Superoxide dismutase inhibits the superoxide-driven Fenton reaction at two different levels

Implications for a wider protective role

John M.C. Gutteridge

National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, England

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Superoxide dismutase (SOD) completely inhibits the damage caused by a ferric-EDTA chelate in the presence of a superoxide-generating system. In this reaction superoxide is enzymically dismuted to hydrogen peroxide. Since hydrogen peroxide and a ferric-EDTA chelate are themselves a hydroxyl radical-generating system, it follows that SOD must also protect against damage done by this reaction. The ability of SOD to inhibit damage to deoxyribose caused by hydrogen peroxide and a ferric-EDTA chelate is experimentally demonstrated in this paper.

Superoxide dismutase *Fenton reaction* *Hydroxyl radical* *Iron chelate* *Deoxyribose degradation*

1. INTRODUCTION

The Fenton reaction (eqn 3) is the most likely mechanism for the generation of the highly reactive hydroxyl radical ($OH^{\cdot}$) in a biological system. Essential requirements for this reaction are hydrogen peroxide and a reduced iron complex. Iron complexes capable of decomposing H_2O_2 are present in vivo [1,2]; copper, cobalt and titanium are also effective in forming $OH^{\cdot}$ from H_2O_2.

A Fenton reaction in biological systems could result from (A) the direct 'activation' of molecular oxygen by Fe^{2+}, (B) the generation of superoxide radicals in the presence of a suitable iron complex or, (C) the decomposition of hydrogen peroxide by iron complexes. Reaction B is widely known as the 'iron-catalysed Haber-Weiss' reaction or can be called a superoxide-driven Fenton reaction.

(A) Fe^{2+}-complex + $O_2 \rightleftharpoons$ Fe^{3+}-complex + $O_2^{\cdot -}$ (1)

$2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$ (2)

Fe^{2+}-complex + $H_2O_2 \longrightarrow$ Fe^{3+}-complex + OH^- + $OH^{\cdot}$ (3)

(B) Fe^{3+}-complex + $O_2^{\cdot -} \rightleftharpoons$ Fe^{2+}-complex + O_2 (4)

$2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$ (2)

Fe^{2+}-complex + $H_2O_2 \longrightarrow$ Fe^{3+}-complex + OH^- + $OH^{\cdot}$ (3)

(C) Fe^{3+}-complex + $H_2O_2 \longrightarrow$ Fe^{2+}-complex + $2H^+$ + $O_2^{\cdot -}$ (5)

Fe^{2+}-complex + $H_2O_2 \longrightarrow$ Fe^{3+}-complex + OH^- + $OH^{\cdot}$ (3)

Hydroxyl radical production by these systems is inhibited by the removal of hydrogen peroxide, the chelation of iron by desferrioxamine and, depending on the type of iron complex, by the ferrox-

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idase activity of caeruloplasmin [3–7]. Superoxide dismutase (SOD) will substantially inhibit reaction B in which $O_2^{\cdot -}$ is acting as a reducing agent for Fe^{3+} [3–8]. However, if SOD were only preventing the initial reduction of the Fe^{3+}-complex (eqn 4) it would not prevent $OH^{\cdot}$ formation, as the product of the SOD catalysed dismutation would be:

$$Fe^{3+}\text{-complex} + 2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} Fe^{3+}\text{-complex} + H_2O_2 + O_2 \quad (6)$$

and Fe^{3+}-complex plus hydrogen peroxide are known to be a good $OH^{\cdot}$-generating system [9,10] able to degrade deoxyribose [11]. To explain the observed inhibition of the iron-catalysed Haber-Weiss reaction by SOD (B), the SOD must operate at two different inhibitory levels.

The protective role of SOD in preventing $OH^{\cdot}$ formation by a ferric complex and hydrogen peroxide is here demonstrated and its possible biological implications are discussed.

2. MATERIALS AND METHODS

2.1. *Materials*

SOD (bovine erythrocyte), 2-deoxy-D-ribose, catalase (bovine liver, thymol-free), albumin (human, fatty acid-free) and diethylenetriaminepentaacetic acid (DETAPAC) were from Sigma. All other chemicals were of the highest purity available from BDH, Poole, Dorset.

2.2. *Preparation of iron-EDTA complex*

2 mM ferrous ammonium sulphate and EDTA solutions were freshly prepared and mixed in the ratio 1:1.1. This solution was left to stand for 1 h at room temperature before use to form a ferric-EDTA complex. Ferric-EDTA was also prepared directly by mixing freshly prepared ferric chloride with EDTA in the proportions described above.

