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C4a-Hydroperoxyflavin Formation in *N*-Hydroxylating Flavin Monooxygenases Is Mediated by the 2′-OH of the Nicotinamide Ribose of NADP$^+$

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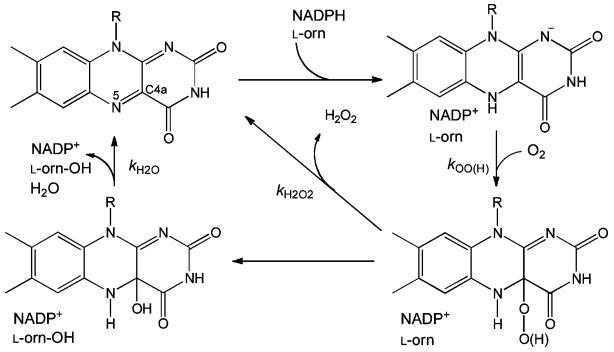
Supporting Information

ABSTRACT: Flavin-dependent monooxygenases must stabilize a C4a-hydroperoxyflavin intermediate to hydroxylate their respective substrates. Formation and decay of the C4a-hydroperoxyflavin were monitored under rapid reaction kinetic conditions in SidA, an *N*-hydroxylating monooxygenase involved in siderophore biosynthesis. Solvent kinetic isotope effect studies of flavin oxidation indicate that both hydrogen peroxide elimination and water elimination occur via abstraction of hydrogen from the N5 of the flavin. Kinetic isotope effect and density functional theory results are consistent with the transfer of a proton from the 2′-OH of the nicotinamide ribose of nicotinamide adenine dinucleotide phosphate (NADP$^+$) to the C4a-peroxyflavin to form the C4a-hydroperoxyflavin. This represents a novel role for NADP$^+$ in the reaction of flavin-dependent enzymes.

Flavin-dependent monooxygenases catalyze the addition of an oxygen atom in hydroxylation, epoxidation, and ester bond formation reactions. The catalytic cycle includes reduction of the flavin cofactor by NAD(P)H, activation of molecular oxygen, and formation of covalent oxygen−flavin intermediates.[1,2] Depending on the specific chemical reaction, a C4a-peroxyflavin (FAD_{OO^-}) or C4a-hydroperoxyflavin (FAD_{OOH}) intermediate is stabilized. Siderophore A (SidA) is a member of the class B flavoprotein monooxygenases that catalyze the N5 hydroxylation of ornithine in the biosynthesis of siderophores in *Aspergillus fumigatus*.[3,4] In the catalytic cycle of SidA, NADP$^+$ is the last product released. The presence of NADP$^+$ in the active site is required for the stabilization of the oxygenated flavin intermediate.[5,6] Biochemical and structural studies of SidA suggest that the role of NADP$^+$ in the stabilization of the flavin intermediate involves movement of the nicotinamide ring after hydride transfer to create a site for the $FAD_{OO(H)}$ intermediate to form and for the assembly of hydrogen bond interactions required for its stabilization.[7] This role for NADP$^+$ is conserved in other members of the class B flavoprotein monooxygenases.[8,9] Activation of molecular oxygen by flavin monooxygenases is initiated by the transfer of a single electron from reduced flavin to oxygen, resulting in a flavin semiquinone and superoxide radical pair that collapses to form the FAD_{OO^-} intermediate. SidA and other hydroxylating flavoprotein monooxygenases require the protonated form of this intermediate (FAD_{OOH}) for catalysis. The mechanism by which FAD_{OOH} forms in SidA is not well-understood.

Formation and decay of FAD_{OOH} in SidA were probed by measuring solvent kinetic isotope effects (SKIEs) in a stopped-flow spectrophotometer. In the absence of ornithine, the formation of the intermediate ($k_{OO(H)}$) was monitored at 372 nm. The intermediate is very stable and decays by the elimination of hydrogen peroxide ($k_{H_2O_2}$) to form the oxidized flavin. This process is monitored at 452 nm. In the presence of ornithine, hydroxylation takes place and the oxidized flavin is formed via the elimination of water (k_{H_2O}), which is also monitored at 452 nm (Scheme 1). In the absence of ornithine,

Scheme 1. Catalytic Cycle of SidA



a SKIE value of ∼1 was determined for $k_{OO(H)}$. In contrast, a SKIE value of ∼1.5 was determined for $k_{H_2O_2}$. In the presence of ornithine, the SKIE value on $k_{OO(H)}$ was also ∼1 (Table 1). The lack of a SKIE on $k_{OO(H)}$ is consistent with there being no protons in flight in the transition state for formation of the oxygenated flavin intermediate or protonation occurring very fast. In the presence of ornithine, a significantly higher SKIE value for k_{H_2O} was determined. This is consistent with previous stopped-flow experiments that showed flavin oxidation to be partially rate-limiting in the catalytic cycle of SidA.[6,10]

