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Copper + zinc and manganese superoxide dismutases inhibit deoxyribose degradation by the superoxide-driven Fenton reaction at two different stages

Implications for the redox states of copper and manganese

John M. C. GUTTERIDGE* and Joe V. BANNISTER†

*National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, U.K., and †Inorganic Chemistry Laboratory, University of Oxford, Oxford OX1 3QR, U.K.

1. When $OH^{\cdot}$ radicals are formed in a superoxide-driven Fenton reaction, in which $O_2^{\cdot -}$ is generated enzymically, deoxyribose degradation is effectively inhibited by CuZn- and Mn-superoxide dismutases. The products of this reaction are H_2O_2 and a Fe^{3+}–EDTA chelate. 2. The mixing of H_2O_2 and a Fe^{3+}–EDTA chelate also generates $OH^{\cdot}$ radicals able to degrade deoxyribose with the release of thiobarbituric acid-reactive material. This reaction too is inhibited by CuZn- and Mn-superoxide dismutases, suggesting that most of the $OH^{\cdot}$ is formed by a non-enzymic $O_2^{\cdot -}$-dependent reduction of the Fe^{3+}–EDTA chelate. 3. Since the reaction between the Fe^{3+}–EDTA chelate and H_2O_2 leads to a superoxide dismutase-inhibitable formation of $OH^{\cdot}$ radicals, it could suggest a much wider protective role for the superoxide dismutase enzymes in biological systems. 4. Urate produced during the reaction of xanthine oxidase and hypoxanthine limits deoxyribose degradation as well as the effectiveness of the superoxide dismutase enzymes to inhibit damage to deoxyribose by H_2O_2 and the Fe^{3+}–EDTA chelate. Some of this damage may result from an $O_2^{\cdot -}$-independent pathway to $OH^{\cdot}$ formation in which urate reduces the ferric complex.

INTRODUCTION

In the absence of high-energy radiation, hydroxyl radicals ($OH^{\cdot}$) are probably formed in biological systems only when transition-metal ions participate in a Fenton-type reaction. In this reaction suitable reduced metal complexes (M^+) decompose H_2O_2 to form the $OH^{\cdot}$ radical (eqn. 1):

$$M^+ + H_2O_2 \rightarrow M^{2+} + OH^- + OH^{\cdot} \quad (1)$$

In biological systems transition-metal ions are complexed, with various affinities, to other molecules. This usually dictates that Fenton reactions will take place at the site of metal binding (Samuni *et al.*, 1981; Gutteridge, 1984*a*). Such site-specific reactions often make $OH^{\cdot}$ radicals difficult to detect and scavenge (Gutteridge & Halliwell, 1982; Gutteridge, 1984*a*).

Recently it was observed that the 'superoxide-driven Fenton reaction' could be inhibited by CuZn-SOD at two different stages (Gutteridge, 1985). The enzyme inhibits the first stage by preventing $O_2^{\cdot -}$ from reducing the metal complex and in the second stage prevents formation of $OH^{\cdot}$ from complexed Fe^{3+} ions and H_2O_2 (a product of the dismutation reaction):

Stage 1:

$$Fe^{3+}\text{–EDTA} + O_2^{\cdot -} \xrightarrow[SOD]{2H^+} Fe^{3+}\text{–EDTA} + H_2O_2 \quad (2)$$

Stage 2:

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

$$Fe^{2+}\text{–EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{–EDTA} + OH^- + OH^{\cdot} \quad (4)$$

The superoxide dismutases so far isolated are known to contain copper + zinc, iron and manganese at their active sites (Fridovich, 1975, 1983). With the copper + zinc- and iron-containing enzymes their catalytic mechanisms have been shown to involve alternate substrate one-electron oxidation and reduction steps (eqn. 5):

$$\begin{aligned} Enz_{ox.} + O_2^{\cdot -} &\longrightarrow Enz_{red.} + O_2 \\ Enz_{red.} + O_2^{\cdot -} &\xrightarrow{2H^+} Enz_{ox.} + H_2O_2 \end{aligned} \quad (5)$$

However, such a simple scheme does not appear to apply to the manganese-containing enzyme (Pick *et al.*, 1974; McAdam *et al.*, 1977*a*). The copper, iron and manganese ions of all three types of SOD can be reduced with H_2O_2, although, unlike CuZn-SOD and Fe-SOD, Mn-SOD is not inactivated by H_2O_2 (McAdam *et al.*, 1977*b*). The following experiments were carried out to see whether the copper and manganese enzymes differed in their protective activities when added to an iron–H_2O_2 chelate reaction generating $OH^{\cdot}$ radicals.

