account for the observed substrate inhibition.

\[ v_o = \frac{v_{max}[S]}{K_m + [S] + \frac{([S]^2)}{K_I}} \]  

Each experiment was repeated at least three times and error was calculated as the standard deviation of the parameters determined from each trial.

*NADPH binding by fluorescence –* The binding of NADPH to SaODH was measured as an increase in fluorescence emission at 450 nm on a stopped-flow spectrometer at 24 °C using a WG360 longpass cut-off filter. SaODH was mixed with NADPH in the stopped-flow cell to give final concentrations of 15 µM SaODH and 2.5 µM to 80 µM NADPH.
Fluorescence emission was measured for 10 s in logarithmic timescale mode. Fluorescence intensity was corrected to account for the inner filter effect caused by increasing concentrations of NADPH using the following equation (24):

\[ F_c = F \cdot 10^{\frac{\varepsilon c}{2}} \]  

(2)

where \( F_c \) is the corrected intensity for fluorescence \( (F) \), \( \varepsilon \) is the extinction coefficient of NADPH at the excitation wavelength of 340 nm (6220 M\(^{-1}\)cm\(^{-1}\)), and \( c \) is the concentration of NADPH. Duplicate data sets were collected, averaged and fit to a one-step equilibrium binding model using numerical integration in Kintek Explorer 8.0 (25,26). Fit was evaluated by FitSpace using a Chi\(^2\) threshold limit of 0.91 from a data set with 10,240 data points. The original data and optimized simulations were replotted in Kaleidagraph.

**Transient state kinetics of the forward reaction** – Reaction progress in the transient state was followed as NADPH was oxidized to NADP\(^+\) at 24 °C using the stopped-flow spectrometer by measuring the loss of absorbance at 340 nm or the loss of total fluorescence emission beyond a 360 nm cutoff filter. SaODH was mixed in one step with all three substrates. For reactions measuring the rate of absorption change with varied D-HisNA, the concentrations were 15 µM SaODH, 1 mM pyruvate and 160 µM NADPH with D-HisNA varied from 7.8 to 62.5 µM. For reactions measuring the rate of absorption change with varied pyruvate, the concentrations were 15 µM SaODH, 500 µM D-HisNA and 160 µM NADPH with pyruvate varied from 3.9 to 4000 µM. Concentrations for reactions measuring the rate of fluorescence change in the forward direction were 15 µM SaODH, 500 µM D-HisNA and 1 mM pyruvate with NADPH varied from 5 to 240 µM. In each experiment, data were collected for 100 s using logarithmic time-base mode. Data fitting was performed in Kintek Explorer 8.0 using the model and constraints described above. Data for both varied NADPH and varied (R)-staphylopine were fit simultaneously to this model. The original data and optimized simulations were replotted in Kaleidagraph.

**The source of metal inhibition by zinc(II), copper(II), nickel(II) and cobalt(II)** – Transient state kinetic assays were performed by measuring absorbance at 340 nm as described above. Cobalt(II) chloride, copper(II) chloride, zinc(II) chloride, or nickel(II) chloride were preincubated with either SaODH or with D-HisNA at room temperature for five minutes prior to initiating the reaction. In each experiment, SaODH was combined with NADPH and pyruvate in one syringe and mixed with D-HisNA from another
syringe. For reactions with metal ion preincubated with D-HisNA, the concentrations were 15 µM SaODH, 1 mM pyruvate, 180 µM NADPH and metal ion varied from 12.5 to 100 µM (Cu(II) and Ni(II)) or 6.3 to 100 µM (Co(II) and Zn(II)), and D-HisNA at 100 µM. For reactions with metal ion preincubated with SaODH, the concentrations were 15 µM SaODH, 1 mM pyruvate, 180 µM NADPH, 25 µM metal ion (Co(II)) or 50 µM metal ion (Cu(II), Ni(II) and Zn(II)), and 100 µM D-HisNA (Cu(II), Ni(II) and Zn(II)) or 6.3 to 100 µM D-HisNA (Co(II)). Negative controls without added metal ions were performed for each experiment. Each experiment was performed twice with representative examples shown.

