

Flavin oxidation in flavin-dependent *N*-monooxygenases

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Abstract: Siderophore A (SidA) from *Aspergillus fumigatus* is a flavin-containing monooxygenase that hydroxylates ornithine (Orn) at the amino group of the side chain. Lysine (Lys) also binds to the active site of SidA; however, hydroxylation is not efficient and H₂O₂ is the main product. The effect of pH on steady-state kinetic parameters was measured and the results were consistent with Orn binding with the side chain amino group in the neutral form. From the pH dependence on flavin oxidation in the absence of Orn, a p*K*_a value >9 was determined and assigned to the FAD-N5 atom. In the presence of Orn, the pH dependence displayed a p*K*_a value of 6.7 ± 0.1 and of 7.70 ± 0.10 in the presence of Lys. Q102 interacts with NADPH and, upon mutation to alanine, leads to destabilization of the C4a-hydroperoxyflavin (FAD_{OOH}). Flavin oxidation with Q102A showed a p*K*_a value of ~8.0. The data are consistent with the p*K*_a of the FAD N5-atom being modulated to a value >9 in the absence of Orn, which aids in the stabilization of FAD_{OOH}. Changes in the FAD-N5 environment lead to a decrease in the p*K*_a value, which facilitates elimination of H₂O₂ or H₂O. These findings are supported by solvent kinetic isotope effect experiments, which show that proton transfer from the FAD N5-atom is rate limiting in the absence of a substrate, however, is significantly less rate limiting in the presence of Orn and or Lys.

ABBREVIATIONS: FAD, flavin adenine dinucleotide; Orn, ornithine; PDB, Protein Data Bank; SidA, ornithine N⁵-monooxygenase siderophore A; SKIE, solvent kinetic isotope effects.

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Short statement for broader audience: Iron-binding molecules, known as siderophores, are required for the pathogen *Aspergillus fumigatus* to scavenge iron during infection in humans. Siderophore A (SidA) is an enzyme that plays an essential role in the biosynthesis of siderophores. Oxidation of the flavin cofactor of SidA was probed using pH studies, site-directed mutagenesis, and kinetic solvent isotope effects. Results indicate that the N5-atom of the flavin cofactor is responsible for regulating the stability of the hydroxylating flavin intermediate and for oxidation. This information that is important for drug design.

Keywords: flavin-dependent monooxygenases; solvent kinetic isotope effect; pH profile; oxidation; siderophore; ornithine hydroxylase; hydroperoxyflavin

Introduction

Flavin-dependent monooxygenases that catalyze the hydroxylation of nitrogen atoms (*N*-monooxygenases, NMO) are important in the biosynthesis of isoxazolidinone rings and hydroxamate functional groups in siderophores.^{1,2} Because siderophores are essential virulence factors in bacterial and fungal pathogens, NMOs have been identified as potential drug targets.^{3,4} NMOs catalyze the NADPH- and oxygen-dependent hydroxylation of ornithine, lysine, histamine, and propyl or butyl-diamines.^{5,6} Siderophore A (SidA) from *Aspergillus fumigatus* is the most biochemically and structurally characterized member of this family of enzymes. The reaction begins with binding of NADPH to SidA with the FAD in its oxidized form (FAD_{OX}) [Fig. 1(A)].^{9,10} This is followed by stereospecific (*pro-R*) transfer of a hydride equivalent,

resulting in NADP^+ and reduced FAD (FAD_{red}), which completes the reductive half-reaction (Fig. 1).¹¹ In the oxidative half-reaction, the $\text{FAD}_{\text{red}}\text{NADP}^+$ complex reacts with molecular oxygen forming the C4a-hydroperoxyflavin (FAD_{OOH}) intermediate.^{7,12} In the absence of ornithine (Orn), this intermediate is stable and decays to hydrogen peroxide very slowly ($k_{\text{H}_2\text{O}_2} \sim 0.01 \text{ s}^{-1}$). However, in the presence of Orn, the enzyme is “activated” and rapid turnover is observed ($k_{\text{cat}} \sim 1.0 \text{ s}^{-1}$).⁷ Stabilization of the FAD_{OOH} in the absence of Orn ensures that oxidation of NADPH is coupled to the hydroxylation of Orn, thereby, preventing the production of H_2O_2 and the wasting of NADPH. The active site architecture provides high specificity for Orn, with almost 100% efficient hydroxylation by the FAD_{OOH} intermediate, with little or no H_2O_2 being released ($\sim 100\%$ coupled). SidA, however, can also bind lysine (Lys). This amino acid is hydroxylated with lower efficiency than Orn, with most of the FAD_{OOH} releasing as H_2O_2 ($\sim 10\%$ coupled).¹³

Previously, we studied the mechanism of formation and stabilization of FAD_{OOH} using a combination of rapid reaction kinetics, isotope effects, and density functional theory. These studies showed that NADP^+ plays several key roles in this process. First, NADP^+ is involved in the protonation of the distal oxygen of FAD_{OO} to form FAD_{OOH} via the 2'-OH of the nicotinamide ribose [Fig. 1(B)]. Second, NADP^+ provides binding interactions that are essential for stabilization of this intermediate, including hydrogen bonding of the NADP^+ amide carbonyl with the FAD_{OOH} -

N5-H and hydrogen bonding between the 2'-OH of the NADP^+ ribose and the distal oxygen of FAD_{OOH} . Third, NADP^+ allows Orn to be in proper orientation for hydroxylation [Fig. 1(B)].^{7,8,13}

Here, we present steady-state, rapid reaction kinetics, pH studies, and solvent kinetic isotope effects, focusing mainly on decay of FAD_{OOH} in the absence and presence of Orn or Lys. The data are consistent with Orn binding with the side chain amino group in the deprotonated form. Flavin oxidation in the absence of Orn substrate is very slow due to stabilizing interactions with the FAD-N5-H atom, which results in a pK_a value >9 . When Orn or Lys are present, the pK_a value decreases to ~ 7 and flavin oxidation no longer limits the catalytic cycle. We present a complete description of the oxidative half-reaction of SidA and briefly discuss previous studies that show conflicting results.

