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Biomimetic Oxidation Studies. 5. Mechanistic Aspects of Alkane Functionalization with Fe, Fe_2O, and Fe_4O_2 Complexes in the Presence of Hydrogen Peroxide[1]

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The biomimetic oxidation reactions of a variety of hydrocarbons with iron complexes, $Fe_2O(OAc)_2(bpy)_2Cl_2$ (**1**), $Fe_4O_2(OAc)_7(bpy)_2(ClO_4)$ (**2**), $Fe_2O(OAc)(tmima)_2(ClO_4)_3$ (**3**) (tmima = tris[(1-methylimidazol-2-yl)methyl]amine), and $Fe(ClO_4)_3 \cdot 6H_2O$ (**4**), using H_2O_2/O_2 as the oxidant were studied. Functionalization of cyclohexane gave cyclohexanol (CyOH) and cyclohexanone (CyONE). Complex **4** was the most effective and selective, 15-20 mmol of CyOH, 5-8 mmol of CyONE/mmol of Fe complex, and CyOH/CyONE ratios of ~2; however, the pseudo-first-order rate constants for the formation of CyOH and CyONE for **1** and **2** were ~1.4-1.6 times greater than those for **4**. Complexes **1-3** gave 2-7 and 3-8 mmol of product/mmol of Fe complex, respectively, with CyOH/CyONE ratios of 0.6-1.1. The presence of an oxidizing intermediate was suggested by iodometric titration in the functionalization of cyclohexane with complexes **1-4** and H_2O_2. This intermediate was isolated from the reaction mixture and identified by ^{13}C NMR as cyclohexyl hydroperoxide (CyOOH) as compared to an independently prepared sample. The decomposition of CyOOH by **1-4** and H_2O_2 gave CyOH/CyONE ratios of 0.7, 0.9, 0.7, and 2.8, respectively, in the ranges observed in the actual cyclohexane oxidation reactions. These hydrocarbon oxidation reactions were also inhibited by 2,4,6-tri-*tert*-butylphenol. Reactions run under a sweep of argon gave mmol of product/mmol of Fe complex 0-31% of the normal values. These results are consistent with a free-radical chain mechanism in which an initially formed cyclohexyl radical is trapped by oxygen gas to give a cyclohexyl peroxyl radical, which abstracts a hydrogen atom to give CyOOH and carry the chain. The tertiary hydrogen of adamantane was selectively abstracted with complexes **1-4** to obtain normalized C^3/C^2 values of 3.5, 3.3, 3.4, and 5.6, respectively. Toluene was transformed to a mixture of benzyl alcohol, benzaldehyde, and *o*; *m*; and *p*-cresols with benzylic/aromatic activation ratios of 3.4, 4.2, 0.9, and 20, respectively, and indicate that hydroxyl radicals (aromatic C-H functionalization) may also participate. Functionalization of methane, ethane, and propane was also observed.

Introduction

Recent spectroscopic studies on methane monooxygenases (MMO) have shown that the active center has a diiron-μ-oxo or diiron-μ-hydroxo structure [$Fe_2(\mu\text{-O})$, $Fe_2(\mu\text{-OH})$] and that histidine may be a terminal ligand.[2] These enzymes are active in the conversion of a variety of alkanes, including methane, to their respective alcohols.[3] This type of diiron-μ-oxo(hydroxo) structure has also been proposed for other iron-containing biomolecules such as hemerythrin,[4] purple acid phosphatase,[5] and ribonucleotide reductase.[6] More importantly, we have shown that this type of possible biomimetic structure, FeOFe, was capable of initiating hydrocarbon functionalization via the synthesis of a MMO active site model, $Fe_2O(OAc)_2(bpy)_2Cl_2$ (**1**), for the conversion of ethane, propane, and cyclohexane to their corresponding alcohols in the presence of *tert*-butyl hydroperoxide (TBHP).[1a]