To determine if H_2O_2 or H_2O elimination involves abstraction of a hydrogen atom from the N5 of FAD, we performed experiments in which SidA was reduced with (4*R*)-4-^{2}H-NADPH (NADPD). Upon reduction, ^{2}H-N5-FAD forms and a kinetic isotope effect (KIE) would be measured if the breaking of this bond were rate-limiting during flavin oxidation,

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Table 1. KIEs on the Oxidative Steps of SidA[a]

	$k_{OO(H)}$ (s^{-1})	
	without ornithine	with ornithine
D_2O	1.2 ± 0.2	1.0 ± 0.1
NADPD	1.1 ± 0.1	1.2 ± 0.2
NADPD with D_2O	1.1 ± 0.2	1.1 ± 0.1
D_2O, incubation for 600 s	1.3 ± 0.1	1.2 ± 0.1
	$k_{H_2O_2}$ (s^{-1})	k_{H_2O} (s^{-1})
D_2O	1.55 ± 0.09	2.97 ± 0.09
NADPD	1.01 ± 0.05	1.18 ± 0.03
NADPD with D_2O	2.3 ± 0.1	4.3 ± 0.2
D_2O, incubation for 600 s	2.5 ± 0.1	4.2 ± 0.1

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

by H_2O or H_2O_2 elimination. A KIE value close to 1 was measured for $k_{H_2O_2}$, and an only slightly higher value was measured for k_{H_2O} (Table 1). We then measured the KIE on oxidation where SidA was reduced with NADPD in D_2O. In this experiment, the KIEs for both $k_{H_2O_2}$ and k_{H_2O} were significantly higher than that with either D_2O or NADPD alone. We hypothesized that the difference in the KIE values might originate from buffer exchange with the N5 of FAD. To test whether the observed lower SKIE value for oxidation is an apparent effect influenced by proton exchange, SidA was mixed with NADPH in D_2O for 600 s (instead of 60 s) before being reacted with oxygen. Under these conditions, the SKIE values for $k_{H_2O_2}$ and k_{H_2O} were equal to the KIE values with NADPD and D_2O. These results indicate that the observed lower SKIE on oxidation is due to relatively slow buffer exchange between D_2O and the H-N5-FAD. Similarly, the observed KIE values with NADPD are lower due to exchange of the ^{2}H-N5-FAD with H_2O. As expected, the KIE values with NADPD alone or in D_2O on $k_{OO(H)}$ were close to 1. In summary, the results indicate that (1) the observed KIEs for $k_{H_2O_2}$ and k_{H_2O} originate from the abstraction of a hydrogen atom from the N5 of FAD, as shown for other flavoenzymes,[11] and (2) the transition state for cleavage of this bond is more rate-limiting for H_2O elimination than for H_2O_2 elimination.

Density functional theory (DFT) analysis was performed to provide insight into the protonation mechanism of FAD_{OO}^- for the formation of FAD_{OOH} in SidA. The DFT analysis of FAD_{OO}^- (no ornithine bound) shows that the proton between the distal oxygen and the oxygen of the 2′-OH is shared on the basis of the bond distance (Figure 1A). Similarly, in the FAD_{OO}^-·Orn complex (N5-ornithine uncharged), the 2′-OH proton is also clearly shared with the distal oxygen (Figure 1B). Results with the FAD_{OO}^-·Orn^+ (N5-ornithine positively charged) complex show that at the very early stages of the simulation, a proton is transferred from the N5 atom of ornithine to the distal oxygen of FAD_{OO}^- to form a FAD_{OOH}·Orn complex (Figure S5 of the Supporting Information). A mechanism evoking the transfer of a proton from ornithine to the FAD_{OO}^- has been proposed in PvdA, a related N5-ornithine flavoenzyme monooxygenase from *Pseudomonas aeruginosa*. The authors proposed that an enhancement of $k_{OO(H)}$ (80-fold) in the presence of ornithine might be coupled to the transfer of a proton from ornithine to FAD_{OO}^- in PvdA.[13] In SidA, the presence of ornithine also enhances $k_{OO(H)}$. We previously attributed this enhancing effect, as well as the effect observed with lysine and arginine, to the presence of the positive charge

