Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 338 (2005) 240–249
BBRC
www.elsevier.com/locate/ybbrc

Review

Oxygen-18 tracer studies of enzyme reactions with radical/cation diagnostic probes

Luke A. Moe, Brian G. Fox *

Department of Biochemistry, University of Wisconsin, Madison, WI 53706, USA

Received 18 July 2005
Available online 8 September 2005

Abstract

This review considers reactions of enzymes with the cyclopropanoid radical/cation diagnostic probes norcarane, 1,1-dimethylcyclopropane, and 1,1-diethylcyclopropane as elaborated by the use of $^{18}O_2$ and $^{18}OH_2$ to trace the origin of O-atoms incorporated during catalysis. The reactions of soluble and integral membrane diiron enzymes are summarized and compared to results obtained from cytochrome P450 studies. Norcarane proved to be an excellent substrate for the diiron enzyme toluene 4-monooxygenase and its engineered isoforms, with k_{cat} and coupling between NADH utilization and total hydroxylated products comparable to that determined for toluene, the natural substrate. Results obtained with toluene 4-monooxygenase show that the un-rearranged and radical-rearranged alcohol products have a high percentage of O-atom incorporation (>80–95%) from O_2, while the cation-derived ring-expansion products have O-atom incorporation primarily derived from solvent water. Mechanistic possibilities accounting for this difference are discussed.

Keywords: Diiron enzyme; Norcarane; Oxygen-18; Hydroxylation mechanism; Radical clock; Catalysis

The study of hydrocarbon metabolism began in earnest during the late 1950s. Soil microbes were identified that efficiently used these abundant compounds, which are ultimately derived from photosynthesis, naturally converted into petroleum, or subsequently modified by the chemical industries. The work of Stanier and Hayaishi [1], Evans [2], Dagley [3], and many others revealed an elaborate network of regulated, specialized pathways evolved for this process. Thus, the coordinated goal of hydrocarbon metabolism is to channel these metabolites toward major biochemical pathways such as the tricarboxylic acid cycle, glycolysis, and fatty acid β-oxidation.

As the specific enzymes became known, it was clear that the first steps of these pathways were often the hardest chemical transformations to accomplish, and that these steps often required the use of O_2. Furthermore, many of the enzymes responsible contained non-heme iron at the catalytic center. Fig. 1 shows representative iron-dependent reactions of significance in hydrocarbon metabolism, including the scission of the aromatic ring of catechol and the stereo- and regiospecific hydroxylation of aromatics and alkanes, which all occur in aqueous solution and at room temperature.

Fig. 1 also summarizes the fate of $^{18}O_2$ as a tracer of the stoichiometry and fidelity of these reactions. This groundbreaking approach originated with Mason's studies of phenolase ([4], see below) and Hayaishi's studies of catechol dioxygenase (CTD, Fig. 1A, originally called pyrocatechase, [5]). The CTD reaction is catalyzed by an Fe^{3+}-containing enzyme that carries out intradiol cleavage of the aromatic ring to yield a *cis*-dicarboxylic acid product. Hayaishi's use of $^{18}O_2$ as a reaction tracer established the dioxygenase stoichiometry by proving that both O-atoms in the product arose from O_2 [5]. This high fidelity for O-atom transfer is one of the hallmarks of oxygenase reactions and their underlying mechanisms. Under exceptional circumstances this high fidelity can break down, however, as Que demonstrated by studies of CTD with the slow

* Corresponding author. Fax: +1 608 262 3453.
E-mail address: bgfox@biochem.wisc.edu (B.G. Fox).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.217

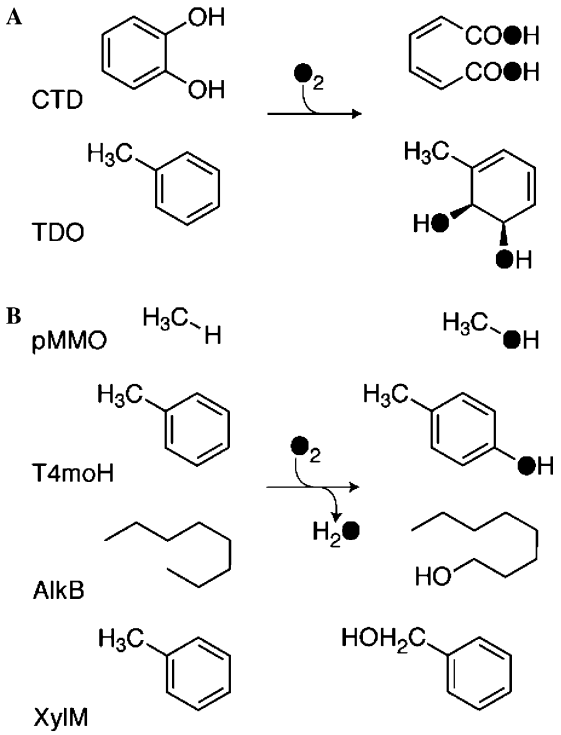

Fig. 1. Representative O_2-dependent enzyme reactions with demonstrated oxygen-18 transfer from $^{18}O_2$ indicated by black spheres. (A) Dioxygenases incorporate both atoms from O_2 into the product. Reactions shown are catechol 1,2-dioxygenase (CTD, pyrocatechase, [5]) and toluene dioxygenase (TDO, [7]). CTD requires no additional reactants. TDO requires $2e^-$ provided from NAD(P)H via a protein electron transfer complex. (B) Monooxygenases incorporate one O-atom from O_2 into the product, while the other O-atom is converted into water. Monooxygenase reactions also require NAD(P)H or another exogenous electron donor. The reactions shown are methane monooxygenase (pMMO, [12]), toluene-4-monooxygease (T4moH, [17]), alkane hydroxylase (AlkB), and xylene monooxygenase (XylM). Oxygen-18 tracer studies have not been published for AlkB and XylM, and so the O-atoms are not shaded black in these reactions.

alternative substrate pyrogallol [6]. This suggests the timing or geometry of the access of solvent molecules to reactive intermediates can be perturbed by an alternative substrate.

Also in the early years of oxygenase discovery, Gibson showed that another class of dioxygenases gives stereospecific *cis*-dihydrodiol products containing 2 mol of ^{18}O derived from O_2 [7,8]. Fig. 1A shows the product of the toluene dioxygenase reaction, (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene. Naphthalene dioxygenase, another enzyme of this versatile class, was capable of dioxygenation, monooxygenation, and desaturation when presented with the appropriate substrate [9]. This versatility arises from the high reactivity of the enzyme-bound iron oxidant coupled with the potential for minor positional variations in the approach of the alternative substrate molecules. Thus, the outcome of the reaction can be dependent on the nature of the substrate utilized and upon its interactions with the enzyme active site.

Mason's original work on phenolase (a multicopper enzyme) established that a single ^{18}O-atom was transferred from $^{18}O_2$ during the hydroxylation of 3,4-dimethylphenol [4]. This was originally termed a mixed function oxidase reaction [10], but now is more commonly called a monooxygenase or hydroxylase reaction. The ^{18}O tracer approach was subsequently used to establish the O-atom transfer stoichiometry in collagen proline hydroxylase [11], particulate methane monooxygenase [12], the P450 enzyme 1α-25-hydroxycholicalciferol hydroxylase [13], and many others.

