



## Dual role of NADP(H) in the reaction of a flavin dependent N-hydroxylating monooxygenase<sup>☆</sup>

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### ABSTRACT

*Aspergillus fumigatus* siderophore A (*Af SidA*) is a flavin-dependent monooxygenase that catalyzes the hydroxylation of ornithine, producing *N*<sup>5</sup>-hydroxyornithine. This is the first step in the biosynthesis of hydroxamate-containing siderophores in *A. fumigatus*. *Af SidA* is essential for virulence, validating this enzyme as a drug target. *Af SidA* can accept reducing equivalents from either NADPH or NADH and displays similar kinetic parameters when using either coenzyme. When the enzyme is reduced with NADPH and reacted with molecular oxygen, a C4a-hydroperoxyflavin intermediate is observed. When the enzyme is reduced with NADH, the intermediate is 2-fold less stable. Steady-state kinetic isotope effect values of 3 and 2 were determined for NADPH and NADH, respectively. The difference in the isotope effect values is due to differences in the rate of flavin reduction by these coenzymes. A difference in the binding mode between these coenzymes was observed by monitoring flavin fluorescence. Limited proteolysis studies show that NADP<sup>+</sup>, and not NAD<sup>+</sup>, protects *Af SidA* from proteolysis, suggesting that it induces conformational changes upon binding. Together, these results are consistent with NADPH having a role in flavin reduction and in the modulation of conformational changes, which positions NADP<sup>+</sup> to also play a role in stabilization of the C4a-hydroperoxyflavin.

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### 1. Introduction

Fungi from the genus *Aspergillus* cause deadly infections, such as allergic bronchopulmonary aspergillosis (ABPA) and invasive pulmonary aspergillosis (IPA), in immunocompromised persons such as those suffering from cystic fibrosis, HIV/AIDS, and patients undergoing organ transplant [1]. In addition, patients in intensive care units are at a higher risk of *Aspergillus* infections [2,3] and the mortality rate has been reported to be as high as 50% [2,4]. Thus, more efficacious treatment of infections by this eukaryotic pathogen is necessary. A possible mechanism to prevent microbial pathogen proliferation during infections is to block the intake of essential nutrients. Iron is an essential nutrient that is present at low concentrations due to its poor solubility in water. In humans, the concentration of free iron is further decreased

due to the action of iron-binding proteins [5]. To establish infection, *Aspergillus* must obtain iron from the mammalian host, which is accomplished through the synthesis, secretion, and re-uptake of iron-binding molecules known as siderophores [4,6].

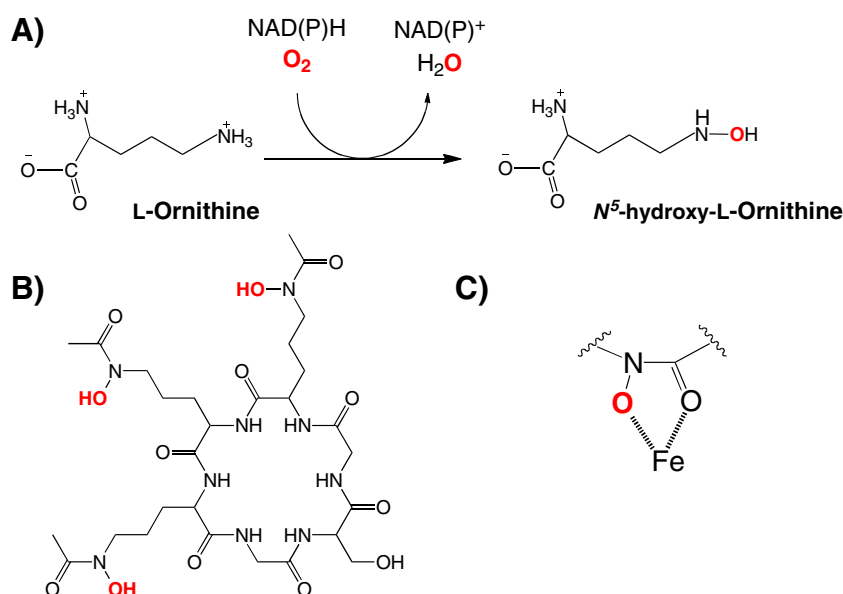
The siderophore, ferricrocin, is an important virulence factor in *Aspergillus fumigatus* and *Aspergillus nidulans* [7,8]. This siderophore contains hydroxamate functional groups that form the iron-binding site. *A. fumigatus* siderophore A (*Af SidA*) is a flavin-dependent enzyme that catalyzes the NAD(P)H- and oxygen-dependent hydroxylation of the amino group on the side chain of ornithine, forming the hydroxamate functional group in the mature ferricrocin (Scheme 1) [7,9]. Deletion of the *Af sidA* gene results in mutant *A. fumigatus* that are unable to cause infection in mice, suggesting that this enzyme is an attractive drug target since its function is essential for virulence [7].

*Af SidA* is a member of the Class B flavin-dependent monooxygenase family of enzymes [10]. These enzymes utilize reducing equivalents from either NADH or NADPH to reduce the flavin cofactor. The reduced flavin then reacts with molecular oxygen forming a C4a-hydroperoxyflavin, which is required for hydroxylation. Here, we present kinetic, spectroscopic, and biochemical data that show that in this flavin-dependent monooxygenase, NADP(H) plays a dual role in flavin reduction and stabilization of flavin intermediates.

