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Desferrioxamine (Desferal) and superoxide free radicals

Formation of an enzyme-damaging nitroxide

Michael J. DAVIES, Rose DONKOR, Christina A. DUNSTER, Carolyn A. GEE, Sonja JONAS and Robin L. WILLSON
Department of Biochemistry, Brunel University, Uxbridge, Middx. UB8 3PH, U.K.

In neutral solutions, desferrioxamine (Desferal) can react with the superoxide free radical, $O_2^{\cdot -}$ (possibly through its protonated form $HO_2^{\cdot}$), to form a relatively stable nitroxide free radical, which can have a half-life of approx. 10 min at room temperature. The formation of the radical can be largely prevented by the presence of superoxide dismutase. The radical reacts rapidly with cysteine, methionine, glutathione, vitamin C and a water-soluble derivative of vitamin E. It also reacts rapidly with alcohol dehydrogenase, causing a loss of enzyme activity. The implications of these findings for mechanistic free-radical biochemistry and iron-chelation therapy could be considerable.

INTRODUCTION

During the last decade interest in the role of iron ions in free-radical-mediated tissue injury has grown considerably, and many investigations continue to take place into the free-radical biochemistry of iron-ion-chelating agents (Wills, 1969; Marklund & Marklund, 1974; Halliwell, 1975, 1987; Fong *et al.*, 1976; Willson, 1977*a*,*b*,*c*, 1982, 1987; Ilan & Czapski, 1977; Buettner *et al.*, 1978; Blake *et al.*, 1981; Butler & Halliwell, 1982; Rice-Evans *et al.*, 1985; Aust, 1985; Kontoghiorghes *et al.*, 1986). In this respect the drug desferrioxamine (Desferal; CIBA–GEIGY), widely used since 1962 in the treatment of haemochromatosis and iron poisoning, has attracted much attention both as a laboratory tool and as a possible ameliorator of rheumatoid disease and reperfusion injury (Wills, 1969; Gutteridge *et al.*, 1979; Hoe *et al.*, 1982; Willson, 1982; Sinaceur *et al.*, 1983, 1984; Aust & White, 1985; Blake *et al.*, 1985; Halliwell, 1985; Nayini *et al.*, 1985; Ribiere *et al.*, 1986; Bernier *et al.*, 1986; Rice-Evans *et al.*, 1986).

Such applications of metal-chelating drugs, however, are fraught with difficulties. Visual and auditory neurotoxicity have been observed in patients receiving desferrioxamine (Simon *et al.*, 1983; Arden *et al.*, 1984; Olivieri *et al.*, 1986). In the treatment of inflammatory joint disease, continued use of such a drug may cause unacceptable levels of side effects in what generally would be non-life-threatening disorders. Indeed, in a recent pilot study coma and serious retinal pigmentation occurred when desferrioxamine was administered to a rheumatoid patient being simultaneously treated with prochlorperazine (Blake *et al.*, 1985).

Similarly, as a tool in mechanistic free-radical biochemistry, the drug may not just act as an iron chelator but may also scavenge free radicals directly. The drug does react rapidly with the hydroxyl radical, $OH^{\cdot}$ (Hoe *et al.*, 1982; Willson, 1982), and studies have indicated that it can also react with the superoxide free radical, $O_2^{\cdot -}$ (Sinaceur *et al.*, 1983, 1984). Although it has been suggested that this latter reaction may be insignificant in biochemical systems (Halliwell, 1985), we now report studies that confirm that such reactions can occur under conditions where $OH^{\cdot}$ radicals do not react with the drug to any extent. Furthermore, a nitroxide free radical formed in such reactions is sufficiently long-lived to enable it to inactivate alcohol dehydrogenase. This is so even when the irradiated desferrioxamine solutions are added to the enzyme long after the initiating $O_2^{\cdot -}$ radicals have disappeared.