Previous biomimetic oxidation studies with mononuclear and, more pertinently, Fe cluster complexes and hydrogen peroxide (H_2O_2), a monooxygen transfer reagent that can replace oxygen gas,[7] have shown functionalization of alkanes and other substrates,[8] but clearly, mechanistic details are still not totally defined with regard to the active Fe oxidant and the identity of any definitive organic intermediate that may provide both alcohol and ketone or aldehyde. In this paper, we report on kinetic and mechanistic aspects of the functionalization of several hydrocarbons by **1**, its synthetic precursor $[Fe_4O_2(OAc)_7(bpy)_2](ClO_4)$ (**2**), a polyimidazole complex, $[Fe_2O(OAc)(tmima)_2](ClO_4)_3$, (**3**) (tmima = tris[(1-methylimidazol-2-yl)methyl]amine),[9] and, for com-

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Table I. Comparison of Complexes **1–4** in the Functionalization of Cyclohexane with Hydrogen Peroxide[a]

entry	complex	[complex], mM	$[H_2O_2]$, mM	mmol of product/mmol of complex[b,c]		OH/(O) ratio
				c-$C_6H_{11}OH$	c-$C_6H_{10}(O)$	
1	1	1.0	150	5.0–6.3[d]	5.2–6.9[d]	0.8–1.1[d]
2	2	1.0	150	1.7–2.9[d]	2.7–3.0[d]	0.6–1.0[d]
3	3	1.0	150	6.6–7.0	7.0–7.5	0.9–1.0
4	4	0.5	150	37	19	1.9
5	4	1.0	150	15–20[d] (20)	4.9–8.2[d] (3.9)	1.9–2.6[d] (5.1)
6	4	2.0	150	8.0	3.5	2.3
7	4	1.0	38	4.0 (4.2)	1.1 (1.1)	3.6 (3.8)
8	4	1.0	75	8.9 (9.7)	2.7 (2.7)	3.3 (4.4)
9	4	1.0	300	12 (45)	9.7 (3.8)	1.3 (12)

[a] Reactions were carried out in Schlenk flasks with stirring at ambient temperature under air in CH_3CN at a cyclohexane concentration of 900 mM. Reactions times were typically 6–18 h. Control experiments indicate that the reactions were complete in 1–4 h with mass balances of >98%. [b] The products were identified by GC and GS/MS analyses; amounts (mmol) of products were determined by GC using cyclopentanone as an internal standard. [c] The numbers in parentheses were obtained upon reanalysis after the addition of saturated NaI in 2-propanol. [d] Range of values observed.

parison purposes, the iron salt $Fe(ClO_4)_3 \cdot 6H_2O$ (**4**),[8a-f] with H_2O_2 (30%, diluted with CH_3CN to 1.5 M) as the monooxygen source *and show unequivocally, with these systems, the intermediacy of an alkyl hydroperoxide in the formation of both alcohol and either ketone or aldehyde during the alkane functionalization process.* Moreover, we believe that other mechanistic pathways previously postulated for alkane functionalization with biomimetic Fe complexes, using H_2O_2/O_2 as the oxygen source, can now be better explained by invoking alkyl hydroperoxides as the most logical intermediates to the oxidation products.[7,8h-j]

Results and Discussion

Table I compares the results obtained for the functionalization of cyclohexane that provides cyclohexanol (CyOH) and cyclohexanone (CyONE) with complexes **1–4**. Under our standard conditions (1 mM Fe complex, 900 mM C_6H_{12}, 150 mM H_2O_2, CH_3CN solvent), the iron salt, **4**, provided more mmol of product/mmol of Fe complex than any of complexes **1–3** and gave as well a greater CyOH/CyONE ratio (entries 1–3 and 5). All the cyclohexane oxidation products were accounted for with mass balances of >98%.