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**Scheme 1.** A) Reaction catalyzed by *Af SidA*. B) Structure of ferricrocin. C) The hydroxylation product of *Af SidA* forms part of the iron binding hydroxamate functional group in *A. fumigatus* siderophores.

## 2. Materials and methods

### 2.1. Materials

Glucose oxidase from *Aspergillus niger* (type X-S), alcohol dehydrogenase from *Thermoanaerobium brockii*, formate dehydrogenase from *Candida boidinii*, NADP<sup>+</sup>, NAD<sup>+</sup>, protease inhibitor cocktail, DNase I, RNase, and lysozyme were purchased from Sigma-Aldrich (St. Louis, MO). NADPH and NADH were purchased from MP Biomedicals (Solon, OH) and Acros Organics (Pittsburgh, PA), respectively.

### 2.2. Protein expression and purification

*Af SidA* was expressed as a fusion to maltose binding protein (His<sub>8</sub>-MBP) as previously described [9]. Cells containing recombinant His<sub>8</sub>-MBP-*Af SidA* were resuspended in buffer A (25 mM HEPES, 300 mM NaCl, 25 mM imidazole, pH 7.5) and incubated at 4 °C in the presence of protease inhibitor cocktail, DNase I, RNase, and lysozyme (25 µg/mL each). All subsequent steps were performed at 4 °C. Cells were lysed by sonication and the resultant lysate was clarified by centrifugation (34,500 g for 45 min). The clarified lysate was loaded onto a HisTrap column (GE Healthcare) equilibrated with buffer A. The column was washed with buffer A and *Af SidA* was eluted with a 200 mL imidazole gradient (25–300 mM) at a flow rate of 5 mL/min. Fractions containing His<sub>8</sub>-MBP-*SidA* were pooled, then diluted with buffer A and concentrated to a 50 mL volume using an Amicon stirred cell concentrator (Billerica, MA). *Af SidA* was liberated from the His<sub>8</sub>-MBP tag by an overnight incubation with the addition of tobacco etch virus (TEV) protease (approx. 0.1 U/mg *SidA*). After cleavage, the protein solution was centrifuged (34,500 g for 15 min) to remove traces of precipitate. The resulting sample was loaded onto a HisTrap column equilibrated with buffer A. In this step, the His<sub>8</sub>-MBP remains bound to the column and the free *Af SidA* elutes in the flow-through. The protein was pooled and concentrated to a 2 mL volume using a stirred cell concentrator. After removal of precipitate by centrifugation, the sample was loaded onto a Superdex S-200 column (GE Healthcare) equilibrated with 25 mM HEPES, 125 mM NaCl, pH 7.5, and eluted at a flow rate of 2 mL/min. *Af SidA* concentration was measured using the bound flavin extinction

coefficient ( $\epsilon_{450} = 13,700 \text{ M}^{-1} \text{ cm}^{-1}$ ). Aliquots were frozen in liquid nitrogen prior to storage at  $-80 \text{ }^{\circ}\text{C}$ .

### 2.3. Synthesis of deuterated reduced dinucleotides

[4R-<sup>2</sup>H]NADPH (NADPD) and [4R-<sup>2</sup>H]NADH (NADD) were prepared with alcohol dehydrogenase from *T. brockii* and formate dehydrogenase from *C. boidinii*, respectively [11]. Control samples of NADPH and NADH were prepared with an equivalent procedure to that used for the deuterated samples. On the basis of an A<sub>260</sub>/A<sub>340</sub> ratio of 3.0 and 2.6, the purity of NADPD and NADD was estimated to be 90 and 100%, respectively.

### 2.4. Oxygen consumption assay

The oxidation of the reduced flavin was monitored by directly measuring the rate of oxygen consumption. The assays were performed on a Hansatech oxygen monitoring system (Norfolk, England). The activity was initiated by addition of 2 µM of *Af SidA* to a final volume of 1 mL of 100 mM potassium phosphate, pH 7.5. Concentrations of ornithine and reduced nicotinamide were varied from 0.1 to 15 mM and 0.01 to 3 mM, respectively.

### 2.5. Detection of flavin intermediates

In studies of the reaction of *Af SidA* with reduced coenzymes under aerobic conditions, 100 mM potassium phosphate, pH 7.5 was used as the buffer. The spectra were recorded on an Agilent 8453 spectrophotometer (Santa Clara, CA). *Af SidA* was then added to the solution and a spectrum of the oxidized enzyme was recorded. The reaction was then initiated by the addition of 1.5-fold excess of NADPH or NADH. The spectra were recorded every 0.5 s for 6 min.

### 2.6. Rapid reaction kinetics

Anaerobic reduction of *Af SidA* was carried out at 15 °C using the single-mixing mode of a SX20 stopped-flow apparatus (Applied Photophysics, UK) installed in an anaerobic glove box. The reaction mixtures contained 14 µM of enzyme and varied concentrations of either NADPH

or NADH (100 and 50–950  $\mu\text{M}$ , respectively). To study substrate kinetic isotope effects, the same concentrations of the deuterated dinucleotides were used as reductants. All solutions were prepared in anaerobic 100 mM potassium phosphate buffer, pH 7.5. The buffer (250 mL) was made anaerobic by repeated cycles of vacuum and flushing with oxygen free argon for 5 h at room temperature, followed by overnight incubation in the glove box. The same procedure was performed for 1 h to make anaerobic enzyme and dinucleotide stock solutions ( $\sim 1$  mL), except that this was carried out immediately prior to the start of the experiment and the solutions were placed on ice during the process. Subsequently, enzyme and dinucleotide stock solutions were diluted to the required concentrations with an oxygen scrubbing solution of 14.84 U/ml glucose oxidase and 100 mM glucose. The flow system of the stopped-flow apparatus was made anaerobic by an overnight incubation with the oxygen scrubbing solution.