EXPERIMENTAL

Materials

Desferrioxamine was supplied as the methanesulphonate (Desferal) by CIBA-GEIGY. Yeast alcohol dehydrogenase, sodium DTPA and sodium EDTA were supplied by Sigma Chemical Co. Trolox C, a water-soluble derivative of vitamin E, and t-butyl hydroperoxide were supplied by Aldrich Chemical Co. Bovine CuZn superoxide dismutase (activity 3000 units/mg) was kindly donated by Grunethal G.m.b.H. KH_2PO_4 and K_2HPO_4 were of AnalaR grade and supplied by BDH Chemicals. Sodium formate was supplied by Merck. All solutions were prepared in doubly distilled water and adjusted to pH 7.0 ± 0.1 with NaOH or HCl.

$O_2^{\cdot -}$ generation

$O_2^{\cdot -}$ radicals were generated by exposing N_2O/O_2-(5:1)-saturated aqueous solutions of sodium formate to ionizing radiation from the Brunel Biochemistry ^{60}Co cave-type γ-radiation source using a dose rate of between 1 and 30 Gy/min calibrated by using $FeSO_4$ dosimetry (Willson, 1985).

Enzyme inactivation studies

Samples of solutions of yeast alcohol dehydrogenase corresponding to a final activity concentration of 4 units/ml (13.8 μg/ml) were included in the irradiated solutions or added to the solutions after irradiation had ceased. Enzyme activity was determined by adding 0.2 ml samples of the enzyme solution to 2.8 ml of a solution containing NAD^+ (1.25 mM), semicarbazide (73 mM), ethanol (180 mM) and phosphate buffer (12 mM), pH 8.5 in a 1 cm cuvette and measuring the increase in

Abbreviation used: DTPA, diethylenetriaminepenta-acetate.

absorption at 340 nm over a period of approx. 1 min (Gee *et al.*, 1985).

E.s.r. studies

Irradiated solutions were quickly transported (less than 2 min) to a Bruker ER200D e.s.r. spectrometer equipped with 100 kHz frequency modulation and gaussmeter. Resonance spectra were measured with the use of a flat cell either immediately or after the addition of various antioxidants. Where necessary, spectra were scanned repeatedly for up to 40 min.

RESULTS

Inactivation of alcohol dehydrogenase

On irradiation of N_2O/O_2-saturated solutions of alcohol dehydrogenase, inactivation of the enzyme occurred exponentially with radiation dose. In the additional presence of formate (10 mM) little inactivation was observed over the same dose range (0–4 Gy). When the above experiments were repeated in the presence of EDTA or DTPA (20 μM), similar results were obtained. However, when desferrioxamine was included rather than EDTA or DTPA, considerable enzyme inactivation was observed even in the presence of excess formate (Fig. 1). Similar effects were also observed where air-saturated rather than N_2O/O_2-saturated solutions were irradiated. The extent of inactivation increased if higher desferrioxamine concentrations were present, but decreased significantly if superoxide dismutase (0.038 μg/ml, 0.11 unit/ml), ascorbate, cysteine, methionine or Trolox C was also included in the irradiated solutions (Table 1). Negligible protection was observed, however, if bovine serum albumin at a similar concentration to superoxide dismutase was included.

Surprisingly, enzyme inactivation also occurred if irradiated solutions containing formate and desferrioxamine, but not EDTA or DTPA, were added to the non-irradiated enzyme immediately after irradiation ceased (Table 1). All the antioxidants again protected if present at the time of irradiation. Cysteine, GSH and methionine also protected if present at the time of adding the enzyme post-irradiation (Table 1).

