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Regulated O₂ Activation in Flavin-Dependent Monooxygenases

Rosanne E. Frederick, Jeffery A. Mayfield, and Jennifer L. DuBois*

Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556, United States

Abstract

Flavin-dependent monooxygenases (FMOs) are involved in important biosynthetic pathways in diverse organisms, including production of the siderophores used for the import and storage of essential iron in serious pathogens. We have shown that the FMO from *Aspergillus fumigatus*, an ornithine monooxygenase (Af-OMO), is mechanistically similar to its well-studied distant homologues from mammalian liver. The latter are highly promiscuous in their choice of substrates, while Af-OMO is unusually specific. This presents a puzzle: how do Af-OMO and other FMOs of the biosynthetic classes achieve such specificity? We have discovered substantial enhancement in the rate of O₂ activation in Af-OMO in the presence of L-arginine, which acts as a small molecule regulator. Such protein-level regulation could help explain how this and related biosynthetic FMOs manage to couple O₂ activation and substrate hydroxylation to each other and to the appropriate cellular conditions. Given the essentiality of Fe to Af and the avirulence of the Af-OMO gene knock out, inhibitors of Af-OMO are likely to be drug targets against this medically intractable pathogen.

Flavin-containing monooxygenases (FMOs) catalyze the oxygenation of diverse small organic molecules using O₂, NADPH, and the flavin adenine dinucleotide (FAD) cofactor.^{1,2} Genome sequencing has shown this family of enzymes to be widespread in bacteria, fungi, plants, and animals, though the mammalian FMOs are the only ones to have received significant prior attention.^{3–9} Like the cytochrome P450s, the mammalian liver FMOs are involved in the degradation of xenobiotics. FMOs from other sources, by contrast, appear to be primarily involved in the biosynthesis of diverse and important natural products. These include the siderophores upon which many pathogenic microorganisms depend for the uptake and storage of nutritionally essential iron, and without which they can lose their virulence. These FMOs have consequently been proposed as promising drug targets,^{11–16} particularly against the medically intractable fungal pathogen *Aspergillus fumigatus* (Af), which depends on a single FMO (SidA, Af-OMO) for all siderophore production and for virulence.¹⁶ FMOs likewise play key roles in the biosynthesis of the primary plant hormone, auxin.¹⁰ The spatial and temporal regulation of auxin biosynthesis is important for controlling plant growth, though how this is achieved at the transcript and protein levels is yet unclear.

Like all monooxygenases, the biosynthetic FMOs must avoid uncoupling O₂ activation from substrate hydroxylation, which would result in the waste of reducing equivalents and release of toxic H₂O₂. At the same time, FMOs must direct the oxidizing power of activated O₂

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Corresponding Author: jdubois@nd.edu.

ASSOCIATED CONTENT

Supporting Information. Experimental details, transient kinetic data, steady state kinetic parameters, and rate constants. This material is available free of charge via the Internet at <http://pubs.acs.org>.

toward the correct substrate. It was previously shown that Af-OMO activates O_2 to generate an FAD-C4a-hydroperoxide intermediate (FAD-OOH) which acts as the hydroxylating agent (Scheme 1).^{14,17} As with the well-characterized mammalian liver enzymes, this species is strikingly stable in the presence of bound $NADP^+$ ($t_{1/2} = 33\text{min}$, 25°C , pH 8). Similar results were obtained for the OMO from the bacterium *Pseudomonas aeruginosa*, PvdA.¹³ Such a “bold” catalytic strategy is consistent with the biodegradative role of the liver FMOs, which hydroxylate literally hundreds of structurally variable nucleophiles including amines, thiols, and halogenated compounds.^{2,9} The FMOs are constitutively expressed; the FAD-OOH forms and simply persists until intercepted by a substrate. Continuous delivery of substrates to the liver minimizes the steady release of H_2O_2 from FAD-OOH that otherwise would occur.

In spite of sharing a bold mechanism, OMO exhibits high substrate specificity.^{12–14} Hydroxylation of the L -Orn side chain amine by Af-OMO is almost completely coupled to NADPH oxidation (Table 1). D -Orn and 1,4-diaminobutane, isosteric with or smaller than L -Orn, have coupling ratios near 60%. L -Lys, though one methylene unit longer than L -Orn, is not hydroxylated, nor are any of a series of structurally related compounds (Table S1). Similar stringency in substrate preference appears to be common in FMOs involved in siderophore biosynthesis.^{12–14,21–23} We have therefore sought to understand how Af-OMO achieves such specificity in spite of its bold mechanism. Notably, no crystal structure for a substrate-stringent FMO yet exists (see refs 24 and 25). (The indole monooxygenase from the bacterium *Methylophaga* sp. strain SK1 has recently been structurally characterized, but its substrate promiscuity was described as comparable to that of the human liver FMO.^{24,25} Substrate preferences for the structurally characterized *Schizosaccharomyces pombe* FMO have not been described. Hence, it is not yet possible to explain substrate specificity or allosteric regulation (described herein) in simple structural terms. However, given the long-lived FAD-OOH observed in Af-OMO, it potentially shares important features with the *Methylophaga* enzyme: an $NADP^+$ -binding site in an interdomain cleft that protects the FAD-OOH from solvent, and an Asn residue that stabilizes the hydroperoxide via H-bonding.)