After mixing equal volumes of enzyme and dinucleotide in the stopped-flow apparatus, measurements were performed in triplicate using a diode-array detector and data were collected in logarithmic mode. The observed rate constants for flavin reduction were determined using the program KaleidaGraph (Synergy Software, Reading, PA) by fitting the data to Eq. (1), which describes a double exponential decay.

$$A = B_1 e^{(-k_{\text{obs}1}t)} + B_2 e^{(-k_{\text{obs}2}t)} + C \quad (1)$$

where  $A$  is the absorbance value at 452 nm,  $k_{\text{obs}1}$  and  $k_{\text{obs}2}$  are the apparent first-order rate constants for the observed phases of the reaction,  $B_1$  and  $B_2$  are the amplitude of the phases, and  $C$  is the final absorbance value. Dependence of the rate constants as a function of NADPH concentration was difficult to accurately calculate since the dissociation constant is  $\sim 1$   $\mu\text{M}$  [12]. With NADH, the concentration dependence was determined since the affinity is much lower than when using NADPH. The rate of the fast phase increased hyperbolically with the dinucleotide concentration and the data were fit to Eq. (2), where  $k_{\text{red}}$  is the maximum rate of flavin reduction and  $K_d$  is the dissociation constant.

$$k_{\text{obs}} = \frac{k_{\text{red}}[S]}{K_d + [S]} \quad (2)$$

### 2.7. Limited proteolysis analyses

To assess the effect of conformational changes induced by ligand binding, *AfSidA* (5 mg mL<sup>-1</sup>) was reacted with trypsin (0.0001 mg mL<sup>-1</sup>) in a final volume of 200  $\mu\text{L}$  with 100 mM HEPES, 125 mM NaCl, pH 7.5, along with either 5 mM NAD(P)<sup>+</sup>, 15 mM L-ornithine, or a combination of these compounds at 37 °C. At specific time intervals, aliquots (15  $\mu\text{L}$ ) were removed and immediately heated to 95 °C to stop the reaction. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli on 12% polyacrylamide gels [13].

### 2.8. Flavin fluorescence

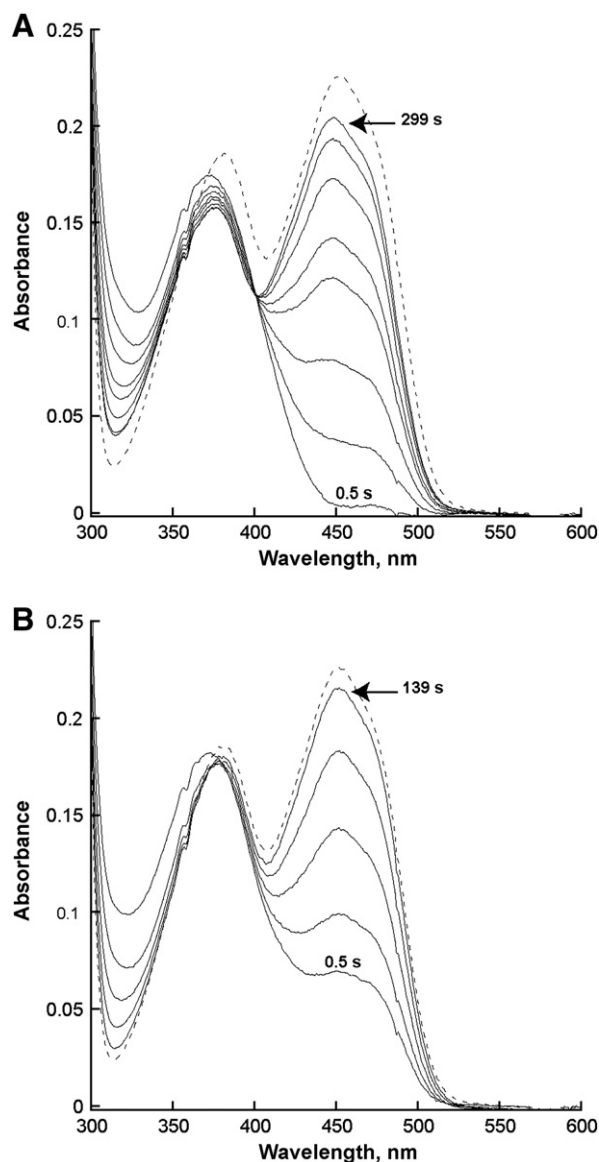
The binding of oxidized coenzyme was determined by monitoring the changes in flavin fluorescence at 520 nm (excitation at 450 nm) on a SpectraMax M5e plate reader (Molecular Devices, Sunnyvale, CA). The  $K_d$  values were obtained by fitting the data to Eq. (3), where  $F_{\text{Nox}}$  is the fluorescence value at each oxidized nucleotide concentration [ $N_{\text{ox}}$ ].