Results

k_{cat} and k_{cat}/K_m pH profiles

The pH profile of SidA activity under steady-state conditions was determined using oxygen consumption assays at a fixed concentration of NADPH (1 mM) and varying Orn. The data show that as the pH increases, the k_{cat} value of SidA slightly increases with a single pK_a value of 7.0 ± 0.2 [Fig. 2(A)]. The dependence of k_{cat}/K_m in Figure 2(B) shows a similar trend with increasing values as the pH increases with a single pK_a value of 8.0 ± 0.2 . The amplitude of the k_{cat}/K_m pH profile is greater than the k_{cat} pH profile because, as the pH increases, the apparent K_m for

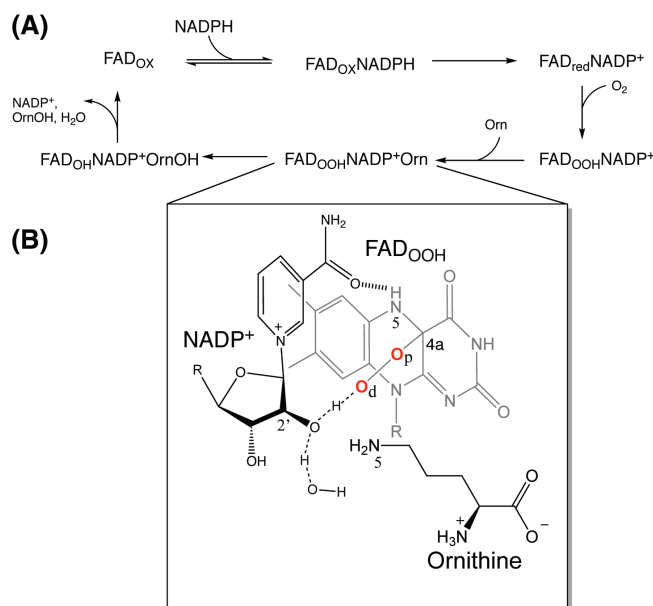


Figure 1. (A) Kinetic mechanism for the reaction catalyzed by SidA. The enzyme begins with the flavin in the oxidized form (FAD_{Ox}). NADPH binds and reduces the flavin (FAD_{red}). The reduced FAD/NADP⁺ complex reacts with oxygen, forming the C4a-hydroperoxyflavin (FAD_{OOH}). This is followed by Orn binding and hydroxylation at the Orn N5-atom. The next step is release of H_2O from the hydroxyflavin (FAD_{OH}). Release of products is shown to occur in one step. (B) Scheme of the FAD_{OOH} , NADP^+ , Orn complex obtained from previous molecular dynamics simulation and density functional theory studies.^{7,8}

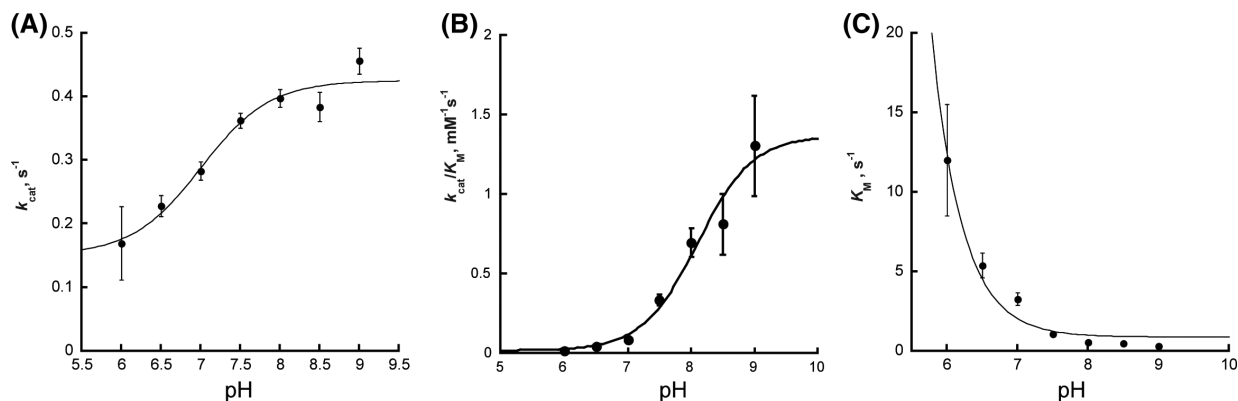


Figure 2. Effect of pH on the steady-state oxygen consumption activity of SidA. (A) Effect of pH on the k_{cat} value. (B) Effect of pH on the k_{cat}/K_m value. The pH profiles were analyzed using Eq. 3 and $\text{p}K_a$ values of 7.0 ± 0.2 and 8.0 ± 0.2 were obtained for k_{cat} and k_{cat}/K_m , respectively. (C) Changes in the K_m value as a function of pH. Values used in all plots are listed in Table S1. The line on panel C connects the points for viewing purposes.

