Models for non-heme iron containing oxidation enzymes
Roelfes, Gerard

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CHAPTER 3

Characterization and Reactivity of Iron-Peroxide Intermediates

3.1 Iron-Peroxide Intermediates

Iron-peroxo species have been established or postulated in the chemistry of dioxygen activation at mononuclear iron centers in biology. For example, activated BLM, the form of the antitumor drug bleomycin responsible for its DNA cleavage activity, is formulated as a low-spin Fe$^{III}$-$\eta^1$-OOH species. Similarly, an Fe$^{III}$-$\eta^1$-OOH moiety is strongly implicated in the mechanism of cytochrome P450 and considered to be the key intermediate immediately prior to the cleavage of the O-O bond to generate the high valent iron-oxo oxidant. Both Fe$^{III}$-$\eta^1$-OOH species are proposed to derive from the one-electron reduction of oxy adducts ([Fe-O$_2$]$^{2+}$) and subsequent protonation, possibly via an [Fe$^{III}$-$\eta^2$-O$_2$]$^+$ species. Such [Fe$^{III}$-$\eta^2$-O$_2$]$^+$ species are also postulated to be the source of nucleophilic peroxides, which are implicated in substrate oxidations by heme enzymes such as aromatase. A related [Fe$^{III}$-$\eta^2$-O$_2$]$^+$ species has been suggested to account for the cis-dihydroxylation chemistry of the Rieske dioxygenases. Significant effort has been devoted to the characterisation of small molecule analogues of such peroxo intermediates. Two high-spin [Fe$^{III}$-$\eta^2$-O$_2$]$^+$ species, namely [Fe$^{III}$-(EDTA)(O$_2$$^2$-)]$^+$ and [Fe(porphyrin)O$_2^{-}$], have been identified, whereas in the last few years the first examples of synthetic Fe$^{III}$-$\eta^1$-OOH species have been reported. In this chapter the interconversion between an [Fe$^{III}$OOH]$^{2+}$ species and its deprotonated form [Fe$^{III}$O$_2$]$^+$, implicit in the mechanisms of bleomycin and cytochrome P450, is described. Furthermore, the spectroscopic characterization of the [Fe$^{III}$O$_2$]$^+$ intermediate, the resonance Raman spectra of both intermediates, and the implications of this interconversion for the mechanism of catalytic oxidation are discussed.

3.2 Interconversion between [(N4Py)Fe$^{III}$OOH]$^{2+}$ and [(N4Py)Fe$^{III}$O$_2$]$^+$

The purple [(N4Py)Fe$^{III}$OOH]$^{2+}$ complex (1, $\lambda_{max}$ 547 nm, $\varepsilon_M$ 1300) is generated at -20$^\circ$ C by addition of 5 equiv. H$_2$O$_2$ to a methanolic solution of [(N4Py)Fe$^{III}$OMe]$^{2+}$, obtained by pretreating [(N4Py)Fe$^{II}$(CH$_3$CN)]$^{2+}$ with 5 equiv. H$_2$O$_2$ at room temperature. Upon addition of 5 equiv. NH$_4$OH at -45$^\circ$ C, the purple chromophore is converted to a blue species 2 with an absorption maximum at 685 nm ($\varepsilon_M$ 520) (figure 1a). Addition of perchloric acid leads to reversal of this color change and the original purple solution is quantitatively regenerated. The conversion of 1 to 2 can also be monitored by EPR spectroscopy. Addition of base converts the characteristic low-spin Fe$^{III}$ EPR spectrum of 1 with $g$ values at 2.3, 2.1, and 1.9 to one with an intense isotropic signal at $g = 4.3$, which is typical of a mononuclear rhombic high-spin Fe$^{III}$ center (E/D = 1/3). In the case of the Fe$^{III}$-(N-R-trispicen) complex, the addition of base to the Fe$^{III}$OOH species converts it to a high-spin Fe$^{III}$ complex with a more axial EPR spectrum (E/D $\approx$ 0.08). Thus the addition of base converts low-spin 1 into a species with a high-spin Fe$^{III}$ centre.
Electrospray ionisation mass spectrometry provides the elemental composition of 2. As reported earlier, the ES/MS of 1 shows a prominent feature at m/z 555, whose mass and isotope distribution pattern lead to unequivocal assignment of this ion as $[(N4Py)Fe^{III}(OMe)]ClO_4^+$. The ES/MS spectrum of 2 (Figure 1b), generated with NH$_4$OAc as base, exhibits a prominent feature at m/z 455. This mass corresponds to the loss of HClO$_4$ relative to that of 1. The isotope distribution pattern associated with this feature matches well with that calculated for $[(N4Py)Fe(OO)]^+$, thus supporting the notion that 2 is the conjugate base of 1. Besides the major peak at m/z 455 two minor peak clusters were observed at m/z 485 and 513. These singly charged species presumably originate from the decomposition of 2, since their intensity increases in time as the peak at m/z 455 decreases. The species at m/z 485 is tentatively formulated as $[(N4Py)Fe^{III}(OMe)_2]^+$ and the species at m/z 513 as $[(N4Py)Fe^{III}(OMe)(OAc)]^+$. Support for this formulation was obtained by use of methanol-d$_4$ as solvent, which caused these peaks to shift to m/z 491 and 516, respectively. Furthermore, when ND$_4$OAc-d$_3$ was used as base the peak at m/z 513 was shifted to m/z 516. The peak cluster at m/z 455 was not affected by the use of methanol-d$_4$ or ND$_4$OAc-d$_3$.