$$F = \frac{F_{\text{Nox}}[N_{\text{ox}}]}{K_d + [N_{\text{ox}}]} \quad (3)$$

## 3. Results

### 3.1. Stability of flavin-dioxygen adducts

The oxidation of *AfSidA* after reduction by NADPH was monitored in a spectrophotometer equipped with a photodiode array. After mixing (0.5 s), a stable intermediate with an absorbance peak at 380 nm was detected. The spectrum is characteristic of a C4a-hydroperoxyflavin intermediate (Fig. 1) [9]. In separate experiments, NADPH was substituted with NADH and a similar spectrum was observed, however, it decayed more rapidly. Spectra of the fully oxidized flavin were observed after  $\sim 400$  s when NADPH was used to reduce *AfSidA*. With NADH as



**Fig. 1.** Formation and decay of C4a-hydroperoxyflavin intermediate. A) Decay of the C4a-hydroperoxyflavin intermediate when *AfSidA* is reduced by NADPH. B) Decay of C4a-hydroperoxyflavin intermediate when the enzyme is reduced by NADH. The C4a-hydroperoxyflavin intermediate has a peak maximum at 382 nm. Formation of the fully oxidized flavin is monitored by the appearance of a peak at 450 nm. With NADPH, the spectra were recorded at: 0.5, 20, 40, 70, 95, 140, 209, and 299 s. The spectra of the C4a-hydroperoxyflavin can clearly be observed in the first spectrum recorded. With NADH the spectra were recorded at: 0.5, 20, 40, 70, and 139 s. Under this condition, significant oxidation in the first spectrum is observed and complete oxidation was achieved 2-fold faster.

**Table 1**  
Steady state kinetic parameters and isotope effects for *Af* SidA.

| Dinucleotide | $k_{cat}$ ,<br>min <sup>-1</sup> | $K_M$ , $\mu$ M | $k_{cat}/K_M$ ,<br>min <sup>-1</sup> $\mu$ M <sup>-1</sup> | $^Dk_{cat}$ | $^D(k_{cat}/K_M)$ |
|--------------|----------------------------------|-----------------|--|-------------|-------------------|
| NADPH        | 36 ± 1                           | 12 ± 0.9        | 3.0 ± 0.15   | 3.1 ± 0.2   | 2.7 ± 0.2         |
| NADPD        | 11 ± 0.4                         | 10 ± 0.9        | 1.14 ± 0.07  |             |                   |
| NADH         | 44 ± 1                           | 100 ± 10        | 0.43 ± 0.03  | 2.0 ± 0.1   | 2.5 ± 0.3         |
| NADD         | 22 ± 1                           | 132 ± 11        | 0.17 ± 0.02  |             |                   |

substrate, oxidation of the flavin was observed in less than 200 s. The rate of decay (measured at 452 nm) with NADPH was 0.013 s<sup>-1</sup> and with NADH was 0.025 s<sup>-1</sup>.

### 3.2. Primary kinetic isotope effects

The steady-state kinetic parameters with unlabeled and deuterated dinucleotides as the variable substrates were determined and the data summarized in Table 1 (Figure S1). Differences in the rates when NADPH is used as substrate versus NADPD results in a primary isotope effect of ~3 for both  $k_{cat}$  and  $k_{cat}/K_M$ . When NADH/D is used as substrate, a kinetic isotope effect of ~2 was determined (Table 1). These results clearly illustrate that hydride transfer is partially rate limiting in the catalytic cycle of *Af* SidA and that some differences in the transition state or commitments to catalysis are present between the two coenzymes that lead to the different isotope effect values.

### 3.3. Reductive half-reaction

The reduction of the flavin cofactor can be directly measured by monitoring the bleaching of the absorbance at 452 nm, which corresponds to the absorbance changes from oxidized to reduced flavin. The data is summarized in Table 2. With NADH, the flavin reduction trace best fit Eq. (1). The rate constant of the fast phase increased as a function of NADH concentration. By fitting the data to Eq. (2) a  $k_{red}$  value of 1.55 ± 0.01 s<sup>-1</sup> and a  $K_d$  value of 56 ± 4  $\mu$ M were determined. In contrast, the rate constant of the slow phase was independent of the concentration of NADH (Fig. 2). With NADD, only the fast phase was isotope sensitive, which is consistent with this phase corresponding to flavin reduction. There was no isotope effect for the slow phase (Table 2). Using NADPH, no concentration dependence was measured because the affinity for this coenzyme is very low ( $K_d$  ~ 1  $\mu$ M). Instead, the rate of flavin reduction was measured at 100  $\mu$ M NADPH. The data was best fit to Eq. (1) and the rates were slower than with NADH. Furthermore, the primary kinetic isotope effect value was higher than with NADH. Interestingly, with NADPH both the slow and fast phases were isotope sensitive (Table 2).

### 3.4. Oxidized coenzyme titrations monitored by fluorescence spectroscopy

Taking advantage of the fact that *Af* SidA can be isolated with bound flavin, binding of oxidized coenzyme was monitored by

**Table 2**  
Rate constants of flavin reduction and kinetic isotope effects measured in stopped-flow.

| Dinucleotide | $k_{obs1}$ , s <sup>-1</sup> | $k_{obs2}$ , s <sup>-1</sup> | $^Dk_{obs1}$ | $^Dk_{obs2}$ |
|--------------|------------------------------|------------------------------|--------------|--------------|
| NADPH        | 0.62 ± 0.01                  | 0.220 ± 0.005                | 5.7 ± 0.3    | 5.4 ± 0.9    |
| NADPD        | 0.109 ± 0.006                | 0.041 ± 0.007                |              |              |
| NADH         | 1.55 ± 0.01                  | 0.09 ± 0.003                 | 4.7 ± 0.04   | 1.2 ± 0.04   |
| NADD         | 0.327 ± 0.002                | 0.072 ± 0.001                |              |              |

measuring changes in the flavin fluorescence. When NAD<sup>+</sup> binds to *Af* SidA, the fluorescence of the flavin increases (Fig. 3). Addition of increasing concentrations of NAD<sup>+</sup> results in further increases in flavin fluorescence and levels off at high concentrations. In contrast, binding of NADP<sup>+</sup> causes a decrease in flavin fluorescence (Fig. 3). Changes in fluorescence were plotted as a function of oxidized nucleotide concentration and the data was fit to Eq. (3) (Fig. 3). From this analysis,  $K_d$  values of 90 ± 4  $\mu$ M and 400 ± 80  $\mu$ M were determined for NADP<sup>+</sup> and NAD<sup>+</sup>, respectively.