In order to examine the functionalization of cyclohexane in greater detail, we have measured the rate of appearance of products (mmol product/mmol Fe complex versus time, Figure 1) with complexes **1**, **2**, and **4** and found that the initial rate of formation of CyONE was faster than that of CyOH. Furthermore, comparison of the pseudo-first order rate constants for the formation of CyOH and CyONE for complexes **1**, **2**, and **4** showed that **1** and **2** had rate constants that were 1.4–1.6 times greater than those of **4** for both products. For example, complex **1** had pseudo-first-order rate constants at room temperature for CyOH and CyONE formation of $k_{obs} = 7.6 \times 10^{-7}$ s^{-1} and 8.8×10^{-7} s^{-1}, respectively, while **4** provided rate constants of $k_{obs} = 4.6$ and 6.0×10^{-7} s^{-1}. To determine the origin of the CyONE, we measured the mmol of product/mmol of Fe complex with time as well as the pseudo-first-order rate constant of its formation from cyclohexane in the presence of 900 mM CyOH using complex **2** and obtained a similar rate profile and rate constant (9.7×10^{-7} s^{-1}); this result further substantiates that CyOH was not the precursor to CyONE.

The millimoles of products formed and the CyOH/CyONE ratio in the reaction between H_2O_2 and cyclohexane initiated by **4** was dependent on the initial concentration of H_2O_2 (Table I, entries 5, 7–9). Increasing the concentration of H_2O_2 does increase the total amount of products and also has the effect of decreasing the CyOH/CyONE ratio. However, a substantial amount of oxidant remains after 24 h as determined by iodometric titration. The dramatic increase in the CyOH/CyONE ratio upon addition of I^- (Table I) implies that a peroxide intermediate is formed that reacts with I^- to give predominantly CyOH.

The identity of this intermediate oxidant was perceived to be cyclohexyl hydroperoxide (CyOOH), whose chemistry has been extensively studied.[10] Accordingly, we have *unequivocally identified CyOOH* in our reaction mixture by using extraction and isolation techniques (complex **4**, 900 mM cyclohexane, and 300 mM H_2O_2 provided 50 mg of CyOOH [32%, mmol product/mmol Fe complex = 15]), while its structure was corroborated by ^{13}C NMR spectroscopy in comparison to an independently prepared sample.[11] Additionally, we have examined the decomposition of CyOOH using complexes **1–4** and H_2O_2 in the absence of substrate and obtained *CyOH/CyONE ratios of 0.7, 0.9, 0.7, and 2.8*, respectively, in the ranges observed in the cyclohexane oxidation reactions. Thus, CyOOH decomposes very slowly in comparison to H_2O_2 accounting for the substantial amount of oxidant (CyOOH) remaining after 24 h. We also found that H_2O_2 decomposes to oxygen gas and water faster in the absence of cyclohexane than in its presence.

It is also important to note that CyOOH is an intermediate in Du Pont's air oxidation of cyclohexane and decomposes to give CyOH and CyONE.[10f] Also, Drago and Davis reported that in the air oxidation of cyclohexane to give CyOH and CyONE, using a Ru_3O catalyst, they were able to titrate an oxidizing intermediate and proposed it to be CyOOH.[12a] As well, while our paper was in the review process, Barton and co-workers reported the detection of CyOOH via ^{13}C NMR in a Gif-type oxidation of cyclohexane.[12b]

Further insight into the mechanism of these alkane activation reactions has been obtained by performing the following experiments. The primary kinetic isotope effects for the activation of cyclohexane using C_6H_{12} and C_6D_{12} with complexes **1–4** were

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Table II. Comparison of Complexes **1–4** in the Functionalization of Adamantane and Toluene in the Presence of Hydrogen Peroxide[a]

A. Adamantane

catalyst	% products[b] 1-ol	2-ol	2-one	C_3/C_2 ratio[c]	OH/(O) ratio
1[d]	19	9.1	7.2	3.5	3.9
2[e]	8.7	4.8	3.1	3.3	4.4
3[d]	16.8	8.5	6.4	3.4	3.9
4[e]	19	14	4.1	3.3	8.0

B. Toluene

catalyst	products, mmol of product/mmol of complex PhCHO	$PhCH_2OH$	cresols	benzylic/aromatic activation ratio
1	3.5	1.4	1.5	3.4
2	1.9	0.6	0.6	4.2
3	3.0	0.9	4.2	0.9
4	6.0	2.1	0.4	20

[a] All reactions contained 1 mM Fe complex and 150 mM H_2O_2 and either 20 mM adamantane or 300 mM toluene in CH_3CN solvent at ambient temperature for 16 h. [b] Percent of starting substrate. 1-ol is 1-adamantanol, 2-ol is 2-adamantanol, 2-one is 2-adamantanone. [c] Normalized on a per hydrogen basis. [d] Material balance is 100%. [e] Material balance is 68%.

