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The enzymatic oxidation of Desferal to a nitroxide free radical

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Desferrioxamine mesylate (Desferal), a transition metal ion chelator, has been used to inhibit the in vitro redox cycling of transition metal ions. ESR spectroscopy was utilized to detect and identify Desferal's one-electron oxidation product. We demonstrate that a horseradish peroxidase/H_2O_2 system, a xanthine oxidase/hypoxanthine system, and a hydroxyl radical-generating system are all capable of oxidizing Desferal to a nitroxide free radical. The same 9-line ESR spectrum ($g = 2.0065$, $a^N = 7.85$ G, $a^H(2) = 6.35$ G) was detected in all of the above systems. We, therefore, stress that care must be taken when using Desferal as a transition metal ion chelator to keep its concentration low enough to minimize these reactions, or to use a different metal ion chelator.

Desferrioxamine; Nitroxide free radical; ESR; Horseradish peroxidase; Xanthine oxidase; Hydroxyl free radical

1. INTRODUCTION

Desferal is an excellent chelating agent for Fe^{3+} with a stability constant of 10^{31} and is currently being used clinically to treat iron-overload patients [1]. Apart from iron, Desferal can bind several other transition metals, but with much lower stability constants [2–4]. Since Desferal is able to bind transition metals in such a way that it inhibits their catalytic activity [5,6], this chelator has been used extensively for in vitro experiments to remove metal ions from the reaction system.

It has been reported that Desferal interferes with peroxidase systems [7]. There have also been reports that the hydroxyl ($^{\cdot}OH$) [8,9] and superoxide anion ($O_2^{\cdot-}$) radicals [9–11] are capable of reacting with Desferal. In all of these cases, the Desferal product(s) formed by these reactions was not identified.

We describe here the identification, by ESR, of the one-electron oxidation product of Desferal formed on reaction with hydroxyl and superoxide radicals. We also demonstrate that the same free radical product is produced when Desferal is oxidized by horseradish peroxidase, thereby suggesting that Desferal should not be used as a transition metal chelator in the presence of peroxidases. It should be noted that as long as the concentration of Desferal is kept low ($< 10^{-3}$ M), reactions of the hydroxyl and superoxide radicals with Desferal will be minimal [8,10] and, as such, Desferal can be used as the chelator of choice to inhibit the Fenton reaction.

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2. MATERIALS AND METHODS

Desferal (desferrioxamine mesylate) was obtained from Ciba-Geigy (Pharmaceuticals Division, Suffern, NY). Horseradish peroxidase, xanthine oxidase, hypoxanthine, catalase (thymol-free), diethylenetriaminepentaacetic acid (DTPA), hydrogen peroxide (30%), and 5,5-dimethyl-1-pyroline *N*-oxide (DMPO) were obtained from Sigma. Erythrocyte superoxide dismutase was from

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Diagnostic Data. The DMPO was purified by vacuum distillation. All other chemicals were of the highest purity available and were used as obtained.

ESR observations were made at room temperature with a Varian E-109 or IBM/Bruker ER200-D spectrometer equipped with a TM_{110} cavity. Solutions were transferred to the quartz flat cell by means of a rapid sampling device (Gilford Instruments) [12]. Where appropriate, solutions were deoxygenated by bubbling with nitrogen for 30 min, or oxygenated by bubbling with oxygen for 30 min. The g factor was measured relative to Fremy's salt ($g = 2.00550 \pm 0.00005$) as described [13]. Computer simulation for the determination of the coupling constants, was performed on an HP 9000/236 microcomputer.

Photolytic generation of hydroxyl radicals from H_2O_2 was performed with a Krotos LPS255HR lamp at 280 nm [14].