### 3.5. Limited proteolysis analyses

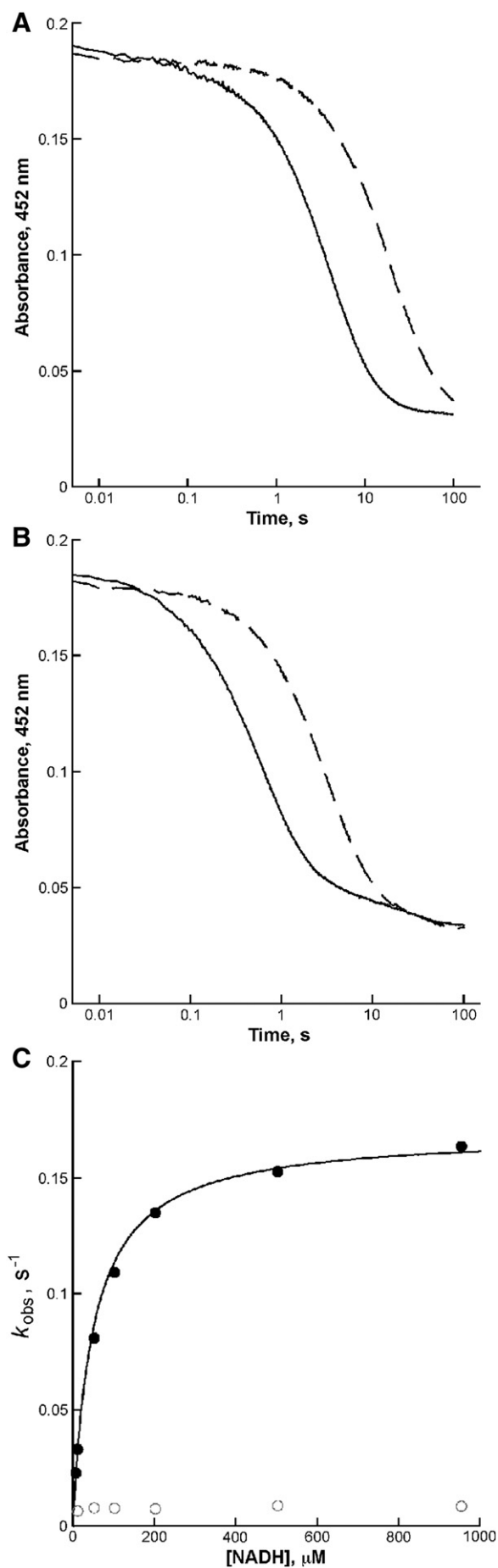
Treatment of *Af* SidA with trypsin resulted in rapid degradation. Similar studies performed in the presence of NADP<sup>+</sup> resulted in significant protection of *Af* SidA against degradation (Fig. 4). Interestingly, NAD<sup>+</sup> (5 mM) was not found to shield *Af* SidA from tryptic digestion as indicated by similar degradation patterns of the enzyme in the absence of ligand. The other participant in the catalytic cycle of *Af* SidA, L-ornithine (15 mM), was also ineffective in preventing the degradation of the enzyme by trypsin. Furthermore, when combined with NADP<sup>+</sup> (5 mM), the pattern of degradation resembled that of NADP<sup>+</sup> alone, suggesting that L-ornithine does not induce conformational changes during the catalytic cycle (Figure S2). It should also be noted that the protective effect exerted by NADP<sup>+</sup> does not appear to be due to the ability to function as an inhibitor of trypsin since Dick and co-workers reported that these compounds have no adverse effect in experiments with azocasein serving as a substrate [14].

## 4. Discussion

Flavin monooxygenases are capable of stabilizing oxygen reactive species utilizing a non-metal organic cofactor [10,15]. The first step in the proposed mechanism of activation of molecular oxygen involves a single electron transfer step from the reduced flavin to oxygen. The resulting superoxide anion and flavin semiquinone rapidly collapses to form the C4a-hydroperoxyflavin intermediate [15]. Flavin monooxygenases must stabilize this intermediate in order to oxygenate the substrate. Otherwise, it will decay to hydrogen peroxide, as in the case of flavin oxidases [16]. Therefore, a molecular mechanism that enables the stabilization of the C4a-hydroperoxyflavin must have evolved in flavin-dependent monooxygenase enzymes.