Our laboratory has worked on reactions of the diiron enzyme toluene 4-monooxygenase. In this work, we consider the reactivity of the hydroxylase component (T4moH, Fig. 1B) of this four-protein complex and engineered hydroxylase isoforms with comparable catalytic parameters (k_{cat}, k_{cat}/K_M, coupling) but with distinct regiospecificities for toluene hydroxylation [14–16]. During reaction in the presence of $^{18}O_2$, the O-atom transfer efficiency determined for the natural T4moH is greater than 95% [17].

T4moH uniquely gives ~96% *para* hydroxylation of both toluene and the challenging alternative substrate 1,3-dimethylbenzene [14], while the relatively well-characterized G103L isoform gives ~55% *ortho* hydroxylation of toluene [16]. The mechanism of *p*-cresol formation has been proposed to include a directed approach of toluene to the diiron oxidant, formation of a transient 3,4-arene oxide, and active site-directed opening of the arene oxide to the final product [18]. Both the natural enzyme and the G103L isoform give similar magnitudes of intramolecular isotope effects and patterns of deuterium retention during the hydroxylation of 4-$^2H_1$- and 3,5-$^2H_2$-toluene, indicating that the active site mutation did not influence the mechanism of hydroxylation. Consequently, alterations in the directed approach of toluene to the diiron oxidant were considered the most likely mechanism for promoting the changes in regiospecificity.

The high substrate specificity and fidelity for O-atom transfer established for T4moH has offered unique opportunities to study how the enzyme active site and various substrates contribute to the outcome of diiron enzyme catalysis. This review will focus on the results of T4moH-catalyzed reactions with the alternative substrates norcarane, 1,1-dimethylcyclopropane (1,1-DMCP), and 1,1-diethylcyclopropane (1,1-DECP), and on the origin of O-atoms present in the diagnostic rearrangement products obtained. Key results from reactions of soluble methane monooxygenase (MmoH), the integral membrane diiron enzymes alkane ω-hydroxylase (AlkB) and xylene monooxygenase (XylM), and various cytochrome P450 isoforms with these substrates are also considered.

Mechanisms of hydroxylation

By virtue of the presence of hydroxyl groups, activated compounds (e.g., methanol, octanol, catechol, and methylphenol) are relatively easily oxidized. In contrast, unactivated hydrocarbons (e.g., methane, octane, benzene, and toluene) present a more rigorous mechanistic challenge arising from the stability of methyl and methylene C–H bonds and aromatic rings. Thus, the aerobic metabolism

of hydrocarbons (both aliphatic and aromatic) proceeds by converting kinetically inert (but thermodynamically reactive) O_2 into an oxidant capable of C–H bond breakage or insertion into π bond systems [19]. These reactions typically require heme P450 or non-heme iron or copper centers in order to generate sufficiently oxidizing species.

In the consensus O_2 activation cycle proposed for the diiron monooxygenases, an exogenous input of $2e^-$ and protons is required for the reduction of O_2 to give an iron-bound (hydroperoxide) intermediate [20,21]. Density functional theory calculations suggest that hydroperoxo intermediates will not be sufficiently electrophilic to perform C–H bond abstraction, but instead would more likely be nucleophilic [22]. Thus, heterolytic cleavage of the peroxide O–O bond is required to generate an oxene-like species suitable for C–H bond oxidation along with a water molecule. As the O-atom transfer efficiency of monooxygenase reactions typically matches the isotopic content of $^{18}O_2$ used in the reaction [23], a high fidelity for the trajectory of interaction between the substrate and one of these two atoms is likely. Furthermore, equilibration of the oxene and water generated from the monooxygenase reaction with solvent-derived water must not occur with normal substrates in the timescale of catalysis. Since it is reasonable to assume that the two chemically distinct species derived from O_2 (oxene and water of reaction) will be generated in close proximity within the active site during the catalysis, experimental approaches that account for the presence of both would seemingly shed further light into oxygenase reaction mechanisms.

Fig. 2 summarizes plausible mechanisms for hydrocarbon oxidation. Fig. 2A shows stepwise $1e^-$ paths that begin with an H-atom abstraction. On the left, recombination of the substrate radical and oxidant corresponds to the "oxygen rebound" mechanism originally introduced by Groves for P450 [24,25]. As the transferred O-atom arises from O_2, the isotopic content of the ROH product must correspond to that established by $^{18}O_2$ tracer studies. On the right, a second $1e^-$ oxidation of the substrate radical to generate a substrate cation is suggested, with the redox potential of the catalytic metal center and the electrophilic nature of the nascent carbon radical contributing to whether oxidation to the cation might occur. In order to achieve the monooxygenase stoichiometry established by the $^{18}O_2$ studies, the cation must capture a water molecule derived from the monooxygenase reaction to yield an isotopically labeled ROH.

Fig. 2B shows a concerted reaction arising from direct insertion of an O-atom into the C–H bond, which would generate the neutral product ROH with high fidelity for isotopic content in the transferred O-atom. Alternatively, Fig. 2C shows that the concerted insertion of ^+OH would generate an R-$^+OH_2$ intermediate that may release a proton to yield the isotopically labeled product ROH or may release H_2O (a "solvolysis" reaction) to yield a cationic intermediate capable of reacting with water to again yield product ROH. If the putative cationic intermediate can discriminate between the water generated by the reaction (isotopically labeled, and presumably in close proximity to the catalytic event) and solvent water (not isotopically labeled but also within the active site), changes in the isotopic content of cation-derived products would indicate that the cation can access different sources of water depending on the proximity to the oxidant.

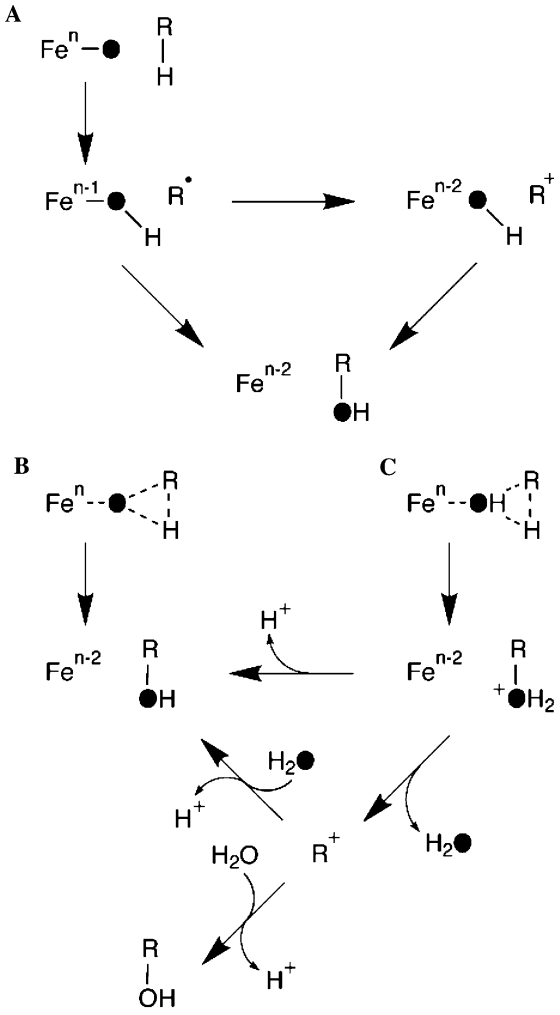

Fig. 2. Plausible pathways of hydrocarbon hydroxylation. (A) Radical "rebound" or $2e^-$ oxidation and recombination of R^+ with metal-bound hydroxide. (B) Insertion of [O] into the C–H bond to yield R-OH. (C) Insertion of [$^+$OH] followed by either release of H^+ to yield R-OH or release of H_2O ("solvolysis) to yield R^+, which then reacts with water.

