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O-Demethylations Catalyzed by Rieske Nonheme Iron Monooxygenases Involve the Difficult Oxidation of a Saturated C−H Bond

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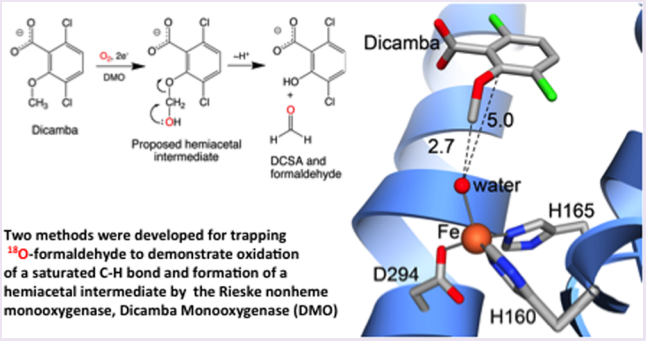
Supporting Information

ABSTRACT: Dicamba monooxygenase (DMO) catalyzes the O-demethylation of dicamba (3,6-dichloro-2-methoxybenzoate) to produce 3,6-dichlorosalicylate and formaldehyde. Recent crystallographic studies suggest that DMO catalyzes the challenging oxidation of a saturated C−H bond within the methyl group of dicamba to form a hemiacetal intermediate. Testing of this hypothesis was made possible by our development of two new independent techniques. As a novel method to allow use of $^{18}O_2$ to follow reaction products, bisulfite was used to trap newly formed ^{18}O-formaldehyde in the stable adduct, hydroxymethanesulfonate (HMS^-), and thereby prevent the rapid exchange of ^{18}O in formaldehyde with ^{16}O in water. The second technique utilized unique properties of *Pseudomonas putida* formaldehyde dehydrogenase that allow rapid conversion of ^{18}O-formaldehyde into stable and easily detectable ^{18}O-formic acid. Experiments using these two new techniques provided compelling evidence for DMO-catalyzed oxidation of the methyl group of dicamba, thus validating a mechanism for DMO [and for vanillate monooxygenase, a related Rieske nonheme iron monooxygenase] that involves the difficult oxidation of a saturated C−H bond.



Two methods were developed for trapping ^{18}O-formaldehyde to demonstrate oxidation of a saturated C-H bond and formation of a hemiacetal intermediate by the Rieske nonheme monooxygenase, Dicamba Monooxygenase (DMO)

Rieske nonheme iron monooxygenases are involved in the synthesis of myriad natural organic compounds as well as in the degradation of these (and xenobiotic) compounds by bacteria and other microorganisms in the environment.[1−4] One such organism is the soil bacterium *Stenotrophomonas* (formerly *Pseudomonas*) *maltophilia*, strain DI-6, that is capable of completely degrading the herbicide dicamba (2-methoxy-3,6-dichlorobenzoic acid) to CO_2, H_2O, and Cl^-.[5,6] The first step in this degradation pathway is catalyzed by dicamba O-demethylase (DOD), which converts the herbicide dicamba to 3,6-dichlorosalicylic acid (DCSA) through the sequential action of three separate proteins.[7,8] In this reaction, electrons originating from NADH are transferred to FAD in a reductase, which then reduces a ferredoxin that, in turn, reduces the terminal monooxygenase (dicamba monooxygenase, DMO). DMO is a nonheme iron Rieske oxygenase that catalyzes the two-electron oxidation of dicamba to produce DCSA and formaldehyde.[8,9] The ability of DMO to inactivate dicamba has been used to create dicamba-resistant transgenic crop plants that soon will be commercially available to farmers.[10,11]