Orn decreases ~39-fold across the pH range studied [Fig. 2(C), Table S1].

pH dependence of FAD_{OOH} formation

The pH dependence of the rate of formation of the FAD_{OOH} intermediate was determined in both the presence and the absence of Orn to determine if any ionizable groups contributed to oxygen activation in SidA. The presence of Orn enhanced the rate constant for formation of the FAD_{OOH} intermediate. However, the rate constant for FAD_{OOH} formation in the presence or absence of Orn did not significantly change as a function of pH, resulting in an almost flat pH profile, as previously observed by our group and others (Supporting Information Fig. S1).^{12,14}

pL dependence and SKIE for flavin oxidation in the absence of substrate

The pL profile for flavin oxidation in the absence of substrate was performed to calculate the $\text{p}K_a$ value and SKIE of flavin oxidation. This experiment reports on the pL dependence of H_2O_2 elimination from FAD_{OOH} . It is essential to calculate SKIEs in the pL-independent region as the $\text{p}K_a$ values of ionizable groups can change in D_2O . The data in the absence of substrate in Figure 3(A) show a single $\text{p}K_a$ value for flavin oxidation that is >9 in H_2O and a $\text{p}K_a$ value >10 in D_2O , where the rate constant for flavin oxidation increases as the pL increases. The $\text{p}K_a$ values could not be accurately determined as SidA is unstable at $\text{pH} > 10$ and the upper rate limit of flavin oxidation could not be defined. These data are consistent with previous experiments with SidA where a high $\text{p}K_a$ value was observed for flavin oxidation in the absence of a substrate.^{14,15} The kinetic traces at 452 nm occurs mainly in a single phase regardless of pH (not shown). To properly measure the SKIE, it is essential to perform the reaction in the region where the kinetic constant is pL independent. This is

necessary to prevent measuring an apparent SKIE that is due to the shift in the $\text{p}K_a$ value in D_2O . For example, in Figure 3(A) at pL of 8.5, a significant difference in the rate constant in D_2O vs. H_2O is observed. However, this is not a KIE, instead it is due to the shift in the $\text{p}K_a$ value. Therefore, the SKIE was calculated at pL 7.0 and a value of 2.30 ± 0.05 was determined. This is consistent with our previous study, where we had a pL of 7.5 (Table I).⁷

pL profile and SKIE for flavin oxidation with Orn

The pL profile for flavin oxidation in the presence of Orn (H_2O elimination from FAD_{OH}) was performed to determine the ionizable groups important for hydroxylation. The data in Figure 3(B) show a significant shift in the pL profile compared with that in the absence of substrate with $\text{p}K_a$ values of 6.7 ± 0.10 in H_2O and 7.18 ± 0.05 in D_2O . A SKIE value of 1.56 ± 0.14 was calculated from the upper limit of pL profiles (Table I).

pL profile and SKIE for flavin oxidation with Lys

SidA is selective for hydroxylation of Orn. Although Lys binds to the active site, hydroxylation is not efficient, and the main outcome is the stimulation of hydrogen peroxide release from FAD_{OOH} .^{9,16} The pL profile of flavin oxidation with Lys was performed to determine if its presence alters the $\text{p}K_a$ value and the SKIE associated with flavin oxidation. Results show a pL profile with $\text{p}K_a$ values of 7.70 ± 0.10 in H_2O and 8.42 ± 0.07 in D_2O [Fig. 3(C), Table I]. A SKIE value of 1.10 ± 0.06 was also calculated from the upper limit of the fits at high pL (Table I).

Characterization of Q102A

The reaction of Q102A with NADPH was characterized in a stopped flow spectrophotometer as previously described.^{9,17} The same experiment was performed on the wild-type enzyme for comparison.

