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CONCENTRATION-DEPENDENT INACTIVATION OF SUPEROXIDE DISMUTASE

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The inactivation yield of superoxide dismutase (superoxide: superoxide oxidoreductase, EC 1.15.1.1) from bovine erythrocytes, when Co^{60}-γ-irradiated in air, N_2 or N_2O-saturated solutions, increases exponentially with the initial enzyme concentration. In aerated solutions at ≤10 μM, the inactivation process continues in a concentration-dependent manner in the subsequent 72 h. This post-irradiation effect is inhibited by catalase. Above 10 μM, radiation-induced inactivation of the enzyme is partially reversed in a concentration-dependent manner and is not affected by catalase. In aerated and N_2O-saturated solutions, competitive scavenging of radiation chemical species by catalase and EDTA in combination reduces the inactivation yield by 80%; the residual yield remains dependent on enzyme concentration. Radiation-induced loss of copper and zinc initially exhibits a linear dose-response relationship and is less severe than the drop in enzyme activity.

Introduction

It is well established that the cupro-zinc superoxide dismutase (superoxide: superoxide oxidoreductase, EC 1.15.1.1) from bovine erythrocytes and other sources catalyzes the dismutation of superoxide radicals (O_2^-) to O_2 and H_2O_2, at a virtually diffusion-controlled rate via a redox couple (reactions 1, 2) involving the cyclic reduction and oxidation of copper atoms at the catalytic site [1–10].

$$Cu^{2+} + O_2^- \rightarrow Cu^{2+} - O_2^- \quad \text{or}$$
$$Cu^{2+} + O_2^- \rightarrow Cu^{+} + O_2 \qquad (1)$$

$$Cu^{2+} - O_2^- + O_2^- \xrightarrow{2H^+} Cu^{2+} + O_2 + H_2O_2 \quad \text{or}$$
$$Cu^{+} + O_2^- \xrightarrow{2H^+} Cu^{2+} + O_2 + H_2O_2 \qquad (2)$$

Pulse radiolysis experiments have given rate constants for reactions 1 and 2 between $1.2–1.4 \cdot 10^9$ and $1.9–2.2 \cdot 10^9$ $M^{-1} \cdot s^{-1}$, respectively [4,5]. Based on these values, the oxidation reaction appears the faster of the two, in agreement with Fielden et al. [11]. However, the results of later work indicate that the two reactions are equally fast with rate constants of $2.4 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ [12]. Hence, the activity of the enzyme is considered to be independent of its copper oxidation state [5]. The high rates support the high turnover of the enzyme approx. $8 \cdot 10^4$ s^{-1}, measured at 50 μM O_2^- [3] and provide a basis for maintaining the enzyme in active form during irradiation at moderate doses. Upon exposure to large radiation doses, the enzyme loses measureable amounts of activity due to reactions with radiolytic products of water which include the hydroxyl radical ($^{\cdot}OH$), the hydroperoxy radical ($HO_2^{\cdot}$), the hydrated electron (e_{aq}^-), the hydrogen atom ($H^{\cdot}$) and H_2O_2 [3,5,11,13]. Some of the ensuing reactions may only involve reduction of the coordinated copper [11] while others lead to the destruction of liganding histidine residues and other amino acids [14]. The first of these effects, copper reduction, continues to progress in the post-irradiation period as observed by a decrease in absorbance at 680 nm [12]. Catalase

inhibits the progression of this post-exposure bleaching and gradually reoxidizes the copper at rates dependent on the amount of catalase present [12].

More recently, radiation-induced inactivation of the enzyme in the liver of X-irradiated rats has been reported [15] and correlated with loss of the cupric electron spin resonance (ESR) signal and bleaching at 680 nm. Recovery of all three parameters was observed following storage of the extracted enzyme at 6°C for 6 days. These authors also found decreased activity and ESR signal of the enzyme when X-irradiated in vitro, but these changes were largely reversible upon storage at 6°C for 48 h. The reversible nature of loss in activity is, however, concentration dependent as will be shown in this report which also presents results of experiments with competitive free radical scavengers.