3. RESULTS AND DISCUSSION

We have investigated the one-electron oxidation of Desferal by horseradish peroxidase, a hydroxyl radical-generating system and a superoxide anion radical-generating system. The oxidation of Desferal leads to a stable nitroxide free radical with a g factor of 2.0065 (fig.1). An acetyl nitroxide nitrogen coupling ($a^N = 7.85$ G) is split by two protons ($a^H = 6.35$ G) from the neighboring CH_2 group giving the 9-line spectra. The unusually small nitrogen coupling of this nitroxide is characteristic of a neighboring carbonyl group [15]. Although Hinojosa and Jacks [9] published this spectrum, their resolution was very poor, and they were unable to assign the spectra they obtained from the reaction of the hydroxyl or superoxide radicals with Desferal. Scheme 1 shows the structure of Desferal and a Desferal free radical. It should be noted that due to the structural similarities of the environment surrounding the nitroxide groups, it is not possible to determine which hydroxamic acid group of the Desferal molecule is under attack. Thus, the scheme shows one possible radical structure which could produce the spectrum shown in fig.1.

As mentioned above, the hydroxyl radical has previously been shown to react with Desferal with a diffusion-controlled rate ($k = 1.3 \times 10^{10}$ $M^{-1} \cdot s^{-1}$) [10], indicating that it is a powerful hydroxyl radical scavenger. However, these authors did not determine the product(s) of this reaction. It was pointed out that at the concentra-

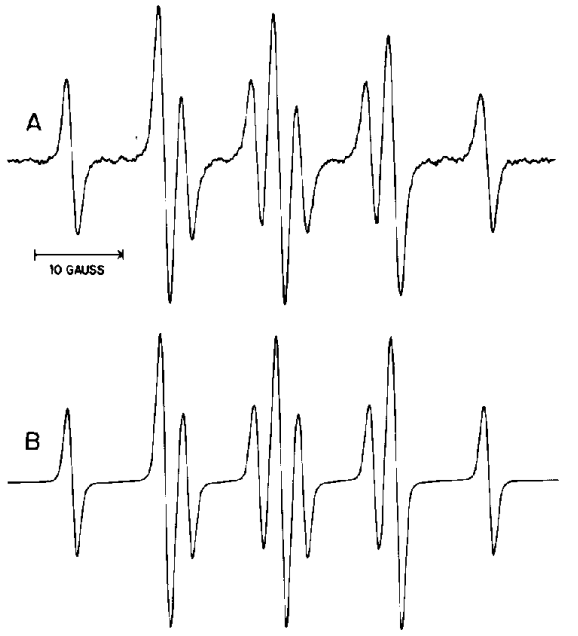
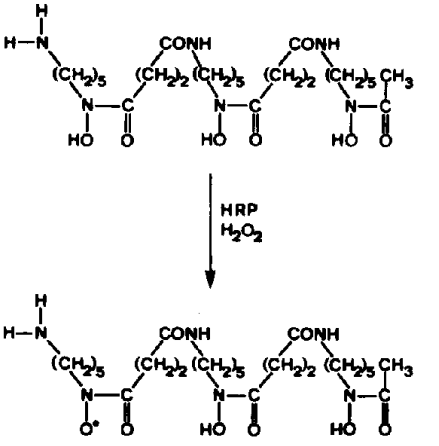


Fig.1. ESR spectrum of the nitroxide free radical generated by one-electron oxidation of Desferal. (A) The solution contained 1 mM Desferal, 0.2 mg/ml horseradish peroxidase and 100 μM hydrogen peroxide in 100 mM phosphate buffer (pH 7.4) containing 0.1 mM DTPA. Spectrometer settings: microwave power, 21 mW; modulation amplitude, 0.2 G; sweep rate, 0.1 G/s; time constant, 1 s. (B) Computer simulation of A, $a^N = 7.85$ G and $a^H(2) = 6.35$ G.



Scheme 1.

tion of Desferal usually used for in vitro experiments (10^{-4} M or less), this reaction would be minimal. Therefore, the use of Desferal as an iron chelator to inhibit Fenton chemistry is appropriate, and has been commonly used. If, however, the concentration of Desferal is increased to 1 mM or greater, Desferal can interfere as a hydroxyl radical scavenger.

A simple way of generating hydroxyl radicals is by photolysis of a dilute hydrogen peroxide solution [14]. If dilute hydrogen peroxide is photolyzed in the presence of DMPO, the DMPO/hydroxyl radical adduct is detected, demonstrating the generation of the hydroxyl radical. When the DMPO is left out of the solution and Desferal is included, the Desferal nitroxide free radical is detected (fig.2A). This signal is dependent on Desferal, H_2O_2 and light (fig.2B–D). From this we can conclude that the hydroxyl radical abstracts hydrogen from the hydroxamic acid portion of Desferal. Although other products may also be formed, these radicals are too unstable to be detected.