Defining substrate interactions with Af-OMO would appear to be critical for understanding specificity. We showed previously that, unlike the liver FMOs, in which the FAD-OOH/substrate reaction is strictly second order, Af-OMO forms a quasi-stable complex with its substrate prior to hydroxylation.^{17,26} This was indicated by competitive inhibition between L -Orn and the N^{ϵ} -hydroxy- L -ornithine (L -Orn- $N^{\epsilon}OH$) product in the steady state. Additionally, the plot of the rate constant for the reaction between FAD-OOH and L -Orn vs $[L\text{-Orn}]$ (k_{FAD} , Scheme 1) saturates with an apparent K_D of 1.1 mM (pH 8), indicating binding. We further demonstrated an interaction between L -Orn and a second, reduced enzyme form (E-FADH⁻, Scheme 1). Specifically, the rate constant for FAD-OOH formation from the reduced enzyme and O_2 ($k_{FAD-OOH}$) increases by about an order of magnitude in the presence of saturating L -Orn (apparent $K_D = 310\text{ }\mu\text{M}$).¹⁷

The observed L -Orn-dependent enhancement in $k_{FAD-OOH}$ led us to look for more potent regulators that could potentially stimulate formation of the reactive species in the presence of the appropriate substrate, or under biologically relevant conditions. We addressed these issues by comparing the effects of L -Orn and a series of related compounds on (1) the rate constant $k_{FAD-OOH}$ (Scheme 1, Figure 1) and (2) the rate constant describing the conversion of FAD-OOH to the oxidized FAD (k_{FAD}) (Scheme 1, Figure 2). The latter occurs along with hydroxylation of the substrate if present, or with the loss of H_2O_2 (see also Table 1). (Substrate hydroxylation involves the intermediacy of a short-lived FAD-OH species that strongly resembles FAD-OOH and which, for the present analysis, is not considered.)

Formation of FAD-OOH from the NADPH-reduced enzyme was monitored via stopped flow UV/vis spectroscopy (Figure 1A), following mixing of the reduced enzyme under Ar with an air saturated solution of buffer containing 5 mM of one of the following: 1,4-diaminobutane (DAB), 5-aminopentanoic acid, L-2,4-diaminobutyric acid, L-citrulline (L-Citr), L-lysine, N^e-tert-butoxycarbonyl-(BOC)-L-lysine, L-glutamine, L-histidine, *n*-octylamine, L-arginine, D-ornithine, L-Orn-N^eOH, or L-ornithine. Of these, only L-Orn, L-Lys, L-Citr, L-Arg, and DAB had an appreciable effect on the observed rate constant for FAD-OOH formation (Tables 1 and S1).

For L-Orn, L-Lys, L-Citr, and L-Arg (Scheme 2), the concentration dependence of k_{obs} was determined, and from this, $k_{\text{FAD-OOH}}$ was extrapolated (Figure 1B, Table 1). The rate constant increases ca. 14-fold in the presence of saturating L-Orn, consistent with prior work.¹⁷ In the presence of either L-Lys or L-Citr, the rate constants increased about half as much. L-Arg, by contrast, has a much more dramatic effect, causing an increase in $k_{\text{FAD-OOH}}$ of greater than 2 orders of magnitude. This demonstrates that L-Arg is a fairly strong effector of O₂ activation.

We subsequently examined the effects of the same full series of compounds on FAD-OOH conversion to FAD (k_{FAD} , Scheme 1; spectra shown in Figure 2A). Except in the presence of L-Orn, D-Orn, and 1,4-diaminobutane, H₂O₂ is overwhelmingly the major product (Tables 1 and S2). L-Lys stimulates the FAD-OOH/FAD conversion at ~60% of the rate constant measured in the presence of L-Orn, and with an identical apparent K_{D} value (Figure 2B). L-Arg, by contrast, has no effect on this step. This strongly suggests that L-Arg discriminates between the reduced and FAD-OOH enzyme forms, selectively stimulating activation of O₂ by the reduced species. L-Arg thus possesses the characteristics of a nonsubstrate allosteric regulator, although it is important to emphasize that in the absence of structural data the actual physical binding sites for L-Arg and L-Orn on the reduced and FAD-OOH enzyme forms, respectively, are unknown and could overlap. It is possible that the positively charged side chain of L-Arg promotes the rate-limiting one-electron reduction of O₂ to superoxide that precedes recombination of the resulting two radicals to form FAD-OOH

