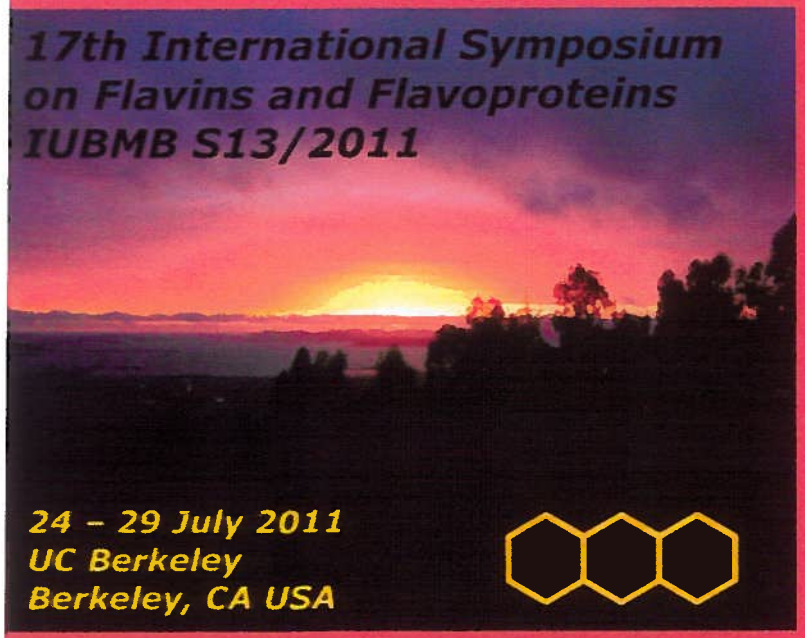

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Effect of pH on the Reductive and Oxidative Half-reactions of *Aspergillus fumigatus* Siderophore A

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Introduction

Aspergillus fumigatus siderophore A (SidA) catalyzes the hydroxylation of ornithine in the biosynthesis of ferricrocin, a siderophore that is essential for virulence [1]. Previous studies revealed that the reaction catalyzed by this monooxygenase involves: i) reduction of the enzyme-bound FAD; ii) reaction with oxygen to form a C4a-hydroperoxyflavin intermediate; iii) transfer of an oxygen atom to the substrate with the formation of a C4a-hydroxyflavin species; and iv) dehydration to yield the oxidized enzyme. In absence of ornithine, H_2O_2 is eliminated from the intermediate to generate oxidized flavin. SidA is able to use both NADH and NADPH as substrate, but the enzyme has higher affinity for the latter (~10 fold) [2,3].

We have investigated here the reactivity of the C4a-hydroperoxyflavin intermediate and the formation of the oxidized SidA under rapid reaction conditions at various pHs and oxygen concentrations. In addition, we have studied the pH dependence of the reductive half-reaction with NADPH or NADH.

Materials and Methods

Enzymes and Reagents. Recombinant SidA was obtained by cloning the corresponding gene into pET-15b vector (Novagen), followed by transformation of BL21(DE3)pLysS-T1^R *Escherichia coli* cells (Sigma Chemical Co.) and expression in autoinduction media. For purification, 5 mL HisTrap columns (GE Healthcare) were used. Enzyme stocks solutions (~400 μM) were prepared in 10 mM potassium phosphate buffer containing 100 mM NaCl and stored at -80 °C.

Pre-steady-state kinetics. All reactions were carried out using a SX20 stopped-flow apparatus (Applied Photophysics, UK) installed in an anaerobic glove box, at 15 °C. Measurements were performed in triplicate using a diode-array detector and data were collected in logarithmic mode. The flow system of the instrument was made anaerobic by following standard procedures [4].

The stopped-flow apparatus was used in single- and double-mixing mode to investigate the reductive and oxidative half-reaction of SidA, respectively. The enzyme was assayed at various pHs and oxygen concentrations. The buffers used were potassium phosphate, pH 6.0, 7.0, and 8.0, and sodium pyrophosphate, pH 9.0 and 10.0. Oxygen concentrations (0.155-0.491 mM) were obtained by mixing the appropriate volumes of air- and oxygen-saturated buffers at 25 °C. NADPH and NADH stock solutions were prepared inside the glove box in anaerobic 10 mM potassium phosphate buffer, pH 7.5. These stocks were diluted in the corresponding 100 mM buffer for studying the anaerobic reduction of SidA. All kinetic data were analyzed with standard equations using the program KaleidaGraph (Synergy Software, Reading, PA).

