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DESFERRIOXAMINE AS AN ELECTRON DONOR. INHIBITION OF MEMBRANAL LIPID PEROXIDATION INITIATED BY H_2O_2-ACTIVATED METMYOGLOBIN AND OTHER PEROXIDIZING SYSTEMS

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Desferrioxamine (DFO) involvement in several peroxidative systems was studied. These sytems included: a) membranal lipid peroxidation initiated by H_2O_2-activated metmyoglobin (or methemoglobin); b) phenol-red oxidation by activated metmyoglobin or horseradish peroxidase (HRP): c) β-carotene-linoleate couple oxidation stimulated by lipoxygenase or hemin. Desferrioxamine was found to inhibit all these systems but not ferrioxamine (FO). Phenol-red oxidation by H_2O_2-horseradish peroxidase was inhibited competitively with DFO. Kinetic studies using the spectra changes in the Soret region of metmyoglobin suggest a mechanism by which H_2O_2 reacts with the iron-heme to form an intermediate of oxy-ferryl myoglobin that subsequently reacts with DFO to return the activated compound to the resting state. These activities of DFO resemble the reaction of other electron donors.

KEY WORDS: Desferrioxamine, membranal lipid peroxidation, phenol-red oxidation, β-carotene oxidation, H_2O_2-activated metmyoglobin, hemin, lipoxygenase.

ABBREVIATIONS: Desferrioxamine (DFO); ferrioxamine (FO); metmyoglobin (MetMb); horseradish peroxidase (HRP).

INTRODUCTION

Desferrioxamine (DFO) is an excellent chelating agent for ferric ion, Fe^{3+}, with a stability constant of 10^{31}. The molecule is composed of acetic, succinic and 1-amino-5-hydroxylaminopentane groups. Early studies examining DFO as a pharmacological compound found that the molecule mobilizes ferritin iron but reacts only very slowly with transferrin iron and does not remove iron from porphyrin systems.[1] As a sideramine, ferrioxamine, the molecule containing iron, seems to exert an important function in the iron metabolism of microorganisms, possibly acting as iron donors in the incorporation of iron in the porphyrin system.[1]

In clinical medicine DFO was proved to be of great value, especially in diseases such

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as β-thalassaemia and in several pathophysiological phenomena which are related to iron-overload[2] or increase in 'free iron', such as during reperfusion following ischemic anoxia.[3] Paraquat toxicity was enhanced by iron and reduced by DFO in laboratory mice.[4]

Under physiological conditions, ferric ions are only very slightly soluble, and at equilibrium are estimated to be 10^{-18} M.[5] A variety of chelating agents greatly increase its solubility and its reactivity with oxygen-active species. The redox potential of the Fe^{+3}/Fe^{+2} pair can vary by complexing ligands.[6-9]

Desferrioxamine totally inactivates iron eliminating 'iron redox cycling' and thus inhibits HO production during catalytic Fenton reactions.[10,11] Compounds such as DFO appear to inhibit Fenton reactions by decreasing the reduction of Fe^{+3} by $O_2^{\cdot-}$.[12] Ferrioxamine chelate does not decompose H_2O_2,[13] perhaps because this chelate lacks a free iron coordination site.[14] In addition, at relatively high concentrations (about 10^{-4} M and above) DFO has been shown to be an effective scavenger of hydroxyl radicals,[15,16] however only a slow reactant with superoxide.[16]

Recently, we reported that H_2O_2-activated metmyoglobin and hemoglobin initiate membranal lipid peroxidation and this reaction was not inhibited by hydroxyl radical scavengers of EDTA at low concentrations. Desferrioxamine was found to inhibit this reaction even at low concentration.

The purpose of this research was to understand the inhibitory effect of DFO on membranal lipid peroxidation initiated by H_2O_2-activated metmyoglobin or methemoglobin, and its effect on other peroxidative systems.

MATERIALS AND METHODS

Hydrogen peroxide (30% for synthesis), ascorbic acid and trichloroacetic acid were purchased from Merck (Darmstadt, West Germany). Myoglobin type I from bovine, hemin potassium chloride, L-histidine free base, thiobarbituric acid, β-carotene, linoleic acid and phenolsulphonophthalein (phenol-red) were all obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.); EDTA and butylated hydroxytoluene (BHT) were purchased from BDH Chemicals Ltd. (Poole, England). Lipoxygenase (soybean) was obtained from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.). Desferrioxamine methanesulfonate (desferal) was obtained from CIBA-Geigy A.G. (Basel, Switzerland).