$(\text{FADH}^- + \text{O}_2 \rightleftharpoons \{\text{FADH} \bullet \text{O}_2^{\bullet-}\} \rightarrow \text{FAD-OOH})$. L-Orn and L-Lys, also positively charged but smaller than L-Arg, have much smaller effects on $k_{\text{FAD-OOH}}$. The roughly isosteric L-Citr and L-Orn-N^eOH by contrast have uncharged side chains at neutral pH. L-Orn-N^eOH has no measurable effect on FAD-OOH formation, and L-Citr has a small effect with a very high apparent K_{D} (4.4 mM, Figure 1B).

In the presence of excess (5 mM) L-Orn and L-Arg together, $k_{\text{FAD-OOH}}$ is smaller than in L-Arg alone. This is expected as the two compete for the regulatory site (on E-FADH⁻) and L-Orn is a much less efficient allosteric effector than L-Arg. The value of k_{FAD} is only slightly diminished, and the reaction remains completely coupled (Table 1). This suggests that L-Arg does not compete strongly with L-Orn for the E-FAD-OOH binding site.

By contrast, L-Lys (Scheme 2) interacts strongly with the E-FAD-OOH enzyme form. L-Lys is not hydroxylated (Table 1) but stimulates the release of H₂O₂ from FAD-OOH with a rate constant (k_{FAD} , uncoupled) that approaches that measured for the FAD-OOH/FAD conversion that occurs with hydroxylation of L-Orn (Figure 2B). It is possible that the E-FAD-OOH substrate-binding pocket is structurally well-defined and that it admits L-Lys but will not accommodate the larger L-Arg.

The mechanism for O₂ control described here differs from that used by the FAD-dependent aromatic hydroxylases, such as *para*-hydroxybenzoic acid hydroxylase (PHBH).^{2,27,28} PHBH is strongly regulated at the level of FAD reduction, which is 10⁴-fold faster when the correct, *para*-hydroxybenzoate substrate is bound. Substrate “proofreading” is followed by

motions of the protein and FAD that alternately expose and protect the cofactor from solvent.²⁹ Interestingly, excess L-Arg and its neutral isostere L-Citr have a moderate (~18- and 10-fold respectively) influence on the rate constant for reduction of FAD (k_{red}), indicating that they bind the oxidized/FAD form of the enzyme, while L-Lys and L-Orn have no effect (Figure S4). Since k_{red} partially limits the rate of turnover of L-Orn, L-Arg and L-Citr are expected to influence the magnitude of k_{cat} , which they indeed do (Table S2). However, k_{FAD} is also partially rate limiting but unaffected by L-Arg or L-Citr. The composite effects of these compounds on k_{cat} are consequently relatively small.

The regulating mechanism described here also differs from that observed in cytochrome P450s, in which substrate binding displaces a water molecule, causing a spin state change in the ferric iron. This allows for a 10⁵-fold more rapid reduction to Fe(II).³⁰ Once reduced, the iron can readily activate O₂. In Af-OMO, the primary locus of regulation is not FAD reduction but O₂ activation by the reduced FADH⁻; the gating molecule is not substrate but an allosteric effector.

The identification of L-Arg as an allosteric regulator of L-Orn hydroxylation has interesting biological implications (Scheme 3). L-Orn is the initial precursor to both the secreted fusarinine and intracellular ferricrocin siderophores in *A. fumigatus*.^{18–20} Under conditions of iron starvation, siderophore production in this organism is prodigious, commandeering up to 10% of the total cellular biomass. The cell consequently requires efficient ways of generating L-Orn and steering it toward siderophore biosynthesis in response to the Fe status. The metabolic commitment required for siderophore production moreover must be balanced against the need to maintain other cellular functions. L-Orn is produced in the mitochondrion, and its pathway for export to the cytosol is upregulated under Fe deprivation. The pathway for converting L-Arg to L-Orn in the cytosol is likewise upregulated in response to Fe stress. Both pathways effectively sacrifice L-Arg to generate L-Orn. Yet, L-Arg itself is vital for protein biosynthesis and cell signaling and must be available in sufficient amounts to sustain the cell. Activation of OMO by L-Arg appears to connect the sensed Fe status and siderophore production to cellular homeostasis: siderophore biosynthesis is stimulated only if the cytosolic L-Arg pool is sufficient for all of the cell's needs. In turn, the cellular L-Arg concentration acts as a metabolic marker of Fe need; recent work by Shrettl et al. has shown that the cellular concentration of L-Arg dramatically increases (> 10-fold) under conditions of iron starvation.¹⁶