Data for SaODH pre-incubated with Co(II) at D-HisNA concentrations from 0 µM to 25 µM were fit in Kintek Explorer to model 1 described above with the addition of a separate one-step equilibrium to account for the binding of D-HisNA by Co(II) (Figure S3). Data for Co(II) pre-incubated with D-HisNA were also fit analytically in Kintek Explorer 8.0 to equations for exponential decay:

\[ [S] = \Delta S_1 \cdot e^{-k_1 \cdot t} + S_{\text{final}} \] (3)
\[ [S] = \Delta S_1 \cdot e^{-k_1 \cdot t} + \Delta S_2 \cdot e^{-k_2 \cdot t} + S_{\text{final}} \] (4)

These analytical fits do not define intrinsic rate constants for reaction steps; they were used to qualitatively compare rates from different experiments. Data and fits were replotted in Kaleidagraph. Complete data sets are shown in Figure S4.

**Protein Crystallization** – All crystals were grown in hanging drops composed of 1.5 µL protein and 1.5 µL well solution at 24 °C. PaODH at 1.2 mg/mL, with its N-terminal hexahistidine tag, was supplemented with 2 mM NADP⁺ prior to crystallization for all structures. PaODH-NADP⁺ crystallized in a well solution of 100 mM BisTris pH 5.45, 200 mM ammonium acetate, and 24% PEG 3350. PaODH-(S)-Pse crystallized in a well solution of 290 mM ammonium formate, and 28% PEG 3350. PaODH-(R)-Pse-2hr crystallized in a well solution of 270 mM ammonium formate, and 28% PEG 3350. PaODH-(R)-Pse-1hr crystallized in a well solution of 260 mM ammonium formate, and 25% PEG 3350. In each condition, rod-shaped crystals grew within two days and reached maximum size within two weeks. Crystals were transferred into a cryoprotectant made by supplementing well solution with 20% ethylene glycol and 2 mM NADP⁺ for PaODH-NADP⁺; 25% ethylene glycol, 2 mM NADP⁺ and 10 mM (S)-pseudopaline for PaODH-(S)-Pse; and 25% ethylene glycol, 2 mM NADP⁺ and 10 mM (R)-pseudopaline for PaODH-(R)-Pse-1hr or 20 mM (R)-pseudopaline for PaODH-(R)-Pse-2hr. PaODH-(S)-Pse crystals were soaked for one hour in their cryoprotectant solution containing 10 mM (S)-pseudopaline prior to flash cooling. PaODH-(R)-Pse-1hr crystals were soaked for one hour in their cryoprotectant solution containing 10 mM (R)-pseudopaline prior to flash cooling. PaODH-(R)-Pse-2hr crystals were soaked for two hours in their cryoprotectant solution containing 20 mM (R)-pseudopaline prior to flash cooling.

**Data Collection and Structure Determination** – Diffraction data were collected remotely using BluIce(27) at the Stanford Synchrotron Radiation Lightsource (SSRL, Menlo Park, CA). For all structures, 360° of data with 0.15° oscillation were collected at a temperature of 100 °K. Statistics for data collection and refinement are listed in Table 1. Data were processed in XDS (28). PaODH-NADP⁺ data were collected on beamline 12-2 at a wavelength of 0.9795 Å and 0.2 s exposure with a detector distance of 325 mm. These data were processed to 1.57 Å. PaODH-(S)-Pse data were collected on beamline 9-2 at a wavelength of 0.9795 Å and 0.31 s exposure with a detector distance of 320 mm. These data were processed to 1.65 Å. PaODH-(R)-Pse-1hr data were collected on beamline 9-2 at a wavelength of 0.9795 Å and 0.2 s exposure with a detector distance of 320 mm. These data were processed to 2.18 Å. PaODH-(R)-Pse-2hr data were collected on beamline 9-2 at a wavelength of 0.9795 Å and 0.2 s exposure with a detector distance of 470 mm. These data were processed to 2.18 Å. Our previously deposited PaODH structure (PDB: 6C4N) was used as a search model for molecular replacement in phenix.phaser(29). The two monomers from the 6C4N dimer were used as separate models in the search, placing one of each. The resulting solution had a LLG = 9,098 and a TFZ = 68.8 for PaODH-NADP⁺, a LLG = 19,571 and a TFZ = 128.9 for
PaODH-(S)-Pse, a LLG = 24,434 and a TFZ = 141.8 for PaODH-(R)-Pse-1hr, and a LLG = 13,989 and a TFZ = 107.8 for PaODH-(R)-Pse-2hr. For each structure, rounds of model building and refinement were completed in Coot(30) and phenix.refine(31). Waters were placed by phenix.refine, corrected manually and verified, using a 2m\textit{Fo-DFc} electron density map contoured at 1.5 \(\sigma\), following a round of refinement. Density was visible for NADP\(^+\) (all four structures), (S)-pseudopaline (PaODH-(S)-Pse), or L-HisNA (PaODH-(R)-Pse-1hr and 2hr) in the initial electron density map. L-HisNA and \(\alpha\)-ketoglutarate density became more apparent through refinement for both PaODH-(R)-Pse structures. Ligands were placed after polypeptide refinement was complete. NADP\(^+\) was added to each model by phenix.ligandfit. (S)-pseudopaline and L-HisNA were built in phenix.elbow. (S)-Pseudopaline was placed in PaODH-(S)-Pse by phenix.ligandfit with CC values of 83.5 and 88.2 for chain A and B respectively. L-HisNA and \(\alpha\)-ketoglutarate were placed by hand in chain A of PaODH-(R)-Pse-1hr, using restraints generated in phenix.elbow. L-HisNA was placed in PaODH-(R)-Pse-2hr by phenix.ligandfit in chain A with a CC value of 77.5. Density for L-HisNA is also visible in chain B for both PaODH-(R)-Pse structures, but is fragmented and was not modeled. Ligand placement was verified following a round of refinement and by generating omit maps and Polder maps in phenix with (S)-pseudopaline, L-HisNA and/or \(\alpha\)-ketoglutarate omitted from the respective model.