Recently, van Berkel et al. classified this growing family of enzymes based on their structure and biochemical properties [10]. According to their classification, *Af* SidA belongs to the class B subfamily. Enzymes in this class are encoded by a single gene, contain two dinucleotide binding domains (for NADPH and FAD binding), and follow a sequential kinetic ordered mechanism where NADP<sup>+</sup> remains bound through the catalytic cycle. Other members of this class are the well-characterized flavin-containing monooxygenases (FMOs) and the Baeyer–Villiger monooxygenases (BVMOs) [17,18]. Recently, we determined that *Af* SidA could be recombinantly expressed and isolated in the active form, with the flavin tightly bound. This enzyme continues to be the only N<sup>5</sup>-ornithine hydroxylase to be isolated in the active form. We also determined that this enzyme follows an ordered kinetic sequential mechanism, where NADP<sup>+</sup> remains bound and is the last substrate to be released, consistent with this enzyme being a class B monooxygenase. Similar to other flavin monooxygenases, *Af* SidA uses NADPH as the coenzyme; however, it can also use NADH (Table 1) [9]. We have previously shown that the rate of hydroxylation of ornithine is independent of the coenzyme used and the regulation of the hydroxylated flavin intermediates varies. With NADPH, the C4a-hydroperoxyflavin is at least 2-fold more stable than when NADH is used as the coenzyme (Fig. 1).

The hydride transfer step between the reduced pyrimidine nucleotide and the flavin was probed with steady-state kinetic isotope