Radical-clock mechanistic probes

Radical-clock compounds were originally developed to probe the chemical mechanisms of radical processes in organic chemistry [26], but were soon adopted by mechanistic enzymologists for studies of hydrocarbon oxidation. Recent efforts have identified additional radical-clock compounds that extend the range of available radical rearrangement rates (e.g., the probe of Fig. 3E has a rearrangement rate of $6 \times 10^{11}\ s^{-1}$, [27]). Probes that provide refined capabilities to undergo intramolecular

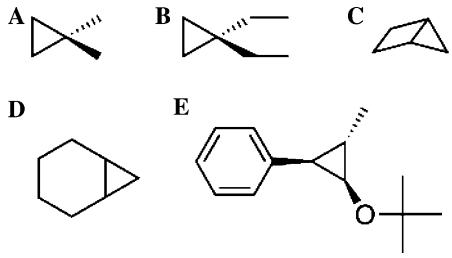

Fig. 3. Radical-clock substrates discussed in this work. (A) 1,1-DMCP; (B) 1,1-DECP; (C) bicyclo[2.1.0]pentane; (D) norcarane; (E) (*trans*, *trans*-2-*tert*-butoxy-3-phenylcyclopropyl)methane. Except for C, the other substrates yield diagnostic products from $1e^-$ (radical rearrangement) or $2e^-$ (cation-derived ring-expansion) oxidation pathways.

rearrangements diagnostic of either radical or cation precursors have also been made [28]. Fig. 3 shows some of the molecules used for these latter studies, where the substituted cyclopropyl ring is the critical functional group [29–31]. Among this entire suite of radical-clock compounds, 1,1-DMCP is the smallest molecule that can undergo diagnostic radical rearrangement and cation ring-expansion reactions. Fig. 4 summarizes these diagnostic reactions. Oxidation of 1,1-DMCP by $1e^-$ gives a primary cyclopropylcarbinyl radical that can undergo either recombination with a quenching agent (k_1) or intramolecular rearrangement (k_2, $0.8 \times 10^8\ s^{-1}$, [32]) to produce a more stable ring-opened 3-methyl-3-buten-1-yl radical before recombination with the quenching agent. Alternatively, oxidation of 1,1-DMCP by $2e^-$ gives a primary methylcyclopropyl cation that can undergo ring-expansion to a more stable

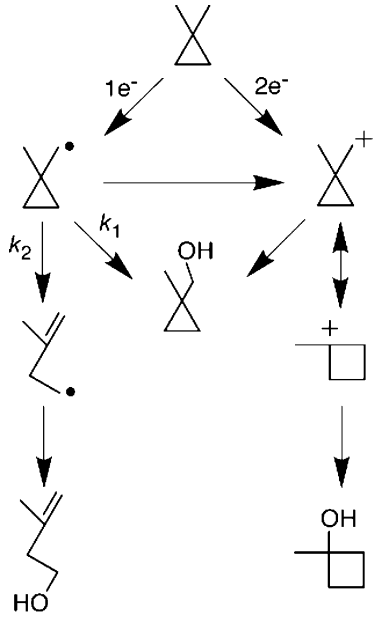

Fig. 4. Rearrangement of 1,1-DMCP. The cyclopropylcarbinyl radical can undergo hydroxylation (k_1) or rearrange into a more stable ring-opened structure (k_2). Upon O-atom transfer, the existence of the methyl radical can be inferred from the presence of the radical-rearranged product. The primary cyclopropylcarbinyl cation can undergo ring-expansion to the more stable tertiary methylcyclobutyl cation. Both cation species are quenched by water.

tertiary methylcyclobutyl cation before reaction with an appropriate quenching agent. To the degree that products arising from the $1e^-$ and $2e^-$ oxidations are distinct, the partition between these reaction pathways is reflected in the product distribution. Furthermore, since k_2 can be independently determined in the gas phase, a radical lifetime can be calculated from the product distribution and the rearrangement rate as

$$\text{radical lifetime} = ([\text{ring} - \text{closed}]/[\text{ring} - \text{opened}] \times k_2)^{-1}.$$

Radical-clock probes have been used to infer the presence of radical intermediates in C–H bond oxidation reactions and to estimate the lifetime of the radical. For example, Ortiz de Montellano and Stearns [33] found hydroxylation products resulting from ring opening of the radical-clock-type substrate bicyclo[2.1.0] pentane. However, a solvolysis study indicated that the same diagnostic product could arise from a $2e^-$ oxidation pathway [34], so an unequivocal assignment to a radical precursor could not be made. Subsequent studies with a series of calibrated radical-clock substrates failed to produce consistent values for the radical lifetime during P450 catalysis, with some probe studies indicating that hydroxylation occurred by a concerted process on the same rate of bond vibrations [27], that only cations were involved and radicals were not [35], or again, that radicals were involved [23,36]. It is clear that an enzyme active site is not necessarily well approximated by the gas phase, and the evidence for the influence of active site residues on the outcome of hydroxylation reactions continues to accumulate. Consequently, the functional group alterations required to obtain the fastest radical rearrangements (e.g., Fig. 3) may present significant challenges to productive binding and other essential spatial and temporal aspects of efficient enzyme catalysis. This problem may be reflected in the negligible rates of oxidation observed for many radical-clock probes, in addition to concerns about effects on the coupling between the NADH consumed, O_2 utilized, and hydroxylated products obtained [16].

By contrast, a study by Frey and co-workers [37] using 1,1-DMCP concluded that products derived from both radical and cation reaction pathways could arise from the same reaction. This study also provided the first use of oxygen-18 to track the fate of O_2 in radical-clock studies of an enzyme reaction. As expected for a monooxygenase, the un-rearranged 1-methylcyclopropanemethanol had the O-atom exclusively derived from O_2, while the cation-derived ring-expansion product 1-methyl-cyclobutanol had the O-atom derived from both O_2 and solvent. The isotopic content of O-atom in the radical-rearranged product 3-methyl-3-buten-1-ol was not determined in this study.