Materials and Methods

Chemicals. Superoxide dismutase from bovine erythrocytes was purchased from Dr. J.V. Bannister, University of Malta, Msida, Malta. Its activity was at least 3 600 units/mg as assayed by the nitroblue tetrazodium method (vide infra). Catalase from bovine liver (13 000 units/mg), glycine and riboflavin was obtained from Sigma Chemical Co., St. Louis, MI. EDTA was from J.T. Baker Chemical Co., Phillipsburg, NJ and nitroblue tetrazolium was from Eastman Chemicals. High purity gases used in the irradiations were supplied by Matheson, Whitby, Ontario.

Irradiation. Aliquots (0.5 ml) of freshly prepared solutions of superoxide dismutase in double-distilled water were irradiated at 21°C with Co^{60} γ rays (Gammacell 220, Commercial Products, Atomic Energy of Canada Ltd., Ottawa) at a dose rate of 46 Gy/min. The samples were gassed by bubbling air, N_2 or N_2O at a controlled flow rate before (3 min) and during irradiation. Each concentration of enzyme was irradiated to four graded doses selected such that the largest dose resulted in greater than 50% inactivation. The pH of representative enzyme solutions was measured before and after irradiation. Before the irradiation, the pH of superoxide solutions saturated with air, N_2 or N_2O was 7.29, 8.20 and 8.18, respectively, and in each case decreased $\leq$0.04 after exposure to 50% inactivation doses. With 100 μM EDTA present in the enzyme solutions, the pH in air, N_2 and N_2O was 7.37, 8.05 and 8.10, respectively, and after exposure to 50% inactivation doses decreased by 0.17 of a unit in the aerated solutions only. These decreases are not expected to affect enzyme activity [3] and the absence of a buffer avoided adding unwanted adventitious metal ions. All solutions were assayed for activity as soon as possible after irradition (0 h) and in some cases again 72 h later. The activities were graphically related as a function of dose to that in unirradiated samples. The dose-response curves were exponential in nature with an initial shoulder. The shoulder became progressively larger with increases in the initial enzyme concentration. The inactivation yield, that is, the number of molecules of enzyme inactivated per 100 eV of radiant energy adsorbed, was, unless indicated otherwise, calculated from the 50% inactivation doses read off from the dose-response curves. In all cases, the 50% inactivation dose fell within the exponential part of the dose-response curve and increased with the initial enzyme concentration.

Enzyme activity assay. Superoxide dismutase activity was measured by the photochemical nitroblue tetrazolium technique as previously described [16].

Metal analysis. Irradiated and control samples of enzyme were dialyzed for 72 h at 4°C against double-distilled water and analyzed (courtesy of Perron, G.) for Cu and Zn by atomic absorption as previously described (Lepock, J.R., Arnold, L.D., Petkau, A. and Kelly, K., unpublished data).

Results

In Table I the inactivation yields are listed as a function of the superoxide dismutase concentration and time of assay after irradiation of aerated solutions. The data show that the inactivation yield immediately after irradiation is strongly dependent on the enzyme concentrations, being low at 0.5 μM and much (113-times) higher at 1 000 μM. Moreover, at or below 10 μM, the yield increases in a concentration-dependent manner in the subsequent 72 h while at 50 μM, or above, the yield decreases within the same period. Thus, post-irradiation progression of the inactivation and reactivation processes is concentration dependent. These opposite trends greatly diminish the influence of concentration on the

TABLE I

Concentration-dependent inactivation yield of Co^{60}-irradiated (in air) bovine superoxide dismutase and its partial reactivation at higher concentration with storage at room temperature for 72 h. Inactivation yield is the number of enzyme molecules inactivated per 100 eV absorbed and this is based on the 20% inactivation doses.