Although Rieske nonheme iron monooxygenases are known to catalyze the O-demethylation of several aromatic compounds, the mechanisms by which oxygen is activated and attacks the enzyme substrate remains uncertain. The recent determination of the crystal structure of DMO[12] has revealed that the carbon atom of dicamba's O-methyl group is the portion of the substrate closest to O_2 in the active site of the enzyme. This and previous observations[2] that the carbon closest to O_2 is the most likely target for oxidation have provided strong suggestive evidence that molecular oxygen, activated by transfer of electrons from the enzyme's mononuclear iron atom, contributes one of its oxygen atoms to break a C−H bond in the O-methyl group of dicamba with no oxidation of the C2 carbon of the dicamba ring structure. This oxygenation is proposed to form an unstable hemiacetal intermediate that would likely decompose to release formaldehyde and DCSA (Figure 1a and b). A possible mechanism for this oxidation in DMO is illustrated in Supporting Figure S1. Furthermore, 4-toluene sulfonate methyl monooxygenase, a homologue of DMO that acts on a substrate that cannot be O-demethylated as can dicamba, produces 4-sulfobenzyl alcohol as a product.[13] This stable primary alcohol product of 4-toluene sulfonate methyl monooxygenase is similar to the proposed hemiacetal intermediate of DMO and suggests that hemiacetal formation is plausible in related Rieske O-demethylases. The comparative difficulty of inserting an oxygen atom into a saturated C−H bond suggests that either a high valency iron-oxo species such as HO-Fe(V)═O or an isoelectronic oxygen radical species are likely involved in DMO-catalyzed oxygenation.[14,15] However, to date no direct evidence for the attack of

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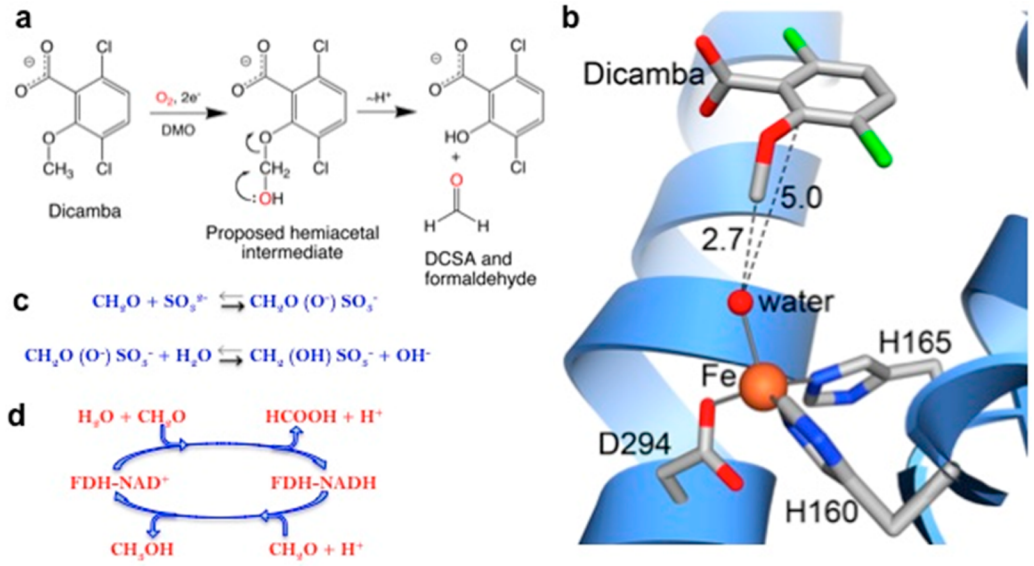

Figure 1. Proposed mechanism for DMO-catalyzed conversion of dicamba to DCSA and formaldehyde and methods for trapping formaldehyde as a reaction product. (a) The proposed mechanism for the reaction catalyzed by DMO. Atoms contributed by molecular oxygen are labeled in red, and the flow of electrons in the spontaneous decomposition of the proposed hemiacetal intermediate is indicated by curved arrows. (b) Structural analysis of dicamba-bound DMO supports oxygenation at the exocyclic methyl group. The 2.1 Å resolution crystal structure of dicamba-bound DMO (PDB 3GL2) is shown with key residues in the mononuclear iron active site labeled. The water coordinating the iron atom was observed to be substituted by dioxygen in higher resolution structures of DMO alone and bound to product.[12] Therefore its position is a reliable indicator of the binding site for molecular oxygen. The distances between the water and the two possible target carbon atoms of dicamba are indicated with dotted lines, with distances given in angstroms. The exocyclic methyl group of dicamba is well-positioned for attack by reduced oxygen, while the aromatic carbon atom appears too distant. (c) Scheme for trapping formaldehyde with bisulfite and the formation of the formaldehyde-sodium bisulfite adduct, $CH_2(OH)SO_3^-$ (hydroxymethane sulfonate, HMS^-). (d) Scheme for trapping formaldehyde as formic acid in the presence of *P. putida* C83 formaldehyde dehydrogenase (FDH) with tightly bound NAD+/NADH as a cofactor.

an oxygen atom on a C−H bond in the O-methyl group of dicamba or of the resulting hemiacetal intermediate is available, and the partitioning of the atoms derived from molecular oxygen into the products is unknown.