Chemical reactions of norcarane

Fig. 5 shows the diagnostic radical/cation rearrangements of norcarane, which can also be understood with reference to Fig. 4. Chemical studies of these reactions have

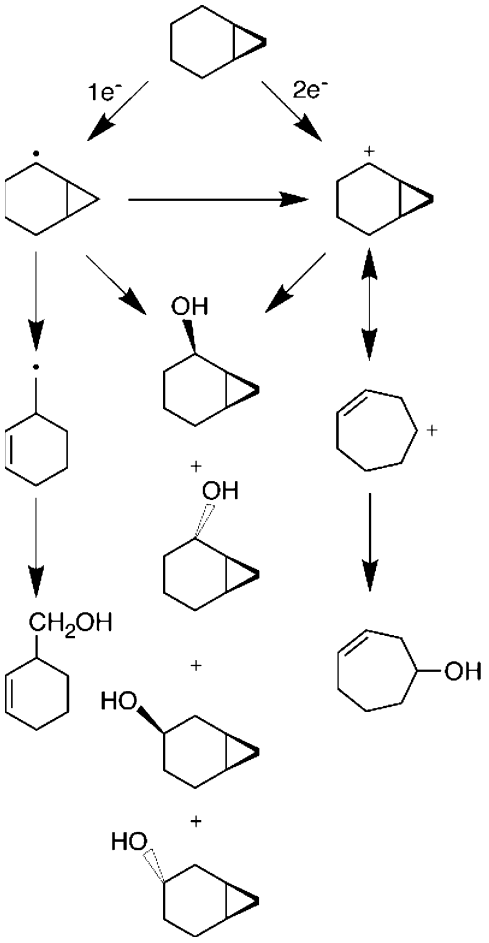

Fig. 5. Products derived from hydroxylation of norcarane. Upon formation of either a transient radical or transient cation at the C-2 position, intramolecular rearrangements can occur to produce more stable species. The stable hydroxylated products are used to deduce the formation of the transient species during the reaction. The diagnostic product of the $1e^-$ radical rearrangement and oxygen rebound pathway is cyclohex-2-enyl-methanol. The un-rearranged norcaranol products shown in the center are, from the top, 2-*endo*-norcaranol, 2-*exo*-norcaranol, 3-*endo*-norcaranol, and 3-*exo*-norcaranol. The diagnostic product of the $2e^-$ cation-derived ring expansion is cyclo-hept-3-enol.

provided important insight into the patterns of reactivity that might be expected from the enzyme reactions. Treatment of 2-chlorononorcarane with tri-*n*-butyltin hydride induced radical formation at C-2 and yielded 3-methyl-cyclohexene, the product from reductive quenching of a cyclohex-2-enyl-methyl radical. Thus, products derived from rearrangement to a cyclohex-2-enyl-methyl moiety are considered diagnostic for the formation of a norcarane C-2 radical [38]. The radical rearrangement rate, $2 \times 10^8\ s^{-1}$, is relatively slow when placed on the complete timescale of radical-clocks [39]. Groves et al. [40] later determined that homolytic C–H bond breakage at the C-2 position of norcarane by a synthetic Mn-substituted protoporphyrin complex yielded cyclohex-2-enyl-methanol during a hydroxylation reaction.

Fig. 5 also shows that $2e^-$ oxidation at the C-2 position would give the 2-norcaranyl cation, which is in resonance with the ring-expanded cyclohept-3-enyl cation. Thus formation of cyclohept-3-enol would be diagnostic for the formation of a C-2 cation precursor. There is no measurable "rate" for cation ring-expansion, however, numerous studies of the solvolysis of 2-norcaranol derivatives [41–43] established that un-rearranged 2-norcaranol is the major product derived from C-2 cation formation, with only ~5–10% of the product balance resulting from ring-expansion. After accounting for the majority fraction of un-rearranged alcohol, it was also shown that acetolysis of 2-deuterio-2-norcaranol [41] gave 96% of cyclohept-3-enol acetate from ring-expansion and 4% of cyclohex-2-enyl-methanol acetate, the product also observed from radical rearrangement. Due to this possible overlap in products from the $1e^-$ and $2e^-$ oxidation pathways, Newcomb et al. [43] suggested caution in the use of norcarane as a mechanistic probe. With this caveat acknowledged and discussed further below, the above-cited solvolysis experiments suggest that formation of 2-norcaranyl cation will predominantly yield an un-rearranged alcohol (90–95% of mass balance), and that the cation-derived ring-expansion products (5–10% of mass balance) should exhibit a ~25-fold excess of cyclohept-3-enol relative to cyclohex-2-enyl-methanol. This distribution possibly corresponds to the higher stability of a secondary cation as compared to a primary cation.

Enzyme reactions

We have proposed that the high regiospecificity of the T4moH reaction with monosubstituted benzenes (e.g., toluene, nitrobenzene, chlorobenzene, and methoxybenzene [14–16]) involves a well-defined binding interaction between the substrate and enzyme active site. Furthermore, this regiospecificity appears to be influenced by protein interactions between T4moH and the catalytic effector protein, T4moD [16]. Upon consideration of the various radical-clocks, norcarane has a size and shape that most closely matches that of toluene. Indeed, the solvent-exposed surface areas are 399 $Å^2$ for toluene and 471 $Å^2$ for norcarane. Based on this similarity, we hypothesized that norcarane might bind in the T4moH active site in a manner like that of toluene, and thus maintain crucial enzyme–substrate interactions and distance constraints during catalysis [17].

The availability of purified, highly active enzyme was an essential component of this work, as it provided well-defined reaction mixtures and low background contamination during product analyses by GC/MS methods. Using these preparations, norcarane was found to be an excellent substrate for T4moH, as k_{cat} of $0.5\ s^{-1}$ was remarkably close to the k_{cat} of $\sim 2.0\ s^{-1}$ measured for toluene. Moreover, norcarane oxidations had a comparable percentage of coupling between NADH utilized and hydroxylated products (72% and 95%, respectively [17]). For comparison, T4moH has k_{cat} and coupling values of $0.2\ s^{-1}$ and 65% for the *para* hydroxylation of nitrobenzene, and $0.13\ s^{-1}$ and ~3% for aliphatic hydroxylation of cyclohexane [16].

Table 1 shows product distributions obtained from reactions of natural T4moH and three active site isoforms (T201A, T201S, and G103L) with norcarane. The engineered variants exhibited nearly equivalent k_{cat} and k_{cat}/K_M values when compared to those of the natural enzyme [14–16], but each of them also showed a unique regiospecificity for toluene hydroxylation. Correspondingly, each of the enzyme isoforms tested gave a distinct distribution of the un-rearranged 2- and 3-norcaranol isomers as the majority of products, indicating that changes in active site architecture are relevant to both aromatic and aliphatic hydroxylation reactions. Notably, the four isoforms also gave distinct amounts of cyclohex-2-enyl-methanol ranging between 1.4% and 5.8% of the total products. Furthermore, none of the isomers yielded a detectable amount of the cation-derived ring-expansion product cyclohept-3-enol to the limit of detection (~0.1% of total products). Based on the gas-phase radical rearrangement rate of $2 \times 10^8\ s^{-1}$ [38] and the observed variations in product distributions, a radical lifetime of ~100–400 ps could be calculated for reactions of the various, catalytically comparable T4moH isoforms. These results indicate that the enzyme active site can indeed affect radical lifetime. Moreover, the absence of the cyclohept-3-enol (expected in a 25-fold excess over cyclohex-2-enyl-methanol derived from cationic pathways) indicated that cyclohex-2-enyl-methanol arose exclusively from a T4moH-generated radical intermediate.

Since T4moH was apparently unable to transform norcarane into a characteristic cation product, we were interested to learn whether other radical-clock probes might allow products from both radical and cation precursors. T4moH was able to oxidize 1,1-DMCP with $k_{cat} = 0.04\ s^{-1}$, a ~50-fold decrease in rate relative to toluene, and the un-rearranged product 1-methylcyclopropane-methanol represented ~93% of the total products. In addition, both 3-methyl-3-buten-1-ol (~0.2%) and 1-methyl-cyclobutanol (~2.1%) were detected from the T4moH-catalyzed reactions. These products arise from radical-rearranged and cation ring-expansion intermediates, respectively [37]. Likewise, T4moH oxidized 1,1-DECP and the un-rearranged 1-(1-ethylcyclopropyl)-ethanol was the major product (89.7%), while 3-methyl-3-penten-1-ol (~1.8%) and 1-methyl-2-ethylcyclobutanol (8.5%) were also observed. By analogy to the 1,1-DMCP assignments, these products also arise from radical-rearranged and cation ring-expansion intermediates, respectively.

