Oxidizing Intermediates in the Reaction of Ferrous EDTA with Hydrogen Peroxide

REATIONS WITH ORGANIC MOLECULES AND FERROCYTOCHROME C*

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The reaction between hydrogen peroxide and ferrous EDTA generates an oxidizing intermediate (I1) which is not the hydroxyl radical. It oxidizes ferrocytochrome c and also reacts with hydrogen peroxide ($k_3 = 3.2 \times 10^9$ M$^{-1}$ s$^{-1}$) to form a second oxidizing transient (I2). I2 is not scavenged by t-butyl alcohol whereas I3 is. I3 is found to be significantly less reactive than the hydroxyl radical toward benzoate ion, t-butyl alcohol, acetate ion, arginine, and serine, but is scavenged by compounds with readily oxidizable functional groups such as ethanol and isopropyl alcohol. This indicates that I3 does not undergo the characteristic reactions of the hydroxyl radical but shows a pattern of reactivity more associated with a metal ion oxidant like a ferryl(FeO2$^+$)-EDTA complex.

The nature of the oxidizing intermediate produced by Reaction 1, whether a hydroxyl radical or a ferryl-(FeO2$^+$) complex, has not been resolved because of its high reactivity.

\[
\text{Fe(II)-EDTA} + H_2O_2 \rightarrow P \quad (1)
\]
\[
P + \text{Fe(II)-EDTA} \rightarrow 2 \text{Fe(III)-EDTA} + 2 OH^- \quad (2)
\]
\[
P = \text{FeO(II)-EDTA} + H_2O, \text{or} P = \text{Fe(III)-EDTA} + \cdot OH + OH^- \quad (3)
\]

The resolution of this question is biologically important since the reaction between hydrogen peroxide and non-haem ferric complexes is thought to initiate damaging free radical reactions in the cell (1). The potential for toxicity arising from the hydroxyl radical is limited by its high reactivity toward all organic molecules, and thus the likelihood that it will react at a site where cell damage will occur is small. A ferryl complex, though unstable, is likely to be much more selective in its reactions. It has been estimated that the reduction potential of a ferryl-ferric complex is about 1 volt, 1.3 volts lower than that of the couple 'OH/H$_2$O at pH 7 (2). Recently, it has been shown that ferryl complexes liganded by hydroxide and EDTA have appreciable lifetimes in aqueous solution (3).†

The EDTA ligand is widely used to sequester iron in biological model systems (4–7) and in Fenton systems employing an excess of hydrogen peroxide (8). In the latter systems there is substantial evidence that hydroxyl radicals are formed. However, catalytic hydroxylations of organic substrates such as alcohol or phenol derivatives by hydrogen peroxide and ferric chelates occur with a site selectivity which cannot be entirely due to the reactions of the hydroxyl radical (9, 10). Recently, it has been found (11) that Fe(III)-EDTA catalyzes the oxidation of ferrocytochrome c by dilute hydrogen peroxide without degradation of the protein as observed upon reaction with hydroxyl radicals generated by y-radiolysis (12). The rapid oxidation of a small part of the ferrocytochrome c seen at the beginning of the reaction is the subject of this paper.

The high molar absorptivity of ferrocytochrome c at 550 nm relative to the ferric form makes it an excellent molecular probe for the oxidizing intermediates produced in Reaction 1. We report here on some properties of the oxidizing intermediates produced in this reaction using stopped-flow spectrophotometry. Our results indicate that the transient produced in Reaction 1 shows patterns of reactivity inconsistent with the hydroxyl radical.

MATERIALS AND METHODS

Rapid mixing experiments were performed on a Kinetic Instruments, Inc. stopped-flow apparatus designed by Drs. D. Ballou and C. Bull. The mixing cell and holding syringes were thermostatted at 25°C. The optical system was supplied by On-Line Instruments Systems, Inc. The signal from the photomultiplier tube was converted to digital form, acquired, displayed, and analyzed using a microcomputer-based A/D interface and software developed by OLIS. The data-fitting routines were supplied by OLIS and use a nonlinear least squares method of fitting multieponential rate expressions. Goodness of fit was checked by visual comparison of the raw and calculated data and by use of the Durbin-Watson statistic which is a measure of the randomness of residuals. Rate and stoichiometric data are the average of at least five measurements. Kinetic measurements of the reaction between hydrogen peroxide and ferrous EDTA were made at 350 nm, while cytochrome c was monitored at 550 nm (10). Stoichiometry data for Reaction 1 were taken at 300 nm ($f_{300} = 8500$ M$^{-1}$ cm$^{-1}$ for Fe(III)-EDTA).

All inorganic and nonbiological organic chemicals were Baker analyzed reagents, except ethanol (Warner-Graham, U.S.P.) and t-butyl alcohol which was recrystallized twice from the Baker A.C.S. grade. Amino acids (>98% purity) were from Aldrich or Sigma. Solutions were made with double-distilled water, and NaClO$_3$ (0.1 M) was used to maintain constant ionic strength. Hydrogen peroxide stock solutions were prepared from Baker 30% hydrogen peroxide containing 1 ppm of Na$_2$S$_2$O$_3$ as stabilizer and standardized by potassium permanganate titration. Ferrous EDTA solutions were prepared from Fe(NH$_4$)$_2$(SO$_4$)$_2$·6H$_2$O with a 1:5 excess of the ligand in thoroughly degassed (N$_2$) 1 mM phosphate buffer. Ferricytochrome c (type VI, Sigma) was reduced with ferrous EDTA. All solutions were purged with nitrogen gas (99.996%) and transferred to the flow machine in syringes with gas-tight fittings.

