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Oxidizing Intermediates in the Reaction of Ferrous EDTA with Hydrogen Peroxide

REACTIONS WITH ORGANIC MOLECULES AND FERROCYTOCHROME *C**

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The reaction between hydrogen peroxide and ferrous EDTA generates an oxidizing intermediate (I_1) which is not the hydroxyl radical. It oxidizes ferrocytochrome *c* and also reacts with hydrogen peroxide ($k_5 = 3.2 \times 10^3$ M^{-1} s^{-1}) to form a second oxidizing transient (I_2). I_1 is not scavenged by *t*-butyl alcohol whereas I_2 is. I_1 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 I_1 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(FeO^{2+})-EDTA complex.

The nature of the oxidizing intermediate produced by Reaction 1, whether a hydroxyl radical or a ferryl-(FeO^{2+}) complex, has not been resolved because of its high reactivity.

$$\text{Fe(II)-EDTA} + H_2O_2 \rightarrow P \quad (1)$$

$$P + \text{Fe(II)-EDTA} \rightarrow 2\ \text{Fe(III)-EDTA} + 2\ OH^- \quad (2)$$

$$P = \text{FeO(II)-EDTA} + H_2O, \text{ or } P = \text{Fe(III)-EDTA} + {}^{\cdot}OH + OH^-$$

The resolution of this question is biologically important since the reaction between hydrogen peroxide and non-haem ferrous 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 ${}^{\cdot}OH/H_2O$ at pH 7 (2). Recently, it has been shown that ferryl complexes liganded by hydroxide and EDTA have appreciable lifetimes in aqueous solution (3).[1]

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 γ-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 multiexponential 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 (13). Stoichiometry data for Reaction 1 were taken at 300 nm ($\epsilon_{255\text{ nm}} = 8500$ 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 $NaClO_4$ (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_2SnO_3 as stabilizer and standardized by potassium permanganate titration. Ferrous EDTA solutions were prepared from $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ with a 1.5–1 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_2O_2—Deaerated hydrogen peroxide (2.75×10^{-4} M $\leq [H_2O_2] \leq 2.75 \times 10^{-3}$ M) was reacted with 1×10^{-4} M Fe(II)-EDTA at pH 7.3 in the

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[1] J. D. Rush and B. H. J. Bielski, submitted to *Inorganic Chemistry*.

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).

$$\frac{d\ \text{Absorbance}}{dt} = Ae^{-k_a t} + Be^{-k_b t} \qquad (3)$$

The observed rate constants, k_a and k_b, are linearly dependent on $[H_2O_2]$ and, from the plots of Fig. 2, $k_a/[H_2O_2] = (1.75 \pm 0.10) \times 10^4\ M^{-1}\ s^{-1}$ and $k_b/[H_2O_2] = (3.2 \pm 0.2) \times 10^3\ M^{-1}\ s^{-1}$. The ratio $A/B \simeq 3$ 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),

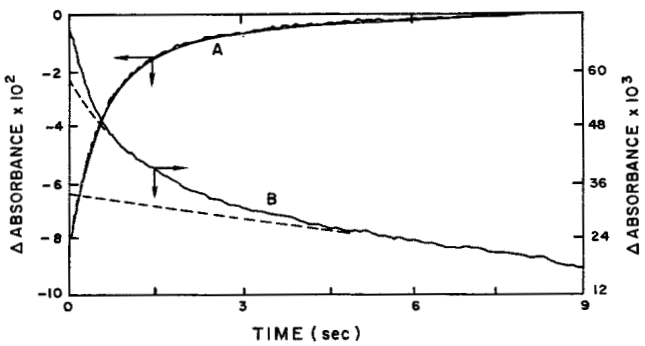

FIG. 1. *A*, kinetic trace of the formation of Fe(III)-EDTA ($[H_2O_2]_o = 2.75 \times 10^{-4}$ M; $[Fe(II)\text{-}EDTA]_o = 1 \times 10^{-4}$, pH 7.3) showing the biphasic rate of absorbance change. Superimposed on the data is the fit obtained with Equation 3 where $k_a(obs) = 4.29\ s^{-1}$ and $k_b(obs) = 0.74\ s^{-1}$. *B*, kinetic trace at 550 nm of the oxidation of ferrocytochrome *c* (6 μM) under the same conditions as for trace *A*. The *dashed lines* show the relative contributions of Reactions 6 and 7 to the total oxidation.