Results and Discussion

Reduction of SidA at various pHs. The anaerobic reduction of SidA was carried out in the stopped-flow apparatus using saturating concentrations of either NADPH or NADH at various pH values. The apparent pseudo-first-order rate constants (k_{obs}) for the reduction were determined by fitting the plot of the absorbance at 452 nm versus time to a double exponential function (Figure 1A). According to the pH profile obtained from these data (Figure 1B), the rate of the first phase of the reduction is slightly increased by the deprotonation of a group with a pK_a value of 7.0 ± 0.2 and 8.7 ± 0.7 with NADPH or NADH, respectively. However, the second phase of reduction is independent of pH for both dinucleotides.

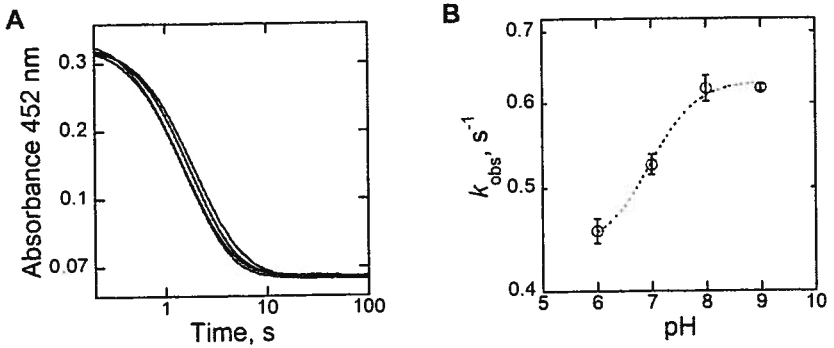

Figure 1. Effect of pH on the reduction of SidA. A) Absorbance traces of SidA reduction with 100 μM NADPH at pH 6.0, 7.0, 8.0 or 9.0. B) Plot of the k_{obs} values for the first phase of the reduction versus pH.

Reaction of SidA with oxygen at various pHs. To study the oxidation of SidA at various pHs and oxygen concentrations, the enzyme was first anaerobically reduced in the aging loop of the stopped-flow apparatus in 10 mM buffer, and then the fully reduced enzyme was mixed with an equal volume of oxygenated 100 mM buffer at the required pH.

At all pH values tested, the C4a-hydroperoxyflavin intermediate is observed after the first phase of the reaction and presents an absorbance maximum at 382 nm, suggesting that the pK_a for the deprotonation of its terminal peroxide group is higher than 10.0 (Figure 2). During the second phase of the reaction, an increase in absorbance at 452 nm is detected, consistent with the formation of oxidized enzyme (Figure 2).

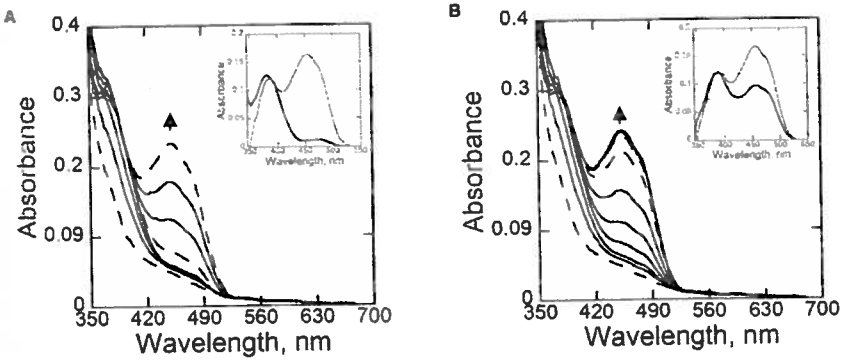

Figure 2. Oxidation of fully reduced SidA in the stopped-flow apparatus at pH 7.0 (A) and 9.0 (B). The reaction mixes contained 15 μM SidA, 22 μM NADH and 0.491 mM oxygen. The spectra at 0.005, 30 and 666 s are shown as dashed curves (from bottom to top). Insets show spectra at 30 and 666 s corrected by subtracting the spectrum at 0.005 s.

The stopped-flow traces at 382 and 452 nm recorded during the oxidation of SidA at various pHs (Figure 3A) were fit to single exponential functions to determine the k_{obs} values. As shown Figure 3B, the plot of k_{obs} values for the formation of the intermediate versus pH is bell-shaped, whereas for the formation of the oxidized flavin only an ionizable group is involved in catalysis. pK_a values were similar at all oxygen concentrations tested (Figure 3C and 3D).