MATERIALS AND METHODS

Materials

CuZn-SOD (bovine erythrocyte), 2-deoxy-D-ribose, catalase (bovine liver, thymol-free), albumin (human, fatty acid-free), xanthine oxidase (type 1) and hypoxanthine were from the Sigma Chemical Co., Poole, Dorset, U.K. Units of catalase and xanthine oxidase activity were as defined in the Sigma catalogue. Mn-SOD was a gift from Dr. J. Walker, M.R.C. Molecular Biology

Abbreviations used: SOD, superoxide dismutase; TBA, thiobarbituric acid.

Laboratory, Cambridge, U.K., and was further purified by gel filtration on Sephadex G-75. Mn-SOD was standardized by using $A^{1\%}_{280} = 13.2$ (Harris, 1977). All other chemicals were of the highest grade available from BDH Chemicals, Poole, Dorset, U.K.

The Fe^{3+}–EDTA complex was prepared as previously described (Gutteridge, 1984*b*). Reaction mixtures contained the reagents shown in appropriate Tables at the final reaction concentrations stated. The xanthine oxidase solution, containing 0.7 unit/ml, was treated with Chelex-100 resin to remove contaminating trace metals.

Deoxyribose degradation

The release of TBA-reactivity by an iron salt from deoxyribose (Gutteridge, 1981) was used as the assay procedure for the formation of OH$^{\cdot}$ radicals (Halliwell & Gutteridge, 1981). After damage to deoxyribose, TBA-reactivity was developed by adding 0.5 ml of TBA reagent (1%, w/v) in 0.05 M-NaOH and 0.5 ml of 2.8% (w/v) trichloroacetic acid. The tube contents were heated for 10 min at 100 °C and the resulting absorbance was read at 532 nm. Results shown are the means for three or more separate assays, which differed by less than 5%, and were calculated after the subtraction of appropriate blanks.

RESULTS

The addition of iron, copper, cobalt, manganese and titanium salts to H_2O_2 in the presence of deoxyribose showed that, of these transition-metal ions, only Mn^{2+} did not bring about OH$^{\cdot}$ formation (Table 1). This suggests that H_2O_2 does not react with Mn^{2+} to form OH$^{\cdot}$. However, both the CuZn-SOD and Mn-SOD inhibited $O_2^{\cdot-}$- and H_2O_2-dependent deoxyribose degradation in the presence of an Fe^{3+}–EDTA chelate (Tables 2 and 3).

An attempt to demonstrate, within a single experiment, the two SOD-inhibitable stages of the $O_2^{\cdot-}$-driven Fenton reaction are shown in Tables 2 and 3. After 1½ h the $O_2^{\cdot-}$-generating reaction had gone to completion, since there was no further production of urate (measured at 290 nm), allowing the spontaneous dismutation of $O_2^{\cdot-}$ to H_2O_2 to occur. When an Fe^{3+}–EDTA chelate

Table 1. Effect of transition-metal ions on H_2O_2-dependent deoxyribose degradation

Final reaction concentrations were: deoxyribose, 2.8 mM; H_2O_2, 1.1 mM; phosphate buffer, pH 7.4, 20 mM; metal ions, 0.1 mM.

	TBA-reactivity after deoxyribose degradation at 37 °C for 30 min (A_{532})
Control (phosphate buffer, pH 7.4 + deoxyribose + H_2O_2)	0.050*
+ Fe^{2+}	0.270
+ Fe^{3+}	0.110
+ Cu^{2+}	0.100
+ Co^{2+}	0.170
+ Mn^{2+}	0
+ Ti^{2+}	0.400

* Blank value, subtracted from other values listed in the Table.