\textit{Crystallographic models} – Summary data for the models is provided in Table 2. Ramachandran analysis was performed by MolProbity (32) showing good geometry with no outliers for each structure. A comparison of structures and calculation of rmsd values were performed using PDBBeFold(33). Solvent accessible surface was calculated by CASTp (Computed Atlas of Surface Tomography of proteins)(34). Structure figures were generated in PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.). The atomic coordinates and structure factors have been deposited in the Protein Data Bank (accession codes in Table 2).

\textbf{ACKNOWLEDGEMENTS}

This publication was made possible by funds from NIH Grant R01 GM127655 and P20 GM103418. J.S.M. was supported by the NIH Graduate Training Program in the Dynamic Aspects of Chemical Biology Grant T32 GM008545 and an American Heart Association Predoctoral Fellowship PRE33960374. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health. Lei and co-workers thank the financial support from the NNSFC (21625201, 21661140001, 91853202 and 21521003), National Key Research and Development Program of China (2017YFA0505200) as well as a special research grant from Roche Pharma Research & Early Development. Use of the Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, is supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract DE-AC02-76SF00515. The Stanford Synchrotron Radiation Lightsource Structural Molecular Biology Program is supported by the U.S. Department of Energy Office of Biological and Environmental Research and by NIGMS, National Institutes of Health Grant P41GM103393. We thank the staff at the Stanford Synchrotron Radiation Laboratory for their support and assistance.

\textbf{NOTE}

The authors declare that they have no conflicts of interest with the contents of this article.
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All data were collected on beamline 12-2 or 9-2 at the Stanford Synchrotron Radiation Lightsource. Values in parentheses are for the highest resolution shells.

