Determination of the Carbon Kinetic Isotope Effects on Propane Hydroxylation Mediated by the Methane Monooxygenases from *Methylococcus capsulatus* (Bath) by Using Stable Carbon Isotopic Analysis

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Authentic propane with known position-specific carbon isotope composition at each carbon atom was subjected to hydroxylation by the particulate and soluble methane monooxygenase (pMMO and sMMO) from Methylococcus capsulatus *(Bath), and the corresponding position-specific carbon isotope content was redetermined for the product 2-propanol. Neither the reaction mediated by pMMO nor that with sMMO showed an intermolecular $^{12}C/^{13}C$ kinetic isotope effect effect on the propane hydroxylation at the secondary carbon; this indicates that there is little structural change at the carbon center attacked during formation of the transition state in the rate-determining step. This finding is in line with the concerted mechanism proposed for pMMO (Bath), and suggested for sMMO (Bath), namely, direct side-on insertion of an active "O" species across the C–H bond, as has been previously reported for singlet carbene insertion.*

KEYWORDS:

enzyme catalysis · hydroxylation · isotope effects · mass spectrometry · methane monooxygenases

Introduction

Methanotrophic bacteria utilize methane as their sole source of carbon and energy.[1–3] Associated with these organisms is the enzyme methane monooxygenase that catalyzes the incorporation of one atom of oxygen from O_2 into one of the C–H bonds of methane to form methanol; the other oxygen atom is converted in to water in the presence of protons. There are two distinct forms of this enzyme. All methanotrophic bacteria use the copper-containing particulate methane monooxygenase (pMMO).[4] Several methanotrophs also express the iron-containing soluble methane monooxygenase (sMMO) under copper-limiting growth conditions.[5, 6] The type X methanotroph *Methylococcus capsulatus* (Bath) is one of the few species that expresses both proteins.[7–10] The study of the C–H bond activation by these proteins has become an area of significant current interest.[5–10] For an excellent overview of the sMMO-catalyzed methane hydroxylation reaction, the interested reader is referred to a recent review by Lippard and co-workers.[11]

Several possible mechanisms for the catalytic function of MMO have been considered.[12–15] One involves the radical mechanism, wherein an activated "oxygen" species in the enzyme abstracts a hydrogen atom from the substrate methane molecule, followed by radical-rebound chemistry of the methyl radical with the "hot" hydroxyl radical to form the product.[15–18] The other mechanism invokes anchoring of the methane molecule at an activated metal cluster and concerted oxenoid or "oxene" insertion across one of the C–H bonds via a four- or three-centered transition state.[14, 19] Radical clock experiments with the sMMO (Bath) system failed to reveal ring-opened products; this fact suggests either formation of a radical species with an extremely short lifetime ($t < 100$ fs) or no formation of a substrate radical during the course of the hydroxylation reaction.[17, 18] Similarly, analysis of the products from the sMMO-catalyzed methylcubane hydroxylation lead to the conclusion that cationic species were produced without forming radical intermediates.[20] The limited substrate range of the pMMO has obviated analogous radical clock studies on the hydroxylation mediated by this enzyme.[19] However, experiments on cryptically chiral ethanes, [1-$^1H_1$,1-$^3H_1$]ethane, showed that the hydroxylation mediated by pMMO[14] and sMMO (Bath)[16] proceeded with 100% and 72% retention of config-

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uration at the carbon attacked, respectively. Accordingly, a concerted reaction mechanism was proposed for the pMMO-mediated reaction,[14] and a nonsynchronous mechanism involving an extremely short-lived alkyl radical in the transition state was proposed for the sMMO (Bath).[15]