Table 2. Inhibitory activity of CuZn-SOD and Mn-SOD on $O_2^{\cdot-}$ + Fe^{3+}–EDTA-chelate-stimulated deoxyribose degradation before and after spontaneous dismutation has occurred

The final reaction concentrations were: deoxyribose, 1.4 mM; phosphate buffer, pH 7.4, 29 mM; hypoxanthine (HXn), 0.67 mM; xanthine oxidase (XOD), 0.05 unit; proteins, 0.04 mg/ml; Fe^{3+}–EDTA (1:1.1), 0.14 mM.

	TBA-reactivity after deoxyribose degradation at 37 °C for 2 h	
	(A_{532})	[Inhibition (%)]
1. Blank 1 (deoxyribose + XOD)	0.079	
1. Blank 2 (deoxyribose + XOD + Fe^{3+}–EDTA)	0.087*	
3. Control (deoxyribose + XOD + HXn + Fe^{3+}–EDTA)	0.212	
+ CuZn-SOD	0.056	74
+ Mn-SOD	0.064	70
No further urate production (A_{290}) after 1½ h incubation		
Reaction mixture 3 incubated at 37 °C for 1½ h, then the following proteins added for a further 30 min incubation:		
+ CuZn-SOD	0.204	4
+ Mn-SOD	0.189	11
+ Catalase	0.189	11

* Blank value, subtracted from other values listed in the Table.

Table 3. Inhibitory activity of CuZn-SOD and Mn-SOD on deoxyribose degradation stimulated by an $O_2^{\cdot -}$-generating system after spontaneous dismutation had been allowed to occur, followed by the addition of an Fe^{3+}–EDTA chelate

The final reaction concentrations were: deoxyribose, 1.4 mM; phosphate buffer, pH 7.4, 29 mM; hypoxanthine (HXn), 0.67 mM; xanthine oxidase (XOD), 0.05 unit; proteins, 0.04 mg/ml; Fe^{3+}–EDTA (1:1.1), 0.14 mM.

	TBA-reactivity after deoxyribose degradation at 37 °C for 2 h	
	(A_{532})	[Inhibition (%)]
1. Blank (deoxyribose + XOD + Fe^{3+}–EDTA)	0.087*	
2. Control 1 (deoxyribose + XOD + HXn + Fe^{3+}–EDTA)	0.212	
3. Control 2 (deoxyribose + XOD + HXn)	0.002	
No further urate production (A_{290}) after 1½ h incubation		
Reaction mixture 3 (control 2) incubated at 37 °C for 1½ h, then the following reagents added for a further 30 min:		
+ Fe^{3+}–EDTA	0.196	
+ CuZn-SOD + Fe^{3+}–EDTA	0.137	30
+ Mn-SOD + Fe^{3+}–EDTA	0.124	37
+ Catalase + Fe^{3+}–EDTA	0.026	87
+ Albumin + Fe^{3+}–EDTA	0.187	5

* Blank value, subtracted from other values listed in the Table.

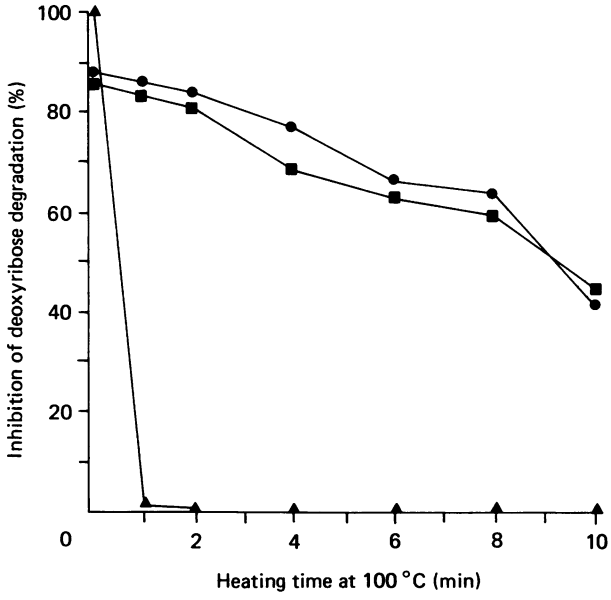

Fig. 1. Heat-denaturation of CuZn-SOD, Mn-SOD and catalase in 98 mM-phosphate buffer, pH 7.4, at 100 °C

Protein concentrations were 0.04 mg/ml. Percentage inhibition of deoxyribose degradation was determined as described in the Materials and methods section. Final reaction concentrations for deoxyribose and H_2O_2 were as shown in Table 4. ●, Mn-SOD; ■, CuZn-SOD; ▲, catalase.