Isolation of the microsomal fraction from muscle tissue was by a procedure described previously.[17] Microsomes for lipid peroxidation assay were incubated in a shaking water bath at 37°C. The reaction mixture contained 1 mg microsomal proteins/ml, the MetMb + H_2O_2 complex, and 0.12 M KCl, 5 mM histidine buffer, or 100 mM acetate buffer adjusted to pH 7.0. The reactions were initiated by the addition of MetMb + H_2O_2.

Thiobarbituric acid-reactive substances were determined by the procedure of Bidlack *et al.*[18] One ml from the incubation system was reacted with 1 ml of TCA 35%. The mixture was centrifuged for 10 min at 4000 × g. From the supernatant, 1.5 ml was removed and added to 2 ml of 0.35% TBA. The tubes were heated in boiling water for 20 min, cooled to room temperature and the absorbance at 532 nm recorded. The results were reported as nanomoles malondialdehyde-reactive substances (TBA-RS) per milligram protein, using a molar extinction coefficient of $E_{532} = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Protein determination was conducted by the modified Lowry procedure, using BSA as standard.[19] The method has been modified by the addition of sodium dodecyl suphate in the alkali reagent and an increase in the amount of copper tartrate reagent. These alterations allowed the method to be used with membranes and lipoproteins preparations without prior solubilization or lipid extraction and with samples containing sucrose or EDTA.

β-Carotene cooxidation in 100 mM acetate buffer adjusted to pH 7.0 and 37°C was determined by a method described previously.[20] Briefly, the technique consists of monitoring the decreased in absorbance at 460 nm in a cuvette containing the reactants. The sample contained 1.5 ml of buffered carotene and linoleate solubilized using Tween-20, 0.1–0.4 ml catalysts and distilled water to a final volume of 2.0 ml. The concentration of the initial reaction mixture was: β-carotene, 14 μM; linoleate, 180 mM; Tween-20, 0.05%; sodium acetate buffer, 100 mM. The sample in the control curvette contained all the reagents except linoleate. The initial linear rate of decrease in absorbance was computed from a recorder tracing and converted into the rate of carotene disappearance in nmoles/min.

Phenol-red peroxidation by peroxidase and metmyoglobin was conducted using a method developed by Pick and Keisari[21] for the measurement of hydrogen peroxide produced by cells in culture. The assay is based on the oxidation of phenol-red by H_2O_2-activated HRP or metmyoglobin which results in the formation of a compound demonstrating increased absorbance at 610 nm. The results are means of triplicates.

RESULTS

Membranal lipid peroxidation initiated by H_2O_2-activated metmyoglobin and methemoglobin was found to be inhibited almost completely by 10 μM of DFO (Fig. 1). The same inhibition was not observed in the model system containing ferrioxamine. The addition of ferric chloride or cupric sulfate to activated metmyoglobin or in the presence of H_2O_2 alone did not change significantly the initiation of membranal lipid peroxidation. Similar results were obtained if buffer histidine was replaced by buffer acetate adjusted to pH 7.0 (Table I). Desferrioxamine was found to inhibit also phenol-red oxidation by activated metmyoglobin. Almost similar inhibition was found during the oxidation of phenol-red by horseradish peroxidase (Fig. 2). Horseradish peroxidase oxidizes phenol-red and this reaction was inhibited competitively by DFO (Fig. 3). It was found that addition of ferric ion at concentrations similar to those of DFO prevents completely the inhibitory effect of DFO on phenol-red oxidation by both H_2O_2-activated metmyoglobin and HRP-compound I.