E.s.r. detection of nitroxide

On irradiation of similar N_2O/O_2-saturated solutions containing sodium formate (100 mM) and desferrioxamine (170 μM), a strong but slowly decaying e.s.r. signal with a half-life of approx. 10 min was observed

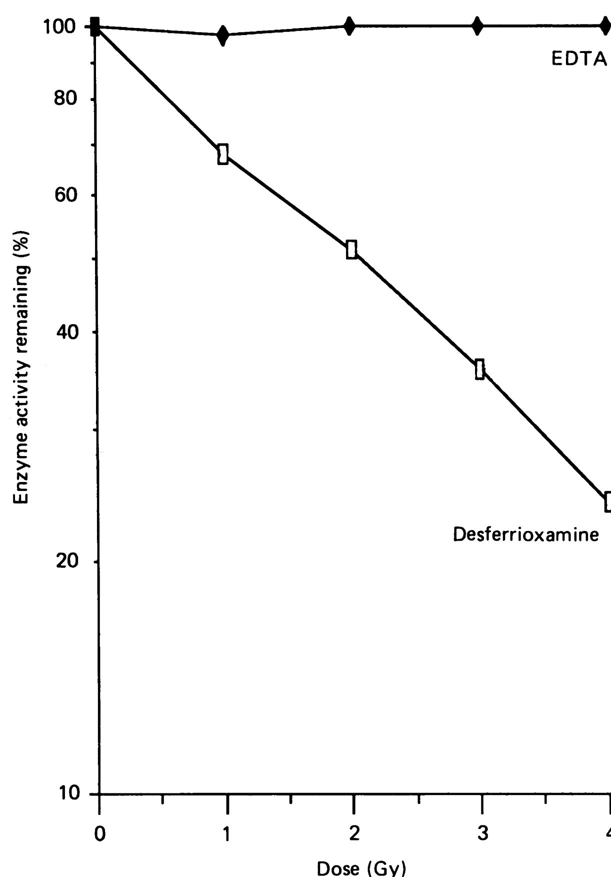


Fig. 1. Inactivation of yeast alcohol dehydrogenase (13.8 μg/ml) on irradiation in N_2O/O_2-saturated solution in the presence of formate (10 mM) and desferrioxamine or EDTA (20 μM) (dose rate approx. 1 Gy/min)

Table 1. Percentage of yeast alcohol dehydrogenase activity remaining after irradiation (4 Gy) of N_2O/O_2-saturated solutions of desferrioxamine (50 μM) and formate (10 mM) in the absence and in the presence of the enzyme and various antioxidants: column (a) all present at time of irradiation; column (b) enzyme added post-irradiation; column (c) enzyme and antioxidant added post-irradiation

Results are given as means ± S.E.M. for the numbers of completely separate experiments given in parentheses.

	Enzyme activity remaining (%)		
Antioxidant	(a) All irradiated	(b) Enzyme added post-irradiation	(c) Enzyme and antioxidant added post-irradiation
None (control)	4±1 (8)	24±4 (6)	28±3 (6)
Superoxide dismutase (0.038 μg/ml = 0.11 unit/ml)	73±5 (5)	86±3 (6)	28±4 (5)
Bovine serum albumin (0.038 μg/ml)	4±4 (3)	21±10 (2)	11±1 (2)
Ascorbate (100 μM)	93±5 (2)	96±1 (2)	28±1 (2)
Trolox C (100 μM)	72±1 (2)	86±4 (2)	24±4 (2)
Cysteine (100 μM)	94±4 (3)	99±1 (2)	96±5 (3)
GSH (100 μM)	77±3 (3)	100±1 (2)	78±0 (2)
Methionine (100 μM)	57±6 (3)	92±1 (2)	65±4 (3)