was present at the start of the reaction addition of SODs and catalase after 1½ h had no significant inhibitory effects on deoxyribose degradation (Table 2), confirming that the reaction had essentially gone to completion. However, when Fe^{3+}–EDTA chelate was added after 1½ h incubation and incubated for a further 30 min, deoxyribose degradation was increased (Table 2), and this increase could be partly inhibited by the addition of SODs and substantially inhibited by the addition of catalase (Table 2). Albumin added as a control for non-specific radical-scavenging effects showed no significant inhibitory activity (Table 3). In this reaction the SODs and catalase were acting catalytically, since heat-denaturation in the presence of phosphate buffer substantially decreased their activities (Fig. 1). When urate, which is present in the xanthine oxidase $O_2^{\cdot -}$-generating system but not in the H_2O_2 reaction, was added to the H_2O_2 system lower inhibitory activities were seen for the SOD enzymes (Table 4). This decrease in activity was not protected against by the inclusion of albumin (0.15 mg/ml) (Table 4) or by the presence of other proteins such as γ-globulin, alcohol dehydrogenase and glutathione reductase (results not shown).

DISCUSSION

Mn^{2+} ions do not appear to react with H_2O_2, to form $OH^{\cdot}$, which presumably explains why Mn-SODs are not inactivated by H_2O_2 and also why *Lactobacillus plantarum* can accumulate Mn^{2+} to an internal concentration of some 25 mmol/l (Archibald & Fridovich, 1982). The CuZn-SODs function with the copper in the Cu^{2+} resting state and undergo univalent reduction and oxidation during the dismutation reaction (eqn. 5). The Mn-SOD has an Mn^{3+} resting state that also undergoes reduction and oxidation during dismutation, although the mechanisms are considerably more complex than those occurring with the CuZn- or Fe-SODs (for a brief review see Halliwell, 1984).

When $O_2^{\cdot -}$ is generated in the presence of an Fe^{3+}–EDTA chelate then the later addition of SODs or catalase does not influence the observed deoxyribose degradation, indicating that all the H_2O_2 formed had been decomposed. However, when $O_2^{\cdot -}$ generation is complete in the absence of an Fe^{3+}–EDTA chelate, then H_2O_2 formed by the spontaneous dismutation reaction can be decomposed by the addition of Fe^{3+}–EDTA

Table 4. SOD activity in the presence and in the absence of urate when deoxyribose degradation is stimulated by H_2O_2 + Fe^{3+}–EDTA chelate

The final reaction concentrations were: deoxyribose, 1.4 mM; phosphate buffer, pH 7.4, 29 mM; H_2O_2, 0.14 mM; proteins (except albumin), 0.04 mg/ml; albumin, 0.15 mg/ml; Fe^{3+}–EDTA (1:1.1), 0.14 mM; urate (if present), 0.14 mM.

	TBA-reactivity after deoxyribose degradation at 37 °C for 1½ h			
	With urate		Without urate	
	(A_{532})	[Inhibition (%)]	(A_{532})	[Inhibition (%)]
Blank (deoxyribose + Fe^{3+}–EDTA)	0.063*		0.096†	
Control (deoxyribose + Fe^{3+}–EDTA + H_2O_2)	0.253		0.700	
+ CuZn-SOD	0.145	57	0.191	84
+ Mn-SOD	0.130	65	0.184	85
+ Catalase	0.050	100	0.060	100
+ Albumin	0.305	0	0.757	0
+ CuZn-SOD + albumin	0.149	55	0.190	84

* Blank value, subtracted from other values listed in the Table for experiments performed in the presence of urate.
† Blank value, subtracted from other values listed in the Table for experiments performed in the absence of urate.