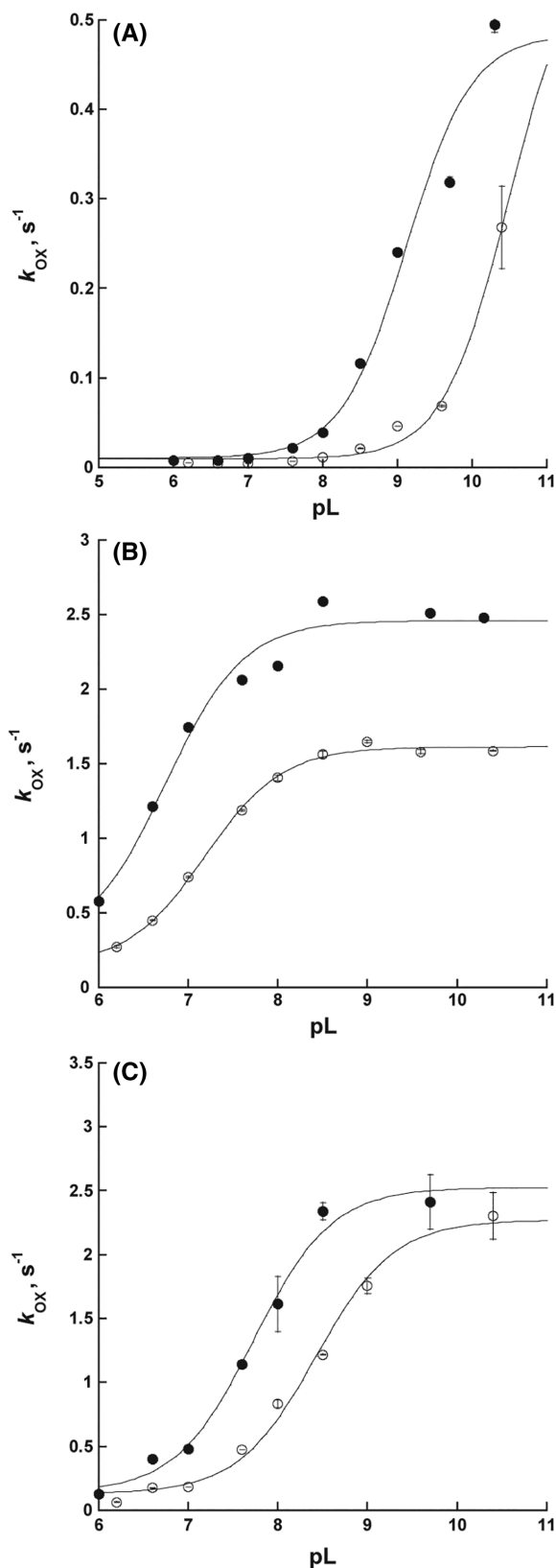


Figure 3. Rate constant for flavin oxidation (k_{OX}) of SidA monitored at 452 nm as a function of pL in the absence or presence of amino acid substrates in H_2O (●) or D_2O (○). (A) Changes in the oxidation rate constant as a function of pH in the absence of amino acid substrate (H_2O_2 elimination). (B) In the presence of Orn (100 mM). (C) In the presence of 15 mM Lys.

Reduction occurred in two phases. A fast phase corresponding to flavin reduction and a slow phase that is independent of NADPH concentration has been suggested to be product release or a subpopulation of slow enzyme.^{11,16} The affinity for NADPH is high, which prevents accurate calculation of the K_D value using the stopped-flow (changes in flavin absorbance are very small at low concentrations of NADPH). Thus, the reaction and apparent binding affinity for NADPH are not negatively affected in the Q102A enzyme (Table S2). The steady-state kinetic parameters for Q102A were determined by monitoring the rate of oxygen consumption and the formation of hydroxylated Orn, as previously described.^{9,17} The oxygen consumption for Q102A was determined using Orn as the variable substrate at fixed saturated concentrations of NADPH. The k_{cat} value was ~45% higher than the value for SidA, while the K_m value was ~30-fold lower. This is an apparent K_m value as the enzyme was highly active in the absence of Orn, indicative of high oxidase activity. The steady-state analysis of Orn hydroxylation as a function of Orn concentrations under saturating concentrations of NADPH resulted in a k_{cat} value of 0.32 s^{-1} , which is ~50% lower than for SidA. The lower k_{cat} value for hydroxylation compared with the value obtained in the oxygen consumption assay indicates that the mutant protein is only ~37% coupled compared with ~100% for SidA (Table II). The low coupling in Q102A indicated that this enzyme is not efficient in stabilizing the FAD_{OOH} .

pH profiles for flavin oxidation with Q102A

Having established that the main effect of Q102A is on coupling, we measured the pH profile of the elimination of H_2O_2 from the FAD_{OOH} (e.g., in the absence of Orn). The increase in absorbance at 452 nm showed a single exponential rise, which was analyzed as described above with SidA. The results are shown in Figure 4 along with the pH profile for SidA for comparison. In the absence of Orn, k_{OX} increases as a function of pH with a pK_a value of 8.55 ± 0.05 , which is lower than for the wild-type enzyme. In the presence of Orn, k_{OX} also increases as a function of pH with a pK_a value of 6.4 ± 0.2 , which is very similar to the value obtained for SidA (Table I).

Discussion

Flavin-dependent monooxygenases are important for adding chemical diversity to natural products as they are responsible for hydroxylating aromatic rings, aliphatic chains, sulfur, and nitrogen atoms.^{6,18} In addition, the formation of epoxides, esters, or lactones are also outcomes of flavin-dependent monooxygenation reactions.^{19–21} Our research group has been interested in the function of flavin-dependent monooxygenases involved in the biosynthesis of hydroxamate-containing siderophores.^{7–9,11,13,22–25} In this work, we

Table I. Observed pK_a Values and Solvent Kinetic Isotope Effect Values for SidA Oxidation

| Parameter | No substrate | Orn | Lys |
|-----------------------------|------------------|--------------------------|--------------------------|
| pK_a , H ₂ O | >9 | 6.7 ± 0.10 | 7.70 ± 0.10 |
| pK_a , D ₂ O | >10 | 7.18 ± 0.05 | 8.42 ± 0.07 |
| k_{ox} , H ₂ O | 0.0100 ± 0.00020 | 2.50 ± 0.07 | 2.52 ± 0.10 |
| k_{ox} , D ₂ O | 0.0043 ± 0.00010 | 1.60 ± 0.02 | 2.30 ± 0.08 |
| SKIE | 2.30 ± 0.05* | 1.56 ± 0.14 [†] | 1.10 ± 0.06 [†] |

All pL experiments were performed at 25°C.

* The SKIE values for flavin oxidation with no substrate were calculated at pL 7.0.