Here we describe development and use of two new methods to follow the fate of $^{18}O_2$ in the DMO-catalyzed monooxygenation of dicamba. The first approach takes advantage of the ability of bisulfite to rapidly bind with formaldehyde to form the stable adduct hydroxymethanesulfonate (HMS^-)[16] (Figure 1c). In our unique application of this reaction, HMS^- acts as a trap for ^{18}O incorporated into formaldehyde by preventing rapid exchange of ^{18}O in formaldehyde with ^{16}O in water, a process that occurs through the reversible formation of methanediol [methylene glycol, $H_2C(OH)_2$]. The second independent technique used to document ^{18}O incorporation into formaldehyde exploited the unique ability of *Pseudomonas putida* formaldehyde dehydrogenase (FDH)[17,18] (Figure 1d) to rapidly convert newly formed $^{18}O^-$formaldehyde to a 1:1 combination of ^{18}O-methanol and ^{18}O-formic acid, with the latter being easily detected by standard mass spectrometry (MS) procedures. Results using these new techniques are presented and discussed in regard to the mechanism of the DMO catalyzed O-demethylation of dicamba and the substrate specificity of activated oxygen in this reaction. These techniques have provided evidence for the existence of a hemiacetal intermediate in the oxidative demethylation of dicamba by DMO as well as in the structurally similar enzyme vanillate monooxygenase (VMO).

Trapping of Formaldehyde with Sodium Bisulfite. To demonstrate the detection of formaldehyde using sodium bisulfite to form the adduct hydroxymethane sulfonate (HMS^-, $CH_2(OH)SO_3^-$) in a complete reaction mixture containing the three components of dicamba O-demethylase (DOD) and dicamba, reactions were carried out in the absence of bisulfite and in the presence of 0.5 mM and 2 mM bisulfite (Supporting Figure S2). Although a small peak of m/z = 111, the expected mass of the HMS^- ion, was observed in the control reaction containing no bisulfite (Supporting Figure S2a), addition of increasing amounts of bisulfite resulted in the production of increasing amounts of the m/z = 111 ion, suggesting that bisulfite was reacting with formaldehyde produced by the DOD reaction to form HMS^-. Further verification of the formation of HMS^- in the complete DOD reaction containing 2 mM bisulfite was obtained in reactions performed either in the absence of added ^{13}C-labeled formaldehyde or in the presence of 0.5 mM and 1 mM $^{13}CH_2O$ (Supporting Figure S3a, b, and c, respectively). In addition to the expected HMS^- ion (m/z = 111) formed with formaldehyde produced during the O-demethylation of dicamba, an ion of m/z = 112 was observed when exogenous $^{13}CH_2O$ was provided in the reaction mixture, verifying that the HMS adduct is being formed between formaldehyde and bisulfite in the reaction mixture.

To determine if added bisulfite inhibited the DOD-catalyzed conversion of dicamba to DCSA, we carried out the reaction in the absence of bisulfite and in the presence of 0.5 mM and 2 mM bisulfite (Supporting Figure S4a, b, and c, respectively) and with a reaction time that allowed for the detection of both substrate and product. In all cases approximately equal proportions of the two dicamba ions [m/z = 219 (Cl isotope 35) and m/z = 221 (Cl isotope 37)] and DCSA ions (m/z = 205 and m/z = 208) were produced, indicating no obvious inhibition by bisulfite under these conditions.

Detection of ^{18}O-Labeled Formaldehyde, but No Detection of ^{18}O-Labeled DCSA. If our model of substrate

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oxygenation based on the DMO crystal structure is correct, the DOD reaction carried out in the presence of $^{18}O_2$ should yield formaldehyde with a mass of 32 and a subsequent HMS^- ion with m/z = 113. During MS analysis of products from a DOD reaction performed in the presence of $^{18}O_2$ but lacking dicamba, a "background" ion of m/z = 113 was detected (Figure 2a). [This background compound was determined to be a consistent contaminant in the acetonitrile used as a solvent for MS samples and when subjected to MS/MS analysis produced two product ions of m/z = 69 and m/z = 83 and, importantly, no observable m/z = 81 corresponding to HMS^- (Supporting Figure S5)]. The same DOD reaction carried out in the presence of dicamba apparently resulted in slightly more of the m/z = 113 peak (Figure 2b). To determine if either ion with (m/z = 113) contained ^{18}O-labeled HMS^-, the m/z = 113 peak was subjected to MS/MS (Figure 2, lower panels). Collision induced dissociation of the m/z = 113 ion from the reaction lacking dicamba produced an abundance of m/z = 63 and m/z = 83 ions from the background compound and a small peak of m/z = 81 (Figure 2a). In contrast, the reaction containing dicamba produced a significantly greater abundance of ion m/z = 81 (Figure 2b) ($P < 0.01$, Supporting Figure S6), suggesting the reaction conducted in the presence of dicamba produced $CH_2{}^{18}O$, as predicted. Conversely, when similar reactions were conducted in the presence of $^{18}O_2$ (Supporting Figure S7a) or in the presence of air ($^{16}O_2$) (Supporting Figure S7b), there were no differences in the masses (m/z = 205 and m/z = 207) of the product, DCSA, verifying that oxygen is not incorporated into the ring structure of dicamba during dicamba O-demethylation.