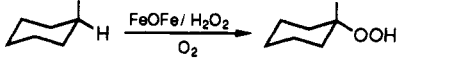
determined. Complexes **1–4** gave nearly identical primary kinetic isotope effects for the formation of cyclohexanol, 1.4, 1.5, 1.6, and 1.5, respectively, and very similar values for the formation of cyclohexanone, 3.1, 3.0, 3.3, and 2.6, respectively. These latter numbers are quite similar to the isotope effect observed in the Gif[IV] system for CyONE formation,[8i] 2.5, and are comparable with those for other metallo-non-porphyrin systems studied (1–10).[8a,13] As well, these numbers are smaller than the primary isotope effects observed in metalloporphyrin systems for alcohol formation (4–13), where metal–oxo species are the putative catalytic intermediates.[14] While the numbers we generate are helpful for comparison purposes, it should be understood that these values may represent several alkane functionalization steps.

We have also examined the effect of the free-radical trap 2,4,6-tri-*tert*-butylphenol (TBP)[13a] on the oxidation of cyclohexane with complexes **1–4**. For clusters **1–3**, the effect of 10 mM TBP substantially decreases (70–100%) the mmol of CyOH and CyONE/mmol of Fe complex. The formation of products was also dramatically affected by the absence of oxygen. In fact, when the reactions were carried out while argon was bubbled through the solutions, the mmol of product/mmol of Fe complex was found to be decreased by 69–100% in comparison to those reactions that were run without an argon purge. These results appear to be consistent with a mechanism in which oxygen traps a cyclohexyl radical as part of a radical-chain process to give CyOOH (autoxidation).[10,15]

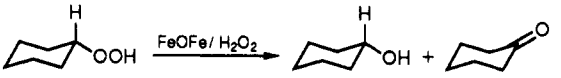
We have also performed alkane functionalization experiments using adamantane, toluene (Table II), and several small hydrocarbons as substrates. The adamantane C_3/C_2 selectivity ratios (normalized) for complexes **1–4** are 3.5, 3.3, 3.4, and 5.6, respectively. The adamamtane C_3/C_2 selectivity ratios are in the range found for other non-porphyrin systems,[13] including the Gif[IV] system (2.6, normalized)[16] and, more importantly, the MMO enzyme (3.0, normalized),[17] which further substantiates a free-radical mechanism for the alkane functionalization step. The toluene selectivity results show that clusters **1–3** have benzylic-/aromatic C–H activation ratios of 3.4, 4.2, and 0.9 respectively, while **4** has a ratio of 20. These results imply that hydroxyl radicals may also be present (aromatic C–H functionalization) in the reaction mixture.[18]

We also examined the activity of clusters **1** and **2** with small hydrocarbon gases such as methane (<1 [mmol of product/mmol of Fe complex], CH_3OH; CD_4 also was converted to CD_3OH and verified by GC/MS as the benzoate ester), ethane (~3, CH_3CH_2OH, and ~5.7, CH_3CHO), and propane (~3–5, CH_3COCH_3, <1, $CH_3CH(OH)CH_3$, <1, $CH_3CH_2CH_2OH$, and ~2–3, CH_3CH_2CHO); however, the systems were difficult to quantify even by GC/MS because of the low conversions and GC separation problems (for example, methanol or any oxidation product that has a value of <1) for an accurate account of the millimoles of product formed.