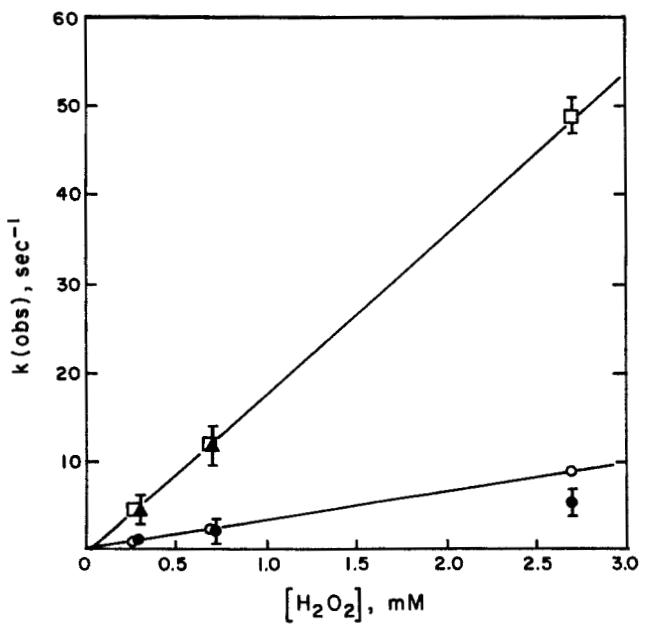

FIG. 2. **Plots of observed rate constants for Fe(III)-EDTA formation at 350 nm obtained from Equation 3 and for ferrocytochrome *c* at 550 nm as a function of $[H_2O_2]$.** □, k_a(obs), 350 nm; ○, k_b(obs), 350 nm; ▲, *k*(obs), fast phase of ferrocytochrome *c* oxidation; ●, *k*(obs), slow phase of ferrocytochrome *c* oxidation.

$$Fe(II)\text{-}EDTA + H_2O_2 \xrightarrow{k_4} I_1 \xrightarrow{Fe(II)\text{-}EDTA} Fe(III)\text{-}EDTA \qquad (4)$$

$$I_1 + H_2O_2 \xrightarrow{k_5} I_2 \xrightarrow{Fe(II)\text{-}EDTA} Fe(III)\text{-}EDTA \qquad (5)$$

where $k_4 = 1.75 \times 10^4\ M^{-1}\ s^{-1}$ and $k_5 = 3.2 \times 10^3\ M^{-1}\ s^{-1}$. 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_2O_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 ferricytochrome *c*. Fe(II)-EDTA solutions containing 12 μM 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_a and k_b for the Fe(II)-EDTA/H_2O_2 reaction, as shown in Fig. 2. We conclude that intermediates in this reaction are being scavenged by ferrocytochrome *c* (Reactions 6 and 7).

$$\text{Ferrocytochrome } c + I_1 \rightarrow \text{ferricytochrome } c + I_1\ (\text{red}) \qquad (6)$$

$$\text{Ferrocytochrome } c + I_2 \rightarrow \text{ferricytochrome } c + I_2\ (\text{red}) \qquad (7)$$

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,

$$Fe(II)\text{-}EDTA + I_1 \rightarrow Fe(III)\text{-}EDTA + I_1\ (\text{red}) \qquad (8)$$

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_2O_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 (5.0×10^{-4} or 1.0×10^{-3} M) was

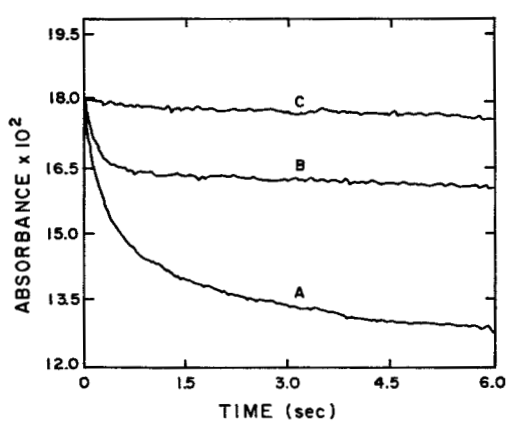

FIG. 3. **Kinetic traces of the oxidation of ferrocytochrome *c* at 550 nm.** *A*, $[Fe(II)\text{-}EDTA]_o = 1.0 \times 10^{-4}$ M, $[H_2O_2]_o = 6.75 \times 10^{-4}$ M, no added alcohol; *B*, 0.1 M *t*-butyl alcohol added; and *C*, 0.1 M ethanol added.

