HYDROXYL RADICAL FORMATION IN BIOLOGICAL SYSTEMS

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From thermodynamic and kinetic considerations it is concluded that the Fenton reaction occurs via an activated complex of Fe^{2+} and H_2O_2. The stoichiometric amount of OH species is spin-trapped by DMPO (5,5-dimethyl-1-pyrroline-N-oxide) when the Fe^{2+} concentration is below 1 μM. Two oxidizing species are detected in the Fenton reaction under normal experimental conditions: one is spin-trapped as DMPO-OH but the other is not. I also discuss one-electron reduction of H_2O_2 by semiquinones and a role of hemoglobin as a Fenton reagent.

Keywords: stoichiometry of the Fenton reaction; spin-trapped hydroxyl radical; one-electron reduction of H_2O_2; oxygen reduction.

INTRODUCTON

The Fenton reaction had been studied mostly by inorganic and physical chemists[1], and before 1970 no one could imagine that the reaction occurs in our bodies under physiological conditions. In 1894 Fenton found that ferrous ion strongly promotes the oxidation of organic compounds by hydrogen peroxide[2] and the combination of ferrous salts and hydrogen peroxide was called Fenton's reagent. Forty years later, Haber and Weiss proposed that the hydroxyl radical (OH) is the actual oxidant in the Fenton reaction[3].

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^- + OH \qquad (1)$$

This reaction which is involved in the iron-catalyzed decomposition of H_2O_2 attracted considerable attention of chemists. Although free radicals including oxygen species were found to be formed during enzymatic reactions[4] and the biological effect of oxygen free radicals was an important subject in the field of radiology[5], it was the finding of superoxide dismutase[6] that stimulated the study of oxygen free radicals in the field of biological and medical science. The hydroxyl radical is now believed to be crucial in oxygen toxicity in biology.

Reaction 1 denotes a simple one-electron reduction of H_2O_2 to form the hydroxyl radical. Therefore, it seems that strong one-electron reductants such as superoxide anion ($O_2^{\cdot-}$) and semiquinone anion ($Q^{\cdot-}$) which are easily formed in biological oxidation-reduction[4] may also reduce H_2O_2 to yield the hydroxyl radical.

$$H_2O_2 + O_2^{\cdot-} \longrightarrow HO^- + OH + O_2 \qquad (2)$$

$$H_2O_2 + Q^{\cdot-} \longrightarrow HO^- + OH + Q \qquad (3)$$

Reaction 2 is called the Haber-Weiss reaction. However, reaction 2 is now thought to be too slow to have a role in the OH formation[7-9]. Although many biochemists reported that semiquinones reduce H_2O_2, results reported on reaction 3 are still controversial and it may be concluded from recent observation[10-12] that reaction 3 is very slow and is accelerated by the iron ion. Since $O_2^{\cdot-}$ and $Q^{\cdot-}$ have much lower reduction potentials than Fe^{2+}, the inability of these reductants to reduce H_2O_2 remains to be solved. For this apparently simple one-electron reduction of H_2O_2, various inconsistent results have been reported. In this paper I will discuss the Fenton reaction from thermodynamic and stoichiometric points of view.

THERMODYNAMIC CONSIDERATION

The reduction of molecular oxygen to water is an important chemical reaction occurring in nature. The reaction, consisting of 4 single-electron steps, has never been elucidated completely.

$$O_2 \xrightarrow{e^-} O_2^{\cdot-} \xrightarrow[2H^+]{e^-} H_2O_2 \xrightarrow[H^+]{e^-} OH + H_2O \xrightarrow[H^+]{e^-} 2\,H_2O$$

The reduction potentials for the O_2/H_2O, O_2/H_2O_2 and H_2O_2/H_2O couples have been reported in text books[13] or review articles[14]. These are listed in Table I. It is, in general, difficult to measure the reduction potential for one-electron steps in overall two-electron reduction. It was recently reported by many scientists that the reduction potential for the $O_2/O_2^{\cdot-}$ couple is -0.33 V[15-18]. With a slight modification of Michaelis theory[19], reduction potentials for the first (E_1) and the second (E_2) one-electron couples in overall two-electron reduction are:

$$E_1 = E_m + RT/2F \ln K_s \qquad (4)$$

$$E_2 = E_m - RT/2F \ln K_s \qquad (5)$$

where, E_m is the potential for overall two-electron reduction and K_s is a semiquinone formation constant. For the $O_2/O_2^{\cdot-}/H_2O_2$ system,

$$K_s = [O_2^{\cdot-}]^2[H^+]^2 / [O_2][H_2O_2] \qquad (6)$$

Table I. Potentials (V) of O_2 reduction at pH 7

		Ref. 13	Ref. 14
Four-electron reduction	O_2/H_2O	0.815	0.82
Two-electron reduction	O_2/H_2O_2	0.268	0.3
	H_2O_2/H_2O	1.356	1.35
One-electron reduction	$O_2/O_2^{\cdot-}$		-0.33
	$O_2^{\cdot-}/H_2O_2$		0.94
	H_2O_2/OH		0.38
	OH/H_2O	2.2	2.33

K_s can be measured for quinone/semiquinone/quinol systems by analyzing potentiometric titration curves[20] and more directly by ESR methods[21]. Although it is difficult to measure the K_s value in the O_2 reduction system, the reduction potential for the O_2/O_2^- couple can be measured kinetically by combining with one-electron redox systems with known reduction potentials such as semiquinones[15-18] or cytochromes[17]. Once the E_1 value is measured, the E_2 value is calculated according to Equation 7,

$$E_1 + E_2 = 2\,E_m \qquad (7)$$

This kinetic method which was successfully applied to the O_2/O_2^- couple, however, has never been successful in the measurement of the reduction potential for the H_2O_2/OH couple. About 2.3 V has been given as reduction potential for the OH/H_2O couple from theoretical calculation[13,14]. The reduction potential for the H_2O_2/OH couple is then calculated to be about 0.38 V according to equation 7 (Table I). From recent calculation, a value of 2.59 V was proposed for the OH/H_2O couple[22].

I will now point out two contradictory facts to be solved in the one-electron reduction of H_2O_2. Table II shows that O_2 is easily reduced by several semiquinones[23-27]. Our recent study has shown the following stoichiometry in the one-electron reduction of O_2 by the paraquat free radical (PQ^+)

$$PQ^+ + O_2 \longrightarrow PQ^{2+} + O_2^- \qquad (8)$$

but no indication of the one-electron reduction of H_2O_2 by this radical in the absence of iron[28]. Since the ratio of one-electron transfer rates of forward and backward reactions (equilibrium constant) is directly related to the difference in the one-electron reduction potentials for two redox couples involved in the reaction[15-17,23,29], it might be concluded that the one-electron reduction potential of H_2O_2 is lower than that of O_2, namely, -0.17 V which is the one-electron reduction potential of O_2 on the molar basis[15]. Then, the reduction potential for the OH/H_2O couple would be at least 0.55 (0.38 + 0.17) V higher than a reported value of 2.3 V.

Table II. Rate constant for O_2 reduction by semiquinone

	$M^{-1}s^{-1}$	Ref.
Benzoquinone	4.5×10^4	17
Duroquinone	$(2 \pm 0.5) \times 10^8$	25
Anthraquinone-2,6-disulphonate	5×10^8	25
Mitomycin	$(2.2 \pm 0.2) \times 10^8$	27
Adriamycin	$(3.0 \pm 0.2) \times 10^8$	27
Menadione	5×10^6	24
Paraquat	7.7×10^8	26

Reaction 1 clearly implies that ferrous ion reduces H_2O_2, overcoming an unfavorable potential gap between the Fe^{3+}/Fe^{2+} and the H_2O_2/OH couples. It should be noted that other reductants having lower reduction potential than Fe^{2+} can hardly reduce H_2O_2 (Table III). Therefore, I conclude that, contrary to other reductants, Fe^{2+} ion reduces H_2O_2 through the formation of an activated complex. There is no thermodynamic contradiction in this mechanism because the potential for the overall two-electron reduction of H_2O_2 to H_2O is much higher than that for the Fe^{3+}/Fe^{2+} couple (see Tables I and III). In Fig. 1, a thermodynamic sketch for processes of the O_2 reduction in the presence and absence of a catalyst is shown. Without the activation mechanism, the first reduction step in each two-electron reduction process (from O_2 to H_2O_2 or from H_2O_2 to H_2O) has lower reduction potentials, usually being rate-limiting. These barriers are eliminated in the presence of a catalyst (here, horseradish peroxidase) by averaging out the four potentials in the reduction of O_2 to H_2O[23,30].