RESULTS AND DISCUSSION

Reaction of Fe(II)-EDTA with Excess H$_2$O$_2$—Deaerated hydrogen peroxide ($2.75 \times 10^{-3}$ M $\leq [H_2O_2] \leq 2.75 \times 10^{-1}$ M) was reacted with $1 \times 10^{-4}$ M Fe(II)-EDTA at pH 7.3 in the
stopped-flow apparatus, and the absorbance change at 350 nm was monitored. The overall reaction results in formation of Fe(III)-EDTA. However, the observed kinetics are biphasic, indicating that an intermediate formed by Reaction 1 reacts with a second molecule of hydrogen peroxide. A trace of absorbance versus time is shown in Fig. 1. The kinetics were resolved using a sum of two exponential rate expressions derived for sequential first-order processes (Equation 3).

\[
\frac{d \text{Absorbance}}{dt} = Ae^{-k_1t} + Be^{-k_2t}
\]  

The observed rate constants, \(k_1\) and \(k_2\), are linearly dependent on \([H_2O_2]\) and, from the plots of Fig. 2, \(k_1/[H_2O_2] = (1.75 \pm 0.10) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}\) and \(k_2/[H_2O_2] = (3.2 \pm 0.2) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}\). The ratio \(A/B = 3\) is independent of \([H_2O_2]\). These kinetics are consistent with the formation of Fe(III)-EDTA by the following processes (Reactions 4 and 5),

\[
\text{Fe(II)-EDTA} + H_2O_2 \rightarrow \text{Fe(II)-EDTA} \rightarrow \text{Fe(III)-EDTA}
\]

\[
\text{I}_1 + H_2O_2 \rightarrow \text{I}_2 \rightarrow \text{Fe(III)-EDTA}
\]

where \(k_1 = 1.75 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}\) and \(k_2 = 3.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}\). Evidence that two oxidizing intermediates are formed, \(I_1\) and \(I_2\), with distinct properties will be presented below.

The Oxidation of Ferrocytochrome c Induced by Fe(II)-EDTA/H\(_2\)O\(_2\)—In the presence of ferrocytochrome c, the reaction of Fe(II)-EDTA with hydrogen peroxide causes a decrease in absorption at 550 nm associated with the oxidation to ferriytochrome c. Fe(II)-EDTA solutions containing 12 \(\mu\)M ferrocytochrome c and hydrogen peroxide were reacted as before, and the absorbance changes at 550 were monitored. A reaction trace is shown in Fig. 1 superimposed on the trace obtained at 350 nm in the absence of cytochrome c. The oxidation occurs in two sequential first order steps, followed by a much slower steady-state process after all the Fe(II)-EDTA has been consumed. The rate constants of the faster processes correspond to the values of \(k_1\) and \(k_2\) for the Fe(II)-EDTA/H\(_2\)O\(_2\) reaction, as shown in Fig. 2. We conclude that intermediates in this reaction are being scavenged by ferrocytochrome c (Reactions 6 and 7).

\[
\text{Fe(II)-EDTA} + I_1 \rightarrow \text{ferriytochrome c} + I_1 \text{ (red)}
\]

\[
\text{Fe(II)-EDTA} + I_2 \rightarrow \text{ferriytochrome c} + I_2 \text{ (red)}
\]

Under the experimental conditions only about 20% of total ferrocytochrome c reacts via Reactions 6 and 7. This might be explained by competition for \(I_1\) and \(I_2\) from Reaction 8,

\[
\text{Fe(II)-EDTA} + I_1 \rightarrow \text{Fe(III)-EDTA} + I_1 \text{ (red)}
\]

and a similar reaction for \(I_2\). A distinction between the two oxidizing intermediates can be made on the basis of their reactivities toward t-butyl alcohol and ethanol which are commonly used as hydroxyl radical scavengers. A comparison of the effect of these alcohols on the oxidation of ferrocytochrome c was made in the presence of 0.1 M alcohol. As shown by the traces in Fig. 3, t-butyl alcohol completely inhibits the process ascribed to Reaction 7 but has no effect on Reaction 6, whereas ethanol appears to scavenge both \(I_1\) and \(I_2\).