2.3. *Deoxyribose degradation*

The reaction mixture contained the following reagents added in the order indicated: 0.2 ml distilled water treated with Chelex resin, 0.2 ml phosphate-saline buffer, pH 7.4 (0.1 M NaH_2PO_4/Na_2HPO_4 in 0.15 M NaCl), 0.1 ml deoxyribose (10 mM). Inhibitors were added at concentrations shown in appropriate tables, followed by 0.05 ml hydrogen peroxide solution (10 mM) freshly prepared in Chelex resin-treated distilled water and 0.1 ml of the iron-EDTA complex. Tube contents were mixed and incubated at 37°C for 30 min. Thiobarbituric acid (TBA) reactivity, following damage to deoxyribose, was developed by adding 0.5 ml of 2.8% (w/v) trichloroacetic acid and 0.5 ml TBA reagent (1%, w/v) in 0.05 M NaOH. The tube contents were heated at 100°C for 15 min

Table 1

Inhibitory activity of SOD on iron salt and iron-EDTA catalysed deoxyribose degradation

Reaction	TBA reactivity (A_{532nm}, 0.5 h at 37°C)		
	Control	Control + SOD (0.03 mg/ml)	% inhibition by SOD
Deoxyribose (1.3 mM) only	0.006		
Deoxyribose + Fe^{3+}-EDTA (0.13 mM) (prepared from a ferrous salt)	0.021 (subtracted)		
Deoxyribose + ferrous salt (0.13 mM)	0.178	0.163	8.4
Deoyribose + Fe^{2+} (0.13 mM) + H_2O_2 (0.6 mM)	0.257	0.284	0
Deoxyribose + Fe^{3+}-EDTA (0.13 mM) (prepared from a ferrous salt) + H_2O_2 (0.6 mM)	0.689	0.088	87.0
Deoxyribose + Fe^{3+}-EDTA (0.13 mM) (prepared from a ferric salt) + H_2O_2 (0.6 mM)	0.133	0.012	90.0
Deoxyribose + Fe^{3+}-EDTA (0.13 mM) (prepared from a ferrous salt) + H_2O_2 (0.6 mM). Incubated under a reduced concentration of oxygen	0.639	0.074	88.0

Reaction mixtures contained 27 mM phosphate. Final reaction concentrations are shown

and the resulting absorbance was read at 532 nm. The results shown were calculated after the subtraction of appropriate blanks and are the mean of 3 or more separate assays which differed by less than ± 5%.

3. RESULTS

SOD inhibits deoxyribose degradation resulting from the mixing of hydrogen peroxide and a ferric-EDTA complex. The EDTA complex formed by pre-mixing a ferrous salt with EDTA promoted considerably more deoxyribose degradation than the complex prepared by mixing EDTA with a ferric salt. The iron complex prepared from the ferrous salt had completely auto-oxidised to the ferric state before use since it did not bring about deoxyribose degradation in the absence of added hydrogen peroxide (table 1). This would also indicate that other radicals, such as an EDTA-peroxy radical, if present in the iron complex did not degrade deoxyribose. However, there were no apparent differences in the catalytic properties of the two iron complexes when they were reacting in a superoxide-generating system (not shown).

Table 2

Effect of hydrogen peroxide concentration and SOD on deoxyribose degradation catalysed by the Fe^{3+}-EDTA complex

Reaction	TBA reactivity (A_{532nm}, 0.5 h at 37°C)		
	Control	Control + SOD (0.03 mg/ml)	% inhibition by SOD
Deoxyribose (1.3 mM) + Fe^{3+}-EDTA (0.13 mM, prepared from a ferrous salt)	0.021 (subtracted)		
+ H_2O_2 (0.60 mM)	0.689	0.088	87
+ H_2O_2 (0.50 mM)	0.603	0.101	83
+ H_2O_2 (0.30 mM)	0.578	0.110	81
+ H_2O_2 (0.17 mM)	0.440	0.090	79
+ H_2O_2 (0.07 mM)	0.207	0.092	56

All reaction mixtures contained 27 mM phosphate. Final reaction concentrations are shown

Table 3

Effect of catalase and albumin on deoxyribose degradation catalysed by Fe^{3+}-EDTA and H_2O_2

Reaction	TBA reactivity (A_{532nm}, after incubation at 37°C for 0.5 h)				
	Control	Control + albumin (0.03 mg/ml)	% inhibition	Control + catalase (0.03 mg/ml)	% inhibition
Deoxyribose (1.3 mM) + Fe^{3+}-EDTA (0.13 mM, prepared from a ferrous salt)	0.021 (subtracted)				
+ H_2O_2 (0.6 mM)	0.689	0.670	2.8	0	100
+ H_2O_2 (0.5 mM)	0.603	0.575	4.6	0	100
+ H_2O_2 (0.3 mM)	0.578	0.558	3.5	0	100
+ H_2O_2 (0.17 mM)	0.440	0.404	8.2	0.004	99
+ H_2O_2 (0.07 mM)	0.207	0.207	0	0.002	99

All reactions contained 27 mM phosphate. Final reaction concentrations are shown

Increase in concentration of hydrogen peroxide added to the reaction produced two effects: increase in the formation of OH· radicals (and hence deoxyribose degradation) and increase in the proportion of the reaction inhibited by SOD. Albumin added as a control for non-specific radical scavenging effects, showed no significant inhibitory properties, whereas catalase was markedly inhibitory (table 3). The activity of catalase was substantially decreased by heat denaturation of the protein (not shown).