TABLE I

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

Scavenger	[S]	pH	Δ[Fe(III)]/Δ[H_2O_2] limiting (observed)	$k_9(I_1 = {}^{\cdot}OH)$[a]	$\alpha({}^{\cdot}OH)$[b]	α(experimentally)
	M			$M^{-1}\ s^{-1}$		
Benzoate[c]	0.01	6	2 (1.9)	5.5×10^9	0.045	≥9
Mannitol	0.01	6	1 (0.9)	1.8×10^9 [d]	0.14	≤0.1
Formate	0.01	6	1 (0.92)	2.8×10^9	0.09	≤0.1
Acetate[c]	0.05	6	2 (1.94)	9.2×10^7	0.54	>15
Serine[c]	0.004	6	1.6 (1.6)	3.2×10^8	0.48	1.5
Serine	0.01	6	1 (1)	3.2×10^8	0.48	≥1
Serine	0.025	6	1 (1)	3.2×10^8	0.48	≥1
Glycine	0.1	6	2 (≥1.6)	1.0×10^7	2.5	≥1.5
Tyrosine	0.002	6	1 (1.0)	1.0×10^9	0.125	≤0.1
t-Butyl alcohol[c]	0.1	3, 6	2 (2.0)	5.2×10^8	0.325	≳20
Ethanol	0.1	3, 6	1 (0.8)	1.8×10^9	0.025	≲0.1
2-Propanol	0.1	3	1 (0.8)	1.9×10^9	0.025	≲0.1
Imidazole	0.004	6	1 (0.95)	8.0×10^9	0.078	≤0.1
Arginine[c]	0.01	6	2 (1.8)	3.5×10^9	0.006	≥15
Arginine[c]	0.025	6	2 (1.8)	3.5×10^9	0.006	≥15

[a] Rate constants obtained from Ref. 14. Most recent values are used and pulse-radiolysis values are preferred to other methods.

[b] $\alpha({}^{\cdot}OH) = 5.0 \times 10^9\ M^{-1}\ s^{-1}$ [Fe(II)-EDTA]/$k_9(I_1 = {}^{\cdot}OH)[S]$.

[c] Scavengers with significantly lower reactivity toward I_1 than the hydroxyl radical.

[d] From Ref. 16.

reacted in the stopped-flow apparatus with 3.8×10^{-5} M H_2O_2 in the presence and absence of a scavenger, *S*. Under these conditions Reaction 5 which yields I_2 is expected to be negligible. The exponential increase in absorbance at 300 nm yields a final absorbance proportional to the number of moles of Fe(III)-EDTA formed per mol of H_2O_2 reacted. The observed stoichiometry depends on the competition between Reaction 8 and 9, in which *S*(ox) is incapable of oxidizing Fe(II)-EDTA.

$$I_1 + S \rightarrow I_1\ (\text{red}) + S(\text{ox}) \qquad (9)$$

It can be shown that for a system of Equations 4, 8, and 9 where [*S*] and [Fe(III)-EDTA] are in excess that

$$\frac{\Delta[\text{Fe(III)-EDTA}]}{\Delta[H_2O_2]} = 1 + \frac{\alpha}{1+\alpha} \qquad (10)$$

in which $\alpha = k_8[\text{Fe(II)-EDTA}]/k_9[S]$, irrespective of whether $I_1 = {}^{\cdot}OH$ or FeO(II)-EDTA. For the scavengers used in this study $k_9(I_1 = {}^{\cdot}OH)$ is known (14) and $k_8(I_1 = {}^{\cdot}OH) = 5 \times 10^9\ M^{-1}\ s^{-1}$ (15). In Table I we have listed the stoichiometries (Δ[Fe(III)-EDTA]/Δ[H_2O_2]) measured for various concentrations of scavengers. Also listed are values of α computed for the hydroxyl radical and those obtained experimentally. The experimental stoichiometries are frequently less than the limiting 2:1 or 1:1 values due probably to a slight loss of hydrogen peroxide when it was added to the scavenger solution. Therefore, α(experimental) is frequently given as a limiting value in Table I.

Four molecules in Table I have values of α(experimental) greater by an order of magnitude than expected for the ·OH radical. Thus, I_1 is significantly less reactive toward benzoate, acetate, *t*-butyl alcohol, and arginine relative to Fe(II)-EDTA. This cannot be accounted for by assuming that the oxidizing intermediate is the hydroxyl radical. The remaining compounds, except serine, do fall within the range expected for the hydroxyl radical at the concentrations used. We did not attempt to resolve some of these (*e.g.* glycine) because at very high scavenger concentrations the introduction of impurities limits the accuracy of our present method.

Although the list of compounds is not extensive, preliminary conclusions about the intermediate can be made. The phenol derivative (tyrosine) reacts with I_1, but the benzoate ion does not. The tertiary alcohol is unreactive, but scavengers with primary and secondary hydroxyl functions, such as ethanol, isopropyl alcohol, mannitol, and serine are effective scavengers. Arginine, which contains an imine bond is unreactive toward I_1, but imidazole is reactive, probably because it contains a basic amine group. I_1 does not readily 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.* Cu^{2+} in Fehling's test) show a similar selectivity among the hydroxyl functions. The inertness of I_1 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 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. The varying efficiencies of scavenger molecules in biological model systems of lipid peroxidation have been noted (17, 18).

The reaction of I_1, which may be the ferryl-EDTA complex, with hydrogen peroxide leads to the formation of a transient I_2 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.

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