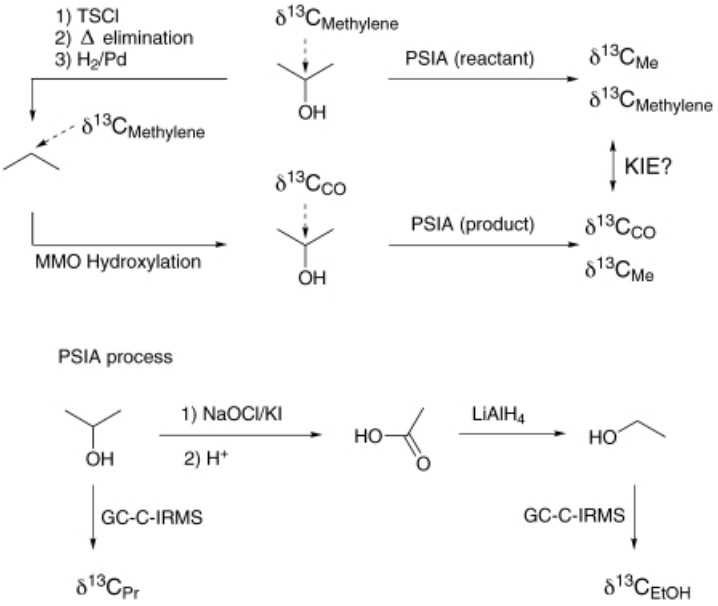
The kinetic isotope effect (KIE) of the hydroxylation of various alkanes mediated by these enzymes has also been studied intensively.[21–23] Unfortunately, these studies have not yielded a consistent picture. Steady state experiments with sMMO (Bath) have revealed a small k_H/k_D ratio (1.7) when the V_{max} value were compared between CH_4 and CD_4 (k_H = rate constant for hydroxylation of CH_4, k_D = rate constant for hydroxylation of CD_4, V_{max} = maximum reaction velocity).[22] Also, both individual and competitive reactions with the nondeuterated and trideuterated methyl group of *trans*-2-phenylmethylcyclopropane by sMMO (Bath) yielded a k_H/k_D ratio of 1.[18] On the other hand, intramolecular k_H/k_D ratios of 3 – 4 have been determined from the products formed in chiral ethane experiments in the case of sMMO (Bath).[15] Similarly, intramolecular k_H/k_D ratios of 5.2 – 5.5 have been deduced from experiments on the cryptically chiral ethanes in the case of pMMO.[14]

In this study, we report the $^{12}C/^{13}C$ KIE on the sMMO- and pMMO-mediated hydroxylation of propane. Recently, we have analyzed the carbon isotope compositions of individual carbon positions of several C-3 species, including acetone, isopropanol, and propane, at natural abundance levels with great accuracy and high precision by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). We exploited online combustion of the compounds separated by gas chromatography to measure the isotope distribution of each of these C-3 species, before and after their degradation to the corresponding C-2 species with one of the terminal methyl groups removed.[24] The results of the compound-specific isotope analysis of the C-3 and C-2 species were then combined to yield position-specific carbon isotope distributions at the two unique carbon atoms. This same approach is used in the present study to determine the carbon KIE during the MMO-mediated propane hydroxylation reactions.

Results

Preparation of authentic propane

Preparation of the authentic propane with known isotopic signatures of individual carbon positions is critical for the outcome of this study. The carbon skeleton of propane could be synthesized by condensing smaller molecules such as methylmagnesium chloride and methyl formate. The advantage of the condensation process is that the isotopic compositions of individual carbon positions within propane might be determined from the starting molecules. However, isotopic fractionation often occurs during the condensation reaction, and if the reaction is incomplete, the outcome becomes obscured. For example, upon the synthesis of carboxylic acids from the reaction of Grignard reagents with CO_2, Vogler and Hayes[25] observed isotopic fractionation, with $k_{12C}/k_{13C} = 1.059$. In contrast, propane prepared from a precursor with the desired carbon skeleton requires only a suitable degradation process to isolate the different molecular positions. Recent experiments in our laboratory indicate that no carbon isotopic fractionation occurs during the iodoform reaction of isopropanol.[24] Accordingly, we took advantage of this result to synthesize the propane in quantitative yields from isopropanol, in a reaction sequence involving tosylation, elimination, and hydrogenation as outlined in Scheme 1. Since the tosylation reaction takes place only at the oxygen atom of the alcohol moiety, the oxygenated carbon center in the isopropanol remains untouched. Thus, no carbon KIE is expected to occur during the tosylation. The subsequent elimination of isopropyl tosylate was carried out under pyrolytic condition. The tosylate was heated gently above the decomposition temperature (128 °C). Propene was produced quantitatively and no side reactions were observed according to the GC studies. Since the reaction was allowed to run to completion, no carbon KIE could be observed in the elimination step. Finally, the hydrogenation of the propene was carried out in an apparatus designed specially for this purpose (Figure 1). Under these