STOICHIOMETRY OF THE FENTON REACTION

Controversial results have been reported in the Fenton reaction, mostly because of difficulty of direct detection of the

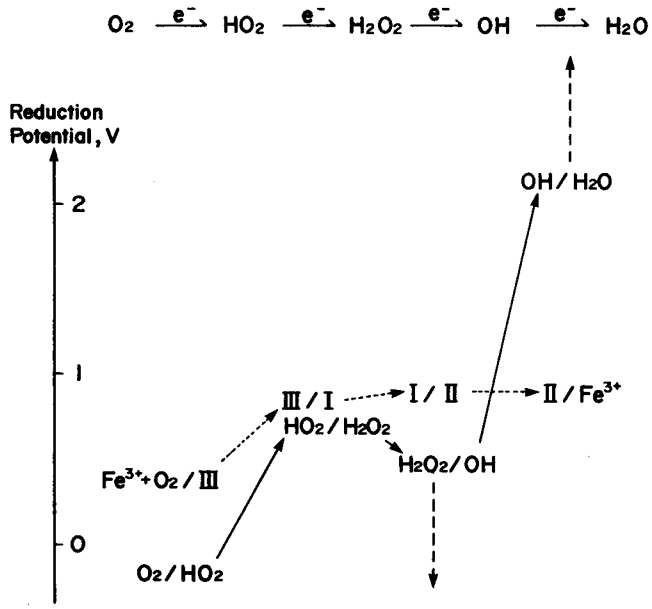


Figure 1. Approximate reduction potential of four single-electron steps from O_2 to H_2O in the free state (solid lines) and in the bound state to horseradish peroxidase (dotted lines). Here, 10^{-10} M is used for dissociation constant for ferroperoxidase-O_2 complex (compound III). Broken lines show that reduction potentials for the H_2O_2/OH and the OH/H_2O couples may shift downward and upward at least by 0.6 V, respectively.

Table III. Rate constants for H_2O_2 reduction

Reductant	Reduction Potential (V)	H_2O_2 reduction ($M^{-1}s^{-1}$)	Ref.
O_2^-	-0.17	3.0 ± 0.6	7
		2-8	8
Paraquat radical	-0.43	6.7	10
Anthrasemiquinone -2-sulphonate	-0.380	< 1	12
Ferrous ion	0.771	*ca* 10^4	(Table IV)

product, the OH radical[31]. At the moment, ESR spin-trapping techniques provide the most direct method to detect such free radical intermediates[32-36]. When 5,5-dimethyl-1-pyrroline N-oxide (DMPO) is used as a spin-trapping reagent,

$$DMPO + OH \longrightarrow DMPO\text{-}OH \quad (9)$$

The product, DMPO-OH is relatively stable and the quantitative analysis of the Fenton reaction becomes possible. Stoichiometry of the Fenton reaction has been determined by careful analysis[37]. In reaction 1, the molar ratio of Fe^{2+} added to DMPO-OH formed is nearly unity at Fe^{2+} concentrations below 1 μM and in the presence of 90 μM H_2O_2. The ratio decreases as the Fe^{2+} concentration is increased (Fig. 2). This decrease is not due to the reduction of OH by Fe^{2+} because of the presence of adequate amounts of DMPO. The second order rate constant of the reaction of DMPO-OH with Fe^{2+} has been measured to be the order of 10^3 $M^{-1}s^{-1}$, varying slightly with iron chelates present in the solution[37]. The loss of spin-adduct at higher concentrations of Fe^{2+}, can be partially recovered as DMPO-Et (spin adduct of ethanol free radical) when ethanol (EtOH) is added to the Fenton system. The formation of DMPO-Et via OH is formulated as,