[†] The SKIE values for flavin oxidation with Orn and Lys were calculated using the upper limits obtained from the fits of the pL profiles.

were interested in determining whether functional groups with specific protonation states were required for the reaction catalyzed by SidA. The pH profile of k_{cat}/K_M showed that an ionizable group needs to be deprotonated for activity with a pK_a value of ~8.0. We focused on the k_{cat}/K_M pH profile, since it reports on the protonation states of the free enzyme and substrate.²⁶ The main effect observed was a decrease in the K_M value for Orn as the pH of the solution was increased [Fig. 2(C)]. Thus, as an ionizable group becomes deprotonated, Orn binding becomes favorable. The structure of SidA in complex with Orn revealed that Lys107, Asn105, Ser469, and Asn323 are in hydrogen bonding distance to Orn (Fig. 5).¹³ Site-directed mutagenesis showed that these residues are important for Orn binding.¹⁷ Asn105, Ser469, and Asn323 do not contain a side chain with a pK_a value close to ~8.0. The pK_a value of ~10.5 for the side chain of Lys107 could be lowered in the active site; however, the K_m value for Orn would increase as Lys107 becomes deprotonated. Therefore, the ionizable group that needs to be deprotonated for Orn binding does not appear to belong to SidA.

Alternatively, the ionizable group could be assigned to one of the amino groups of Orn. Although the pK_a value of both groups is ~9, higher than the values observed in the k_{cat}/K_M pH profile, it has been well established that flavoproteins' active sites modulate the protonation states of substrates to facilitate the chemical steps. For instance, *p*-hydroxybenzoate hydroxylase (PHBH) modulates the protonation state of the *p*-hydroxyl group such that it becomes deprotonated upon binding. This deprotonation is important

to facilitating the nucleophilic aromatic hydroxylation.^{28,29} Another well-established modulation of the protonation state of the substrate is the case of amino acid oxidases. It has been shown that the amino acid binds with the α -amino group in the deprotonated form, where deprotonation facilitates hydride transfer to the flavin.³⁰ Thus, it is not unreasonable that SidA is capable of decreasing the pK_a of the N^6 -Orn atom so that it binds in the deprotonated form. Previous computational studies have suggested that binding of Orn with the side chain in the deprotonated neutral form is more favorable.^{7,8} Another ionizable group present in Orn is the α -amino group. However, this group hydrogen bonds with Asn293 and deprotonation is not required for this interaction to occur (Fig. 5).^{13,17} Our conclusions are different from that of Fredrick et al., who concluded that Orn binds in the protonated form and then becomes deprotonated in the ES complex. Their conclusion was mainly based on the pH profile of flavin oxidation that displayed a pK_a value of ~7, which they assigned to Orn. However, as we will discuss below, we believe that this pK_a value reports on the protonation state of the FAD-N5 atom instead.

The effect of pH on flavin oxidation in SidA was also studied. In this case, the FAD_{red}:NADP⁺ complex reacts with molecular oxygen to form the FAD_{OOH} intermediate, which decays to FAD_{ox} and H₂O₂ if Orn is absent. Hydroxylation occurs in the presence of Orn and H₂O is eliminated to form FAD_{ox}. The effect of pH on the formation and decay of the FAD_{OOH} intermediate was determined first. We observed that formation of the FAD_{OOH} is pH independent in the

Table II. Steady-State Kinetic Characterization

| Parameter | SidA | Q102A |
|--|--------------------------|---------------|
| | Oxygen consumption assay | |
| k_{cat} , s ⁻¹ | 0.59 ± 0.01 | 0.86 ± 0.03 |
| K_m (Orn), mM | 1.1 ± 0.3 | 0.035 ± 0.02 |
| k_{cat}/K_m (Orn), M ⁻¹ s ⁻¹ | 500 ± 100 | 24,700 ± 1400 |
| | Orn hydroxylation assay | |
| k_{cat} , s ⁻¹ | 0.62 ± 0.02 | 0.32 ± 0.04 |
| K_m (Orn), mM | 1.0 ± 0.2 | 0.64 ± 0.03 |
| k_{cat}/K_m (Orn), M ⁻¹ s ⁻¹ | 600 ± 100 | 510 ± 20 |
| Coupling, % | 105 ± 4 | 37.2 ± 0.4 |

Conditions: 100 mM sodium phosphate, pH 7.5, and 25°C.

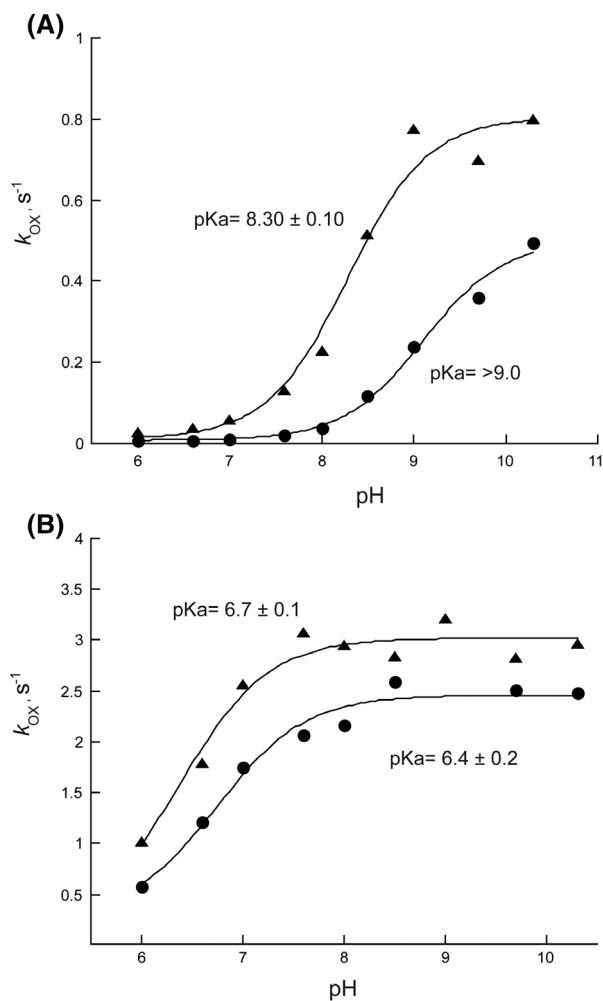


Figure 4. Effect of pH on the oxidation of Q102A (●) in the absence of Orn (A) or in the presence of 100 mM Orn (B). In both panels, SidA (▲) is shown for comparison.