We believe that complexes **1–4**, using H_2O_2/O_2 as the oxidant and CH_3CN as the solvent, functionalize hydrocarbons through a well-known free-radical-chain process to initially generate an alkyl radical.[10,15,19] This radical is trapped by oxygen to give a peroxyl radical, which abstracts hydrogen from the hydrocarbon, or other hydrogen sources, to give alkyl hydroperoxide and carry the chain (eq 1).[19] We believe that likely candidates for the radical initiation process include Fe—OO• or Fe=O species[20] (all our attempts to trap and identify this species have been unsuccessful to date), which could abstract a hydrogen atom to generate the alkyl radical. The Fe complex/H_2O_2 then has the additional role of catalyzing the decomposition of CyOOH to CyOH and CyONE (eq 2).[10f]



(1)



(2)

We are continuing to perform additional experiments to elucidate the nature of the initial hydrogen atom abstracting species using H_2O_2/O_2 as the oxidant and are also examining these Fe complexes and their Mn analogues with *tert*-butyl hydroperoxide (TBHP),[1a,b] to provide evidence for similar pathways (we now known that there is also an O_2 dependence in the oxidation of hydrocarbons with complexes **1–4** and their Mn analogues with TBHP).[21] Finally, we wish to reiterate that current thinking on mechanisms of hydrocarbon functionalization with Fe cluster complexes that possibly structurally model the active site of MMO and utilize H_2O_2/O_2, irrespective of solvent differences, should now consider alkyl hydroperoxides (as well as alkyl peroxy–metal complexes) as viable intermediates and a simplified pathway to alcohols and to ketones that are not formed directly from alcohols.[7,8h-j,16]

Experimental Section

Instrumentation. Gas chromatography was done on a Hewlett-Packard 5880 instrument with an FID detector and a J&W DB5 Carbowax capillary column, while GC/MS analyses were performed on a Hewlett-Packard MSD 5971A instrument in the EI mode with the same capillary column. ^{13}C NMR (500 MHz) spectra were obtained at the

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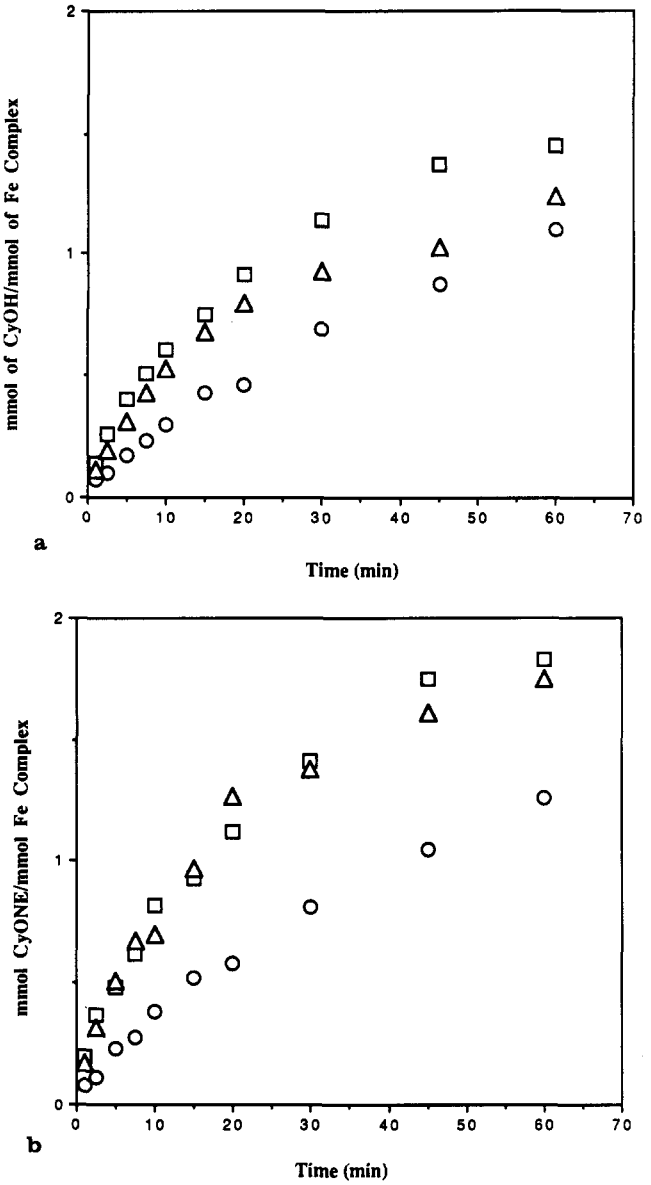