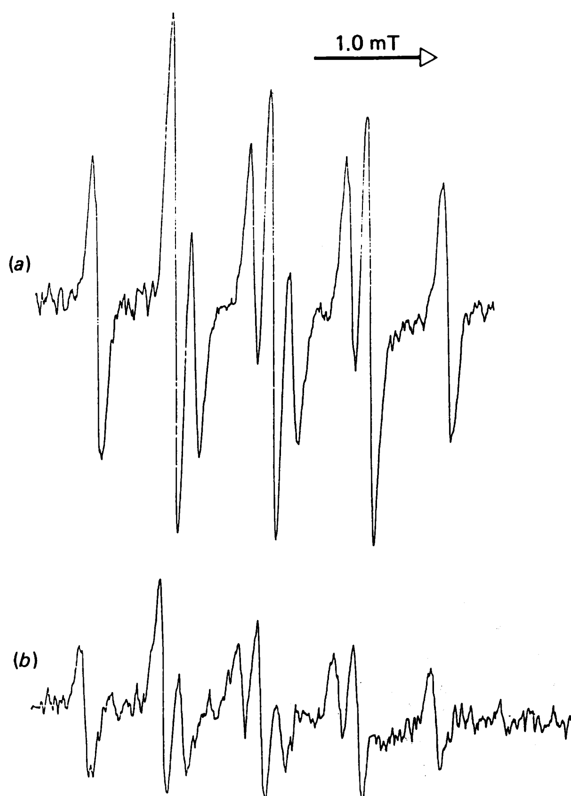


Fig. 2. E.s.r. spectra (a_N 0.780 mT, a_{2H} 0.628 mT) assigned to the desferrioxamine nitroxide free radical observed (*a*) 2 min and (*b*) 16 min after irradiation of an N_2O/O_2-saturated solution containing 200 μM-desferrioxamine and 100 mM-sodium formate (dose 15 Gy; 3 Gy/min)

Spectrometer conditions: Gain 2×10^6, modulation amplitude 0.10 mT, time constant 0.5 s, scan time 200 s, field 347.5 mT, field scan 0.60 mT, power 13 dB, frequency 9.73 GHz, room temperature.

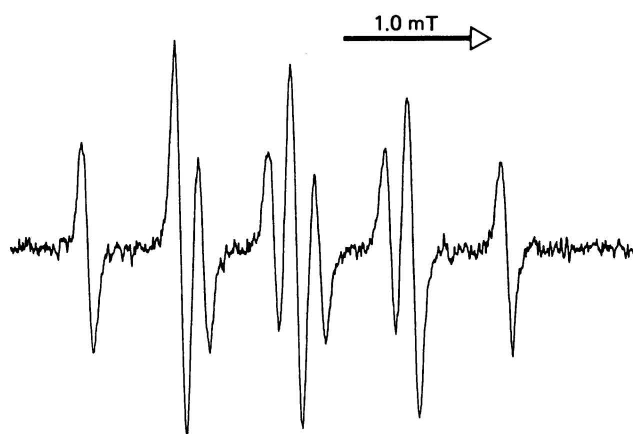


Fig. 3. E.s.r. spectrum (a_N 0.780 mT, a_{2H} 0.628 mT) observed on mixing 500 μM-desferrioxamine with 10 mM-t-butyl peroxide in borate buffer, pH 7.8

Spectrometer conditions: as in Fig. 2 except gain 3.2×10^5, modulation amplitude 0.05 mT, scan time 500 s. Marker signals at 1.0 mT intervals.

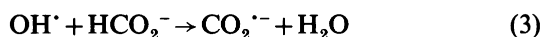
(Fig. 2). The absorption, consisting of a triplet of triplets characteristic of a carbonyl-conjugated nitroxide (nitroxyl or aminoxyl) free radical, was also observed in irradiated air-saturated solutions of the drug and formate (10 mM) and when non-irradiated solutions of desferrioxamine and t-butyl hydroperoxide were mixed (Fig. 3). The absorption was not observed in the irradiated solutions containing EDTA or DTPA instead of desferrioxamine, and was diminished to 25% of its magnitude when superoxide dismutase (0.55 unit/ml) was included at the time of radiation. If Trolox C or ascorbate (final concentrations 100 μM) were added to the irradiated drug/formate solutions immediately after the radiation ceased, the nitroxide absorption was absent but was replaced by signals consistent with the simultaneous formation of the ascorbate or Trolox C free radicals respectively. The nitroxide absorption was also absent when GSH (final concentration 100 μM) was added immediately after irradiation ceased.