absence of Orn, whereas the presence of Orn enhanced the rate constant for formation of the FAD_{OOH} intermediate. However, this effect is also pH independent, as previously shown (Supporting Information Fig. S1).^{14,15} This is also consistent with our previous work that showed that this enhancement is independent of the amino group of the side chain of Orn, since norvaline (which lack an amino group in the side chain) can also enhance the formation of FAD_{OOH} , albeit to a lesser extent.⁷

In contrast, decay of FAD_{OOH} in the absence of Orn was very slow at low pH values and accelerated as the pH of the solution was increased. A pK_a value above 9 could only be approximated from our data (Fig. 3 and Table I). It has been proposed that stabilization of the FAD_{OOH} intermediate is mediated by hydrogen bonding interactions with the FAD-N5-H atom, as this atom is released with H_2O_2 or H_2O during oxidation.^{31,32} We have shown that the carbonyl oxygen of the nicotinamide moiety of NADP^+ in SidA plays a key role in the stabilization of this intermediate (Fig. 1).¹³ Thus, the pK_a value might be assigned

to the FAD-N5-H atom, which is tuned to prevent elimination of H_2O_2 in the absence of Orn. pH studies in other monooxygenase systems have shown similar high pK_a values, which have also been assigned to the FAD-N5-H atom.³² We then measured the effect of pH on decay of FAD_{OOH} in the presence of Orn or Lys. Here, the amino acid binds either to the $\text{FAD}_{\text{red}}:\text{NADP}^+$ or the $\text{FAD}_{\text{OOH}}:\text{NADP}^+$ complex in a rapid equilibrium process.^{7,16} Hydroxylation of Orn takes place and the FAD_{OH} intermediate is formed. This is followed by elimination of H_2O , which yields the oxidized FAD_{ox} . A pK_a value of 6.7 was obtained from the k_{OX} pH profile, indicating that a group in the ES complex needs to be deprotonated for optimal activity (Fig. 3 and Table I). This pK_a value is very similar to the pK_a value of 7.0 obtained from the k_{cat} pH profile, which reports ionizable groups in the enzyme-substrate complex.²⁶ In the presence of Lys, where hydroxylation occurs less than 10% of the time, a pK_a value of ~ 7.7 was measured. We believe that this pK_a value is for the $\text{FAD}_{\text{OH}}\text{-N5-H}$, which is tuned to a lower value to accelerate turnover. SKIE was used as an indirect measure of the relative strength of the FAD-N5-H bond. In the absence of Orn, the SKIE value is 2.3, suggesting that elimination of the step that involves the hydrogen atom equivalent from the FAD-N5 is significantly rate limiting. The SKIE decreases to 1.6 in the presence of Orn (Table I) and further decreases to a value of 1.1 when Lys is present, suggesting that breaking of the FAD-N5-H bond is facilitated in these cases. It is worth noticing that a previous work reported a SKIE value >6 with Lys. This high value most likely originated from performing the assay in the pH sensitive region of the curve and not in the pH independent region.¹⁴

Evidence of modulation of the FAD-N5-H atom comes from studies on flavin-dependent oxidases, where flavin oxidation appears to be pH independent or more likely that the pK_a value is much lower than what can be tested *in vitro* (possibly lower than 5). The decrease in the pK_a value of FAD-N5-H allows

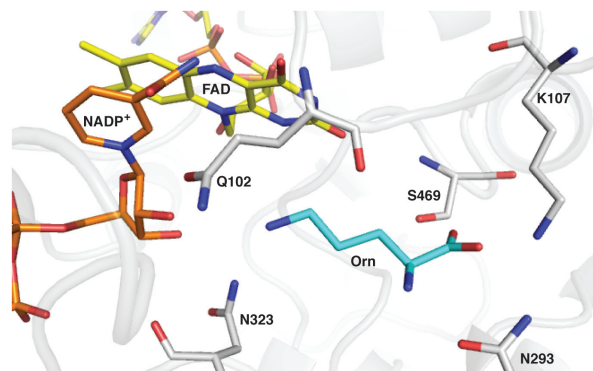


Figure 5. Active site of SidA showing the FAD (yellow carbons), NADP^+ (orange carbons), and Orn (cyan carbons) (PDB code 4B63). The figure was made using PyMol.²⁷