3.3 Resonance Raman Spectroscopy of $[(N4Py)FeOOH]^2^+$

Laser excitation into the 548 nm absorption band of 1 in methanol at 77 K gives the resonance Raman spectrum shown in figure 2, with four enhanced features at 632, 650, 672, and 790 cm$^{-1}$. Intermediate 1 represents the first low-spin mononuclear iron hydroperoxide species to be characterized by resonance Raman spectroscopy. The use of H$_2^{18}$O$_2$ causes only the features at 632 and 790 cm$^{-1}$ to shift significantly, to 615 and 744 cm$^{-1}$, respectively. The downshift by -46 cm$^{-1}$ for the 790 cm$^{-1}$ feature is close to the value of -45 cm$^{-1}$ predicted.
by Hooke's Law for a pure O-O stretch, leading to its assignment as $\nu$(O-O). However, the downshift of the 632 cm$^{-1}$ feature by only -17 cm$^{-1}$ is significantly lower than predicted for a pure Fe-O vibration (-28 cm$^{-1}$). Inclusion of the other peroxo oxygen, \textit{i.e.} $\nu$(Fe-OO), in the calculations affords a downshift (-23 cm$^{-1}$) that approaches the experimental value, and the further addition of a proton, \textit{i.e.} $\nu$(Fe-OOH), makes the downshift (-22 cm$^{-1}$) even closer to the experimental value.

![Resonance Raman spectra of 1.](image)

**Figure 2.** Resonance Raman spectra of 1. (a) 5 eq H$_2$O$_2$ was added to 10 mM [(N4Py)Fe$^{II}$(CH$_3$CN)]$^{2+}$ in methanol at -10°C. The spectrum was obtained with 615 nm laser excitation at 20 mW power at the sample. (b) Same as (a), except H$_2^{18}$O$_2$ and acetone were used. (c) Same as (a), except [(N4Py)$^{54}$Fe$^{II}$(CH$_3$CN)]$^{2+}$ was used. (d) Same as (a), except H$_2$O$_2$ diluted in D$_2$O was used.