The regulatory mechanism identified here could also be critical for understanding regulation of other biosynthetic pathways. In particular, protein-level regulation could play a role in how plants fine-tune the time and place of auxin production, via the plant FMOs (YUC proteins).³¹ Future work will address these important pathways and the structural basis for the unique allosteric mechanism identified here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Reaction of reduced OMO with O_2 to form FAD-OOH in the presence of saturating (5 mM) ι -Arg, as monitored by stopped flow UV/vis spectroscopy over time (measurement times listed). The initial spectrum is shown in red, and the final in blue. Spectra measured in between are shown in gray. See ref 17 for analogous data for ι -Orn and Figure S1 (ι -Lys). (B) Concentration dependence of k_{obs} for this conversion (fit to a single exponential at 370 nm; see Figure S2) in the presence of ι -Orn, ι -Lys, ι -Arg, and ι -Citr. These have measured apparent K_D values of 310 ± 40 μ M, 130 ± 30 μ M, 620 ± 70 μ M, and 4.4 ± 0.9 mM respectively. Associated values for $k_{FAD-OOH}$ extrapolated from these data at saturating concentrations for each compound are listed in Table 1.

Figure 2.

(A) Conversion of the OMO FAD-OOH to oxidized FAD and H_2O_2 in the presence of saturating (5 mM) l-Arg as monitored by stopped flow. (B) Concentration dependence of k_{obs} for the conversion in (A) (fit to a single exponential at 450 nm; see Figure S3) in the presence of l-Orn or l-Lys . These have measured apparent K_D values of 1.1 ± 0.09 and 1.2 ± 0.1 mM respectively. Note that $\text{l-Orn-N}^{\text{e}}\text{OH}$ is the major product in the presence of l-Orn , while the enzyme converts O_2 to H_2O_2 in l-Arg and l-Lys . Values for k_{FAD} extrapolated from these data at saturating concentrations for each compound are listed in Table 1.

Scheme 1.
Reactions Catalyzed by OMO

Scheme 2.

Amino Acids and Related Compounds Discussed in the Text^a

^a These are drawn in the protonation states expected at neutral pH.

Scheme 3.

Pathways for L-Orn Production and Conversion into Siderophores (*mito* = mitochondrion; *cyto* = cytoplasm)^a

^a Steps upregulated under Fe-deficient conditions are indicated by blue arrows. This scheme is adapted from ref 16.

Table 1

Rate Constants, Apparent Rate Constants, and Coupling Ratios Measured in the Presence of the Given Compounds^a

compd ^b	k _{red} (s ⁻¹)	k _{FAD-OOH} (s ⁻¹)	k _{FAD} (s ⁻¹)	compd-OH: NADPH ^c
None	<u>1.21</u> (0.03)	<u>1.28</u> (0.13)	<u>0.041</u> (0.00)	0
L-Orn	<u>1.46</u> (0.16)	<u>19.8</u> (0.5)	<u>2.58</u> (0.05)	0.87 (0.06)
D-Orn	ND ^d	1.78 (0.01)	0.142 (0.004)	0.63 (0.03)
DAB	ND	4.74 (0.16)	0.151 (0.001)	0.59 (0.02)
L-Lys	<u>2.26</u> (0.05)	<u>9.0</u> (0.3)	<u>1.98</u> (0.04)	0.03 (0.01)
L-Arg	<u>20.3</u> (0.8)	<u>144</u> (4.5)	<u>0.038</u> (0.00)	0.06 (0.03)
L-Citr	<u>13.3</u> (0.7)	<u>16.9</u> (1.0)	0.157 (0.002)	0.10 (0.001)
L-Orn-N ^e OH	ND	1.13 (0.02)	0.04 (0.00)	ND
L-Orn+L-Orn-N ^e OH	ND	13.5 (0.4)	0.34 (0.01)	ND
L-Orn+L-Lys	1.74 (0.12)	15.6 (0.3)	0.157 (0.002)	0.89 (0.06)
L-Orn+L-Arg	13.4 (0.66)	48.5 (1.1)	1.75 (0.07)	1.03 (0.07)

^a Concentration dependencies were measured for rate constants when 5 mM of the added compound was observed to have a >5-fold effect on the step of interest relative to no-compound-added controls (see Figures 1B and 2B). Extrapolated rate constants obtained from such data are underlined. Otherwise, apparent rate constants at 5 mM each of the given compounds are reported. The average of three measurements is given with the standard deviation as the error in parentheses. All measurements were made at 25 °C in 100 mM Tris-SO₄ buffer, pH 8.

^b Several additional compounds not shown here were examined for their effects on the reaction kinetics. See text and Supporting Information.

^c Coupling ratios were measured in the presence of limiting amounts of NADPH.

^d ND = not determined.