$$EtOH + OH \longrightarrow \text{ethanol free radical} + H_2O \quad (10)$$

$$DMPO + \text{ethanol free radical} \longrightarrow DMPO\text{-}Et \quad (11)$$

When 4 μM Fe^{2+} is present, DMPO-Et formed is greater than the loss of DMPO-OH in the presence of ADP but not EDTA (Fig. 3). At $[Fe^{2+}]$ = 100 μM, the efficiency of DMPO-OH formation is much greater in the presence of DETAPAC than in the presence of EDTA, but the addition of ethanol slightly decreases the total spin adduct in the case of DETAPAC while it greatly increases the total spin adduct in the case of EDTA (Fig. 4). The slight loss in the total spin adduct is ascribable to loss in the spin conversion by reactions 10 and 11. The significant increase in the total spin adduct in the presence of ethanol (Fig. 3B and Fig. 4B) can be explained by assuming that ethanol is oxidized not only by OH but also by other species that does not form DMPO-OH.

COMPARISON BETWEEN REACTIONS OF H_2O_2 WITH HEMOPROTEINS AND IRON IONS

It is widely accepted that H_2O_2 is formed in our body and removed through the scavenging functions of catalase and peroxidases[38,39]. In the preceding section, I assumed that H_2O_2 is resistant to reduction as compared with O_2 in their free state. H_2O_2, however, undergoes a variety of reactions with hemoproteins (PrPoFe), where Pr and Po denote protein and porphyrin, respectively.
For peroxidases[40], myoglobin[41] and hemoglobin,

$$PrPoFe^{2+} + H_2O_2 \rightarrow PrPoFeO^{2+} \text{ (comp. II)} + H_2O \quad (12)$$

For catalase and peroxidases,

$$PrPoFe^{3+} + H_2O_2 \rightarrow PrPo^{+}FeO^{2+} \text{ (comp. I)}$$
$$\text{or } Pr^{+}PoFeO^{2+} + H_2O \quad (13)$$

where, Po^+ and Pr^+ denote cation radicals of porphyrin and amino acid residues, respectively.
For catalase and peroxidases[42],

$$PrPoFeO^{2+} + H_2O_2 \rightarrow PrPoFeO_2^{2+} \text{ (comp. III)} + H_2O \quad (14)$$

For catalase and chloroperoxidase,

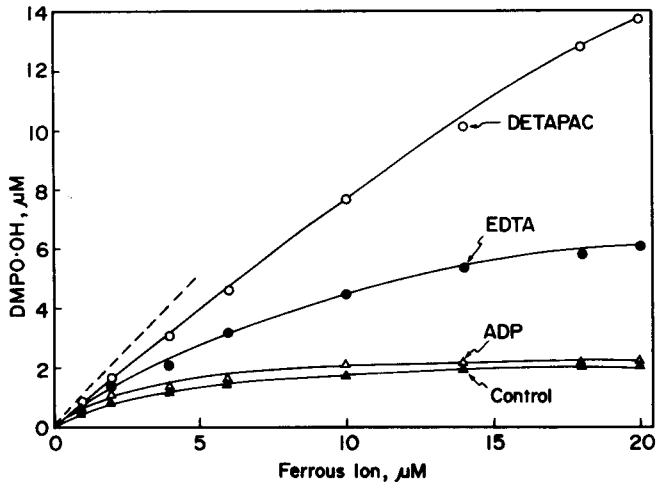


Figure 2. Stoichiometry of the Fenton reaction. Broken lines show the 1 ; 1 stoichiometry (37). 90 μM H_2O_2 and 40 mM DMPO.

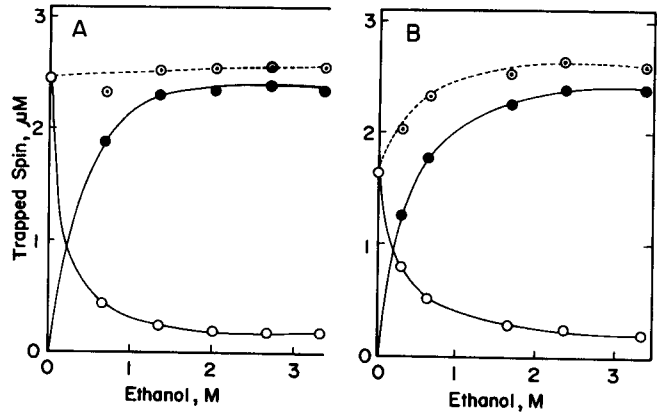


Figure 3. Effect of ethanol concentration on the DMPO-spin adducts. 4 μM Fe^{2+} and 90 μM H_2O_2. ○, DMPO-OH and ●, DMPO-Et. The dotted lines show the sum of the spin adducts. Iron chelator was EDTA in A and ADP in B. See ref. 37.