The use of the $^{54}$Fe complex, prepared according to scheme 1, does not affect the 790 cm$^{-1}$ vibration, thus supporting its assignment as a pure O-O vibration.

\[
\begin{align*}
^{54}\text{Fe} & \stackrel{1)}{\xrightarrow{70 \% \text{ HClO}_4}} [\text{(N4Py)}^{54}\text{Fe}(\text{CH}_3\text{CN})](\text{ClO}_4)_2 \\
& \stackrel{2)}{\xrightarrow{\text{N4Py, MeOH/CH}_3\text{CN}} \text{87 %}} [\text{(N4Py)}^{54}\text{Fe}(\text{CH}_3\text{CN})](\text{ClO}_4)_2
\end{align*}
\]

**Scheme 1.** Synthesis of [(N4Py)$^{54}$Fe(CH$_3$CN)](ClO$_4$)$_2$
Chapter 3

However, the 632 cm\(^{-1}\) vibration is upshifted by 2 cm\(^{-1}\) in the \(^{54}\)Fe complex, which best approaches the calculated shift for a \(\nu(\text{Fe-OOH})\) (\(\nu_{\text{calcd}} +4 \text{ cm}^{-1}\)). Additional support for the involvement of the hydroperoxy group in the 632 cm\(^{-1}\) deformation comes from the -5 cm\(^{-1}\) downshift observed upon \(^2\)H labeling, close to the calculated shift of -6 cm\(^{-1}\) for a \(\nu(\text{Fe-OOH})\). Thus the \(^{18}\)O and \(^2\)H effects on the 632 cm\(^{-1}\) feature suggest that this feature arises from a coupled mode of the Fe-OOH unit.

The Raman features at 790 and 632 cm\(^{-1}\) associated with 1 are distinct from other iron-peroxo species, which in general have \(\nu(\text{O-O})\)'s at 806-900 cm\(^{-1}\) and \(\nu(\text{Fe-O})\)'s at 421-503 cm\(^{-1}\) (table 1).\(^7,13,14\) Some of these are (\(\mu\)-1,2-peroxo)diiron(III) species, so the most salient comparisons are with oxyhemerythrin (844, 503 cm\(^{-1}\))\(^13\) and [Fe\(^{III}\)(EDTA)(\(\eta^2\)-O\(_2\))]\(^3-\) (816, 459 cm\(^{-1}\)),\(^7\) which are the only complexes with terminally bound peroxide that have been characterized by resonance Raman spectroscopy. Oxyhemerythrin, like 1, has a terminal hydroperoxide ligand, but [Fe\(^{III}\)(EDTA)(\(\eta^2\)-O\(_2\))]\(^3-\) has a side-on bound peroxide. The most dramatic difference among these three iron peroxo species is observed for the lower frequency vibration, which for 1 is at least 129 cm\(^{-1}\) higher than the corresponding vibrations in the other two complexes.

**Table 1.** Comparison of resonance Raman vibrations for iron-peroxide complexes.

<table>
<thead>
<tr>
<th>Complex/Peroxide Binding Mode</th>
<th>(\nu_{\text{Fe-L}}) (cm(^{-1}))(^a)</th>
<th>(\nu_{\text{O-O}}) (cm(^{-1}))</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Fe(N4Py)(OOH)](^{2+})</td>
<td>632(^b)</td>
<td>790</td>
<td>this chapter</td>
</tr>
<tr>
<td>Fe-O</td>
<td>O-H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[(N4Py)Fe(OO)](^+)</td>
<td>478</td>
<td>827</td>
<td>this chapter</td>
</tr>
<tr>
<td>Fe-O</td>
<td>O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Fe(III)(EDTA)((\eta^2)-O(_2))](^3-)</td>
<td>459</td>
<td>816</td>
<td>7</td>
</tr>
<tr>
<td>Fe-O</td>
<td>O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxyhemerythrin</td>
<td>503</td>
<td>844</td>
<td>13</td>
</tr>
<tr>
<td>Fe(_2)((\mu)-1,2-O(_2)) species</td>
<td>415-476</td>
<td>848-900</td>
<td>14</td>
</tr>
</tbody>
</table>

\(^a\) L = O or OOH. \(^b\) Observed feature for the coupled Fe-OOH mode.
The significantly higher energy of this vibration in 1 may arise from the presence of a low-spin Fe$^{III}$ center in 1, the other two complexes having high-spin Fe$^{III}$ centers. Furthermore, recent calculations using nonlocal density function theory on the putative low-spin Fe$^{III}$-OOH intermediate in cytochrome P450 suggest that the protonation of the Fe$^{III}$-OO$^{-}$ intermediate results in the strengthening of the Fe-O bond and the weakening of the O-O bond.\textsuperscript{5} This proposed effect of protonation is entirely consistent with the high energy observed for the coupled Fe-OOH vibration in 1 and the fact that 1 has the lowest reported ν(O-O) of all the Fe-peroxide complexes. Therefore the distinctive Raman features observed in 1 may serve as the Raman signature for a low-spin iron(III)-hydroperoxide intermediate.