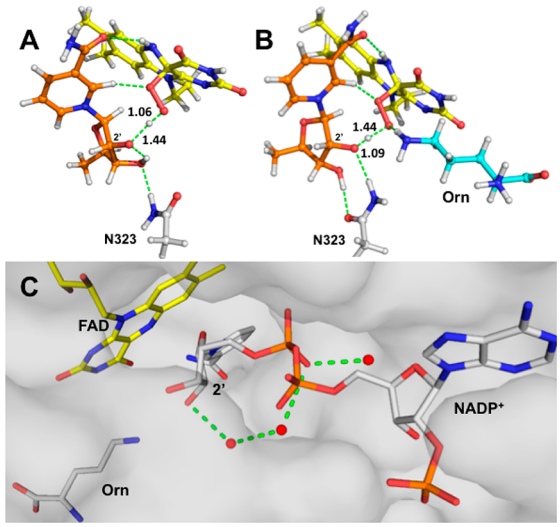

Figure 1. (A and B) Density functional theory-optimized coordination of the intermediate formed in SidA shows that the 2′-OH of the nicotinamide ribose is in transition to the distal oxygen when the starting complex is either FAD_{OO}^- (A) or the FAD_{OO}^-·Orn complex (B) (distances in angstroms). (C) Top view of SidA highlighting the proposed water (red spheres) and phosphate-mediated proton shuttle that will facilitate protonation of FAD_{OO}^-.

from N5-ornithine, or other ligands, being in the proximity of C4a-FAD. This would stabilize the flavin semiquinone and superoxide radical pair that forms prior to formation of the covalent oxygenated flavin intermediate.[7] To determine if a positive charge close to C4a-FAD is in fact required to enhance $k_{OO(H)}$, we tested the effect of norvaline on this step. Because norvaline does not have a positive charge on the side chain, it should not have an enhancement effect. However, norvaline enhances $k_{OO(H)}$ by ∼7-fold. This is smaller than the enhancement determined for arginine (∼200-fold) but similar to the enhancement observed with ornithine (∼20-fold) and lysine (∼10-fold) (Table S2 of the Supporting Information). Clearly, a positive charge is not absolutely necessary to enhance the formation of FAD_{OOH} in SidA. This is inconsistent with the mechanism of protonation of FAD_{OO}^- by ornithine. Similarly, the SKIE studies presented here do not support the transfer of a proton from ornithine (or solvent) to the FAD_{OO}^- to form FAD_{OOH}. In addition, pH studies of SidA have shown that $k_{OO(H)}$ is pH-independent, and no changes in the spectra of the intermediate characteristic of protonation of FAD_{OO}^- were observed.[12] We interpreted these data as suggesting that the intermediate observed in SidA is FAD_{OOH}. Furthermore, the spectra of the intermediate are identical in the presence of ornithine, norvaline, or in the absence of a ligand, suggesting that formation of the intermediate is independent of ornithine (or analogue) binding and the intermediate is most likely FAD_{OOH} (Figure S4 of the Supporting Information). Structural analysis of the SidA active site fails to identify a residue at the proper distance to function as an acid to protonate the flavin intermediate. Similarly, in the FAD_{OOH}·$NADP^+$·Orn complex, there is not enough space to accommodate a water molecule to donate a proton (Figure S7 of the Supporting Information). Together, the data do not support the protonation of FAD_{OO}^- by ornithine, solvent, or other amino acids in the active site of SidA and suggest that the intermediate observed in SidA is the FAD_{OOH}.

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We favor the alternative mechanism suggested by the DFT results, where ornithine binds with the N5 in the catalytically competent neutral form and formation of FAD_{OOH} is mediated by the transfer of a proton from the 2′-OH of the nicotinamide ribose of $NADP^+$. Proton transfer might be facilitated by a water and β-phosphate-mediated proton shuttle network that connects the 2′-OH of the nicotinamide ribose to the solvent (Figure 1C). We propose that the enhancement of $k_{OO(H)}$ in the presence of ornithine observed in SidA and PvdA originates from substrate-induced preorganization of the active site. This would include alignment of the water molecules to form the proton shuttle network, and proper hydrogen bonding interactions to position the 2′-OH of the nicotinamide ribose in an optimal orientation for the transfer of a proton to the distal oxygen of FAD_{OO^-}.

In summary, the biochemical and computational results described here support a novel role for $NADP^+$ in the formation of FAD_{OOH} by donating a proton from the 2′-OH of the nicotinamide ribose. This is a previously unrecognized role for this cofactor in the mechanism of flavin-dependent monooxygenases and might be a general mechanism utilized for hydroxylating flavin monooxygenases that require $NADP^+$ for stabilization of the FAD_{OOH} as they all have a highly conserved mode of coenzyme binding.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental methods, stopped-flow kinetic traces, rate constants, and results of DFT analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

#R.R. and S.B. contributed equally to this work.

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Notes

The authors declare no competing financial interest.

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