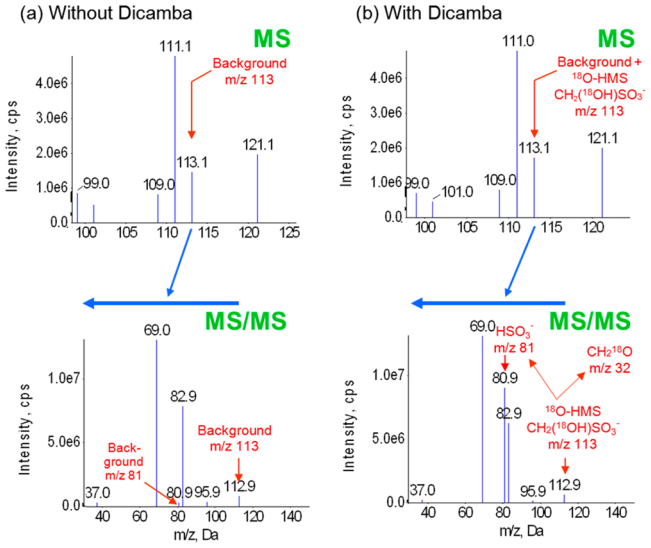

Figure 2. Mass spectrometry MS and MS/MS analyses of ^{18}O-labeled formaldehyde-bisulfite adduct (HMS, m/z = 113) formed in a DOD reaction (a) without dicamba and (b) with dicamba. A mixture of 21% $^{18}O_2$ and 79% of N_2 was injected into the reaction mixture in an evacuated reaction vessel along with bisulfite (2 mM final concn) to trap ^{18}O-labeled formaldehyde as ^{18}O-HMS with a m/z of 113. Upper scans, MS analyses of reaction contents and products with m/z between 99 and 121. Lower scans, MS/MS analyses of products from bombardment of the m/z 113 ion selected from the initial MS ion separations resulting in detection of HSO_3^- (m/z 81), a product derived from bombardment of ^{18}O-HMS (m/z = 113) and the release of $H_2C^{18}O$ (m/z 32) and HSO_3^- from the formaldehyde/sulfite adduct formed in the DOD reaction containing dicamba. cps: counts per second.

Independent Verification of Incorporation of ^{18}O into Formaldehyde, but Not DCSA. As an independent method for verifying that the reaction of activated ^{18}O with the carbon atom of the dicamba O-methyl group produced ^{18}O-labeled formaldehyde, we utilized formaldehyde dehydrogenase (FDH) from *Pseudomonas putida* C83.[17,18] Each subunit of this enzyme has the unique characteristic of tightly binding a single oxidized nictotinamide adenine dinucleotide molecule (NAD^+) and converting it as a cofactor (not as a coenzyme) to NADH during dismutation of formaldehyde to formic acid (an irreversible reaction). Once NADH is formed, the enzyme is then capable of converting the next incoming formaldehyde molecule to methanol and simultaneously converting NADH to NAD^+, without the release of the cofactor[17,18] (Figure 1d). Because the DOD reaction must be conducted in the presence of high levels of NADH (2.5 mM) and this concentration drives the conversion of formaldehyde to methanol in reactions catalyzed by most other types of FDHs,[19,20] the *P. putida* FDH offered an opportunity to capture at least a portion of ^{18}O-formaldehyde as formic acid that, unlike formaldehyde, does not readily exchange oxygen with water.