Scheme 1. *Determination of the carbon $^{12}C/^{13}C$ KIE in the propane hydroxylation reaction mediated by methane monooxygenases (MMO). $\delta^{13}C_{Pr}$ and $\delta^{13}C_{EtOH}$ values were determined by GC-C-MS; other $\delta^{13}C$ values were calculated from these two values. The $\delta^{13}C$ values are calculated with the equations found in the Experimental Section.*

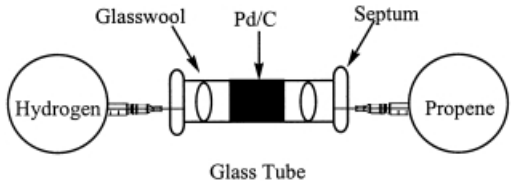


Figure 1. *The apparatus for propene hydrogenation.*

reaction conditions the yield of propane was also quantitative. Only slight amounts of hydrogen gas remained after the process.

In this manner prepared propane was used without further purification. Further verification of the lack of isotope fractionation during the overall synthetic process was concluded by GC-C-IRMS analysis. Both the propane and the precursor isopropanol gave the same overall carbon isotopic composition, $\delta^{13}C‰$ value ($-26.9 \pm 0.1‰$). The $^{13}C/^{12}C$ isotope abundance ratio of a test sample is normally referred to the corresponding isotope abundance ratio of a standard, which is usually PDB (a belemnite from the Cretaceous Peedee Formation), and is expressed as in Equation (1):

$$\delta^{13}C‰ = \left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{PDB}} - 1\right] \times 1000 \quad (1)$$

δ values are given in units of per mil or ‰ relative to the standard. For PDB $^{12}C/^{13}C = 88.99$ and its $\delta^{13}C$ value is set to be 0‰ according to the definition. Therefore, the isotope compositions of the individual carbon positions of the authentic propane must mirror the position-specific isotope analysis (PSIA) in the corresponding isopropanol precursor. The PSIA of the isopropanol was then carried out as described previously[24] to yield $\delta^{13}C_{Me}$ ($-26.2 \pm 0.4‰$) and $\delta^{13}C_{CO}$ ($-28.2 \pm 0.5‰$) for the methyl and methylene carbons in the propane, respectively.

$^{12}C/^{13}C$ Kinetic isotope effect on the propane C-2 hydroxylation

To determine the $^{12}C/^{13}C$ KIE on the sMMO- and pMMO-mediated hydroxylation chemistry, the synthesized authentic propane was incubated with MMOs, NADH, and O_2. When whole-cell incubation was used, a suitable amount of sodium formate (1 mM) was also added to the reaction mixture. Since the comparison of intermolecular KIEs at a single substrate concentration could be misleading, the hydroxylation was studied over a wide range of propane concentrations. The reaction mixtures after 2 h incubation at 45 °C were centrifuged and the isopropanol-containing supernatant was subjected to the iodoform reaction. The resulting acetate-containing solution was extracted and reduced to ethanol. The compound-specific carbon isotope abundance ratios of both the ethanol and isopropanol obtained from GC-C-IRMS were used to calculate the isotope compositions of the individual carbon positions as before. The $\delta^{13}C_{CO}$ values of the enzymatically produced isopropanol are shown in Table 1.