Oxygen-18 tracer studies

Table 2 summarizes the results of T4moH reactions with norcarane, 1,1-DMCP, and 1,1-DECP in the presence of either $^{18}O_2$ or $^{18}OH_2$ as tracers of the origin of O-atom incorporation [17]. For all three substrates, the enrichment of ^{18}O in the un-rearranged and radical-rearranged alcohols matched that of the $^{18}O_2$ present in the reaction. Significantly, this included the radical-rearranged product cyclohex-2-enyl-methanol obtained from norcarane oxidation. Complementary studies of the oxidation of norcarane, 1,1-DMCP and 1,1-DECP performed in $^{18}OH_2$-enriched buffer showed negligible incorporation of ^{18}O (1–11%) into the un-rearranged and radical-rearranged alcohols.

A contrasting result was obtained for the cation-derived ring-expansion products. Thus, oxidation of 1,1-DMCP in $^{18}O_2$ gave ~70% enrichment of ^{18}O in the cation-derived ring-expansion product 1-methyl-cyclobutanol, while a similar oxidation of 1,1-DECP in $^{18}O_2$ gave only ~1% enrichment of ^{18}O in the cation ring-expansion product.

Table 1
Product distribution for enzyme-catalyzed hydroxylation of norcarane

Enzyme	*endo*-2-Norcaranol	*exo*-2- and *endo*-3-Norcaranol	*exo*-3-Norcaranol	Radical-rearranged	Cation-derived	lifetime (ps)	Ref.
T4moH	47.5	39.2	8.8	4.5	0	263	[17]
T201A[a]	35.5	48.5	10.2	5.8	0	343	[17]
T201S[a]	35.9	50.4	10.3	3.5	0	200	[17]
G103L[a]	37.6	49.8	11.2	1.4	0	80	[17]
MmoH (OB3b)[b]	57	29	7	1.4	1.1	20	[44]
MmoH (Bath)[b]	55	30 and 3[c]	6	3	3	n.d.[d]	[43]
AlkB[b]	74	6	n.d.	15	2	1000	[54]
XylM[b,e]	73.1	17.1	0	3.9	2.7	200	[55]
$P450_{cam}$[b]	56.7	19.8	9.1	0.9	0.3	52	[36]
$P450_{BM3}$[b]	57.6	28.2	7.5	0.7	0.3	44	[36]
CYPΔ2E1[b]	25	68 and 4[c]	2	0.5	0.16	n.d.	[43]
T303A[b,f]	41	38 and 14[c]	3	3.1	0.6	n.d.	[43]
CYP2B4[b]	69	22 and 4[c]	5	0.3	0.4	n.d.	[43]
CYPΔ2B4[b]	71	20 and 4[c]	4	0.4	0.5	n.d.	[43]
CYP2B1[b]	56.7	33.4	6.2	0.3	0.3	16	[36]
CYP2E1[b]	31.7	60	5.7	0.6	0	35	[36]

[a] Isoforms of T4moH created by site-directed mutagenesis.
[b] In addition, small amounts of 2- and/or 3-norcaranone were detected.
[c] Different GC/MS protocols were used for separation of the two isomers.
[d] n.d., not determined.
[e] Results from studies of *Pseudomonas putida* mt-2; similar results were obtained for all other organisms tested.
[f] Isoform of CYPΔ 2E1 T303A created by site-directed mutagenesis.

Table 2
Origin of oxygen incorporated during T4MO reactions[a]

Substrate	Product percentage	$^{18}O_2$ reactions	$^{18}OH_2$ reactions
Toluene[b]			
p-Cresol	96	>95	n.d.[c]
Norcarane[d,e]			
Total products	95.5	>95[f]	<1
Norcarane/toluene[g]			
Un-rearranged	95.5	68	<1
Radical-rearranged	4.5	62	<1
p-Cresol	96	65[g]	n.d.
1,1-DMCP[d,e]			
Un-rearranged	92.9	99.1 ± 0.8	0.9
Radical-rearranged	0.2	79 ± 6	5.3
Cation-derived ring-expansion	2.1	69 ± 5	23 ± 2
Unidentified product	4.8	99.4	0.3
1,1-DECP			
Un-rearranged	89.7[h]	63.5 ± 0.5	10.6
Radical-rearranged	1.8	65	7.3
Cation-derived ring-expansion	8.5	~1	91.3

[a] Reactions were performed as described in [17]. Standard deviations are the result of three or more separate determinations.
[b] Product percentages from [16]; ^{18}O incorporation data from [58].
[c] not determined.
[d] The isotopic enrichment of the $^{18}O_2$ was 99%.
[e] The reported percentage incorporation values were corrected for an 85% isotopic enrichment of ^{18}O in water.
[f] Isotopic contents of combined 2- and 3-norcaranols.
[g] The isotopic content of the $^{16}O_2/^{18}O_2$ mixture was determined experimentally to correspond to ~65% by reference to previous T4moH studies of *p*-cresol incorporation [58].
[h] The reported percentage includes the contribution of corresponding ketone, ~17%.

For the complementary experiments performed in $^{18}OH_2$-enriched buffer, the cation-derived ring-expansion product from 1,1-DMCP contained ~25% of ^{18}O enrichment, while the corresponding product from 1,1-DECP contained ~90% enrichment in ^{18}O.

These results can be rationalized in light of the different types of O-atoms that might be found in a diiron monooxygenase active site immediately prior to O-atom transfer. Fig. 6 summarizes the possibilities with respect to a discrete substrate radical or a discrete substrate cation. Radical and cation intermediates will have different propensities for reaction with different types of O-atoms. For example, the spin-paired recombination of radicals (Fig. 6A) and the reaction of a carbocation with water (Fig. 6B) are well established.

T4MO-catalyzed reaction at C-2 of norcarane gives rise to isomeric norcaranols and to cyclohex-2-enyl-methanol, with both having the same ^{18}O content as $^{18}O_2$. The minimum distance between the abstracted hydrogen atom and carbon atom that will receive the transferred O-atom in the radical-rearranged product is 2.8 Å. Thus, the relative positions of the C-2 radical and diiron oxidant relative to the bridgehead carbon (which is ultimately hydroxylated in the radical-rearranged product) may contribute to the favorability of O-atom transfer as opposed to radical rearrangement prior to O-atom transfer. This distance constraint also suggests that partial rotation of the substrate relative to the oxidant may be required to achieve the high fidelity observed for O-atom transfer. By comparison, for a

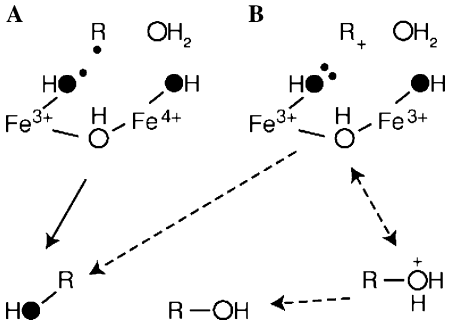

Fig. 6. A schematic representation of O-atoms in the diiron hydroxylase active site. The black spheres represent O-atoms derived from O_2 that may be either incorporated into substrate or converted to water or hydroxide according to the monooxygenase reaction stoichiometry. The open spheres represent solvent water sequestered in the active site. Oxidized substrate intermediates potentially have access to each of these three different types of O-atoms. (A) Rebound reaction of a substrate radical giving rise to the hydroxylated product with high fidelity for O-atom transfer from O_2. (B) Reaction of a substrate cation with any water present in the active site.

cation ring-expansion reaction leading to cyclohept-3-enol (not observed from the T4moH reactions), the minimum distance between the abstracted hydrogen atom and carbon atom that would receive the transferred O-atom is 4.9 Å. Moreover, this carbon atom lies on the opposite side of the substrate. These geometric considerations suggest that substantial motion might be required to incorporate the isotopically labeled water of reaction and instead a solvent

water molecule might be incorporated with high efficiency into this product.