To confirm that *P. putida* FDH could be used successfully to detect formaldehyde when included in a DOD reaction mixture, we first performed reactions containing ^{13}C-labeled formaldehyde. If the reaction was conducted in the absence of $H_2{}^{13}CO$, no ion corresponding to m/z = 46 was observed (Supporting Figure S8a). When increasing amounts of ^{13}C-labeled formaldehyde (i.e., 0.1 mM and 0.5 mM) were added to the reaction, increasing amounts of ions of m/z = 46 were

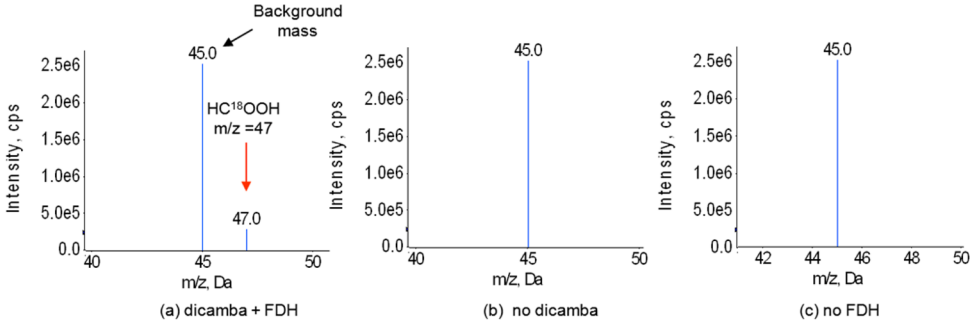

Figure 3. Mass spectrometry detection of ^{18}O-labeled formic acid ($HC^{18}OOH$, m/z = 47) formed in DOD reaction mixtures having both 2.5 mM NADH and 2.5 mM NAD and carried out in the presence of 21% $^{18}O_2$ and 79% N_2 . Reaction mixtures contained (a) dicamba plus 0.1 unit of FDH, (b) FDH but no dicamba, and (c) dicamba but no FDH.

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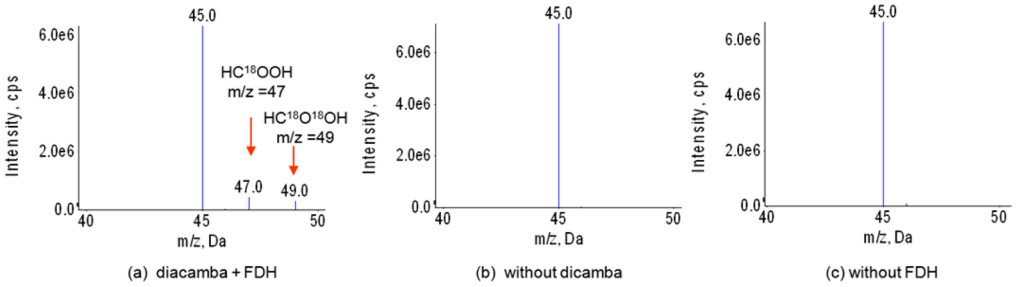

Figure 4. Mass spectrometry detection of ^{18}O-labeled formic acid ($HC^{18}OOH$, m/z = 47; $HC^{18}O^{18}OH$, m/z = 49;) formed in the DOD enzyme reaction system containing $H_2{}^{18}O$, $H_2{}^{16}O$, and $^{18}O_2$ and in the presence or absence of dicamba and the presence or absence of FDH. The reactions were carried out in a 1:1 mixture of $H_2{}^{18}O$ and $H_2{}^{16}O$ in the reaction buffer and with an atmosphere of 21% $^{18}O_2$ and 79% N_2. (a) Reaction containing dicamba plus FDH, (b) reaction containing no dicamba, and (c) reaction containing no FDH.

observed (Supporting Figure S8b and c, respectively), indicating successful conversion of formaldehyde to formic acid in the presence of *P. putida* FDH. Next, we added FDH to a complete DOD reaction mixture containing dicamba and $^{18}O_2$. This reaction produced a product with m/z = 47, the expected mass of formic acid containing a single ^{18}O atom (i.e., $HC^{18}OOH$) (Figure 3a), whereas reactions lacking dicamba or FDH (Figure 3b and c, respectively) lacked such an ion. Similar reactions conducted in the presence of $^{18}O_2$ and in the presence of FDH (Supporting Figure S9a) or in the absence of FDH (Supporting Figure S9b) display no shifts in the masses of ions associated with the reaction product, DCSA, again confirming the lack of an ^{18}O atom addition to the DCSA ring structure. In a modification of these experiments aimed at confirming the identity of the ion with a mass m/z = 47 as ^{18}O-labeled formic acid, similar reactions were conducted in an buffer containing a 1:1 mixture of $H_2{}^{18}O$ and $H_2{}^{16}O$ in an atmosphere of $^{18}O_2$ (Figure 4a). In this reaction two ions with m/z = 47 and m/z = 49 corresponding to the expected masses of $HC^{18}O^{16}OH$ and $HC^{18}O^{18}OH$ were produced. Reactions lacking dicamba or FDH (Figure 4b and c, respectively) produced no detectable formic acid ions.