In the case of pMMO-mediated hydroxylation, the $\delta^{13}C_{CO}$ value ($-27.6 \pm 0.6‰$) is very close to that for the propane standard ($-28.2 \pm 0.5‰$), a fact implying an intermolecular KIE of $^{12}C/^{13}C = 1.000 \pm 0.001$. Similarly, the $\delta^{13}C_{CO}$ value ($-29.8 \pm 1.2‰$) obtained from the sMMO-mediated reaction[26] is only slightly different from that of the propane standard. However, when whole cells were used in the hydroxylation, the $\delta^{13}C_{CO}$ value ($-28.2 \pm 0.5‰$) was in close agreement with that of the propane standard. As any primary isotopic effect on carbon in the range of 1.02 – 1.10 ($^{12}C/^{13}C$) should give a differential $\delta^{13}C_{CO}$ (depletion) in the range of 10 – 50 per mil, it is evident that a $^{12}C/^{13}C$ kinetic isotope effect of this magnitude is not being observed here. Thus, the propane hydroxylation reaction mediated by neither sMMO nor pMMO from *M. capsulatus* (Bath) shows an intermolecular KIE ($^{12}C/^{13}C$) effect.

Table 1. *The $\delta^{13}C_{CO}$ (‰) values of isopropanol from biomediated propane hydroxylation.*[a]

Propane[b] [μmol]	pMMO	sMMO	sMMO-cell	standard
40	-28.3	-30.0	-28.2 ± 0.5	
4	-27.1	-31.0		
0.4	-27.4	-28.6		
average	-27.6 ± 0.6	-29.8 ± 1.2	-28.2 ± 0.5	-28.2 ± 0.5

[a] Bioconversions of propane into isopropanol were performed at 45 °C in 10-mL conical flasks closed with a rubber septum. The products were generated by the incubation of either a pMMO-containing membrane suspension (2 mL, isolated from 2 g of pMMO-cell) or a sMMO-containing crude extract (3 mL, from 2 g of sMMO-cell) with NADH (10 mM) in MOPS buffer (10 mM, pH 7.0) and substrate. In the case of whole cell studies, sMMO-cells (2 g) were suspended in sodium formate (2 mL, 1 mM) containing potassium phosphate buffer (25 mM, pH 7.0) and incubated with NADH and substrates in a shaking incubator. [b] Either pure propane or air-diluted propane (1 mL) was added to the gas phase of incubation.

Discussion

It is possible to rationalize the apparent lack of a $^{12}C/^{13}C$ KIE on the hydroxylation reaction in a number of ways. First, however, it is unlikely that the isotope effect is masked by a slow product release. H/D isotope effects have now been observed for both ethane and butane, and compared with ethane the turnover of the enzyme is only 30% slower for propane. Thus, in our experiments we should be measuring the isotope effect on k_{cat} or k_2 in the Michaelis – Menten kinetics. Since we are obtaining an intermolecular KIE, it should be noted that differential K_M (Michaelis–Menten Constant) effects are not expected, as the K_M of the ^{12}C and ^{13}C propane isotopomers should be the same.

In contrast to the lack of a primary intermolecular H/D isotope effect in the sMMO (Bath) system, a very high intermolecular KIE of $k_H/k_D = 19$ was reported for the sMMO from *Methylosinus trichosporium* OB3b (sMMO (OB3b)) by comparing the products formed from a 50:50 mixture of CH_4 and CD_4 as substrates under single turnover.[23, 27] An even larger isotope effect of 50 – 100 was obtained for the putative hydrogen abstraction step in stopped-flow kinetic experiments comparing CH_4 and CD_4.[23, 27] In addition, radical clock experiments gave ring-opened products (1 – 6%) with the probes 1,1-dimethylcyclopropane[28] and norcarane.[29] However, the same authors also described cationic intermediates for the oxidation of 1,1-dimethylcylclopropane and norcarane.[28, 29] Thus a stepwise reaction mechanism involving an initial substrate radical intermediate ($t > 20$ ps) formed by hydrogen atom abstraction followed by either oxygen rebound or rearrangement followed by oxygen rebound, or loss of a second electron to yield a cationic intermediate was concluded. On the other hand, evidence in support of the formation of a carbocation-type rearrangement products was also obtained by using methylcubane and (*trans*,*trans*-2-methoxy-3-phenyl-cyclopropyl)methane as the substrates, which would seem to exclude the possibility of formation of radical intermediates altogether.[20] Clearly, the sMMOs could exhibit

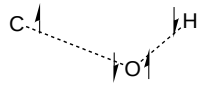
different mechanisms for alkane hydroxylation, and the details are substrate-dependent.