DISCUSSION

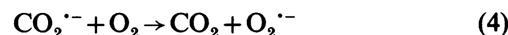
Desferrioxamine contains three ionized hydroxamic acid (–NOH) groups (Moeschlin & Schnider, 1963). The above results can therefore be ascribed to an enzyme-damaging nitroxide free radical being formed in irradiated N_2O/O_2-saturated solutions containing desferrioxamine ($DfNO^-$) and excess formate. The following principal reactions are thought to take place initially:

$$H_2O \rightarrow e^-_{aq.}, OH^{\cdot}, H^+ \quad (1)$$

$$e^-_{aq.} + N_2O \rightarrow N_2 + OH^- + OH^{\cdot} \quad (2)$$



$$OH^{\cdot} + HCO_2^- \rightarrow CO_2^{\cdot -} + H_2O \quad (3)$$



$$CO_2^{\cdot -} + O_2 \rightarrow CO_2 + O_2^{\cdot -} \quad (4)$$

Although the $OH^{\cdot}$ radical can react rapidly with desferrioxamine ($k = 1 \times 10^{10}\ M^{-1} \cdot s^{-1}$), negligible reaction occurs when at least a 200-fold higher concentration of the good $OH^{\cdot}$ scavenger formate ($k_3 = 3 \times 10^9\ M^{-1} \cdot s^{-1}$) is also present (Hoe *et al.*, 1982; Willson, 1982). In air-saturated solution alternative reactions of $e^-_{aq.}$ may also take place to some extent, although the net result, the formation of $O_2^{\cdot -}$, is likely to remain the same:

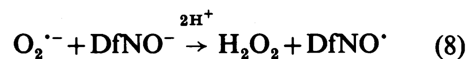
$$e^-_{aq.} + O_2 \rightarrow O_2^{\cdot -} \quad (5)$$

or

$$e^-_{aq.} + DfNO^- \rightarrow DfNO^{\cdot 2-} \quad (6)$$

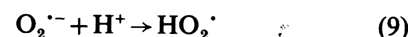
$$DfNO^{\cdot 2-} + O_2 \rightarrow DfNO^- + O_2^{\cdot -} \quad (7)$$

The formation of the nitroxide resonance absorption and the inactivation of yeast alcohol dehydrogenase (YADH) can thus be attributed to the reactions:



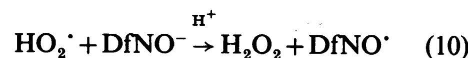
$$O_2^{\cdot -} + DfNO^- \xrightarrow{2H^+} H_2O_2 + DfNO^{\cdot} \quad (8)$$

or



$$O_2^{\cdot -} + H^+ \rightarrow HO_2^{\cdot} \quad (9)$$

and



$$HO_2^{\cdot} + DfNO^- \xrightarrow{H^+} H_2O_2 + DfNO^{\cdot} \quad (10)$$

followed by:

$$DfNO^{\cdot} + YADH \rightarrow \text{inactivation} \quad (11)$$

The spectral shape and the hyperfine coupling constants of the e.s.r. signals are consistent with the presence of the desferrioxamine nitroxide free radical ($DfNO^{\cdot}$) containing the structural component $-CH_2-N(O^{\cdot})-CO-$ and

are similar to those reported for the radical derived from CH_3–N(OH)–$COCH_3$ ($a_N = 0.78$ mT, $a_{3H} = 0.89$ mT) reported previously (Minor *et al.*, 1967). Furthermore, a related nitroxide from the reaction of $O_2^{\cdot-}$ with the hydroxylamine 2-ethyl-2,5,5-trimethyl-3-oxazolidine has already been reported (Rauckman *et al.*, 1979; Rosen *et al.*, 1982).