It has also been shown previously that the superoxide anion radical reacts with Desferal [9–11]. This reaction is very slow ($k = 9 \times 10^2\ M^{-1} \cdot s^{-1}$), and therefore, very little reaction is expected in vitro if the concentration of Desferal used is kept low ($\leqslant$0.1 mM). Once again, the product of the reaction was not determined.

Superoxide anion radicals can be conveniently trapped by DMPO in a xanthine oxidase/hypoxanthine system (fig.3A) [16]. If the system is bubbled with oxygen (fig.3B), the ESR signal becomes more intense and persistent. Due to the large concentration of oxygen present, the ESR signal exhibits line broadening associated with the interaction of the paramagnetic oxygen with the free radical [17]. The addition of catalase to the reaction results in a more persistent radical signal (fig.3C). This persistence is associated with the catalase-driven disproportionation of hydrogen peroxide to oxygen and water (reaction 1).

$$2H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2H_2O \qquad (1)$$

As expected, superoxide dismutase completely inhibited the signal (fig.3D).

We were therefore able to investigate the reaction of the superoxide anion radical with Desferal

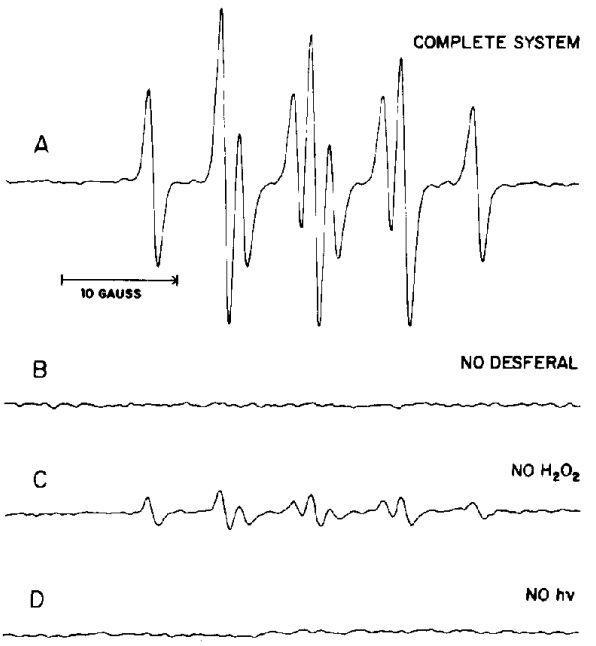


Fig.2. ESR spectrum of the nitroxide free radical obtained upon reaction of the hydroxyl radical with Desferal. (A) Photolysis of a 0.1 mM hydrogen peroxide solution containing 1 mM Desferal in 100 mM phosphate buffer (pH 7.4). (B) Same as A, but in the absence of Desferal. (C) Same as A, but without H_2O_2 added. (D) Same as A, but with the lamp off. Spectrometer settings: microwave power, 21 mW; modulation amplitude, 0.53 G; sweep rate, 0.1 G/s; time constant, 2 s.

(fig.4A). Desferal, hypoxanthine, xanthine oxidase, and oxygen are absolutely required in order to form this free radical species (not shown). Bubbling the sample with oxygen caused the signal to become more persistent (fig.4B) and again broadened the lines. The persistence of this signal is due to an increased generation of superoxide. Fridovich [18] has demonstrated that by increasing the oxygen tension at a fixed pH, the univalent reduction of oxygen to superoxide is favored over the two-electron reduction to hydrogen peroxide. The addition of catalase to the reaction produced a stimulation in the production of the Desferal nitroxide radical (fig.4C). This stimulation is a result of the recovery of oxygen from hydrogen peroxide by catalase, thereby maintaining the production of superoxide. Superoxide dismutase inhibited the ESR signal as expected (fig.4D). The more persistent signal in the presence of catalase is