When the hydrogen peroxide and Fe^{3+}-EDTA complex were mixed in the reaction under low concentrations of oxygen (gassing for 2 min with nitrogen) no change in the amount of deoxyribose degradation or the proportion of the reaction inhibited by SOD was observed (table 1). Replacing EDTA with DETAPAC prevented deoxyribose degradation, which might suggest that the DETAPAC-Fe^{3+} complex was not readily reduced by hydrogen peroxide as has been shown for $O_2^{\cdot-}$ [12,13].

4. DISCUSSION

The iron catalysed Haber-Weiss reaction is essentially a superoxide-driven Fenton reaction [14], in which $O_2^{\cdot-}$ reduces a ferric complex (eqn 4) and serves as a precursor of hydrogen peroxide via the dismutation reaction (eqn 2). Damage to biological molecules resulting from OH· produced in such systems can be completely prevented by the addition of SOD [3–8]. However, it can be seen from the reaction shown in eqn 6 that the addition of SOD will produce hydrogen peroxide in place of $O_2^{\cdot-}$ leaving the ferric complex and hydrogen peroxide to set up a Fenton reaction. Since complete protection by SOD is seen in the $O_2^{\cdot-}$-dependent reaction it follows that SOD must also be protecting against the hydrogen peroxide-stimulated part of the sequence. The ability of SOD to inhibit the damage done in a hydrogen peroxide-Fe^{3+}-EDTA system is experimentally demonstrated here.

The sequence of events which might explain the substantial inhibitory properties of SOD towards the hydrogen peroxide-Fe^{3+}-EDTA system are summarised in eqns 7–11. Fe^{3+}-EDTA would first be reduced by hydrogen peroxide to Fe^{2+}-EDTA and $O_2^{\cdot-}$ (eqn 7). The Fe^{2+}-EDTA complex might then auto-oxidise rapidly (half-life of less than 15 s [15]) before it can react with hydrogen peroxide or, could form a complex with $O_2^{\cdot-}$ [16,17] (eqn 9). The major products resulting from the mixing of hydrogen peroxide and the Fe^{3+}-EDTA complex, which give rise to the OH· radical, appear to be $O_2^{\cdot-}$ and Fe^{3+}-EDTA. SOD inhibits the formation of OH· radicals by again preventing reduction of the ferric complex by $O_2^{\cdot-}$. It will continue to do so in a cyclic way since the products of the reaction will again be hydrogen peroxide and the ferric-EDTA complex.

$$Fe^{3+}\text{-EDTA} + H_2O_2 \longrightarrow Fe^{2+}\text{-EDTA} + 2H^+ + O_2^{\cdot-} \quad (7)$$

$$Fe^{2+}\text{-EDTA} + O_2 \longrightarrow Fe^{3+}\text{-EDTA} + O_2^{\cdot-} \quad (8)$$

$$Fe^{2+}\text{-EDTA} + O_2^{\cdot-} \longrightarrow Fe^{2+}\text{-EDTA} - O_2^{\cdot-} \quad (9)$$

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (2)$$

$$Fe^{3+}\text{-EDTA} + O_2^{\cdot-} \longrightarrow Fe^{2+}\text{-EDTA} + O_2 \quad (10)$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \longrightarrow Fe^{3+}\text{-EDTA} + OH^- + OH^{\cdot} \quad (11)$$

Failure to influence the degradation of deoxyribose by lowering the concentration of oxygen can be explained by the generation of ample oxygen during the dismutation reaction (eqn 2). The difference observed between an Fe^{3+}-EDTA complex prepared from a ferrous salt and that prepared directly from a ferric salt suggests that 'oxygen activation', occurring by the auto-oxidation of the ferrous ions, advantageously alters the catalytic properties of the resulting complex since the two Fe^{3+}-EDTA complexes are markedly different in their reactivity with hydrogen peroxide or possibly deoxyribose. However, no catalytic differences were observed when they were reduced by $O_2^{\cdot-}$.

Hydrogen peroxide is a product of enzymic 2-electron transfers as well as a product of the dismutation reaction. Its removal in vivo depends on both catalase and glutathione peroxidase. However, hydrogen peroxide scavenging systems are known to be absent in certain aerobic bacteria, such as *Bacillus popilliae*, which respond to oxygen

by inducing higher levels of SOD [18]. The biological significance of the ability of SOD to inhibit formation of $OH^{\cdot}$ radicals from hydrogen peroxide and iron complexes will depend on whether naturally occurring iron complexes can act in the same way as EDTA. If they can, then it would add a wider protective role to the functions of the enzyme SOD.

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