Figure 1. Plots of mmol of (a) cyclohexanol and (b) cyclohexanone formed/mmol of Fe complex versus time in the oxidation of cyclohexane by complexes **1** (△), **2** (□), and **4** (○) in the presence of 30% hydrogen peroxide. All reactions were run in air in a constant temperature bath at 25.5 ± 0.5 °C and were 0.22 mM in Fe complex, 200 mM in cyclohexane, and 33 mM in H_2O_2. Each data point is the average of two runs.

NMR facility of the Department of Chemistry, University of California, Berkeley, CA. The Parr kinetic apparatus was described elsewhere.[22]

Materials. Cyclohexane, adamantane, acetonitrile, and toluene were purchased from Aldrich. Cyclohexane was washed with acid and distilled from calcium hydride before use. Acetonitrile was dried over 4A sieves before use. H_2O_2 was purchased as a 30% aqueous solution from Aldrich. Methane, ethane, propane, and methane-d_4 were purchased from Matheson at purities of 99.99%. $Fe_2O(OAc)(bpy)_2Cl_2$, $[Fe_4O_2(OAc)_7(bpy)_2](ClO_4)$, and $[Fe_2O(OAc)(tmima)_2](ClO_4)_3$ (**1–3**) were prepared and characterized by single-crystal X-ray analysis.[1a,9]

Cyclohexane and Cyclohexanol Oxidations. Typical oxidation reaction procedures were as follows: The complexes **1–4** (0.01 mmol) were dissolved in 10 mL of CH_3CN, and 9 mmol of cyclohexane was added. After the mixture was stirred for 15 min, 1.50 mmol of 30% H_2O_2 was then added and the reaction mixture stirred for 6–18 h (ratio of Fe complex/cyclohexane/H_2O_2 = 1/900/150). Prior to analysis of a 1-mL aliquot of the above-mentioned reaction mixture, 5 μL of cyclopentanone was added as an internal standard and the organic products were quantified by GC (R_f values were determined for each product for area corrections) and verified by GC/MS. The cyclohexanol (900 mM) reactions were performed in a similar manner (Table I). When the above-mentioned reactions were continuously purged with argon gas the mmol of product/mmol of Fe complex ratio was dramatically reduced by 70–100%. Similarly, the addition of 10 mM TBP, a free-radical inhibitor, also reduced product formation by 70–100%.

Methane, Ethane, and Propane Oxidation. The reactions of methane, ethane, and propane were carried out in a Parr kinetic apparatus at partial pressures of 1500, 250, and 90 psi, respectively, at room temperature for 24 h in CH_3CN. The apparatus was charged with a CH_3CN solution of the Fe complex (0.0025 M), all accomplished inside an argon atmosphere drybox. The setup was then taken out of the drybox and flushed twice with the appropriate gas. Then H_2O_2 was syringed into the Parr kinetic apparatus and the system pressurized with the desired gas. After the reaction, the solution was transferred to a vial; pyridine and benzoyl chloride were used to derivatize propanols, ethanol, or methanol to their respective benzoate esters for alcohol quantification (GC, *n*-butyl benzoate as the internal standard) and verification (GC/MS). The resulting products were also analyzed directly and quantified by GC (cyclopentanone as the internal standard) and GC/MS (see text for results).

Adamantane Oxidation. 20 mmol of adamantane was added to a 0.0025 M solution of the Fe complex in CH_3CN and the reaction stirred for 15 min. Then 100-mmol of 30% H_2O_2 was added and the reaction stirred for 3 h. The resulting products, adamantan-1-ol, adamantan-2-ol, and adamantan-2-one, were quantified by GC analysis (CyONE as the internal standard) and verified by GC/MS (Table II).