Based on the discussion in the literature,[30] the overall catalytic process involves only one or two steps, depending on whether the "O" insertion step is concerted, or a two-step process involving hydrogen-abstraction followed by methyl/hydroxyl radical rebound. Thus, the $^{12}C/^{13}C$ KIE that we are reporting here pertains to "O" insertion step in the concerted mechanism, or to the slower of the two steps in the hydrogen-abstraction, methyl/hydroxyl-radical rebound mechanism, whichever is slower. Given the nature of the chemistry, however, both steps of the latter mechanism are expected to exhibit a $^{12}C/^{13}C$ KIE.

Thus we surmise that the lack of a $^{12}C/^{13}C$ KIE on the hydroxylation is more in line with the concerted mechanism proposed for pMMO, namely, the direct insertion of an active "O" species across the C—H bond. Within this context, one should expect a fairly normal k_H/k_D ratio as has been reported for the hydroxylation of ethane and butane by pMMO.[14]

In any case, the finding that there is no intermolecular $^{12}C/^{13}C$ KIE on the propane hydroxylation reaction indicates that there is little structural change about the carbon center during formation of the transition state in the rate-limiting step. A side-on "O" insertion across a C—H bond would yield an early transition state in which there would be little carbon motion and no hydrogen tunneling during product formation. In contrast, both the hydrogen-abstraction and methyl/hydroxyl-radical-rebound steps in the classical rebound mechanism are expected to involve significant carbon motion during the formation of the transient state. In other words, taken together, these data point to rather limited stretching of the C—H bond instead of complete bond cleavage in the rate-limiting step of the hydroxylation chemistry. On the other hand, a side-on "O" insertion would only lead to an early transition state.

While an early transition state would be optimum for a "concerted" reaction, this scenario in itself does not rule out formation of an extremely short-lived carbon-center radical. Also, although the mechanisms for sMMO (Bath) and sMMO (OB3b) could be intrinsically different, the different behaviors could just as well be accounted for somewhat different extents of spin-crossover in an otherwise concerted "O" side-on insertion. Suppose the "oxene" attacks side-on as a singlet "O" species (see Scheme 2). The transient OH species that is moving away from the carbon would then be generated with the spin of the odd electron antiparallel to the odd spin of the electron localized on the carbon center. These two spins would be favorable for rapid closure to form the C—O bond upon product formation. In this limit, the chemistry would be unequivocally "concerted". On the other hand, if the two spins could somehow lose "correlation" in the transition state (due to decreasing orbital overlap), then there would be a small probability for the triplet configuration to be formed. If this occurs, C—O closure would not take place until the two spins are reverted back to the original singlet configuration. The likelihood of this scenario would depend on the distance between the carbon and the OH centers and the interactions modulating the exchange interaction between the spin pair. Thus, even within the framework of the concerted mechanism, radical rearrangement products, not to mention configurational inversion at the hydroxylated carbon center,[16] could still be expected if there is "spin-crossover" in the transition-state. In other words, the observation of radical-related rearrangement products is not necessarily diagnostic of a radical mechanism. What is more diagnostic of a radical mechanism would be the extent of radical-related rearrangement products formed. When radical-related products only account for 1%–6%, one is observing, in our judgment, predominantly a singlet "O" side-on attack with a small amount of spin-crossover in the transition state, as has been previously reported for singlet carbene insertion across C—H bonds.[31] Of course, within the framework of this picture, "cross-over" to an ionic state is also possible, and this kinetic channel will lead to a carbocation during product formation.