Given the fact that the few structurally characterized M-OOH complexes all have $\eta^{1}$ (end-on) binding modes,\textsuperscript{15} it is likely that the hydroperoxide in 1 is similarly bound.

### 3.4 Resonance Raman Spectroscopy of [(N4Py)Fe(OO)]$^{+}$

Conversion of 1 into 2 by addition of NH$_4$OH results in a significantly different Raman spectrum with prominent peaks at 495 and 827 cm$^{-1}$ (figure 3).\textsuperscript{16}

![Resonance Raman Spectroscopy of [(N4Py)Fe(OO)]$^{+}$](image)

**Figure 3.** Resonance Raman spectra of 2. (a) 2 generated by the addition of 5 eq NH$_4$OH to a solution of 1, generated by the addition of 5 eq H$_2$O$_2$ to a methanolic solution of [Fe$^{III}$N4Py](OMe)$_2^+$, at -35°C. (b) Same as (a), except H$_2$O$_2$ was used. (c) Same as (a), except that the $^{56}$Fe complex was used. (d) Same as (a), except H$_2$O$_2$ diluted in D$_2$O was used.
The use of \( \text{H}_2\text{^{18}O}_2 \) leads to a shift of the two features to 478 (\( \Delta \nu = -17 \)) and 781 (\( \Delta \nu = -46 \)) cm\(^{-1} \), respectively, which is fully consistent with their assignments as \( \nu(\text{Fe-O}_2) \) (\( \Delta \nu_{\text{calc}} = -18 \) cm\(^{-1} \)) and \( \nu(\text{O-O}) \) (\( \Delta \nu_{\text{calc}} = -47 \) cm\(^{-1} \)), respectively. In accord with these assignments, the introduction of \( ^{54}\text{Fe} \) in \( 2 \) does not affect the 827 cm\(^{-1} \) vibration but upshifts the 495 cm\(^{-1} \) feature by 3 cm\(^{-1} \) (\( \Delta \nu_{\text{calc}} = +3 \) cm\(^{-1} \)). Furthermore, unlike for \( 1 \),\(^{11} \) the use of \( ^2\text{H}_2\text{O}_2 \) does not affect the Raman spectrum of \( 2 \). Thus the Raman spectra support the notion that \( 2 \) is an \([\text{Fe}^{\text{III}}-\text{O}_2]^+\) complex.

Mixed labeled hydrogen peroxide can be used to distinguish end-on binding of the peroxide from side-on binding. In the case of end-on binding two separate signals are expected for the O-O vibration due to the fact the Fe can bind to both the \(^{18}\text{O} \) as the \(^{16}\text{O} \) atom, whereas with side-on binding only one signal will be observed (figure 4).

**Figure 4.** Possible binding modes for end-on and side-on bound mixed labeled peroxide.

Strong evidence for a symmetric \( \eta^2 \)-peroxo binding mode was obtained from the Raman spectrum of \( 2 \) with \( \text{H}_2\text{O}_2 \) containing 61\% \(^{18}\text{O} \) (figure 5). Although the various isotopic components of the \( \nu(\text{Fe-O}_2) \) feature centered at 486 cm\(^{-1} \) are unresolved, three peaks associated with the \( \nu(\text{O-O}) \) feature are readily discerned at 781, 802, and 826 cm\(^{-1} \) and can be fit with peaks having approximately equal linewidths. The fact that the peak arising from the \(^{16}\text{O}^{18}\text{O} \) isotopomer has a linewidth equal to those of \(^{18}\text{O}^{16}\text{O} \) and \(^{18}\text{O}^{18}\text{O} \) isotopomers strongly implies a symmetric \( \eta^2 \)-peroxo binding mode, as found for \([\text{Fe}^{\text{III}}(\text{EDTA})(\eta^2-\text{O}_2)]^+ \).\(^{7a,8} \)