The formation of the C4a-hydroperoxyflavin intermediate is only slightly dependent on pH, suggesting that the pK_a values are not related to the protonation state of the reduced flavin N1. The rate of formation of the oxidized flavin increases at pH values higher than 7.0, indicating that an ionizable group with a pK_a> 9.0 is involved in the release of H_2O_2 to generate the oxidized flavin. In the case of *p*-hydroxyphenylacetate hydroxylase, the same influence of the pH on this phase of the reaction was described and it has been proposed that this pK_a value may be due to the deprotonation at flavin N5 of the

intermediate or may correspond to a residue that is involved in this process [5]. Using NADH, the pK_a values for the formation of the oxidized flavin are slightly higher than with NADPH (Figure 3D). This result may be due to conformational differences among the intermediate-NADH and intermediate-NADPH complexes, the latter being more appropriate for deprotonation.

As is shown in the Table 1, the k_{obs} values for the formation of the oxidized flavin is higher with NADH than with NADPH at all pH values tested. However, in both cases the rate of formation of the oxidized enzyme is slow to prevent wasteful reduced dinucleotide consumption and H_2O_2 generation. This strategy has been described for other monooxygenases that stabilize the intermediate in absence of the oxidant substrate as cyclohexanone monooxygenase and flavin-containing monooxygenase [6,7].

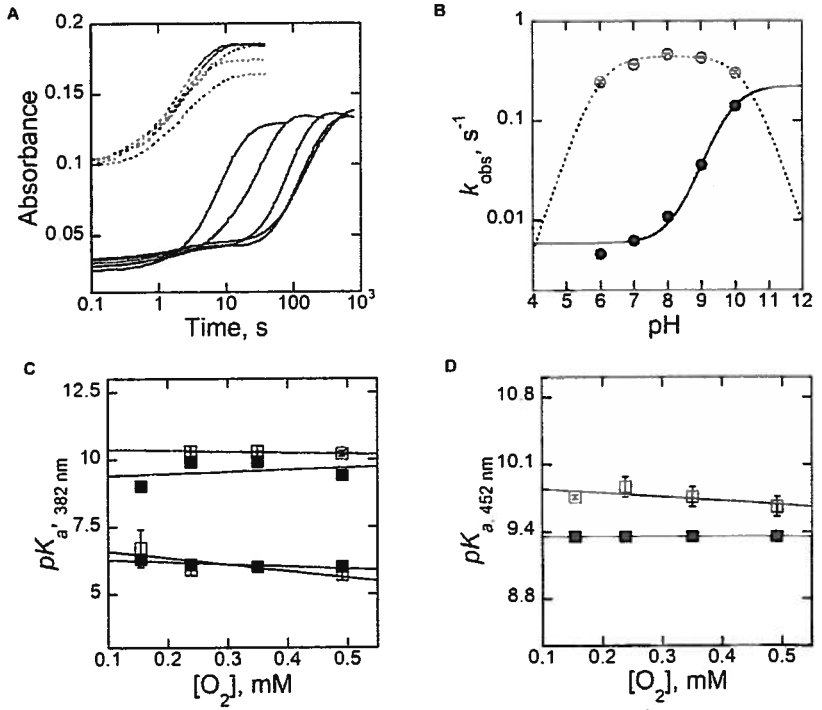

Figure 3. Effect of pH on oxidation of SidA. A) Stopped-flow traces at 382 (····) and 452 (—) nm recorded after mixing the fully reduced SidA (reduced with NADH) with oxygenated buffer (0.35 mM, final concentration) at various pHs. B) k_{obs} values from traces in Figure 3A versus pH. C) pK_a for the formation of the intermediate versus oxygen concentration. D) pK_a for the formation of the oxidized enzyme versus oxygen concentration. In 3C and 3D, results with NADPH (■) and NADH (□) are shown.

The k_{obs} values for the formation of the intermediate are linearly dependent on oxygen concentration, whereas the k_{obs} values for the formation of the oxidized flavin are constant at all oxygen concentrations tested (Figure 4). Determination of the second-order rate constants for the reaction of SidA with oxygen was performed by fitting k_{obs} values as a function of oxygen concentration to a linear equation. The highest value of the second-order rate constant for the formation of the intermediate was obtained at pH 8.0 with NADPH (Table 1). A similar value of this parameter was reported for the ornithine hydroxylase from *P. aeruginosa* (1.8×10^3 M^{-1} s^{-1}) using the same buffer at 22 °C [8].