Scheme 2. *Early transition state for side-on "oxene" insertion across a C—H bond.*

Experimental Section

Gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS):

IRMS Measurements: The molecular stable carbon isotope ratio of propane, isopropanol, and ethanol was measured on a Varian GC interfaced with a Finnigan Mat 252 IRMS and performed by Baseline DGSI Analytical Laboratory in Texas, USA. The precision of the determination is 0.1‰. The isotopic composition of the test sample is normally expressed by δ values (in units of per mil or ‰) relative to the standard, which is usually PDB (a belemnite from the Cretaceous Peedee Formation). For PDB $^{12}C/^{13}C = 88.99$ and its $\delta^{13}C$ value is set to be 0‰. See text for a full definition for $\delta^{13}C$.

Notation: In practice, the molecular or overall isotopic signature of individual compounds determined with GC-C-IRMS is an average of the isotopic compositions of individual carbon positions within that molecule. Thus, in the case of isopropanol or propane Equation (2) applies:

$$\delta^{13}C_{Pr} = (2\delta^{13}C_{Me} + \delta^{13}C_{CO})/3 \quad (2)$$

Here, $\delta^{13}C_{Pr}$ is the overall $\delta^{13}C$ value of isopropanol or propane measured from GC-C-IRMS, $\delta^{13}C_{Me}$ is the $\delta^{13}C$ value of the methyl group of propane or isopropanol, and $\delta^{13}C_{CO}$ is the value of the oxygenated carbon in isopropanol or the methylene in propane. Similarly, if $\delta^{13}C_{EtOH}$ stands for the overall $\delta^{13}C$ value of the ethanol obtained from degradation of the isopropanol or propane, the relationship between $\delta^{13}C_{Pr}$, $\delta^{13}C_{Me}$, $\delta^{13}C_{CO}$, and $\delta^{13}C_{EtOH}$ is as shown in Equation (3):

$$\delta^{13}C_{EtOH} = (\delta^{13}C_{Me} + \delta^{13}C_{CO}/2 \quad (3)$$

From Eqs. (2) and (3), we can compute the isotopic signatures of the oxygenated and methyl carbons:

$$\delta^{13}C_{CO} = 4\delta^{13}C_{EtOH} - 3\delta^{13}C_{Pr} \quad (4)$$

$$\delta^{13}C_{Me} = 3\delta^{13}C_{Pr} - 2\delta^{13}C_{EtOH} \quad (5)$$

Preparation of the authentic propane standard: A solution of *n*-butyl lithium (125 mL, 2.5 M) in hexane was added to a solution of isopropanol (13 mL) in hexane (100 mL) at 0 °C under an argon

atmosphere. The resulting solution was added to *p*-toluenesulfonyl chloride (31.4 g) and stirred for 2 hr in an ice bath. After workup isopropyl *p*-toluenesulfonate was obtained as a pale yellow liquid.

The ester (approximately 5 mL) was placed in a 10 mL round-bottomed flask and heated gently to 100 °C (oil bath) to remove any trace of hexane remaining from the previous workup. The flask was then sealed with a rubber septum and an empty balloon with a needle was stuck to the septum. The ester was decomposed to propene and collected in the balloon by heating to 160 °C (oil bath). The propene balloon together with the needle was then placed in a glass tube prefilled with palladium (5% on charcoal) as shown in Figure 1. Another balloon filled with hydrogen in an equal volume of propene was also inserted at the other end of the glass tube. The two balloons were squeezed back and forth until the total volume of gases was reduced to half of the original amount. A sample of high quality propane was obtained in this manner according to GC analysis.