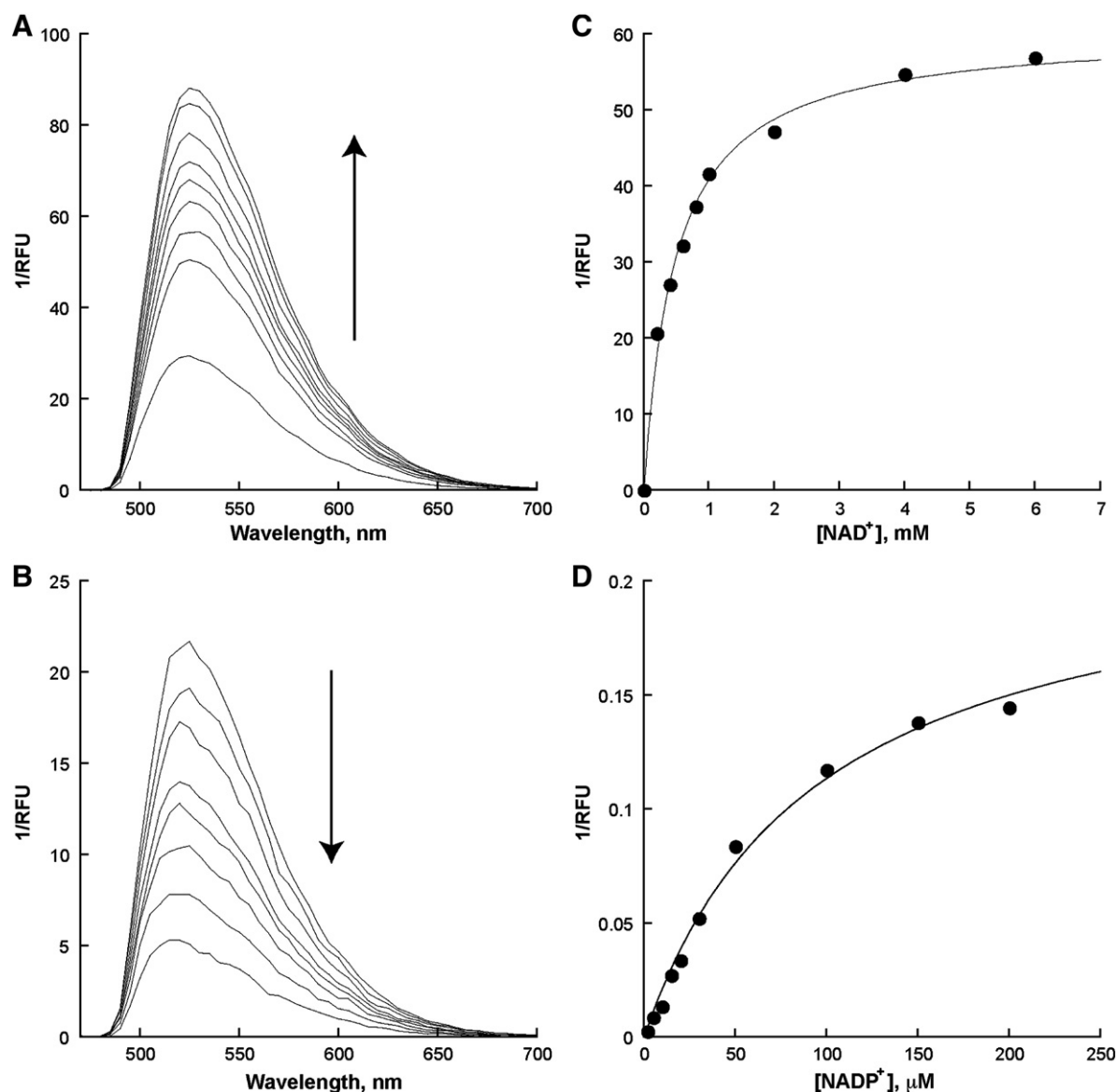


effect experiments. The enzyme was stereo selective for the *pro-R* hydrogen at the C4 carbon in both coenzymes. The stereoselectivity is consistent with other flavin monooxygenases [20]. It was determined that hydride transfer was partially rate limiting for both NADPH and NADH. However, hydride transfer appears to be more rate limiting with NADPH (Table 1). To investigate this further, we measured the rate of flavin reduction by reduced dinucleotide directly in the stopped-flow spectrophotometer. With both coenzymes, flavin reduction occurs in two phases. Because the  $K_d$  value for NADPH is very low, the binding constant is difficult to determine accurately. At saturating concentrations, the rate constant for the fast and slow phases were  $0.62 s^{-1}$  and  $0.22 s^{-1}$ , respectively. In contrast, the rate constant of flavin reduction could be calculated as a function of NADH, since this coenzyme binds with lower affinity (Fig. 2 and Table 2). With NADH, the first phase is fast and is concentration dependent, while the second phase is much slower and does not change with the concentration of NADH. Primary kinetic isotope effects determined by rapid reaction kinetics show that with NADH, the fast phase is isotope sensitive with a primary isotope effect of 4.7, while the second phase does not show significant isotope effect. Using deuterated NADPH, a primary isotope effect  $>5.4$  was observed in both phases (Fig. 2 and Table 2). With pig liver FMO, a biphasic reduction with NADPH was observed and an isotope effect value of 6 was measured in both phases [18]. In this enzyme, it was proposed that NADPH reacts with two different enzyme forms. We propose that, as observed in FMO, reaction with NADPH also occurs with different forms of *Af* SidA. The observed decrease in the kinetic isotope effect values under steady state kinetics compared to the values measured under rapid reaction conditions is due to the fact that flavin oxidation is partially rate limiting [9,12].

The binding of oxidized coenzymes was monitored by measuring the changes in flavin fluorescence. Binding of  $NAD^+$  induces an increase in the flavin fluorescence. The simplest explanation is that  $NAD^+$  binding increases the hydrophobicity of the active site upon binding, thus, increasing the fluorescence of the flavin. In contrast, binding of  $NADP^+$  decreases flavin fluorescence (Fig. 3). A decrease in fluorescence is normally attributed to quenching by adjacent charged atoms. It is possible that the nicotinamide ring of  $NADP^+$  binds closely to the flavin, quenching the fluorescence. Consistent with the conclusion that  $NADP^+$  binds close to the flavin, in the structure of the related ornithine hydroxylase PvdA from *Pseudomonas aeruginosa*, the nicotinamide ring binds adjacent to the flavin [19]. In *Af* SidA, the effect on flavin fluorescence suggests that these coenzymes either bind in different positions or induce/modulate conformational changes in different ways.

The affinity of NAD(H) calculated from fluorescence and kinetic methods clearly shows it binds between 3 and 50 fold less tightly than NADP(H). We and others previously showed that  $NADP^+$  remains bound throughout the catalytic cycle [9,12]. Clearly, this is necessary for stabilization of oxygenated flavin intermediates. Thus, it is possible that the relatively lower stabilization of the C4a-hydroperoxyflavin by  $NAD^+$  might be due to release of the coenzyme. We believe that  $NAD^+$  release before complete turnover does occur and this leads to uncoupling of the reaction, where hydrogen peroxide is formed. We have previously shown that more (~20–40%) hydrogen peroxide is formed when NADH is used as substrate. This leads to a higher apparent  $k_{cat}$  value when the activity of the enzyme is determined by measuring oxygen consumption (Table 1). However, binding of  $NAD^+$  to *Af* SidA, monitored by flavin fluorescence and the results from limited

**Fig. 2.** Flavin reduction monitored in the stopped-flow spectrophotometer with NADPH (A) and NADH (B). Reduction with deuterated coenzyme is shown with dashed lines. (C) Dependence of the two phases on the concentration of NADH. The values of the fast phase are shown in solid circles and of the slow phase in open circles.