When the less-well-matched substrates 1,1-DMCP and 1,1-DECP were oxidized, the product distribution included un-rearranged, radical-rearranged, and cation ring-expansion products. These different classes of products undoubtedly arise from alternative binding configurations available relative to a strong oxidant and intrinsic differences in the chemical properties of the substrates. For example, 1,1-DMCP has an elevated fraction of the water of reaction in the cation ring-expansion product, suggesting formation of cation must occur close to the catalytic center. Also, $2e^-$ oxidation of the mechanistically relevant methylene position in 1,1-DECP to a cation should be easier to achieve than the methyl position in 1,1-DMCP, accounting for the increase in cation ring-expansion product observed from 1,1-DECP. However, this product also has an O-atom content derived entirely from solvent. It is reasonable to conclude that small adjustments of the orientations and distances between substrates, intermediates, and oxidants can have profound influence on the favorability of $1e^-$, $2e^-$, and O-atom transfer reactions. By reference to Fig. 6, it is also plausible that these small adjustments may give access to the different types of O-atoms during the lifetime of a discrete radical or cation intermediate before recombination.

Other enzymes

Table 1 summarizes results obtained from the oxidation of norcarane by other oxygenases. In combination with results obtained from the T4moH isoforms, these studies reveal considerable variability in the distribution of the un-rearranged 2-norcaranol isomers, in the percentage of cyclohex-2-enyl-methanol, and in the appearance of cyclohept-3-enol. This variability emphasizes the influence of the different active sites on the outcome of reaction with norcarane. Table 1 also shows that similar product distributions were obtained from the MmoH-catalyzed oxidation of norcarane in two different laboratories [43,44]. As discussed above, differences in interpretation have arisen from the assessment of whether cyclohex-2-enyl-methanol arises from a radical rearrangement or from a cation-derived process. The near equivalence of the amounts of cyclohex-2-enyl-methanol and cyclohept-3-enol obtained from the MmoH studies seems incompatible with the 25-fold excess of cyclohept-3-enol expected from the acetolysis of 2-deuterio-2-norcaranol [41]. Thus, it is reasonable that radical rearrangement is responsible for most, if not all, of the cyclohex-2-enyl-methanol in the MmoH reactions.

MmoH has yielded a surprising level of detail about the nature of intermediates in the diiron catalytic cycle [45], including the role of a diferrous state [46,47], a peroxodiferric precursor [48], and the kinetically competent high-valent oxidant named compound Q [49]. This detail now includes high level computational studies [23,50]. Transient kinetic studies of the MmoH reaction with norcarane have given important insight into the oxidation of this radical-clock probe. Lipscomb and co-workers [44] showed that hydroxylation of norcarane occurs exclusively through the action of the compound Q, the diiron intermediate that is also responsible for the hydroxylation of methane [45]. In contrast, compound P did not react with either norcarane or methane and thus seems incompetent for aliphatic hydroxylation reactions.

Studies of the reactions of two integral membrane diiron hydroxylases with norcarane have also been undertaken (see Fig. 1B; alkane ω-hydroxylase, AlkB; and xylene monooxygenase, XylM). AlkB catalyzes the oxidation of linear C-6 to C-10 alkanes to the corresponding *n*-alcohols [51]. Mössbauer studies of AlkB provide the best physical evidence that this entire class of integral membrane enzymes contain a diiron center [52]. XylM catalyzes the hydroxylation of the methyl groups on methylbenzenes [53]. This reaction initiates a different pathway for toluene metabolism than that used by T4moH, and thus provides an interesting comparative focus. Owing to the difficulty in obtaining purified preparations of these enzymes, methods to perform the oxidation studies in whole cells were developed, and include both the native pseudomonad strains and recombinant *Escherichia coli* cells expressing the complete gene clusters. With AlkB [54], the majority of products arose from reaction at the C-2 position, and yielded ~85% of 2-norcaranol and ~15% of cyclohex-2-enyl-methanol. As with T4moH, no cyclohept-3-enol was detected, suggesting that catalysis likely proceeds through H-bond abstraction and oxygen rebound. Based on the product distribution, a remarkable radical lifetime of 1000 ps was calculated for the 2-norcaranyl radical. XylM also gave oxidation at the C-2 position [55], with un-rearranged 2-norcaranols providing the majority of products. Furthermore, cyclohex-2-enyl-methanol (~4%) and cyclohept-3-enol (~2%) were also detected, and an average radical lifetime of ~200 ps was calculated. Again, it is notable that an excess of cyclohex-2-enyl-methanol relative to cyclohept-3-enol was observed, supporting the radical-derived origin for the cyclohex-2-enyl-methanol.

The previous results discussed have all been obtained with non-heme, diiron enzymes. Table 1 also includes results from studies of P450-catalyzed oxidations of norcarane. Among the P450 isozymes tested by several different laboratories, all gave measurable amounts of both radical-rearranged and cation-ring-expansion products with the exception of CYP2E1, which did not give detectable amounts of the cation-derived ring-expansion product. Table 1 shows that the radical lifetimes, as calculated by Ortiz de Montellano and Groves [36], varied between 16 and 52 ps, consistent with previous studies implicating a short-lived radical intermediate in P450 catalysis [56,57].

A surprising feature of the results presented in Table 1 is the difference in amounts of radical-rearranged products observed from the diiron systems, MmoH, and the P450 systems. In majority, the diiron systems accumulate higher levels of the radical-rearranged products. This may imply a

longer radical lifetime, less favorability of the $2e^-$ oxidation pathway, a lower redox potential for the oxidizing metallocenter, or a combination of these and other factors. In contrast, MmoH, which is arguably the most powerful biological hydrocarbon oxidation catalyst, and the P450 systems, which have long been regarded as powerful biological oxidants, give lower levels of radical-rearranged products and higher levels of the cation ring-expansion products. Thus, it seems likely that these differences reflect the continuum of oxidative capability available from evolution of these highly specialized catalytic systems.

Acknowledgments

This work was supported by National Science Foundation Grant MCB-0316232 to B.G.F. The authors acknowledge many useful discussions with our patient collaborators John T. Groves Zhengbo Hu, Dayi Deng (Princeton University) and Rachel N. Austin (Bates College) while the oxygen-18 experiments progressed.