Toluene Oxidation. A similar procedure as described above for adamantane was utilized with a substrate/catalyst/oxidant ratio of 300/1/150 (Table II).

Primary Kinetic Isotope Effect. A 0.0025 mmol sample of the Fe complex, **1–4**, in CH_3CN (2.5 mL) was stirred at room temperature with approximately 0.2 mmol each of cyclohexane and cyclohexane-d_{12}. After stirring for 15 min, 0.38 mmol of 30% H_2O_2 was added (ratio of Fe complex/cyclohexane/cyclohexane-d_{12}/H_2O_2 = 1/75/75/150) and the reaction stirred for 1–3 h. The products were then quantified by GC (cyclopentanone as internal standard) and verified by GC/MS. The k_H/k_D was then calculated for CyOH and CyONE by simply dividing the concentrations ratios, CyOH/CyOH-d_{11} and those for CyONE/CyONE-d_{12} (see text for values obtained).

Isolation and Characterization of Cyclohexyl Hydroperoxide from the Oxidation of Cyclohexane with Fe Complexes 1–4 in the Presence of H_2O_2. Complex **4** was used as an example for the isolation and characterization of CyOOH from the oxidation of cyclohexane. Thus, **4** (0.03 mmol), cyclohexane (27 mmol), and H_2O_2 (9 mmol) in a ratio of 1/900/300 were reacted at room temperature in 35 mL of acetonitrile for 9 h. The reaction mixture was placed in a separatory funnel, and 40 mL of H_2O and 50 mL of diethyl ether were added and the layers separated. The aqueous layer was further extracted twice with 25-mL portions of ether. The ether extracts were chilled to 0 °C and extracted thrice with chilled (0 °C) 10-mL aliquots of a 30% solution of NaOH. The aqueous NaOH solution was further extracted with two 25-mL portions of ether. The aqueous layer was then acidified with concentrated HCl (0 °C) and extracted thrice with 25-mL portions of ether (this procedure may be repeated if the ether solution contains CyOH and CyONE). The ether solution is dried over anhydrous $MgSO_4$. Removal of the ether by rotory evaporation gave an oil (50 mg, 32%), whose analysis by ^{13}C NMR (CD_3CN) showed the COOH signal at 83.2 ppm with the other ring carbons at 31, 26.5, and 24.4 ppm and completely consistent with a known sample of CyOOH prepared independently.[11]

Cyclohexyl Hydroperoxide Decomposition in the Presence of Complexes 1–4 and Hydrogen Peroxide. The CyOOH decomposition with Fe complexes **1–4** in the presence of 30% H_2O_2 was done as follows with complex **1** as an example. In a flask was placed 0.0025 mmol of complex **1**, 0.05 mmol of CyOOH, and 0.375 mmol of 30% H_2O_2 in 2.5 mL of CH_3CN. After 18 h, 2 μL of cyclopentanone was added as an internal standard and GC analysis provided a value of 0.7 for the CyOH/CyONE ratio.

Rate Studies of Cyclohexane Oxidation to Cyclohexanol and Cyclohexanone with Complexes 1–4. The following procedure was used for the initial rates of formation of CyOH and CyONE with plots of mmol of product/mmol of Fe complex versus time (Figure 1) and the same data was used to calculate pseudo-first-order rate constants (k_{obs}) for the conversion of cyclohexane with Fe complexes **1**, **2**, and **4** with plots of −ln [cyclohexane (0 time and concentration) − CyOH or CyONE (*t* time)] versus time (rate constants were obtained by least-squares analysis for the slope of the line). In a flask immersed in a constant temperature bath at 25.0 (±0.05) °C was placed 0.22 mmol of Fe complex, **1–4**, 200 mmol of cyclohexane, and 33 mmol of H_2O_2 in 45 mL of CH_3CN. At certain time intervals, a 1-mL aliquot was removed and quenched with 0.5 mL of 0.5 M $Na_2S_2O_3$. Then 1.5 mL of diethyl ether was added and

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the ether layer separated and dried. To this ether solution was added 2 μL of cyclopentanone as an internal standard, and quantitative analysis by GC gave the concentrations of CyOH and CyONE with time.