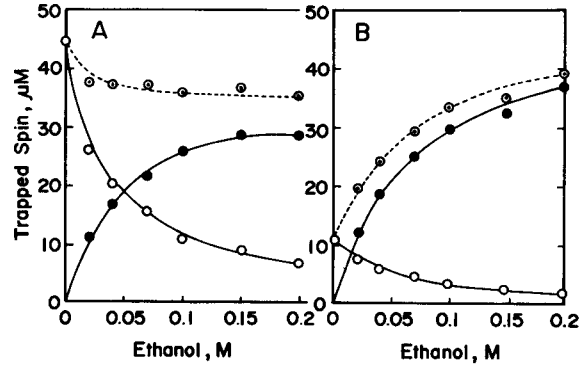


Figure 4. Effect of ethanol concentration on the DMPO-spin adducts. 100 μM Fe^{2+} and 200 μM H_2O_2. ○, DMPO-OH and ●, DMPO-Et. The dotted lines show the sum of the DMPO-spin adducts. Iron chelator was DETAPAC in A and EDTA in B.

$$PrPo^{+}FeO^{2+} + H_2O_2 \rightarrow PrPoFe^{3+} + H_2O + O_2 \quad (15)$$

H_2O_2 acts as an oxidant in reactions 12 and 13, while it acts as a reductant in reaction 15. Two types are mixed in reaction 14[42]. In all cases H_2O_2 appears to form activated complexes with hemoproteins. It is of special interest to note that the rate constant of reaction 12 is approximately the same as that of the reaction of ferrous ion with H_2O_2 (Table IV). In reaction 13, H_2O_2 is activated through interaction not only with the heme iron but also with distal bases and the rate constant is higher. Contrary to the reaction of H_2O_2 with Fe^{2+}, the reaction of H_2O_2 with Fe^{3+} is quite different between simple iron complexes and hemoproteins. Fe^{3+} is oxidized by H_2O_2 in hemoproteins, but is rather reduced at a very slow rate in its complex form[43],

$$Fe^{3+} + H_2O_2 \rightleftharpoons Fe^{2+} + O_2^{\cdot} + 2\,H^{+} \quad (16)$$

probably via a peroxo complex[44],

$$Fe^{3+} + H_2O_2 \rightleftharpoons Fe^{3+}O_2^{2-} + 2\,H^{+} \quad (17)$$

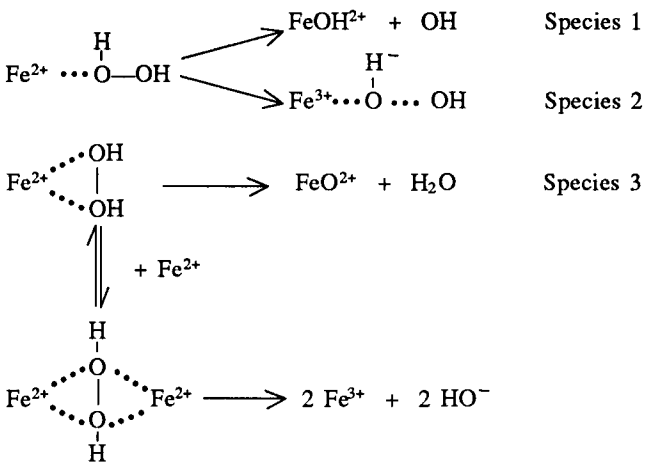
Table IV. Reactions of H_2O_2 with Fe^{2+} in various states

	10^4, $M^{-1}s^{-1}$	Ref.
Fe^{2+}-EDTA	1.4	37
Fe^{2+}-DETAPAC	0.041	37
Fe^{2+}-ADP	0.82	37
Fe^{2+}-phosphate	2.0	37
Myoglobin	0.36	41
Peroxidase	9.0	40