References

[1] R.Y. Stanier, O. Hayaishi, M. Tsuchida, The bacterial oxidation of tryptophan. I. A general survey of the pathways, J. Bacteriol. 62 (1951) 355–366.
[2] H.N. Fernley, W.C. Evans, Oxidative metabolism of polycyclic hydrocarbons by soil pseudomonads, Nature 182 (1958) 373–375.
[3] S. Dagley, W.C. Evans, D.W. Ribbons, New pathways in the oxidative metabolism of aromatic compounds by microorganisms, Nature 188 (1960) 560–566.
[4] H.S. Mason, W.L. Fowlks, E. Peterson, Oxygen transfer and electron transport by the phenolase complex, J. Am. Chem. Soc. 77 (1955) 2914–2915.
[5] O. Hayaishi, M. Katagiri, S. Rothberg, Mechanism of the pyrocatechase reaction, J. Am. Chem. Soc. 77 (1955) 5450–5451.
[6] R.J. Mayer, L. Que Jr., ^{18}O studies of pyrogallol cleavage by catechol 1,2-dioxygenase, J. Biol. Chem. 259 (1984) 13056–13060.
[7] D.T. Gibson, M. Hensley, H. Yoshioka, T.J. Mabry, Formation of (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from toluene by *Pseudomonas putida*, Biochemistry 9 (1970) 1626–1630.
[8] D.T. Gibson, G.E. Cardini, F.C. Maseles, R.E. Kallio, Incorporation of oxygen-18 into benzene by *Pseudomonas putida*, Biochemistry 9 (1970) 1631–1635.
[9] D.T. Gibson, S.M. Resnick, K. Lee, J.M. Brand, D.S. Torok, L.P. Wackett, M.J. Schocken, B.E. Haigler, Desaturation, dioxygenation, and monooxygenation reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp. strain 9816-4, J. Bacteriol. 177 (1995) 2615–2621.
[10] O. Hayaishi, Fifty years of oxygen activation, J. Biol. Inorg. Chem. 10 (2005) 1–2.
[11] D. Prockop, A. Kaplan, S. Udenfriend, Oxygen-18 studies on the conversion of proline to collagen hydroxyproline, Arch. Biochem. Biophys. 101 (1963) 499–503.
[12] I.J. Higgins, J.R. Quayle, Oxygenation of methane by methane-grown *Pseudomonas methanica* and *Methylomonas methanooxidans*, Biochem. J. 118 (1970) 201–208.
[13] T. Madhok, H. Schnoes, H.F. DeLuca, Mechanism of 25-hydroxycholecalciferol 1α-hydroxylation. Incorporation of oxygen-18 into the 1α position of 25-hydroxycholecalciferol, Biochemistry 12 (1970) 2555–2558.
[14] J.D. Pikus, J.M. Studts, K. McClay, R.J. Steffan, B.G. Fox, Changes in the regiospecificity of aromatic hydroxylation produced by active site engineering in the diiron enzyme toluene 4-monooxygenase, Biochemistry 36 (1997) 9283–9289.
[15] J.D. Pikus, K.H. Mitchell, J.M. Studts, K. McClay, R.J. Steffan, B.G. Fox, Threonine 201 in the diiron enzyme toluene 4-monooxygenase is not required for catalysis, Biochemistry 39 (2000) 791–799.
[16] K.H. Mitchell, J.M. Studts, B.G. Fox, Combined participation of effector protein binding and hydroxylase active site residues provide toluene 4-monooxygenase regiospecificity, Biochemistry 41 (2002) 3176–3188.
[17] L.A. Moe, Z. Hu, D. Deng, R.N. Austin, J.T. Groves, B.G. Fox, Remarkable aliphatic hydroxylation by the diiron enzyme toluene 4-monooxygenase in reactions with radical or cation diagnostic probes norcarane, 1,1-dimethylcyclopropane, and 1,1-diethylcyclopropane, Biochemistry 43 (2004) 15688–15701.
[18] K.H. Mitchell, C.E. Rogge, T. Gierahn, B.G. Fox, Insight into the mechanism of aromatic hydroxylation by toluene 4-monooxygenase through the use of specifically deuterated toluene and *p*-xylene, Proc. Natl. Acad. Sci. USA 100 (2003) 3784–3789.
[19] G.A. Hamilton, Mechanisms of biological oxidation reactions involving oxygen and reduced oxygen derivatives, in: C.C. Reddy, G.A. Hamilton, K.M. Madyastha (Eds.), Biological Oxidation Systems, Academic Press, New York, 1990.
[20] B.J. Wallar, J.D. Lipscomb, Dioxygen activation by enzymes containing binuclear non-heme iron clusters, Chem. Rev. 96 (1996) 2625–2657.
[21] M. Merkx, D.A. Kopp, M.H. Sazinsky, J.L. Blazyk, J. Muller, S.J. Lippard, Dioxygen activation and methane hydroxylation by soluble methane monooxygenase: a tale of two irons and three proteins, Angew. Chem. Int. Ed. Engl. 40 (2001) 2782–2807.
[22] S. Shaik, S. Cohen, S.P. de Visser, P.K. Sharma, D. Kumar, S. Kozuch, F. Ogliaro, D. Danovich, The rebound controversy: an overview and theoretical modeling of the rebound step in C–H hydroxylation by cytochrome P450, Eur. J. Inorg. Chem. 2 (2004) 207–226.
[23] V. Guallar, B.F. Gherman, W.H. Miller, S.J. Lippard, R.A. Friesner, Dynamics of alkane hydroxylation at the non-heme diiron center in methane monooxygenase, J. Am. Chem. Soc. 124 (2002) 3377–3384.
[24] J.T. Groves, G.A. McCluskey, Aliphatic hydroxylation via oxygen rebound: oxygen transfer catalyzed by iron, J. Am. Chem. Soc. 98 (1976) 859–861.
[25] J.T. Groves, The bioinorganic chemistry of iron in oxygenases and supramolecular assemblies, Proc. Natl. Acad. Sci. USA 100 (2003) 3569–3574.
[26] D. Griller, K.U. Ingold, Free-radical clocks, Acc. Chem. Res. 13 (1980) 317–323.
[27] M. Newcomb, M.-H. Le Tadic-Biadatti, D.L. Chesney, E.F. Roberts, P.F. Hollenberg, A nonsynchronous concerted mechanism for cytochrome P-450 catalyzed hydroxylation, J. Am. Chem. Soc. 117 (1995) 12085–12091.
[28] M. Newcomb, D.L. Chestney, A hypersensitive mechanistic probe for distinguishing between radical and carbocation intermediates, J. Am. Chem. Soc. 116 (1994) 9753–9754.
[29] M. Newcomb, C.C. Johnson, M.B. Manek, T.R. Varick, Picosecond radical kinetics. Ring openings of phenyl substituted cyclopropylcarbinyl radicals, J. Am. Chem. Soc. 114 (1992) 10915–10921.
[30] J.K. Atkinson, K.U. Ingold, Cytochrome P450 hydroxylation of hydrocarbons: variation in the rate of oxygen rebound using cyclopropyl radical clocks including two new ultrafast probes, J. Am. Chem. Soc. 32 (1993) 9209–9214.
[31] Y. Jin, J.D. Lipscomb, Mechanistic insights into C–H activation from radical clock chemistry: oxidation of substituted methylcyclopropanes catalyzed by soluble methane monooxygenase from *Methylosinus trichosporium* OB3b, Biochim. Biophys. Acta 1543 (2000) 47–59.
[32] V.W. Bowry, K.U. Ingold, A radical clock investigation of microsomal cytochrome P-450 hydroxylation of hydrocarbons. Rate of oxygen rebound, J. Am. Chem. Soc. 113 (1991) 5699–5707.
[33] P.R. Ortiz de Montellano, R.A. Stearns, Timing of the radical recombination step in cytochrome P-450 catalysis with ring-strained probes, J. Am. Chem. Soc. 109 (1987) 3415–3420.