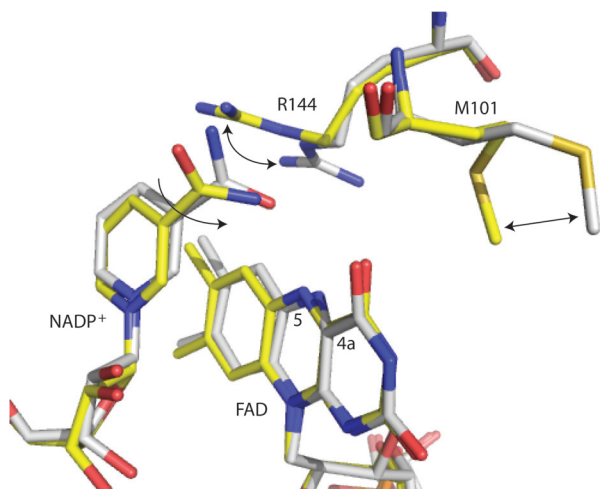


Figure 6. Observed conformational changes in SidA upon reduction. The oxidized form is shown with yellow carbons (PDB code 4B63) and the reduced form with white carbons (PDB code 4B65). The only observed changes are the rotation of residues R144 and M101, which are near the N5-C4a. Typical bending of the FAD in the reduced form is also observed. Rotation of the amide bond is predicted based on the hydrogen bonding interactions with the oxidized flavin (where the amino group acts as a hydrogen bond donor) or reduced flavin (where the carbonyl oxygen acts as a hydrogen bond acceptor).

for the fast elimination of H_2O_2 in oxidases.^{33,34} To provide further evidence that the $\text{p}K_{\text{a}}$ value of the $\text{FAD}_{\text{OOH}}\text{-N5-H}$ intermediate is decreased from >9 to facilitate elimination of H_2O or H_2O_2 , we performed the pH profile of Q102A. In this mutant, interactions with FAD-N5 have been changed, leading to destabilization of the FAD_{OOH} intermediate and, thus, the elimination of H_2O_2 , as suggested by only 37% coupling (Table II). The pH profile of flavin oxidation in the absence of Orn shows a clear shift, with a lower $\text{p}K_{\text{a}}$ value (~ 8.5). These results suggest that in the absence of Orn, the hydrogen bonding network that tunes the $\text{p}K_{\text{a}}$ value of the $\text{FAD}_{\text{OOH}}\text{-N5-H}$ atom to >9 in SidA is changed in the Q102A mutant, presumably by changing the position of NADP^+ . These changes result in a lower $\text{FAD}_{\text{OOH}}\text{-N5-H}$ $\text{p}K_{\text{a}}$ value and, thus, a more facile elimination of H_2O_2 . Binding of Orn and formation of product leads to further changes in the hydrogen bonding of the $\text{FAD}_{\text{OH}}\text{-N5-H}$, which further decreases the $\text{p}K_{\text{a}}$ value to ~ 6.4 (compared with 6.7 for the wild-type). Computational studies of elimination of H_2O_2 or H_2O from FAD_{OOH} have shown the requirement of a proton transfer network.³⁵ Analysis of the structure of SidA: NADP^+ in the oxidized form and SidA: NADP^+ that had been reduced by soaking the crystal in dithionite only show changes in the position of the side chains of R144 and M101. These residues are located above the FAD-N5-H locus and might be involved in the hydrogen binding network that tunes the $\text{p}K_{\text{a}}$ atom, presumably mediating with water molecules (Fig. 6).

The results presented here show that Orn binds with the N5-atom in the deprotonated form, which is required for the activity. The active site of SidA tunes the FAD-N5-H for optimal catalysis. The $\text{p}K_{\text{a}}$ value >9 in the absence of Orn stabilizes the FAD_{OOH} . Binding and hydroxylation of Orn leads to changes in the active site that decrease the FAD-N5-H $\text{p}K_{\text{a}}$ value to ~ 7 .

Materials and Methods

Materials

Buffers and media were obtained from Fisher Scientific (Pittsburgh, PA). BL21(DE3)-T1^R chemically competent cells were obtained from Sigma-Aldrich (St. Louis, MO). NADPH was obtained from EMD4 Biosciences (Billerica, MA). D_2O was obtained from Cambridge Isotope Laboratories (Tewksbury, MA). Chromatography columns were obtained from GE Healthcare. All reagents were used without further purification.

Protein expression and purification

Wild-type SidA (SidA) and Q102A were expressed in *Escherichia coli* BL21(DE3)-T1^R cells and purified as previously described.¹³ In general, ~ 25 mg of protein was obtained per liter of media. The purified proteins were stored in 30 μL aliquots in 100 mM sodium phosphate and 50 mM NaCl, pH 7.5, at -80°C at a concentration of ~ 200 μM . Protein concentration was calculated using the extinction coefficient at 450 nm of 13700 $\text{M}^{-1} \text{cm}^{-1}$ for the FAD bound.⁹

pH effects on steady-state kinetic parameters

The rate of oxygen consumption was measured using a Hansatech Oxygraph system (Norfolk, England). Assay solutions consisted of 1 mL of 100 mM sodium phosphate (pH 6–8) and 100 mM Tris- SO_4 (pH 8.5–9.0) at 25°C . NADPH was kept constant at a concentration of 1 mM while Orn was varied between 0.1 and 10 mM. Reactions were initiated by the addition of 2 μM SidA and monitored for 5 min with constant stirring.

pL (pH or pD) effects on flavin oxidation

All rapid reaction experiments were carried out at 25°C using an SX-20 stopped-flow spectrophotometer (Applied Photophysics, Leatherhead, UK) in an anaerobic glove box (Coy, Grass Lake, MI). The preparation of anaerobic buffer was carried out with five cycles of vacuum (5 min) and flushing with O_2 -free argon (0.5 min) with continuous stirring. This was repeated five times. The enzyme was made anaerobic with 10 cycles of vacuum (2 min) and flushing with O_2 -free argon (10 s). The stopped-flow was made anaerobic by flushing with 1 mL of anaerobic 100 mM sodium acetate, pH 5.0, containing 100 mM D-glucose and 100 $\mu\text{g}/\text{mL}$ glucose oxidase Type-X. Substrates were made anaerobic by dissolving the appropriate mass in anaerobic buffer inside the glove box.