Vanillate Monooxygenase Reaction Also Produces ^{18}O-Labeled Formaldehyde, but Not ^{18}O-Labeled Protocatechuate. Similarity of the structural components of DMO and vanillate monooxygenase (VMO) and their reaction products[21−23] suggests that their reaction mechanisms may be analogous. To determine if VMO directs an activated oxygen atom to the carbon atom of the O-methyl group of vanillate during its conversion to protocatechuic acid (PCA) or, alternatively, to the ring structure of vanillate, we conducted the VMO reaction in the presence of $^{18}O_2$. At the start of the reaction (Supporting Figure 10a), a major ion of m/z = 167 corresponding to the vanillate ion was present. After 2.5 h of reaction, a new ion (m/z = 153) corresponding to nonisotopically labeled PCA was detected (Supporting Figure S10b). When the same reaction was conducted in the presence of bisulfite, an ion with the expected mass m/z = 113 of ^{18}O-containing HMS^- was present both at zero time and after 2.5 h of reaction (Supporting Figure S11a and b). When the m/z = 113 ion from the zero time reaction was subjected to MS/MS analysis, the parent HMS^- ion of m/z = 113 was seen as well as a minor ion of m/z = 81 (Supporting Figure S12a). However, in the reaction lasting 2.5 h, the parent (m/z = 113) ion was degraded during MS/MS bombardment to produce substantial quantities of a m/z = 81 ion, the expected mass of an HSO_3^- ion resulting from the release of $CH_2{}^{18}O$ from the m/z = 113 HMS^- adduct of $H_2C^{18}O$ and HSO_3^- (Supporting Figure S12b). These results suggest the VMO and DMO monooxygenase reactions proceed by similar, if not identical, mechanisms.

Details of the DMO Reaction Mechanism As Related to DMO Structure. The 1.75 Å crystal structure of DMO showed evidence of a diatomic species bound near the catalytic mononuclear iron site that was consistent with oxygen.[12] The proximity of this species to the exocyclic methyl group of dicamba (Figure 1b) suggested that activated oxygen attacks a saturated C−H bond in this moiety, which has been confirmed in this work. The oxygenation of saturated hydrocarbons is known to be one of the most chemically difficult reactions in nature but is catalyzed by at least two other classes of enzymes: methane monooxygenases[24] and the cytochrome P450s.[25] In both cases, highly reactive Fe(IV)- or Fe(V)-oxo species are proposed to be the active oxygenating species. The ability of the Rieske nonheme iron oxygenases to catalyze a similar chemistry suggests that a high valency iron-oxo species may also be the oxygenating species for at least some of these enzymes. This is consistent with recent proposals by Chakrabarty et al.[14] and van der Donk et al.[15]

Implications Regarding the DMO and VMO Enzyme Mechanism to That of Other Rieske Monooxygenases. DMO and VMO are members of a larger group of Rieske oxygenases that includes 4-methoxybenzoate monooxygenase[22] 4-toluene sulfonate methyl monooxygenase[26] and stachydrine demethylase.[27] Our results suggest that other enzymes catalyzing similar reactions may produce the same partitioning of isotopically labeled oxygen into products akin to those generated by DMO and VMO. Although the structures of the related VMO and 4-toluene sulfonate methyl monooxygenase enzymes are not available, a recent structural and spectroscopic study of the more distantly related stachydrine demethylase features a proposed mechanism that is similar to the one we have proposed for DMO, including the production of an intermediate resembling the hemiacetal in the DMO mechanism.[27] The techniques developed in the present studies should facilitate future experiments to determine if these and other Rieske nonheme monooxygenases share a common mechanism. More broadly, these methods should be of significant value to biologists and biochemists analyzing any of the myriad enzyme mechanisms involving formation of formaldehyde.

METHODS

Methods are available in Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

Methods and Supporting Figures S1−S12. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): The University of Nebraska has licensed dicamba resistance gene technology for commercial development and D.P.W. receives royalties from that licensing.

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