[34] K.B. Wiberg, V.Z. Williams Jr., L.E. Friedrich, Solvolysis of bicyclo[2.1.*O*]pentyl 2- (3,5-dinitrobenzoates), J. Am. Chem. Soc. 92 (1970) 564–567.
[35] M. Newcomb, R. Shen, S.-Y. Choi, P.H. Toy, P.F. Hollenberg, A.D.N. Vaz, M.J. Coon, Cytochrome P450-catalyzed hydroxylation of mechanistic probes that distinguish between radicals and cations. Evidence for cationic but not for radical intermediates, J. Am. Chem. Soc. 122 (2000) 2677–2686.
[36] K. Auclair, Z. Hu, D.M. Little, P.R. Ortiz de Montellano, J.T. Groves, Revisiting the mechanism of P450 enzymes with the radical clocks norcarane and spiro[2,5]octane, J. Am. Chem. Soc. 124 (2002) 6020–6027.
[37] F. Ruzicka, D.-S. Huang, M.I. Donnelly, P.A. Frey, Methane monooxygenase catalyzed oxygenation of 1,1-dimethylcyclopropane. Evidence for radical and carbocationic intermediates, Biochemistry 29 (1990) 1696–1700.
[38] E.C. Friedrich, R.L. Holmstead, Cyclopropylcarbinyl-allylcarbinyl radical rearrangements in the simple bicyclo[3.1.0]hexyl and-[4.1.0]heptyl systems, J. Org. Chem. 37 (1972) 2550–2554.
[39] M. Newcomb, P.H. Toy, Hypersensitive radical probes and the mechanisms of cytochrome P450-catalyzed hydroxylation reactions, Acc. Chem. Res. 33 (2000) 449–455.
[40] J.T. Groves, J.W. Kruper, R.C. Haushalter, Hydrocarbon oxidation with oxometalloporphinates. Isolation and reaction of a porphinato-manganese(V) complex, J. Am. Chem. Soc. 102 (1980) 6375.
[41] E.C. Friedrich, J.D. Cooper Jassawalla, Methyl substituent effects on cyclopropylcarbinyl–cyclopropylcarbinyl cation rearrangements in the 2-bicyclo[4.1.0]heptyl system, Tetrahedron Lett. 11 (1978) 953–956.
[42] E.C. Friedrich, J.D. Cooper Jassawalla, Methyl substituent effects upon the chemistry of 2-bicyclo[4.1.0]heptyl 3,5-dinitrobenzoates, J. Org. Chem. 44 (1979) 4224–4229.
[43] M. Newcomb, R. Shen, Y. Lu, M.J. Coon, P.F. Hollenberg, D.A. Kopp, S.J. Lippard, Evaluation of norcarane as a probe for radicals in cytochrome P450- and soluble methane monooxygenase-catalyzed hydroxylation reactions, J. Am. Chem. Soc. 122 (2002) 2677–2686.
[44] B.J. Brazeau, R.N. Austin, C. Tarr, J.T. Groves, J.D. Lipscomb, Intermediate Q from soluble methane monooxygenase hydroxylates the mechanistic substrate probe norcarane: evidence for a stepwise reaction, J. Am. Chem. Soc. 123 (2001) 11831–11837.
[45] S.-K. Lee, J.C. Nesheim, J.D. Lipscomb, Transient intermediates of the methane monooxygenase catalytic cycle, J. Biol. Chem. 268 (1993) 21569–21577.
[46] M.P. Hendrich, E. Münck, B.G. Fox, J.D. Lipscomb, Integer-spin EPR studies of the fully reduced methane monooxygenase hydroxylase component, J. Am. Chem. Soc. 112 (1990) 5861–5865.
[47] B.G. Fox, W.A. Froland, J.E. Dege, J.D. Lipscomb, Methane monooxygenase from *Methylosinus trichosporium* OB3b: purification and properties of a three-component system with high specific activity from a Type II methanotroph, J. Biol. Chem. 264 (1989) 10023–10033.
[48] K.E. Liu, A.M. Valentine, D. Qui, D.E. Edmondson, E.H. Appelman, T.G. Spiro, S.J. Lippard, Characterization of a diiron(III) peroxo intermediate in the reaction cycle of methane monooxygenase hydroxylase from *Methylococcus capsulatus* (Bath), J. Am. Chem. Soc. 119 (1997) 11134–11136.
[49] S.-K. Lee, B.G. Fox, W.F. Froland, J.D. Lipscomb, E. Münck, A transient intermediate of the methane monooxygenase catalytic cycle containing an $Fe^{IV}Fe^{IV}$ cluster, J. Am. Chem. Soc. 115 (1993) 6450–6451.
[50] M.-H. Baik, M. Newcomb, R.A. Friesner, S.J. Lippard, Mechanistic studies on the hydroxylation of methane by methane monooxygenase, Chem. Rev. 103 (2003) 2385–2419.
[51] E.J. McKenna, M.J. Coon, Enzymatic ω-oxidation IV. Purification and properties of the ω-hydroxylase of *Pseudomonas oleovorans*, J. Biol. Chem. 245 (1970) 3882–3889.
[52] J. Shanklin, C. Achim, H. Schmidt, B.G. Fox, E. Münck, Mössbauer studies of alkane ω-hydroxylase: evidence for a diiron cluster in an integral membrane enzyme, Proc. Natl. Acad. Sci. USA 94 (1997) 2981–2986.
[53] S. Harayama, M. Rekik, M. Wubbolts, K. Rose, R.A. Leppik, K.N. Timmis, Characterization of five genes in the upper-pathway operon of TOL plasmid pWW0 from *Pseudomonas putida* and identification of the gene products, J. Bacteriol. 171 (1989) 5048–5055.
[54] R.N. Austin, H.-K. Chang, G.J. Zylstra, J.T. Groves, The non-heme diiron alkane monooxygenase of *Pseudomonas oleovorans* (AlkB) hydroxylates via a substrate radical intermediate, J. Am. Chem. Soc. 122 (2000) 11747–11748.
[55] R.N. Austin, J. Buzzi, E. Kim, G.J. Zylstra, J.T. Groves, Xylene monooxygenase: a membrane-spanning non-heme diiron enzyme that hydroxylates hydrocarbons via a substrate radical intermediate, J. Biol. Inorg. Chem. 8 (2003) 733–740.
[56] J.T. Groves, G.A. McClusky, R.E. White, M.J. Coon, Aliphatic hydroxylation by highly purified liver microsomal cytochrome P-450: evidence for a carbon radical intermediate, Biochem. Biophys. Res. Commun. 81 (1978) 154–160.
[57] J.T. Groves, D.V. Subramanian, Hydroxylation by cytochrome P-450 and metalloporphyrin models. Evidence for allylic rearrangement, J. Am. Chem. Soc. 106 (1984) 2177–2181.
[58] K.S. Lyle, J. Ai, P. Moenne-Loccoz, J. Sanders-Loehr, T.M. Loehr, B.G. Fox, Resonance Raman studies of the stoichiometric catalytic turnover of a substrate-stearoyl-acyl carrier protein Δ^9 desaturase complex, Biochemistry 39 (2000) 10507–10513.