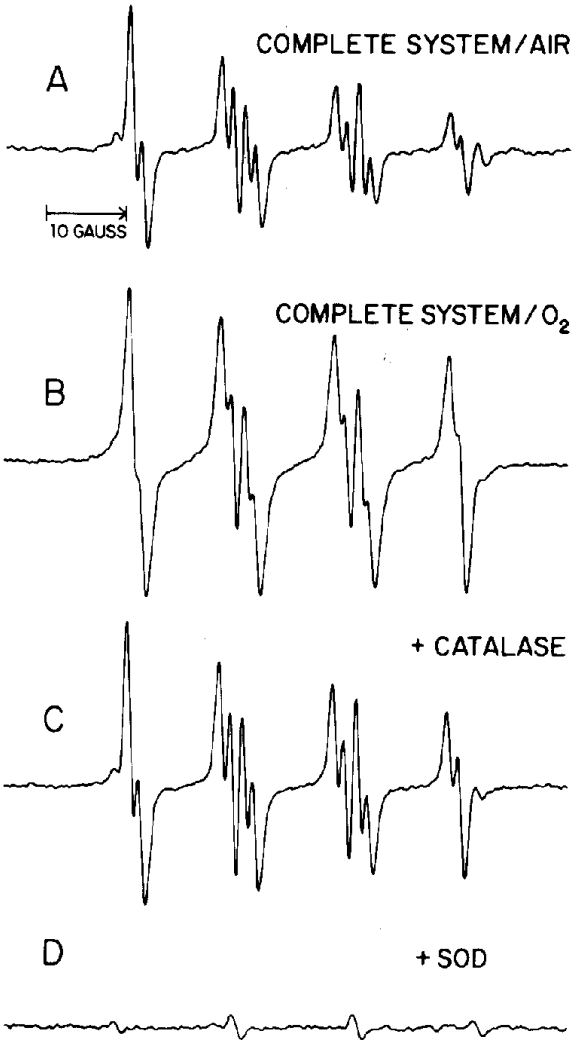


Fig.3. DMPO/superoxide anion radical adduct. (A) An air-saturated solution containing 100 mM DMPO, 0.002 U/ml xanthine oxidase, 0.2 mM hypoxanthine in 100 mM phosphate buffer containing 1 mM DTPA. (B) Same as A, except the solution was oxygen-saturated. (C) Same as A, with 50 μg/ml catalase added. (D) Same as A, with 50 μg/ml superoxide dismutase added. Spectrometer settings: microwave power, 20 mW; modulation amplitude, 0.25 G; sweep rate, 0.5 G/s; time constant, 2 s.

strongly inhibited by superoxide dismutase (fig.4E), showing that superoxide is still the primary oxidizing agent in this system.

The above results demonstrate that, and are consistent with, hydroxyl and superoxide anion radicals oxidizing Desferal to produce the same