We extend our work determining the cooxidation of β-carotene by linoleate, containing traces of hydroperoxides, and catalyzed by hemin.[22] Desferrioxamine at a concentration of 10 μM inhibits more than 40% of this reaction (Fig. 4). Lipoxygenase, which contains non-heme iron in the active site of the molecule, cooxidizes β-carotene during linoleate oxidation.[22] Figure 4 shows that DFO also inhibits β-carotene oxidation by lipoxygenase. The enzymatic activity of lipoxygenase did not diminish during the incubation of the enzyme with DFO. Lipoxygenase incubated with DFO and separated from the chelator by Sephadex G-10 column chromatography showed the same activity as the control (results not shown). It was also found that the addition of equimolar equivalents of ferric ion to DFO decrease its inhibitory

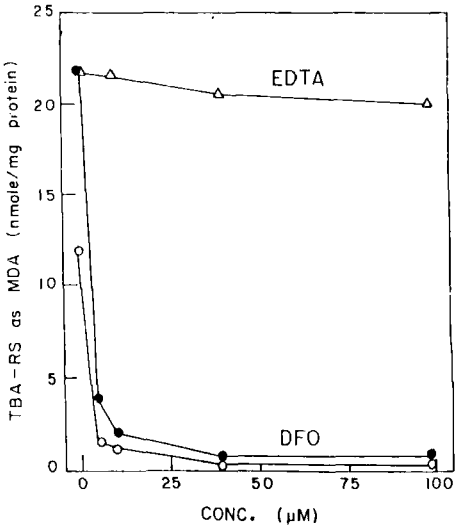

FIGURE 1 The effect of DFO and EDTA concentration on membranal lipid peroxidation by H_2O_2-activated metmyoglobin and methemoglobin. △, metmyoglobin/H_2O_2 (30 μM each); ●, metmyoglobin/H_2O_2 (30 μM each); ○, methemoglobin/H_2O_2 (7 μM/30 μM, respectively).

TABLE I
The effect of iron and copper ions on the activities of activated metmyoglobin and desferrioxamine in membranal lipid peroxidation

Treatments	TBA-RS as MDA nmole/mg prot/30 min
MetMb + H_2O_2	22.1 ± 2.4
MebMb (after Chelex X-100) + H_2O_2	19.5 ± 2.2
MetMb + H_2O_2 + $FeCl_3$	20.8 ± 2.7
MetMb + H_2O_2 + $CuSO_4$	21.5 ± 2.6
MetMb + H_2O_2 + ferrioxamine	21.7 ± 2.1
MetMb + H_2O_2 + DFO	1.2 ± 0.5
$FeCl_3$ + H_2O_2	1.8 ± 0.8
$FeCl_3$ + H_2O_2 + DFO	1.5 ± 0.5
$CuSO_4$ + H_2O_2	1.2 ± 0.4
$CuSO_4$ + H_2O_2 + DFO	1.3 ± 0.3

The reaction mixture contained microsomes (mg/protein/ml); MetMb and H_2O_2, 30 μM each; $FeCl_3$, 30 μM; $CuSO_4$; 10 μM; desferrioxamine, 30 μM; ferrioxamine ($FeCl_3$, 30 μM + DFO, 30 μM incubated before addition); in buffer acetate, 100 mM adjusted to pH 7.0, at 37°C. Each result is the mean of triplicates ± SD.

effect toward β-carotene peroxidation by hemin or lipoxygenase by almost 50% (results not shown).

The reaction of H_2O_2 with resting metmyoglobin was studied using a scan double-beam spectrophotometer in the Soret region of the spectrum. The scans performed were taken at 1-min intervals and show formation of one spectral intermediate (Fig. 5a). The intermediate is formed slowly and shows a bathochromic shift from 408.5 nm. No spectral changes were observed upon reaction of metmyoglobin with

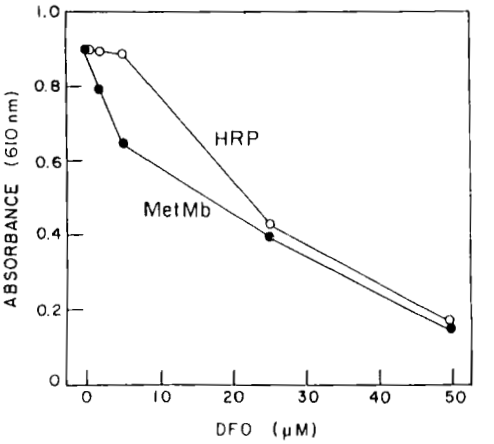

FIGURE 2 The effect of DFO concentration on the oxidation of phenol-red by horseradish peroxidase and metmyoglobin at pH 7.0 and 25°C. ○, horseradish peroxidase/H_2O_2 (30 μM each); ●, metmyoglobin/H_2O_2 (30 μM each).