complex, thereby producing OH· radicals and increasing deoxyribose degradation. This 'second stage' was substantially inhibited by the addition of catalase but less inhibited by the SODs. Poor inhibition by the SODs could be partly related to the presence of urate, since less inhibition by SOD was observed when urate was added to the Fe^{3+}–EDTA chelate/H_2O_2 reaction. It is known that urate radicals can inactivate enzymes (Kittridge & Willson, 1984). However, the addition of several different proteins at 4 times the concentration of SOD failed to increase the percentage inhibition seen in the reaction, and did not, therefore, point to enzyme inhibition. Urate is a strong reducing agent able to reduce ferricyanide, and could, therefore, conceivably be providing an $O_2^{\cdot-}$-independent route to OH· formation. Urate will, of course, be present in the complete reaction system, which is inhibited by some 70% by the two SODs. The loss of inhibition by SODs seems to occur when Fe^{3+} chelate, H_2O_2 and urate are mixed. The fraction of the overall reaction that is not inhibited by SOD appears to increase as the rate of deoxyribose degradation is decreased by the presence of urate, a known radical scavenger (Matsuchita *et al.*, 1963; Ames *et al.*, 1981).

Several different experiments have shown that heat-denaturation of CuZn-SOD is not always a good control for its enzymic participation in reactions (Halliwell & Gutteridge, 1981; Gutteridge *et al.*, 1984), since after being heated it often retains and regains SOD activity. However, when heated for 10 min at 100 °C in 98 mM-phosphate buffer, pH 7.4, a substantial loss of activity was seen for both the SOD enzymes. Catalase, however, was almost completely inactivated by heating for 1 min at 100 °C. These differences confirmed that the SODs were not contaminated with catalase or the catalase with SODs.

These results confirm that the two stages of the $O_2^{\cdot-}$-driven Fenton reaction, when carried out as two separate experiments, are substantially inhibited by both CuZn-SOD and Mn-SOD. This suggests that the Fe^{2+} chelate formed in eqn. (3) in the 'stage 2' reaction does not participate in the Fenton reaction (eqn. 4), and that the ultimate Fe^{2+} reactant is formed by an $O_2^{\cdot-}$-reduction step. However, when inhibition is demonstrated in a single reaction system, with xanthine oxidase-generated $O_2^{\cdot-}$, the presence of urate appears to decrease the SOD-inhibitable part of the 'stage 2' reaction. This then appears to give the stoichiometry for OH· formation that might be expected if the iron(II) complexes were formed both directly (eqn. 3) and indirectly from the $O_2^{\cdot-}$ generated in eqn. (3).

REFERENCES

Ames, B. N., Cathcart, R., Schwiers, E. & Hochstein, P. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 6858–6862

Archibald, F. S. & Fridovich, I. (1982) Arch. Biochem. Biophys. **214**, 452–463

Fridovich, I. (1975) Annu. Rev. Biochem. **44**, 454–458

Fridovich, I. (1983) Annu. Rev. Pharmacol. Toxicol. **23**, 239–257

Gutteridge, J. M. C. (1981) FEBS Lett. **128**, 343–346

Gutteridge, J. M. C. (1984*a*) Biochem. J. **224**, 761–767

Gutteridge, J. M. C. (1984*b*) FEBS Lett. **172**, 245–249

Gutteridge, J. M. C. (1985) FEBS Lett. **185**, 19–23

Gutteridge, J. M. C. & Halliwell, B. (1982) Biochem. Pharmacol. **31**, 2801–2805

Gutteridge, J. M. C., Quinlan, G. & Wilkins, S. (1984) FEBS Lett. **167**, 37–41

Halliwell, B. (1984) Neurotoxicology **5**, 113–118

Halliwell, B. & Gutteridge, J. M. C. (1981) FEBS Lett. **128**, 347–352

Harris, J. I. (1977) in Superoxide and Superoxide Dismutase (Michelson, A., McCord, J. & Fridovich, I., eds.), pp. 151–157, Academic Press, London

Kittridge, K. J. & Willson, R. L. (1984) FEBS Lett. **170**, 162–164

Matsuchita, S., Ibuki, F. & Aoki, A. (1963) Arch. Biochem. Biophys. **102**, 446–451

McAdam, M. E., Fox, R. A., Lavelle, F. & Fielden, M. (1977*a*) Biochem. J. **165**, 71–79

McAdam, M. E., Lavelle, F., Fox, R. A. & Fielden, M. (1977*b*) Biochem. J. **165**, 81–87

Pick, M., Rabani, J., Yost, F. & Fridovich, I. (1974) J. Am. Chem. Soc. **96**, 7329–7333

Samuni, A., Chevion, M. & Czapski, G. (1981) J. Biol. Chem. **256**, 12632–12635

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