INHIBITION BY SUPEROXIDE DISMUTASE OF LINOLEIC ACID PEROXIDATION INDUCED BY LIPOXIDASE

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1. Introduction

The metalloprotein erythrocuprein has been known for 35 years [1], but the discovery of its enzymatic function by McCord and Fridovich [2] as a dismutase of superoxide radicals has occurred only 30 years after. More recently evidence has been produced that singlet oxygen is produced in biological systems [3–6], and that SOD[1], besides its dismutase activity, is capable of transforming singlet oxygen to ground state triplet oxygen [7,8]. Some reports, however, did not confirm the proposed 'singlet oxygen decontaminase' activity of the enzyme [10–12].

The problem has been reinvestigated by studying the effect of SOD on the peroxidation of linoleic acid by lipoxidase. This reaction, in fact, has been demonstrated to yield as an intermediate only O_2* and not superoxide radicals [4,10,11]. Thus an inhibition of linoleic acid peroxidation by SOD would be indicative that this enzyme can catalyze singlet to triplet oxygen transformation. Moreover, since O_2* has a half life of 2 μsec in H_2O [14], but ten-fold as high in D_2O [15], it is expected that a more pronounced inhibition of SOD would be visible in heavy water, as a consequence of a more favourable competition with the process of solvent induced decay of singlet oxygen.

[1] *Abbreviations:* SOD, superoxide dismutase, DPF, diphenylfuran, $Cu(Tyr)_2$, Tyrosyl–Tyrosine copper chelate.

2. Materials and methods

Soybean lipoxidase type I was obtained from Sigma Chemical Company, St. Louis, Mo. U.S.A. and used without further purification. Bovine erythrocuprein (Superoxide dismutase) was from Miles Laboratories, Inc., Kankaee, I11. U.S.A. and dissolved in 50 mM potassium phosphate pH 7.4. Deuterium oxide (approx. 99.7%) was from Sigma and Fluka AG, Buchs, Switzerland. Diphenylfuran was obtained from Eastman Kodak Co., Rochester, N.Y., USA. Linoleic acid (98%) was from Schuchardt, Munich, Germany. 50 μl were dissolved in 50 μl ethanol and mixed with 5 ml borate buffer of a pH or pD = 8.6. Appropriate amounts of this stock solution were diluted into borate buffer (pH or pD = 8.6).

Formation of lipid peroxides was recorded with a thermostated Hitachi-Perkin-Elmer spectrophotometer (Model 124) at 234 nm. The temperature was kept at 36°C.

$Cu(tyr)_2$ was a kind gift of Regina Brigelius, University of Tübingen, Germany. Water was distilled twice. All other reagents employed were of analytical grade.

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Table 1
Effect of different agents on the lipid peroxidation produced by lipoxidase

Additions	pmol peroxides formed/min (initial rate)	%	pmol peroxides formed (5 min incubation)	%
No inhibitor	153.18	100	682.10	100
5 μl DPF 6 mM in MeOH	78.25	51.1	366.30	53.7
5 μl MeOH	128.20	83.7	582.75	85.4
5 μl EtOH	158.17	103.2	715.95	105.0
20 μl boiled SOD 100 μM	149.85	97.8	599.40	87.9
20 μl native SOD 100 μM	103.23	67.8	449.55	65.8
10 μl native SOD 500 μM	59.94	39.1	266.40	39.0
20 μl native SOD 500 μM	49.95	32.6	233.10	34.1

Experimental conditions: Lipid peroxide formation recorded at 234 nm: 2.5 ml D_2O buffer, 0.2 M borate pD 8.6, 2.66 μM linoleic acid, 36°C. The reaction was started by adding lipoxidase to a final concentration of 8×10^{-9} M.

3. Result and discussion

The experiments reported in table 1 were performed in order to establish whether the lipoxidase–linoleic acid system, under our experimental conditions, produced singlet oxygen as detected by the increase in absorbance at 234 nm, due to the formation of hydroperoxides [13]. It can be seen in table 1 that in the presence of 8×10^{-9} M lipoxidase and 2.66×10^{-6} M linoleic acid indeed a time dependent increase in absorbance occurs, indicative of hydroperoxide production as the consequence of the intermediate formation of O_2* in the enzyme system. A confirmation that O_2* was formed under our conditions comes from the effect of diphenylfuran, an efficient and selective O_2* scavenger [16] which produced at a concentration of 1.2×10^{-5} an inhibition of 49%. In this system native SOD, but not the boiled enzyme, appears to be effective at a concentration of 2×10^{-6} M to reduce lipid peroxide formation to 39.1%. Since intermediates of the linoleic acid peroxidation other than O_2* such as peroxides and tetroxides [13] are not likely to react with SOD it appears that the inhibition of the peroxidation is a consequence of a decrease in O_2* levels caused by SOD. Homolytic cleavage of the oxygen-oxygen bond in hydroperoxides occurs easily and yields the highly reactive hydroxyl radical OH· [17]. Ethanol is known to quench this radical effectively with a rate constant of 1.85×10^9 M^{-1} sec^{-1} [18]. In our experiment 2.4×10^{-2} M ethanol did not affect appreciably the initial rate of peroxide formation.