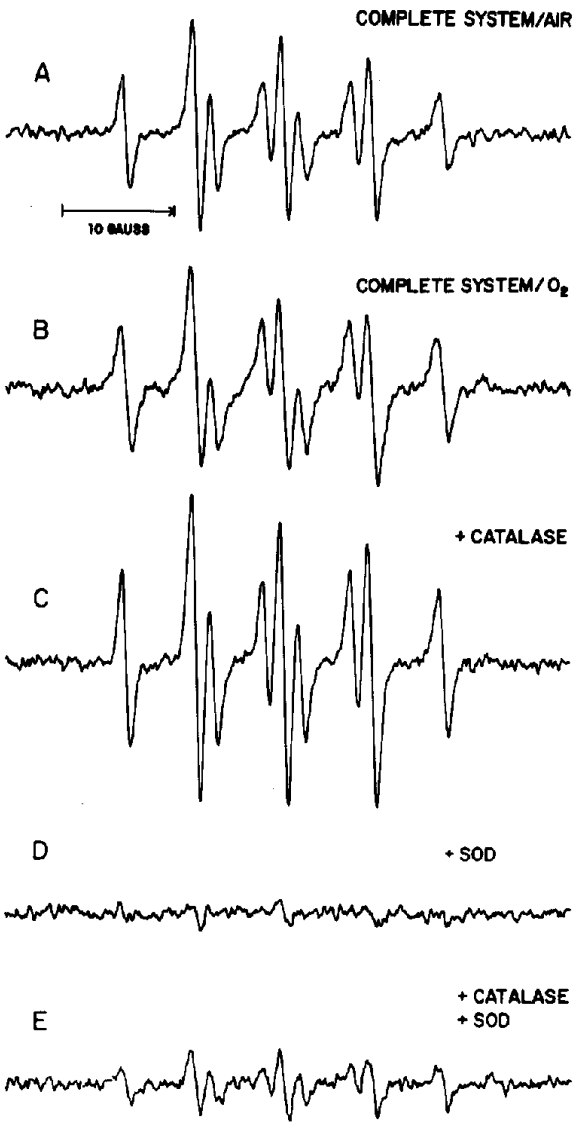


Fig.4. ESR spectra of the nitroxide free radical obtained by reaction of the superoxide anion radical with Desferal. (A) The reaction mixture contained 10 mM Desferal, 0.002 U/ml xanthine oxidase and 0.2 mM hypoxanthine in 100 mM phosphate buffer (pH 7.4), air-saturated. (B) Same as A, except the solution was oxygen-saturated. (C) Same as A, with 50 μg/ml catalase added. (D) Same as A, with 50 μg/ml superoxide dismutase added. (E) Same as A, with 50 μg/ml catalase and 50 μg/ml superoxide dismutase added. Spectrometer settings: microwave power, 21 mW; modulation amplitude, 1 G; sweep rate, 0.1 G/s; time constant, 2 s.

nitroxide free radical. Desferal is used to chelate transition metals, and thereby prevent their reaction with hydrogen peroxide in peroxidase systems [7]. In this vein it was proposed that Desferal can act as an electron donor for peroxidizing systems [7], but the chemical species thus formed was not identified. Fig.5 shows the ESR spectra of the Desferal nitroxide metabolite obtained by the enzymatic one-electron oxidation of Desferal by horseradish peroxidase. The ESR spectrum is dependent on Desferal, horseradish peroxidase and hydrogen peroxide (fig.5B–D) and catalase (50 μg/ml) could not replace horseradish peroxidase (not shown). These results are in agreement with Desferal acting as a substrate for the peroxidases [7]. As such, Desferal should not be utilized as an iron chelator in biological systems using peroxidases. After the completion of this work, Hinojasa et al. [19] identified the Desferal nitroxide as a product of UV irradiation.

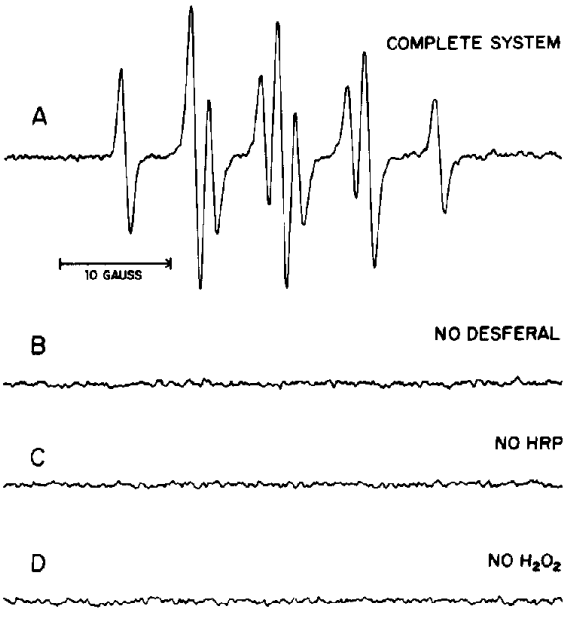


Fig.5. ESR spectra obtained by the one-electron oxidation of Desferal by horseradish peroxidase. (A) The reaction mixture contained 0.1 mM Desferal, 0.1 mg/ml horseradish peroxidase, and 50 μM H_2O_2 in 100 mM phosphate buffer (pH 7.4). (B) Same as A, with Desferal omitted. (C) Same as A, but without horseradish peroxidase. (D) Same as A, but without hydrogen peroxide. Spectrometer settings: microwave power, 21 mW; modulation amplitude, 0.5 G; sweep rate, 0.5 G/s; time constant, 2 s.

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