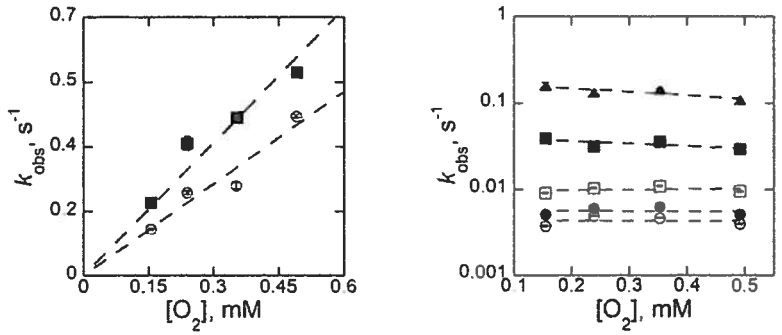

Figure 4. Oxygen concentration dependence on oxidation of SidA at various pHs. A) k_{obs} values for the formation of the intermediate versus oxygen concentration. Only pH 6.0 and 9.0 are shown for clarity. B) k_{obs} values for the formation of the oxidized flavin. NADH was used as reductant. pH 6 (○), 7 (●), 8 (□), 9 (■) and 10 (▲).

Table 1. Pre-steady-state kinetic parameters determined for both the formation of the intermediate and oxidized SidA

pH	NADH		NADPH	
	$k_{obs,\ 382\ nm}$ $M^{-1}s^{-1}$	$k_{obs,\ 452\ nm}$ s^{-1}	$k_{obs,\ 382\ nm}$ $M^{-1}s^{-1}$	$k_{obs,\ 452\ nm}$ s^{-1}
6.0	8.3×10^2	0.004	7.7×10^2	0.001
7.0	1.2×10^3	0.006	1.4×10^3	0.002
8.0	1.4×10^3	0.010	1.7×10^3	0.006
9.0	1.2×10^3	0.034	1.1×10^3	0.031
10.0	9.0×10^2	0.134	6.0×10^2	0.085

Conclusions

This study has revealed several catalytic properties of SidA. Both the reductive and oxidative half-reactions of this monooxygenase are

dependent on pH. During reduction and formation of the C4a-hydroperoxyflavin intermediate, different residues may facilitate binding of the dinucleotides or conformational arrangements of the enzyme, but they are not essential. However, a strong effect of the pH was observed for the formation of the oxidized enzyme, which likely is related with the deprotonation at flavin N5. In addition, our results showed that the pK_a of the C4a-hydroperoxyflavin is higher than 10.0.

Acknowledgments

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Structural Basis of Regioselective Hydroxylation in 3-Hydroxybenzoate Hydroxylases

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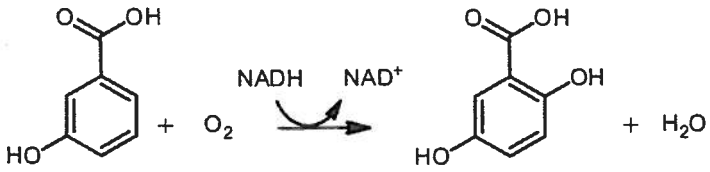
Introduction

Biological processes as lignin and xenobiotics degradation, and synthesis of polyketide antibiotics make use of flavoprotein aromatic hydroxylases in important reaction steps. These enzymes are extremely regioselective performing either *ortho* or *para* hydroxylation [1]. A number of FAD-dependent aromatic hydroxylase structures have been solved, but the structural basis of regioselectivity is still elusive. Here we present the crystal structure of 3-hydroxybenzoate 6-hydroxylase (3HB6H) from *Rhodococcus jostii* RHA1, an enzyme involved in the gentisate pathway of aromatic degradation.

Scheme 1. Reaction performed by 3HB6H



3-hydroxybenzoate 2,5-dihydroxybenzoate

The structure of 3HB6H provides new insights into the structural relationship of flavoprotein phenolic hydroxylases. Furthermore comparison with the structure of 3-hydroxybenzoate 4-hydroxylase (3HB4H) from *Comamonas testosteroni* [2] has offered the opportunity to shine some light on the structural features that allow these enzymes to discriminate between *ortho*- and *para*-hydroxylation.