The rate constant of flavin oxidation (k_{OX} , monitored at 452 nm) as a function of pH was measured in double mixing mode. Anaerobic SidA (60 μM before mixing) was first mixed with an equal volume of NADPH (60 μM before mixing) in 20 mM Tris-Cl, 200 mM NaCl, pL 8.0 [pL refers to either the pH (H_2O) or the pD (D_2O)]. This mixture was incubated in an ageing loop for 60 s until the bound flavin was fully reduced. The reduced SidA–NADP⁺ complex was then allowed to react with air saturated buffer (130 μM oxygen after mixing at 1 atm and 25°C [O_2] = 260 μM). This air-saturated solution contained 200 mM of buffer, which rapidly increased the pL to the desired value in the flow cell. Between pL values of 6 and 8, sodium phosphate was used, between pL values of 8.5–9.0, Tris-SO₄ was used, and between pL values of 9.5–10.5, sodium carbonate/bicarbonate was used. In pL experiments with different amino acids present, 100 mM Orn and 15 mM Lys were used. For the reactions in D_2O , SidA was concentrated to ~400 μM (based on flavin content) and diluted to 60 μM in a 100% D_2O buffer of 20 mM Tris-Cl, 200 mM NaCl, pD 8.0. This gave a concentration of ~85% D_2O and, after two mixes in the stopped-flow with 100% D_2O buffer, a final D_2O concentration of ~96% was achieved. The proper pD of all D_2O buffers was calculated by adding 0.4 to the value on the pH meter, which is the variation from the change in the equilibrium on a hydrogen selective glass electrode.³⁶ All solutions were checked for their proper pL values with a Fisher Scientific Accumet AB15+ Basic pH meter. Spectra were taken on a logarithmic time scale until complete flavin oxidation was observed. The rate constants were calculated from the average of at least three experiments.

Site-directed mutagenesis

Replacement of Gln at Position 102 to Ala was performed using the QuikChange (Agilent Technologies, Santa Clara, CA) method following manufacturer's instructions. The wild-type SidA gene, subcloned into the pET15b plasmid, was used as the template.¹¹ The reaction was done using the forward primer (5'-CCGGGCTCGAAGATGGCTATCAGCTTCATCAAG-3') and reverse primer (5'-CTTGATGAAGCTGATAGC-CATCTTCGAGCCCGG-3'). Mutations were confirmed by DNA sequencing at the Virginia Biocomplexity Institute Core Sequencing Facility.

Characterization of Q102A

Steady-state kinetic parameters were obtained by monitoring the reaction with oxygen using a Hansatech Oxygraph System (Norfolk, England). Reactions consisted of a 1 mL volume of 100 mM sodium phosphate, pH 7.5, at 25°C. NADPH was kept constant at a concentration of 1 mM while Orn was varied between 0.1 and 15 mM. Reactions were initiated by addition of 2 μM of Q102A. Reactions were monitored for 5 minutes

with constant stirring. Hydroxylated Orn was monitored using a variation of the Csaky iodine oxidation assay.^{22,37} The standard assay buffer contained 104 μL of 100 mM sodium phosphate (pH 7.5) with varying concentrations of Orn and NADPH held constant at 1 mM. Reactions were initiated by addition of 2 μM Q102A. Reactions were incubated for 10 min at 25°C with constant stirring at 750 rpm. The rates of flavin reduction with NADPH measured in the stopped-flow spectrophotometer were done as previously described.^{11,38,39} The effect of pH on flavin oxidation in the absence or presence of Orn were performed as described above for wild-type enzyme.

Data analysis

All data were fit using KaleidaGraph (Synergy Software, Reading, PA). For flavin oxidation studies, the increase in absorbance at 372 nm corresponding to formation of the FAD_{OOH} intermediate, was fit to Eq. 1 and describes a single exponential rise. Flavin oxidation in the absence of substrate, measured at 450 nm, was also fit to Eq. 1. In the presence of Orn or Lys the increase in absorbance at 452 nm, was fit to Eq. 2, which describes a double exponential rise. In both of these equations, c is the absorbance value at the beginning of the experiment, a is the total absorbance change, and k_x is the rate constant for the formation of FAD_{OOH} (Eq. 1) or flavin oxidation (Eq. 2) at a particular pH value:

$$v = c + a \left(1 - e^{-(k \times t)} \right) \quad (1)$$

$$v = c + a_1 \left(1 - e^{-(k_1 \times t)} \right) + a_2 \left(1 - e^{-(k_2 \times t)} \right) \quad (2)$$

The pL dependence of flavin oxidation (monitored at 452 nm) and the pH dependences of k_{cat} and k_{cat}/K_m were fit to Eq. 3, which describes a curve with a single $\text{p}K_a$ value with increasing activity as the pL increases and with plateau regions at high and low pL. The upper limits and lower limits for the pL profiles are denoted by C and A , respectively. The solvent kinetic isotope effects were calculated by dividing the value obtained for C in H_2O by the value obtained in D_2O :

$$y = \frac{C + A \left(10^{(\text{p}K_a - \text{pL})} \right)}{1 + 10^{(\text{p}K_a - \text{pL})}} \quad (3)$$

Supporting Information.

Table S1, Figure S1, Table S2.

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