The steady state concentration of $[O_2^*]_{ss}$ in a system in which linoleic acid and lipoxidase is present, and solvent relaxation of $[O_2^*]$ to ground state oxygen occurs, is given by the following equation [21]:

$$[O_2^*]_{ss} = \frac{V_0}{k_1 + k_2\,[L]}$$

where V_0 is the rate of formation of O_2*, k_1 is the first-order rate constant of O_2* relaxation to ground state, and k_2 [L] is the apparent rate constant of the reaction of singlet oxygen with linoleic acid to yield the hydroperoxide. If SOD is also present a decrease in the $[O_2^*]_{ss}$ should be seen as a consequence of the presence in the denominator in equation 1 of the quantity k_3 $[O_2^*]$, the second order rate constant of SOD if this enzyme is able to use O_2* as a substrate. Vice versa if relaxation to ground state occurs in D_2O, it is expected that the first order rate constant k_1 decreases up to ten-fold with the consequence of an increase in the $[O_2^*]_{ss}$. Since the level of $[O_2^*]_{ss}$ can be detected as formation of peroxides this parameter should be sensitive to the nature of the solvent and the presence of SOD. Moreover, the rate of formation of peroxides should be more sensitive to SOD in D_2O than in H_2O.

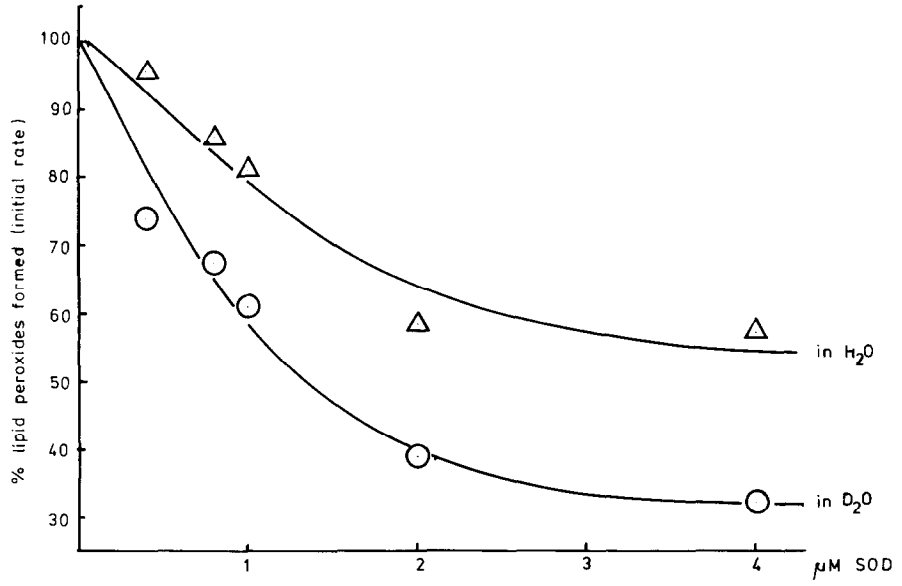

Fig. 1. Inhibition by SOD of lipid peroxide formation in D_2O and H_2O buffer.

Fig.1 shows the inhibition by SOD of lipid peroxide formation in D_2O and H_2O buffer. The initial rate in the absence of SOD (= 100%) was 153 pmol/min in D_2O and 133 pmol/min in H_2O. Qualitatively identical results were obtained determining the amount of hydroperoxides formed during an incubation time of 5 min. In each case there is a stronger inhibition by SOD in heavy water compared to light water. These results are in agreement with the consideration that at a given concentration of linoleic acid there is a competition for O_2* between the solvent and SOD, and that SOD can prevent the formation of hydroperoxides in this system.

Superoxide dismustase activity is not restricted to SOD. The free Cu^{2+}aq complex and cupric–peptide chelates show a very fast dismutation as studied by pulse radiolysis [19]. To investigate whether the reaction of SOD with O_2* is a specific property of the enzyme we tested the effect of the chelate $Cu(Tyr)_2$ in our system. The results are presented in table 2. Using the inhibition of adrenochrome formation in the system xanthine-xanthine oxidase as an indicator for dismutase activity we confirmed that $Cu(Tyr)_2$ is able to dismutase $O_2^{\cdot-}$. 2 μM SOD caused a 48% inhibition of adrenochrome formation.

Using the lipoxidase–linoleic acid system as a generator of O_2* the enzyme as shown above proved to be very effective in competing with linoleic acid for O_2*. The model complex, however, showed only a very low activity, similar to that exhibited by the boiled enzyme. These results strongly support the idea of the specificity of SOD only in the reaction with O_2* and not in that with $O_2^{\cdot-}$ radicals.

In conclusion it appears that the linoleic acid–lipoxidase system is competent in producing O_2* in line with previous evidence. Moreover, the effect

Table 2
$Cu(Tyr)_2$ as a quencher of O_2*·and $O_2^{\cdot-}$

	Lipoxidase system	Xanthine oxidase system
2 μM SOD	40%	48%
1.2 μM $Cu(Tyr)_2$	8%	35%

Experimental conditions: The lipoxidase–linoleic acid system was similar to that reported in table 1, but in H_2O buffer. The xanthine–xanthine oxidase system was that reported previously [20]. The % inhibition of the production of hydroperoxides at 234 nm was determined after 5 min reaction time.

of SOD to inhibit the formation of the peroxide strongly suggest that this enzyme is capable of catalyzing singlet triplet O_2 transformation. Such a conclusion is further substantiated by the effect of D_2O as a solvent, in which the inhibition of the peroxide production by SOD is more pronounced. While SOD reacts with either O_2^- or O_2* species, a copper chelate complex $Cu(Tyr)_2$ appears instead to be competent in catalyzing only the reaction with O_2^- as a substrate. This implies a greater structural complexity for the catalysis of O_2* decay to ground state than for O_2^- dismutation to H_2O_2 and O_2.

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