**Figure 5.** Raman spectrum of \( 2 \) generated with a statistical mixture of \( \text{H}_2\text{O}_2 \) isotopomers (61\% \(^{18}\text{O} \)). The dashed lines represent the curve fit of the features associated with the \( \nu(\text{O-O}) \) peaks. The curve was fitted with three peaks with the \(^{18}\text{O}^{18}\text{O} \) feature constrained to a frequency of 781 cm\(^{-1} \) and a linewidth of 16 cm\(^{-1} \) as found for \([\text{(N4Py)}\text{Fe}(^{18}\text{O}_2)]^+ \). The fitted data gave the other two peaks at 802 and 826 cm\(^{-1} \) with linewidths of 16.3 and 15.9 cm\(^{-1} \), respectively. The latter matched well the properties of the \( \nu(\text{O-O}) \) feature in \([\text{(N4Py)}\text{Fe}(^{18}\text{O}_2)]^+ \).
At this point there is not enough information to determine whether the iron center in 2 becomes seven-coordinate, as illustrated in scheme 2, or remains six-coordinate by a decrease in the denticity of the N4Py ligand. The latter may come about by detachment of one of the pendant pyridines or, alternatively, by breaking the already weak tertiary amine bond as the \( \eta^2 \)-peroxo ligand pulls the iron center further out of the plane defined by the four pyridine ligands to achieve a coordination geometry similar to that found in the structure of \([\text{Mn(TPP)}(\eta^2-O_2)]^+\).^{17}

Scheme 2. Proposed structures for 1 and 2.

3.5 Mechanistic Implications

With the peroxo binding mode of 2 established, it is interesting to compare the relative abilities of 1 and 2 to oxidize substrates. It has been demonstrated that \([(N4Py)\text{Fe}^{II}(\text{CH}_3\text{CN})](\text{ClO}_4)_2\) can catalyze the hydroxylation of cyclohexane with \( \text{H}_2\text{O}_2 \) in acetone or acetonitrile via intermediate 1.\(^{18}\) With 5 eq of \( \text{H}_2\text{O}_2 \) 1.6 and 0.4 turnovers to cyclohexanol and cyclohexane were observed in acetone as solvent (table 2, entry 1).

Table 2. Oxidation of cyclohexane with \([(N4Py)\text{FeOOH}]^{2+}\) (1) and \([(N4Py)\text{Fe}(\text{OO})]^+\) (2).

<table>
<thead>
<tr>
<th>entry</th>
<th>T (°C)</th>
<th>eq ( \text{H}_2\text{O}_2 )</th>
<th>eq base</th>
<th>CyOH(^a)</th>
<th>CyO(^a)</th>
<th>D(_2)CO(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(^b)</td>
<td>25</td>
<td>5</td>
<td>-</td>
<td>1.6</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>5</td>
<td>-</td>
<td>0.8</td>
<td>0.4</td>
<td>1.4</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>100</td>
<td>-</td>
<td>7.5</td>
<td>1.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>5</td>
<td>6(^c)</td>
<td>0</td>
<td>0</td>
<td>1.7</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>5</td>
<td>30(^d)</td>
<td>0</td>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>6(^e)</td>
<td>25</td>
<td>100</td>
<td>100(^d)</td>
<td>0</td>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>7(^e)</td>
<td>25</td>
<td>100</td>
<td>35(^e)</td>
<td>0</td>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>8(^e)</td>
<td>0</td>
<td>100</td>
<td>100(^d)</td>
<td>0</td>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>9(^e)</td>
<td>0</td>
<td>100</td>
<td>35(^e)</td>
<td>0</td>
<td>0</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