| Table 1 Data collection and refinement statistics |
|-----------------------------------------------|
| All data were collected on beamline 12-2 or 9-2 at the Stanford Synchrotron Radiation Lightsource. Values in parentheses are for the highest resolution shells. |
| | PaODH-NADP  | PaODH-(S)-Pse | PaODH-(R)-Pse-1hr | PaODH-(R)-Pse-2hr |
| Data collection | | | | |
| PDB ID | 6PBM | 6PBP | 6PBN | 6PBT |
| Spacegroup | C2 | C2 | C2 | C2 |
| Unit cell (Å, °) | a=180.9 b=53.9 c=96.9 β=99.1 | a=180.6 b=53.8 c=96.8 β=98.7 | a=181.0 b=53.8 c=96.9 β=98.8 | a=181.6 b=53.9 c=96.9 β=98.8 |
| Resolution range (Å) | 38.3 - 1.57 | 39.9 - 1.64 | 40.0 - 1.65 | 39.8 - 2.18 |
| Completeness (%) | 98.0 (95.9) | 97.1 (88.0) | 98.5 (88.0) | 97.9 (88.6) |
| Total reflections | 876,519 | 757,987 | 753,247 | 324,135 |
| Unique reflections | 126,487 | 110,264 | 110,062 | 48,004 |
| I/ σ | 10.2 (2.0) | 12.8 (2.0) | 12.9 (2.0) | 11.5 (2.3) |
| Rmerge | 9.4 (79.4) | 8.1 (69.5) | 7.2 (69.3) | 12.0 (71.9) |
| Rpim | 5.7 (51.8) | 5.0 (47.4) | 4.5 (46.8) | 7.5 (54.3) |
| Multiplicity | 6.9 (6.3) | 6.9 (5.7) | 6.8 (5.7) | 6.8 (5.1) |
| Refinement | | | | |
| Resolution range (Å) | 38.3 - 1.57 | 39.9 - 1.64 | 40.0 - 1.65 | 38.4 - 2.18 |
| No. of reflections | 126,475 | 110,120 | 110,060 | 47,989 |
| Rwork / Rfree | 16.5 / 18.1 | 17.0 / 20.0 | 17.5 / 20.8 | 17.5 / 22.7 |
| No. atoms | | | | |
| Non-hydrogen | 7299 | 7499 | 7379 | 6925 |
| Protein | 6625 | 6612 | 6605 | 6593 |
| Ligand/ion | 132 | 182 | 144 | 122 |
| Water | 542 | 705 | 626 | 210 |
| Ramachandran favored (%) | 98.6 | 98.7 | 98.9 | 98.9 |
| Ramachandran outliers (%) | 0.0 | 0.0 | 0.0 | 0.0 |
| Wilson B | 17.7 | 15.3 | 18.2 | 27.5 |
| Average B (Å²) | 22.8 | 19.3 | 22.8 | 33.9 |
| Protein | 22.2 | 18.6 | 22.0 | 34.0 |
| Ligand/ion | 25.5 | 18.3 | 26.0 | 37.2 |
| R.m.s. deviations | | | | |
| Bond lengths (Å) | 0.011 | 0.011 | 0.013 | 0.014 |
| Bond angles (°) | 1.15 | 1.11 | 1.17 | 1.31 |

All data were collected on beamline 12-2 or 9-2 at the Stanford Synchrotron Radiation Lightsource. Values in parentheses are for the highest resolution shells.

\[ \text{R}_{\text{merge}} = \sum_{hhl} I_{hhl} - \langle I_{hhl} \rangle / \sum_{hhl} I_{hhl} \]

where \( I_{hhl} \) is the intensity of reflection \( hhl \) and \( \langle I_{hhl} \rangle \) is the mean intensity of related reflections.

\[ \text{R}_{\text{pim}} = \sum_{hhl} \sqrt{\frac{1}{n} - 1} I_{hhl} / \sum_{hhl} I_{hhl} \]

where \( n \) is the multiplicity of related reflections.

\[ R = \frac{\sum|F_o| - |F_e|}{\sum|F_o|} \]

where \( F_o \) is the observed structure factors and \( F_e \) is structure factors calculated from the model. 5% of the reflections were initially reserved to create an Rfree test set used during each subsequent round of refinement.
| Protein       | PDB Code | ASU<sup>a</sup> | Ordered Residues | Ligands              |
|--------------|----------|----------------|------------------|----------------------|
| PaODH-6PBM   | 6PBM     | 2              | Chain A: 6-431   | 2 NADP<sup>+</sup>   |
|              |          |                | Chain B: 7-431   |                      |
| PaODH-(S)-Pse| 6PBP     | 2              | Chain A: 7-431   | 2 NADP<sup>+</sup>   |
|              |          |                | Chain B: 7-431   | 2 (S)-Pse            |
| PaODH-(R)-Pse-1hr| 6PBN   | 2              | Chain A: 7-431   | 2 NADP<sup>+</sup>   |
|              |          |                | Chain B: 7-431   | 1 L-HisNA            |
|              |          |                |                  | 1 α-KG<sup>b</sup>   |
| PaODH-(R)-Pse-2hr| 6PBT   | 2              | Chain A: 7-431   | 2 NADP<sup>+</sup>   |
|              |          |                | Chain B: 7-431   | 1 L-HisNA            |

<sup>a</sup> Number of monomers in the asymmetric unit
<sup>b</sup> α-KG is α-ketoglutarate
Figure 1. ODH reaction and product stereochemistry. 

A) Generalized opine metallophore-forming opine dehydrogenase reaction that condenses HisNA and an α-keto acid forming a Schiff base that is reduced by NAD(P)H. 

B) Opine metallophores. The stereo centers derived from amino acids are labeled (L) or (D) to distinguish them from the stereocenter formed by the opine dehydrogenase which is labeled (S) or (R). Numbering for the carbons of (R)-pseudopaline is used to identify specific carbons in the structural studies presented below. 