Bacterial growth and enzyme extraction: The organism *Methylococcus capsulatus* (Bath) was grown in a semicontinuous flow system. In this process cells could be controlled to express either the sMMO or pMMO exclusively. The crude extract of sMMO[32] was used throughout this study without further purification; in the case of pMMO, partially purified, pelleted membranes were employed as described previously.[8]

Hydroxylation of Propane: Bioconversions of the authentic propane to isopropanol were performed at 45 °C in 10-mL conical flasks sealed with a rubber septum. The products were generated by the incubation of either a pMMO-containing membrane suspension (2 mL isolated from 2 g of pMMO-cells) or a sMMO-containing crude extract (3 mL from 2 g of sMMO-cells) with nicotinamide adenine dinucleotide (NADH, 1.0 mM) in MOPS buffer (3-(*N*-morpholino)-propanesulphonic acid, 10 mM, pH 7.0) and substrate (1 mL of propane or diluted propane was added to the gas phase of incubation). The substrate was diluted from one-tenth to one-hundredth concentration with air (v/v) prior to injection. In the case of whole-cell studies, either sMMO-cells or pMMO-cells (2 g) were suspended in sodium formate (2 mL, 1 mM) containing potassium phosphate buffer (10 mM, pH 7.0) and the mixture was incubated with NADH and substrates in a shaking incubator. Each assay was conducted in five flasks and incubated for 2 h. The combined incubated aliquots ("solution A") were centrifuged and the isopropanol was degraded to ethanol according to the procedure described later for PSIA. For determination of the molecular carbon isotope composition of isopropanol, "solution A" was extracted with dichloromethane (2 × 1 mL). The dichloromethane extract was dried (anhydrous calcium chloride) and subjected to GC-C-IRMS analysis ($\delta^{13}C_{Pr}$).

Chemical degradation of isopropanol: Isopropanol was degraded to acetate according to the iodoform reaction as described earlier[4] but slightly modified as shown in Equation (6):

$$CH_3CHOHCH_3 + 4NaOCl + 3\,KI \rightarrow CH_3COONa + HCI_3 + 3\,KCl + 2\,NaOH + NaCl + H_2O \quad (6)$$

A typical reaction was carried out by adding NaOCl solution (25 mL, 10%) and KI solution (10 mL, 40%) to solution A. The mixture was than stirred for 2 h at room temperature. The resulting aliquot mixture was quenched with sodium thiosulfate solution (5 mL, 10%), filtered, and subjected to lyophilization. The salts remaining were dissolved in water (0.5 mL, pH 2.0) and extracted with methyl *tert*-butyl ether (2 × 1 mL). After drying the combined organic layers lithium aluminum hydride (30 mg) was added at 0 °C under an argon atmosphere. After the solution was stirred for 2 h at room temperature, sodium hydroxide aqueous solution (10 mL, 10%) was added. The resulting ethanol solution was dried and subjected to GC-C-IRMS analysis ($\delta^{13}C_{EtOH}$).

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[26] Unlike in the case of pMMO, where isopropanol is the exclusive product in the enzyme-mediated propane hydroxylation, both isopropanol and 1-propanol are generated in the sMMO-mediated reaction. However, if there is a KIE in the competition between the formation of isopropanol and 1-propanol, a depletion of $\delta^{13}C_{CO}$ value would have been expected.
[27] There are good reasons to question the validity of the large $^1H/^2H$ KIE reported here. These data accounted for different on-rates for the substrates in competitive experiments, when the on-rates are rate-limiting. This is likely to be the case in the sMMO(OB3b) hydroxylation experiments, when the rates for CH_4 and CD_4 are compared under nonlimiting substrates conditions, or in the stopped-flow experiments of Nesheim and Lipscomb,[23] where the hydrogen abstraction was presumably being monitored directly under transient conditions. In the former experiment, it seems that the significantly different K_M values for CH_4 and CD_4 (140 μM and 700 μM, respectively, at 30 °C;[33] 12(2) and 184(4) μM at

4 °C[23]) for sMMO(OB3b) had not been taken into consideration in the analysis of the data. In the stopped-flow experiments, the authors analyzed the data in terms of a single-step bimolecular reaction, assuming implicitly that access of the substrate to the active site of the enzyme is intrinsically rapid and the hydrogen-abstraction step is absolutely rate limiting. This would not be the case when the on-rate is the rate-determining step. In consideration of the large difference of the K_M-values of CH_4 and CD_4, the two substrates could exhibit sufficiently different on-rates to yield an "apparent" overall KIE, unless the hydrogen-abstraction step itself is rate-limiting.

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