All reactions were performed in \( \text{CD}_3\text{OD} \), unless noted otherwise. n.d. = not determined. (a) turnovrs = mmol product / mmol catalyst. (b) acetone used as solvent. (c) \( \text{NH}_4\text{OH} \) used as base. (d) \( \text{NH}_4\text{OAc} \) used as base. (e) \( \text{CH}_3\text{OH} \) used as solvent.
In methanol-d4 as solvent, which allows the formation of both 1 and 2, with 5 equiv. H₂O₂, 1.0 and 0.4 turnovers of cyclohexanol and cyclohexanone, respectively, were observed in the case of 1 (table 2, entry 2). With 100 eq H₂O₂ the yield increased to a total of 8.6 turnovers (table 2, entry 3). These yields are quite low due to a competing oxidation of the solvent to D₂C=O (1.4 turnovers). When NH₄OH or NH₄OAc was added prior to or after the formation of 1, both in CH₃OH and CD₃OD no oxidation of cyclohexane was observed, not even using 100 eq H₂O₂ (table 2, entries 4-9). With 5 eq H₂O₂ 1.7 turnovers of D₂C=O were formed, which could be due to the formation of superoxide during the reaction. This was indicated by EPR which sometimes showed the characteristic superoxide signal at g = 2 when 2 was not measured immediately after formation. These results suggest that, whereas 1 is capable of activating the O-O bond to oxidize alkanes, 2 is unreactive towards such substrates, resembling the inertness of other [Fe³⁺(η²−O₂)] species.7a,19 Thus the demonstration of an [Fe³⁺-η¹-OOH]/[Fe³⁺-η²-HO⁻] interconversion supports unequivocally the hypothesis that protonation of a peroxo species is required to facilitate O-O bond cleavage in the mechanisms of oxygen activation by iron enzymes.5,7a

3.6 Conclusions

In this chapter two N4Py-iron peroxide intermediates, e.g. [(N4Py)FeOOH]²⁺ (1) and [(N4Py)Fe(OO)]⁺ (2) and their characterization were described. The resonance Raman spectrum of 1 showed a weakening of the O-O bond of the peroxide compared to other iron-peroxo complexes. This weakened O-O bond correlates well with the high reactivity observed for low-spin iron(III)-hydroperoxide intermediates. Resonance Raman spectroscopy of 2 generated with H₁⁶O₁⁸OH established the η², side-on, binding mode of the peroxide. Intermediate 2 was demonstrated to be unreactive in alkane hydroxylation. These results represent the first experimental proof for the hypothesis that protonation of an iron-peroxide intermediate is required for O-O bond activation.

3.7 Experimental Section

General Information

For general information see also chapter 2.

Mass spectra were recorded on a NERMAG R 3010 triple quadrupole mass spectrometer (Nermag, Argenteuil, France) equipped with a home-built atmospheric pressure ionization source. Samples were introduced by means of a syringe pump. The syringe needle was used as the electrospray emitter, and the syringe pump was positioned such that the syringe needle was at approximately 1 cm from the ion sampling orifice. The Perkin-Elmer-Sciex API 3 data system with a home built analog and digital interface was used for data acquisition and data processing.

Raman experiments were performed by Dr. R.Y.N. Ho (University of Minnesota). Resonance Raman spectra were collected on an Acton AM-506 spectrometer (2400-groove grating) using Kaiser Optical holographic super-notch filters with a Princeton Instruments liquid N₂-cooled (LN-1100PB) CCD detector with 4 cm⁻¹ or 2 cm⁻¹ spectral resolution. Spectra were
obtained by back-scattering geometry on liquid N\textsubscript{2} frozen samples using 568.2 nm laser excitation from a Spectra Physics 2030-15 argon ion laser and a 375B CW dye (Rhodamine 6G). Raman frequencies were referenced to indene.

\(\text{H}_2^{18}\text{O}_2\) (90\% \(^{18}\text{O}\)-enriched, 2\% solution in \(\text{H}_2^{16}\text{O}\)) was obtained from ICON Services Inc. The statistical mixture of \(\text{H}_2\text{O}_2\) (61\% \(^{18}\text{O}\)) was prepared by Dr. R.Y.N. Ho (University of Minnesota) by the reduction of \(\text{O}_2\) (statistical mixture with 61\% \(^{18}\text{O}\) from ICON Services Inc.) following a literature procedure.\textsuperscript{20}

Catalytic reactions were performed using the standard conditions described in chapter 4. GC analyses were performed on a Hewlett Packard 6890 Gas Chromatograph using a HP-5 5\%-phenyl methyl siloxane column. Retention times of cyclohexanol and cyclohexanone were compared with commercial samples.