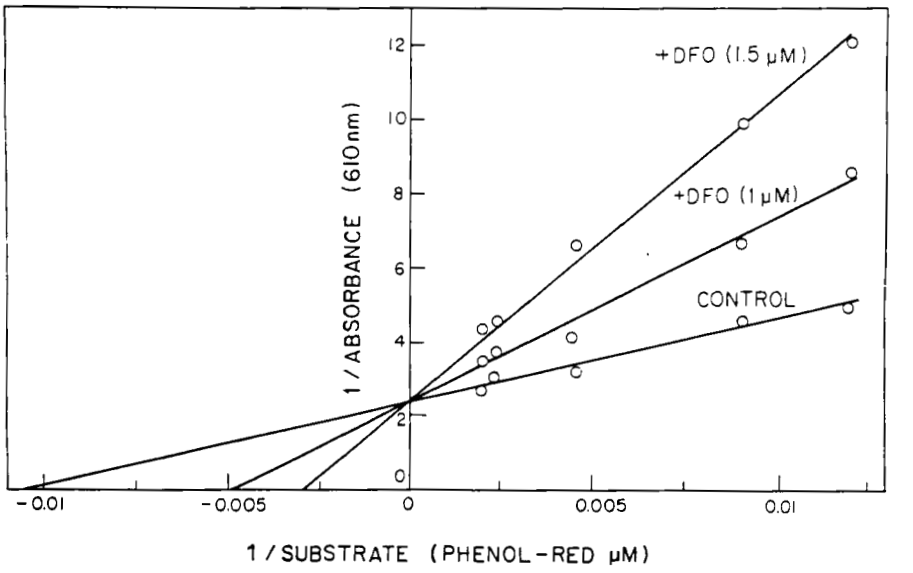

FIGURE 3 Horseradish peroxidase competitive inhibition of phenol-red oxidation by DFO, at pH 7.0 and 25°C.

DFO, in the absence of H_2O_2. We examined the effect of adding a constant amount of DFO to equimolar metmyoglobin (7.5 μM) and H_2O_2 (7.5 μM). If the electron donor (DFO) was added to metmyoglobin 5 min after its incubation with H_2O_2, DFO reduced the intermediate, which shifted back from 413.0 to 408.5 nm and increased the absorbance to 50% of the resting metmyoglobin (Fig. 5b). However, if DFO was added with H_2O_2 to metmyoglobin, the absorbance of MetMb decreased almost at the

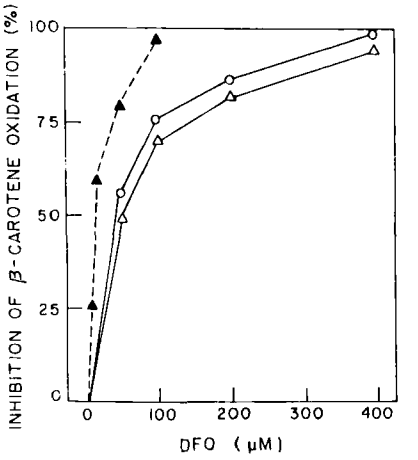

FIGURE 4 Desferrioxamine inhibition of ß-carotene-linoleate cooxidation by hemin (30 nM) and soybean lipoxygenase (30 ng/ml) at pH 7.0 and 25°C.

same rate (Fig. 5c), the shift from 408.5 nm was almost insignificant, and the intermediate was reduced to 90% of the resting metmyoglobin (Fig. 5d).

DISCUSSION

The model compound of metmyoglobin and H_2O_2 was proposed by George and Irvine[23] to be an oxyferryl iron $(Fe^{+4} = O^{-2})^{+2}$ and was recently found to have a structure similar to that of compound II of HRP.[24]