Effects of Scavengers on the Stoichiometry of the Reaction of Fe(II)-EDTA with H\(_2\)O\(_2\)—In order to characterize the reactivity of the intermediate formed in the rate-determining step of Reaction 1, i.e. \(I_1\), we decided to investigate the scavenging efficiencies of some organic compounds. In these experiments an excess of Fe(II)-EDTA (5.0 \(\times\) \(10^{-4}\) or 1.0 \(\times\) \(10^{-3}\) M) was
Hydrogen Peroxide and Ferrous EDTA

Efficiencies and stoichiometries of various hydroxyl radical scavengers and other organic molecules

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>[S]</th>
<th>pH</th>
<th>Δ[Fe(III)-EDTA]/Δ[H₂O₂] limiting (observed)</th>
<th>kₒ(I₁ = 'OH)*</th>
<th>α('OH)*</th>
<th>α(experimentally)</th>
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<tbody>
<tr>
<td>Benzoate*</td>
<td>0.01</td>
<td>6</td>
<td>2 (1.9)</td>
<td>5.5 x 10⁵</td>
<td>0.045</td>
<td>≥9</td>
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<tr>
<td>Mannitol</td>
<td>0.01</td>
<td>6</td>
<td>1 (0.9)</td>
<td>1.8 x 10⁵</td>
<td>0.14</td>
<td>≤0.1</td>
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<tr>
<td>Formate</td>
<td>0.01</td>
<td>6</td>
<td>1 (0.92)</td>
<td>2.8 x 10⁵</td>
<td>0.09</td>
<td>≤0.1</td>
</tr>
<tr>
<td>Acetate*</td>
<td>0.05</td>
<td>6</td>
<td>2 (1.94)</td>
<td>9.2 x 10⁵</td>
<td>0.54</td>
<td>&gt;15</td>
</tr>
<tr>
<td>Serine</td>
<td>0.004</td>
<td>6</td>
<td>1.6 (1.6)</td>
<td>3.2 x 10⁵</td>
<td>0.48</td>
<td>1.5</td>
</tr>
<tr>
<td>Serine</td>
<td>0.01</td>
<td>6</td>
<td>1 (1)</td>
<td>3.2 x 10⁵</td>
<td>0.48</td>
<td>≥1</td>
</tr>
<tr>
<td>Serine</td>
<td>0.025</td>
<td>6</td>
<td>1 (1)</td>
<td>3.2 x 10⁵</td>
<td>0.48</td>
<td>≥1</td>
</tr>
<tr>
<td>Glycine</td>
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<td>6</td>
<td>2 (≥1.6)</td>
<td>1.0 x 10⁵</td>
<td>2.5</td>
<td>≥1.5</td>
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<tr>
<td>Tyrosine</td>
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<td>1 (1.0)</td>
<td>1.0 x 10⁵</td>
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<td>t-Butyl alcohol*</td>
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<td>3, 6</td>
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<td>5.2 x 10⁵</td>
<td>0.325</td>
<td>≥20</td>
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<td>Ethanol</td>
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<td>1 (0.8)</td>
<td>1.8 x 10⁵</td>
<td>0.025</td>
<td>≤0.1</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>0.1</td>
<td>3</td>
<td>1 (0.8)</td>
<td>1.9 x 10⁵</td>
<td>0.025</td>
<td>≤0.1</td>
</tr>
<tr>
<td>Imidazole</td>
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<td>6</td>
<td>1 (0.95)</td>
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<td>0.078</td>
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<td>6</td>
<td>2 (1.8)</td>
<td>3.5 x 10⁵</td>
<td>0.006</td>
<td>≥15</td>
</tr>
<tr>
<td>Arginine*</td>
<td>0.025</td>
<td>6</td>
<td>2 (1.8)</td>
<td>3.5 x 10⁵</td>
<td>0.006</td>
<td>≥15</td>
</tr>
</tbody>
</table>

* Rate constants obtained from Ref. 14. Most recent values are used and pulse-radiolysis values are preferred to other methods.
* α('OH) = 5.0 x 10⁶ M⁻¹ s⁻¹ [Fe(II)-EDTA]/[H₂O₂](I₁ = 'OH)[S].
* Scavengers with significantly lower reactivity toward I₁ than the hydroxyl radical.
* From Ref. 16.

The intermediate produced in the rate-determining step of Reaction 1 is not the hydroxyl radical and is capable of oxidizing ferrocytochrome c. Although it appears to be a strong oxidant, it reacts slowly, if at all, with molecules that do not contain readily oxidizable functional groups and does not undergo the characteristic reactions of the hydroxyl radical, addition to unsaturated bonds and abstraction of hydrogen atoms from aliphatic carbon atoms (16). The inertness of t-butyl alcohol is significant since metal ion oxidants (e.g. Cu²⁺ in Fehling's test) show a similar selectivity among the hydroxyl functions. The inertness of I₁ toward the acetate ion also seems to signify the absence of a readily oxidizable group, since the hydroxyl radical reacts with acetate via H-atom abstraction to form a carbon-centered free radical.

CONCLUSIONS

The reaction of I₁, which may be the ferryl-EDTA complex, with hydrogen peroxide leads to the formation of a transient I₃ capable of reacting with ferrocytochrome c, but which is scavenged by t-butyl alcohol. This latter observation is consistent with the inhibition by t-butyl alcohol of Fe(III)/peroxide systems in which a large excess of hydrogen peroxide is commonly used. Although the mechanism of its formation from ferryl-EDTA and hydrogen peroxide is unclear, it is likely that this intermediate is the hydroxyl radical.

REFERENCES