Formaldehyde was quantified by a colorimetric method, adapted from a literature procedure,\textsuperscript{21} described below.

\([(\text{N}_4\text{Py})^{54}\text{Fe(CH}_3\text{CN})](\text{ClO}_4)\textsubscript{2}\)

A lump of \(^{54}\text{Fe}\) (8.2 mg, 0.15 mmol) was converted to smaller particles using a hardened steel file. The \(^{54}\text{Fe}\) particles were added to 70\% perchloric acid (47 mg, 0.33 mmol) in a micro test tube (no magnetic stirring bar!). The mixture was carefully heated using a heat gun, during which time an orange color appeared. An additional aliquot of 70\% HClO\textsubscript{4} (18 mg, 0.13 mmol) was added and the mixture was heated for 30 min. During the reaction H\textsubscript{2}O (4 \times 20 \(\mu\)l) was added to compensate for evaporation. After standing at room temperature for 1.5 h all iron had disappeared and MeOH (1 ml) was added. A solution of N\text{4Py} (60 mg, 0.17 mmol) in CH\textsubscript{3}CN (1 ml) was added and the resulting dark red solution was placed in an ethyl acetate bath. After standing for 1 night red crystals were formed which were isolated and washed with ethyl acetate. The crystals were redissolved in CH\textsubscript{3}CN and placed in an ethyl acetate bath to afford \([(\text{N}_4\text{Py})^{54}\text{Fe(CH}_3\text{CN})](\text{ClO}_4)\textsubscript{2}\) (86 mg, 0.13 mmol, 87\%) as red crystals after 3 days. \(^{1}\text{H}-\text{NMR (CD}_3\text{CN)} \delta 4.34 \text{ (q (AB), 4H, J = 18.3 Hz)}, 6.34 \text{ (s, 1H)}, 7.06 \text{ (d, 2H, J = 7.9 Hz)}, 7.35 \text{ (m, 4H)}, 7.68 \text{ (m, 2H)}, 7.91 \text{ (m, 4H)}, 8.91 \text{ (d, 2H, J = 5.3 Hz)}, 9.04 \text{ (d, 2H, J = 5.6 Hz)}; \text{ES/MS: m/z 520 [M - (ClO}_4^- -(CH}_3\text{CN)]^+}, 210.5 \text{ [M - 2(ClO}_4^- -(CH}_3\text{CN)]}^2^+.

**Colorimetric quantification of formaldehyde**

A solution of the colorimetric reagent was prepared by dissolving NH\textsubscript{4}OAc (15 g, 0.19 mol), 2,4-pentanedione (0.2 ml, 1.9 mmol) and acetic acid (0.3 ml, 5.2 mmol) in water (100 ml).

A sample (1 ml) was taken from the catalytic reaction and diluted 20 times with water. From this a sample (1 ml) was combined with the colorimetric reagent (1 ml). The mixture was heated at 30 \(\degree\)C, with concomittant monitoring of the UV/Vis spectra, until the absorption from the product 3,5-diacetyl-1,4-dihydrolutidine at 412 nm reached a constant value (typically 1.5 h). From the absorption the concentration 3,5-diacetyl-1,4-dihydrolutidine, which equals the concentration of formaldehyde, can be calculated using \(\varepsilon_M\) (3,5-diacetyl-1,4-dihydrolutidine) 8840.
References and Notes


2. Abbreviations used: BLM = bleomycin; EDTA = N,N,N',N'-ethylenediaminetetraacetate; N4Py = N,N-bis(2-pyridylmethyl)-N-bis(2-pyridyl)methyamine.


16 Aside from the features at 495 and 827 cm⁻¹, a weaker feature at 625 cm⁻¹ is also sensitive to ¹⁸O labeling. However the intensity of this feature varies relative to the intensities of the 495 and 827 cm⁻¹ features with different samples suggesting that the 625 cm⁻¹ feature is not associated with 2.