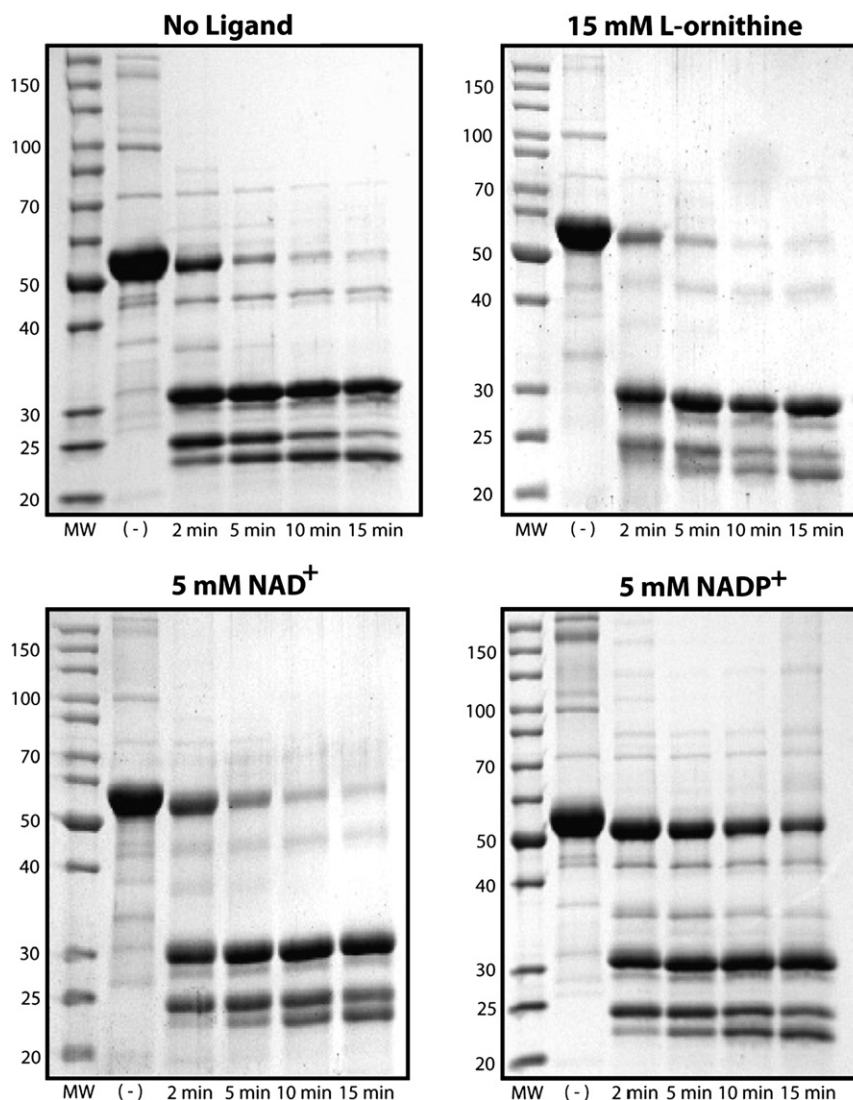


**Fig. 3.** Changes in flavin fluorescence as a function of NADP<sup>+</sup> or NAD<sup>+</sup> binding to Af SidA. A) Increase in relative fluorescence unit (RFU) upon NAD<sup>+</sup> binding. B) Decrease in RFU upon binding of NADP<sup>+</sup>. Panels C and D show the plots of the changes in RFU as a function of oxidized coenzyme concentrations. The lines are fits to Eq. (3). The assay conditions are described in the [Materials and methods](#) section.

proteolysis data, clearly show that this coenzyme is binding in a different conformation than NADP<sup>+</sup>; thus it appears that even minor changes in the position of the nicotinamide ring could influence the stability of the flavin intermediate.

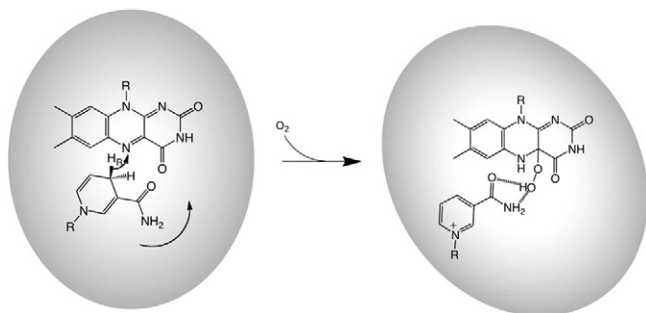
To investigate if conformational changes were modulated by coenzyme binding, limited proteolysis studies were performed. In the absence of coenzyme, Af SidA is rapidly degraded by trypsin and this also occurs in the presence of NAD<sup>+</sup>. However, when NADP<sup>+</sup> is added, Af SidA is protected from proteolysis (Fig. 4). These results suggest that NADP<sup>+</sup> binding changes the conformation (or shifts the equilibrium) of regions of the protein and these changes block trypsin cleavage sites. It is clear that NAD<sup>+</sup> does not cause the same effect. Alternatively, one must consider the possibility that interactions between the phosphate group at the 2'-hydroxyl of NADP<sup>+</sup> with lysine or arginine residues in Af SidA might be producing the observed decrease in the action of trypsin. Although this is a possibility, the results from binding of the oxidized coenzymes monitored by measuring the flavin fluorescence suggest that different binding

modes are utilized. Therefore, the conclusion that conformational changes accompany a different position of NADP<sup>+</sup> in the active site of Af SidA is more realistic. Furthermore, recent kinetic and structural data have provided insights into the mechanism of regulation of flavin intermediates in FMO from the bacterium *Methylophaga* sp. strain SK1 and the BVMO, phenylacetone monooxygenase (PAMO), from the thermophilic bacterium *Thermobifida fusca*. Structural and mutagenesis data suggest that conformational changes occur after flavin reduction resulting in a shift in NADP<sup>+</sup> position in the active site where it would play a role in the stabilization of the oxygenated flavin intermediates [16,20,21]. Similarly, in the 3D-structure of PvdA, the nicotinamide ring is also in a position predicted to interact with the C4a-hydroperoxyflavin [19]. Together, the data presented here are consistent with the mechanism shown in Scheme 2, where NADPH reduces not only the flavin but also the product of this reaction, NADP<sup>+</sup>, is involved in the stabilization of C4a-hydroperoxyflavin intermediate. This mechanism is modulated by conformational changes that place NADP<sup>+</sup> in the correct orientation for this dual role. This is



**Fig. 4.** Trypsin digests of *Af SidA* in the presence of various ligands. *Af SidA* ( $5 \text{ mg mL}^{-1}$ ) was digested with trypsin ( $0.0001 \text{ mg mL}^{-1}$ ) at  $37^\circ \text{C}$ .

specific to NADP(H) as NAD(H) is unable to induce conformational changes and, thus, is less effective in flavin intermediate stabilization. This mechanism of regulation appears to be shared among members of the class B flavin monooxygenases.



**Scheme 2.** Cartoon representation of the proposed coenzyme induced conformational changes in *Af SidA* that leads to the stabilization of oxygenated flavin intermediates. These conformational changes are specific to NADP(H).

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at [doi:10.1016/j.bbapap.2012.03.004](https://doi.org/10.1016/j.bbapap.2012.03.004).

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