C) Saccharopine dehydrogenase (PDB: 3UH1) contains two Rossmann-like fold domains. Characterized members of this enzyme class produce (S)-opine products. PDBeFold calculates a 4.8 Å rmsd over 28% of the Cα residues in comparison to PaODH-NADP⁺ (Panel D). 

D) Pseudopaline dehydrogenase (PaODH-NADP⁺, PDB: 6PBM) is structurally homologous with (R)-opine and not (S)-opine ODHs such as Nα-[1-(R)-(carboxyl)ethyl]-(L)-norvaline dehydrogenase (Panel E). 

E) Nα-[1-(R)-(carboxyl)ethyl]-(L)-norvaline dehydrogenase (PDB: 1BG6). Characterized members of this enzyme class produce (R)-opine products. PDBeFold calculates a 3.4 Å rmsd over 91% of the Cα residues, in comparison to PaODH-NADP⁺.
Figure 2. Steady-state kinetic plots for the reverse reaction. Final concentrations were 150 nM SaODH (A) or PaODH (B) and 112 µM NADP⁺ mixed with varied concentrations of (R)- or (S)-staphylopine or (R)- or (S)-pseudopaline. A) Dependence of the initial rate for (R)-staphylopine (blue, fit to Eq. 1) and (S)-staphylopine (red). Structural formulas for staphylopine accompany each plot. B) Secondary plots of initial rates for (R)-pseudopaline (blue, fit to Eq. 1) and (S)-pseudopaline (red). Structural formulas for pseudopaline accompany each plot.
Figure 3. PaODH structures with (S)-pseudopaline, L-HisNA and α-ketoglutarate bound. PaODH-(S)-Pse at 1.64 Å in panels A-C; chain B shown. Ligand density for PaODH-(R)-Pse-1hr at 1.65 Å in panels D and E. Ligand density for PaODH-(R)-Pse-2hr at 2.18 Å in panel F. (S)-Pseudopaline (green), NADP+ (yellow), L-HisNA (orange) and α-ketoglutarate (cyan). A) PaODH-(S)-Pse ribbon diagram. B) Active site cavity with a CASTp (computed atlas of surface topography of proteins) calculated surface (gray mesh). C) Overlay of PaODH-NADP+ (gray) and PaODH-(S)-Pse (purple). Pink dashed line is the distance between C4 of the nicotinamide ring and C2 of (S)-pseudopaline (both from PaODH-(S)-Pse). Yellow dashed lines indicate hydrogen bonding interactions. Electron density around (S)-pseudopaline and NADP+ is displayed as an mFo-DFc omit map contoured at 3.5 σ. D) PaODH-(R)-Pse-1hr. Ligands overlaid with a 2mFo-DFc electron density map contoured at 1.5 σ as seen following the final round of refinement (dark blue mesh). E) PaODH-(R)-Pse-1hr with an active site overlay with a mFo-DFc difference electron density map contoured at 3.0 σ (negative density is red, positive density is green). A Polder electron density map generated with L-HisNA and α-ketoglutarate omitted from the model and contoured at 4.5 σ is shown in light blue. F) PaODH-(R)-Pse-2hr. L-HisNA overlaid with a mFo-DFc omit map generated with L-HisNA omitted and contoured at 3.5 σ. There is no density for α-ketoglutarate in this structure.
Figure 4. Transient state kinetics of SaODH. The oxidation of NADPH to NADP⁺ was measured at 340 nm by stopped-flow spectrometry. Concentrations were 15 µM SaODH, 1000 µM pyruvate, 220 µM NADPH and varied D-HisNA in panel A. Concentrations were 15 µM SaODH, 500 µM D-HisNA, 160 µM NADPH and varied pyruvate in panel B. Colors indicate equivalent concentrations of substrate; black – 7.8 µM, green - 15.6 µM, red - 31.3 µM and blue - 62.5 µM. Inset is a secondary plot of $k_{obs}$ (s⁻¹) for the slow reaction phase. A) Varied D-HisNA; 7.8, 15.6, 31.3 and 62.5 µM. The global fit to model 1 overlays the data. B) Varied pyruvate; 3.9, 7.8, 15.6, 31.2, 62.4, 124.8, 250, 500 and 1000 µM.