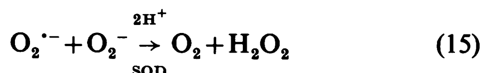
The absence of the nitroxide e.s.r. signal observed when ascorbate (VitCH), Trolox C (TxOH) or GSH was added to the desferrioxamine solutions post-irradiation can be attributed to the overall reactions of the type:

$$DfNO^{\cdot} + GSH \rightarrow DfNO^- + GS^{\cdot} + H^+ \quad (12)$$

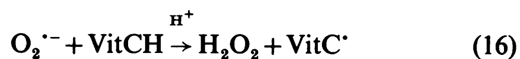
$$DfNO^{\cdot} + TxOH \rightarrow DfNO^- + TxO^{\cdot} + H^+ \quad (13)$$

$$DfNO^{\cdot} + VitCH \rightarrow DfNO^- + VitC^{\cdot} + H^+ \quad (14)$$

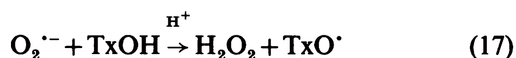
However, only in the case of GSH, cysteine and methionine can such reactions with the nitroxide free radical be responsible for the enzyme protection observed when the antioxidants were included at the time of radiation, since the other antioxidants do not protect when added with the enzyme to desferrioxamine solutions post-irradiation (Table 1, column c). Even in these intances, however, as with the other antioxidants, the protection observed when they are included at the time of irradiation is more likely attributable to the respective reactions of the $O_2^{\cdot-}$ radical or its protonated form competing with reactions (8), (9) and (10), thereby preventing the actual formation of the nitroxide, e.g.:



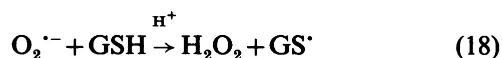
$$O_2^{\cdot-} + O_2^- \xrightarrow[SOD]{2H^+} O_2 + H_2O_2 \quad (15)$$



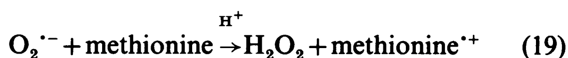
$$O_2^{\cdot-} + VitCH \xrightarrow{H^+} H_2O_2 + VitC^{\cdot} \quad (16)$$



$$O_2^{\cdot-} + TxOH \xrightarrow{H^+} H_2O_2 + TxO^{\cdot} \quad (17)$$



$$O_2^{\cdot-} + GSH \xrightarrow{H^+} H_2O_2 + GS^{\cdot} \quad (18)$$



$$O_2^{\cdot-} + \text{methionine} \xrightarrow{H^+} H_2O_2 + \text{methionine}^{\cdot+} \quad (19)$$

(SOD represents superoxide dismutase). Clearly, whatever the protective mechanism, the nature of the chelating agents present in biochemical systems can have a profound influence on the free-radical processes taking place. Chelating agents may indeed bind what has variously been described as 'decompartmentalized', 'ill-placed', 'adventitious', 'free', 'low-molecular-weight chelatable' or 'misplaced' iron and so prevent deleterious catalytic reactions. However, they may also react with iron or with free radicals directly and form damaging species (Albert, 1973; Willson, 1976*a*,*b*, 1977*a*,*b*). The fact that with desferrioxamine a relatively stable nitroxide free radical is formed is particularly interesting, not only in relation to the occasional disturbing side effects described above, but also in relation to cancer, where nitroxide free radicals are known to be formed during the metabolism of some carcinogens (Floyd *et al.*, 1967; Stier *et al.*, 1972; Fischer & Mason, 1986). The implications of these findings for the development of clinically useful iron-chelating drugs, for our understanding of the biological activity of hydroxamic acid-containing siderophores, and for mechanistic free-radical biochemistry and cancer studies, seem considerable.

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