MECHANISM OF THE FENTON REACTION

As discussed previously it is reasonable to assume that the Fenton reaction takes place via an activated complex of Fe^{2+} ion and H_2O_2. The formation of this activated complex will drastically change the reduction potentials for both the H_2O_2/OH and the OH/H_2O couples (Fig. 1). Then, the reactivity of OH will not be the same as the reactivity in a free state. The OH radical exists as a restricted form, which might also be described as either bound, complexed, caged or crypto OH[45]. This species, however, still yield DMPO-OH upon reaction with DMPO. Figures 3 and 4 clearly show the formation of a non-OH oxidant. This species does not yield DMPO-OH but oxidizes ethanol to the free radical. Therefore, the mechanism of the Fenton reaction can be schematized as shown in Scheme 1. Here, OH is free in Species 1 and restricted in Species 2, but both react with DMPO to yield DMPO-OH. The non-OH oxidant is Species 3.

The question then is whether or not Species 1 is really formed in the Fenton reaction. It is possible to measure rate constants for reactions of various electron donors with free OH formed by photolysis[46]. By ESR spin-trapping techniques, we cannot directly measure rate constants for the reactions of electron donors with OH species formed in the Fenton reaction, but we can measure the ratio of the rate constants to that for the reaction of DMPO with the OH species[47]. On the basis of this measurement we conclude that OH species formed in the presence of phosphate alone, EDTA or DETAPAC is not Species 1, but cannot deny that Species 1 is formed in the Fe^{2+}-ADP system[47]. From thermodynamic considerations, however, it can be safely said that free OH is formed only when H_2O_2 is reduced by way of one-electron reduction without the activation mechanism. In this case, the reductant should have one-electron reduction potential at least below -0.3 V. Then,



Scheme 1

the reduction potential for the free OH/H_2O couple will be higher than 3.0 V.

Reaction 12, which commonly occurs in hemoproteins, may suggest that the ferryl ion is formed in the Fenton reaction. The ferryl form has been observed in a strong alkaline solution for free iron ion[48] or as its pyrophosphate complex at pH 10[49]. Instability of the ferryl ion at neutral pH implies that it acts as a strong oxidant if it occurs in the Fenton reaction under our experimental conditions. Scheme 1 shows that the formation of the ferryl ion (Species 3) and the process which yields no oxidant are favorable at high Fe^{2+} concentrations.

FENTON-TYPE REACTIONS IN BIOLOGICAL SYSTEMS

H_2O_2 is formed in our body under aerobic conditions[38,39] and iron is released from iron proteins under anaerobic conditions[50,51]. It is therefore very likely that the Fenton reaction might occur at the momment of reperfusion after ischemic[52-54]. H_2O_2 is obligatory in the Fenton reaction, but iron can be replaced by other transition metals, such as copper[55]. Fe^{3+} ion can be reduced by $O_2^{\cdot}$ and semiquinone, or directly by some reductases. Then, one may ask what kind of iron complexes may act as a Fenton reagent. Two factors should be considered. The most important factor would be replacement of a ligand of iron complex with H_2O_2, through which H_2O_2 is activated[56]. The second factor would be a suitable reduction potential for the Fe^{3+}/Fe^{2+} couple. Although Fe^{2+}-desferrioxiamine (DF) acts as a Fenton reagent, DF is known as an inhibitor for the Fenton reaction. The problem in this case is the reduction potential for the Fe^{3+}/Fe^{2+} couple is so low that its Fe^{3+} complex cannot be reduced back under physiological conditions. On the other hand, if the Fe^{2+} complex is very stable (high reduction potential), it cannot reduce H_2O_2. When these two factors are satisfied in such systems containing EDTA or ADP, the Fenton reaction proceeds to a significant degree even in the presence of a trace amount (ca. 0.1 uM) of iron[57].

I will now discuss the possibility that Hb acts as a Fenton reagent[58,59]. There is no doubt that nonspecific biological oxidation is accelerated in the presence of Hb in vivo and in the presence of Hb and H_2O_2 in vitro[60]. However, we have failed to detect OH formation in both systems[60]. Similar results have been observed when Hb is replaced by MetHb and hematin[60]. We conclude that oxidizing species formed in the presence of Hb, MetHb and hematin are neither Species 1 nor 2, but probably ferryl complexes (Species 3). It should be noted that free radicals of amino acid residues formed in the reaction of MetMb with H_2O_2 act as strong one-electron oxidants[61].

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