Figure 5. Transient state kinetics for SaODH by fluorescence. Fluorescence emission measured beyond 360 nm cut-off filter. A) SaODH binding NADPH. Final concentrations in the stopped-flow cell were 15 µM SaODH mixed with varied NADPH concentrations in µM as shown. Data were fit to a one-step equilibrium binding model with a forward rate constant of $0.4 \times 10^6$ M$^{-1}$s$^{-1}$ and reverse rate constant of 27 s$^{-1}$; giving a $K_{NADPH}$ of 70 µM. B) Overlay of fluorescence binding and forward reaction data for equivalent enzyme and NADPH concentrations. 15 µM SaODH binding 240 µM NADPH (red trace) and 15 µM SaODH reacting with 500 µM D-HisNA, 1000 µM pyruvate and 240 µM NADPH (gray trace). The gray trace is the same as in panel C. C) SaODH reaction with varied NADPH. 15 µM SaODH reacted with 500 µM D-HisNA, 1000 µM pyruvate and varied NADPH concentrations as shown. Inset shows low concentrations proceeding as a single turnover up to 10 µM NADPH followed by a successive slowing of the progression toward equilibrium at higher NADPH concentrations. D) Comparison of forward reaction data by absorption and fluorescence using the same concentration of enzyme and reactants. 15 µM SaODH reacted with 1 mM pyruvate, 500 µM D-HisNA and 160 µM NADPH. Gray trace is the absorption data from figure 4B. Blue trace is fluorescence data from figure 5C. Dashed line indicates the transition from the fast single-turnover phase to the slow steady-state phase.
Figure 6. Transient state kinetics for the SaODH reverse reaction. Reverse reaction measured as an increase in fluorescence emission at 450 nm as NADP⁺ is reduced to NADPH. A) Varied NADP⁺. B) Varied (S)-staphylopine. Final concentrations in the stopped-flow cell were 15 µM SaODH mixed with 125 µM (R)-staphylopine (A) or varied (S)-staphylopine (B) and varied NADP⁺ (A) or 150 µM NADP⁺ (B). Every 10th data point shown for clarity. Both data sets fit simultaneously to model 1 by numerical integration in Kintek Studio 8.0 (lines).
Figure 7. Inhibition of SaODH reaction by metal ions. Transient state kinetics for SaODH measuring NADPH oxidation. Final concentrations in the stopped-flow cell were 15 µM SaODH, 180 µM NADPH and 1 mM pyruvate mixed with a final concentration of 100 µM D-HisNA. The black traces are 100 µM metal pre-incubated with 100 µM D-HisNA. The green traces are 25 µM (Co(II) or Ni(II)) or 50 µM (Zn(II) or Cu(II)) pre-incubated with D-HisNA. The blue traces are 25 µM (Co(II) or Ni(II)) or 50 µM (Zn(II) or Cu(II)) pre-incubated with SaODH. The red trace is 0 µM metal added. A) Co(II); 1 - Fast phase. 2 - Early steady-state phase. 3 - Late steady-state or inhibited phase. The black trace is fit to Eq. 3 and the red, blue and green traces are fit to Eq. 4. Every 10th data point shown as triangles for clarity. B) Ni(II). C) Zn(II). D) Cu(II).
Figure 8. Overlay of SaODH and PaODH ligand structures. A) Overlay of SaODH structures. Yellow is SaODH with (S)-staphylopine bound (green carbons) from PDB:6H3F. Gray is SaODH with D-histidine bound (yellow carbons) from PDB: 6H3D. Purple mesh is (S)-staphylopine $mF_o-DF_c$ omit map generated in phenix.polder and contoured at 3.5 $\sigma$ using 6H3F structure factors. Blue mesh is D-histidine $mF_o-DF_c$ omit map generated in phenix.polder and contoured at 3.5 $\sigma$ using 6H3D structure factors. The C1 carboxylate of the pyruvate moiety of (S)-Staphylopine is within hydrogen bonding distance of NADP$^+$. B) Overlay of SaODH and PaODH. Yellow is SaODH with (S)-staphylopine bound (green carbons) as for A. Purple is PaODH-(R)-PSE-1hr with L-HisNA (orange carbons) and $\alpha$-ketoglutarate bound (cyan carbons).

Scheme 1
Staphylopine and pseudopaline dehydrogenase from bacterial pathogens catalyze reversible reactions and produce stereospecific metallophores
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*J. Biol. Chem.* *published online October 15, 2019*

Access the most updated version of this article at doi: 10.1074/jbc.RA119.011059

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