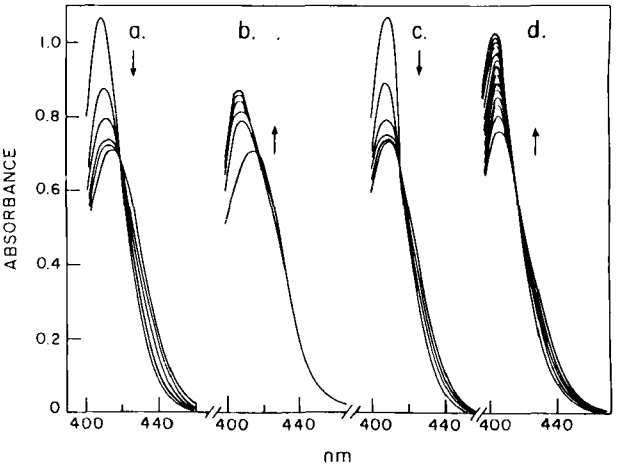

FIGURE 5 The changes in Soret region spectra during the interaction of metmyoglobin (7 μM) with H_2O_2 (7 μM), with and without DFO (100 μM). (a), metmyoglobin + H_2O_2; (b), DFO added to a. after 5 min; (c), metmyoglobin + H_2O_2 + DFO; (d), scanning c. after 5 min.

Hydrogen peroxide activated metmyoglobin or methemoglobin which initiate membranal lipid peroxidation was found to be inhibited by DFO. Gutteridge[25,26] suggested that iron is released from the heme group during the interaction of H_2O_2 with myoglobin or other hemeproteins and affect lipid peroxidation. We assume, however, that such a free iron in our model system has no significant effect on membranal lipid peroxidation. We based our assumption by analyzing the data accepted in this study and from other model systems containing an "iron redox cycle" and EDTA.[27] EDTA (100 μM) did not inhibit membranal lipid peroxidation by H_2O_2-activated MetMb, however it did inhibit those by "iron or copper redox cycles".

We propose that the inhibitory effect of DFO is derived from its properties of acting as an electron or hydrogen donor.

Desferrioxamine was found to inhibit competitively the oxidation of phenol-red by horseradish peroxidase. Desferrioxamine inhibits also the oxidation of phenol-red by activated metmyoglobin. These results support our suggestion that DFO acts as an electron donor which, by competition with phenol-red, interacts with compound I and compound II of peroxidase. Activated metmyoglobin possibly as oxyferryl seems to interact with DFO in the same fashion.

Important information on the properties of HRP was derived by several researchers from optical titration of compound I and compound II to the native enzyme with reductants, such as ferrocyanide.[23] We show that DFO titration of H_2O_2-activated metmyoglobin reverses the optical properties of the intermediate to the native metmyoglobin. These results demonstrated, inter alia, that DFO could act as an electron or hydrogen donor. Desferrioxamine and its complex with ferric ion, ferrioxamine were found to be powerful scavengers of hydroxyl radical.[16] However, in our study only DFO inhibit all the perioxidizing systems. Ferrioxamine was found to inhibit only partially β-carotene linoleate oxidation by hemin or lipoxygenase. The chelation of ferric ion by DFO is by three hydroxyl amines and carboxyl groups. The coordinative bonding of the hydroxyl amines to iron eliminate their possibility to donate electrons. We assume that the inactivation of H_2O_2-activated metmyoglobin and compound I HRP by DFO derived especially from electrons donated by the hydroxyl amine groups. Hydroxyl and unsaturated fatty acid free radicals seem to be at a higher redox potential than activated hemeproteins, and they could interact also with amine groups. This could explain the scavenger capability of hydroxyl radicals and the partial inhibition of β-carotene oxidation by ferrioxamine.

In clinical medicine DFO is given to thalassaemic patients to aid iron excretion which prevents several pathophysiological phenomena related to 'free iron', such as in models of tissue inflammation,[31] brain reperfusion following ischemic anoxia,[3] and paraquat toxicity.[4] There are also reports that DFO stimulates inflammation,[31] alloxan cytotoxicity[32] and, at a high concentration, paraquat toxicity.[33] Osheroff *et al.*[33] suggested that the enhancement of paraquat toxicity was generated from a reductive decomposition of DFO by paraquat semi-quinone. We, however, postulate that DFO cytotoxicity may derive from its oxidative decomposition to a DFO radical.

We found evidence that DFO is a good electron or hydrogen donor. Desferrioxamine seems to have not only the potential to chelate very efficiently ferric ions and to prevent 'iron redox cycle', but also to affect several oxidative reactions, their importance of which 'in vivo' should be systematically determined in the future.

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