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Spectroscopic Studies of the Copper Sites in Wild-Type *Pseudomonas stutzeri* N_2O Reductase and in an Inactive Protein Isolated from a Mutant Deficient in Copper-Site Biosynthesis

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Pseudomonas stutzeri N_2O reductase is a complex multicopper enzyme (approximately 8 Cu ions/protein molecule). Two copper sites appear to be closely similar to the Cu_A-type site in cytochrome *c* oxidase, but relatively little is known about the other copper sites in N_2O reductase. In this paper circular dichroism, magnetic circular dichroism, and X-ray absorption and fluorescence spectroscopy have been used to further characterize the copper sites in native N_2O reductase and in a "mutant" protein isolated from a strain deficient in the biosynthesis of the N_2O reductase copper sites that contains only 2 copper ions/protein molecule. Both magnetic circular dichroism and X-ray absorption (Cu K-edge and EXAFS) data are consistent with the presence of (on average) one Cu_A-type site per protein in the mutant N_2O reductase. Comparisons of the near-infrared circular dichroism spectra of the oxidized native and "mutant" N_2O reductases suggest that transitions at 7200 and 9500 cm^{-1} in the native enzyme are associated with copper sites other than the Cu_A-type sites. To the best of our knowledge, these are the first electronic spectral features that can be attributed to non-Cu_A-type sites in N_2O reductase. The near-infrared bands are significantly less intense in preparations of N_2O reductase that display lower specific activities. Several electronic transitions are resolved in the circular and magnetic circular dichroism spectra of dithionite-reduced N_2O reductase. Notably a near-infrared band is observed in the circular dichroism spectrum at 8200 cm^{-1}. The data are plausibly attributed to a highly covalent [Cu(II)–S^-(cys) ↔ Cu(I)–$S^•$(cys)] site in the *reduced* enzyme. Copper removal from N_2O reductase enhances the tryptophan fluorescence intensity about 3-fold; most of the quenching appears to be associated with occupation of the Cu_A-type sites.

Denitrifying organisms can couple nitrate, nitrite, and nitrous oxide reduction to ATP synthesis,[5] via proton translocation and the formation of a membrane potential.[6] N_2O reductase is generally the terminal enzyme in a complete denitrification pathway; this enzyme catalyzes the two-electron reduction of nitrous oxide (eq 1). The physiological electron donors for various N_2O reductases have not yet been definitively identified, but *c*-type cytochromes are considered likely candidates.[7]

$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \qquad (1)$$

All N_2O reductases isolated to date are complex multicopper enzymes. The enzymes from *Pseudomonas stutzeri*[8-12] and *Paracoccus denitrificans*[13] are the best characterized. Multiple forms of the *P. stutzeri* N_2O reductase have been isolated or prepared; key features of these various forms have been summarized in the literature.[8,9] The native enzyme may be isolated in high-activity or low-activity forms (N_2OR I and N_2OR II, respectively) and contains a maximum of eight copper ions per protein molecule. The *P. stutzeri* protein is a dimer of two identical subunits, and the amino acid sequence has been obtained by translation of the *nosZ* gene in this organism.[14] Comparison of the N_2O reductase sequence to published cytochrome oxidase sequences,[11] together with spectroscopic data,[11,12] strongly indicates that N_2O reductase contains Cu_A-type sites. These sites are prominently reflected in the visible absorption spectrum of the resting enzyme (Figure 1). Resonance Raman spectroscopy has shown that the principal electronic absorption band at 540 nm is a RS^-(cys) → Cu(II) ligand-to-metal charge-transfer (LMCT) transition.[10] A [Cu^{II}(cys-*S*)$_2$(his-*N*)$_2$] site is most consistent with the resonance Raman data;[10] a similar coordination environment has been suggested for Cu_A in cytochrome oxidase.[15] Cu_A is believed to be located in subunit II of cytochrome oxidase (coxII);[16]

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