[Enzyme] (μM)	Inactivation yield		
	0 h *	After 72 h	% Change
0.5	0.003	0.138	+4500
1.0	0.015	0.149	+893
5.0	0.084	0.179	+113
10	0.164	0.230	+40
50	0.20	0.19	−5
100	0.18	0.14	−22
1000	0.34	0.23	−33

* Measured as soon as practicable after irradiation.

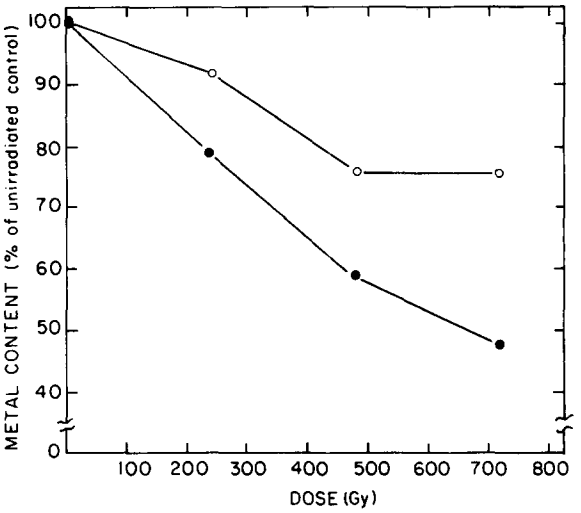

Fig. 1. Decrease with radiation dose in the content of copper (○) and zinc (●) in aerated bovine superoxide dismutase solutions, irradiated at 10 μM.

enzyme inactivation yield at the 72 h mark.

The increase in yield with post-irradiation time did not occur if catalase (100 μg/ml) was either present during the irradiation or added immediately after the exposure. This observation is consistent with the known inactivating action of H_2O_2 on the enzyme [11]. By contrast, addition of catalase before or after an irradiation did not change the post-exposure decrease in yield, seen at the higher enzyme concentrations. Thus, H_2O_2 is not implicated in this reversible component of the inactivation process, a conclusion consistent with the claim that H_2O_2-induced inactivation of the enzyme is not reversible [11].

The foregoing observations appear relevant to radiation effects on the enzyme in vivo. For instance, they suggest that, because tissue and cellular superoxide dismutase concentrations are generally less than 5 μM [17,18], the amount of enzyme inactivated or lost due to irradiation could increase in the immediate post-exposure period. This has been observed for tissues [19] and bone marrow cells [20]. On the contrary, the relatively higher enzyme concentration in liver [17,20] may place it in the range where there is either no change [20] or post-exposure recovery [15].

Radiation-induced inactivation of superoxide dismutase may involve loss of the coordinated copper and zinc since it is well established that the metal ions play important roles with respect to the catalytic activity of the enzyme [6–10]. Radiation-induced loss of copper and zinc in the enzyme at 10 μM is illustrated in Fig. 1. The loss of zinc is approx. linear with dose and more radiation sensitive than that of copper which plateaus after 480 Gy. This greater sensitivity of zinc is not unexpected in view of its weaker binding [21,22]. The copper and zinc levels, respectively, decrease to 76 and 48% of the unirradiated control after 720 Gy, a dose that is approx. 3-times that required for 50% inactivation. At these levels, the copper and zinc equal 1.07 and 0.80 gatoms/molecule enzyme, respectively. Therefore, in quantitative terms, not all of the observed decrease in enzyme activity is due to the concurrent loss of coordinated metal ions. Also, the linear dose dependence of the metal loss contrasts with that for inactivation which, in aerated solutions, was exponential after an initial curvilinearly-downward shoulder.

The concentration dependence of inactivation of irradiated superoxide dismutase distinguishes it from other enzymes such as ribonuclease, where the yield measured chemically is constant [23], and papain, where the yield at first increases rapidly and non-linearly over a narrow concentration range and then

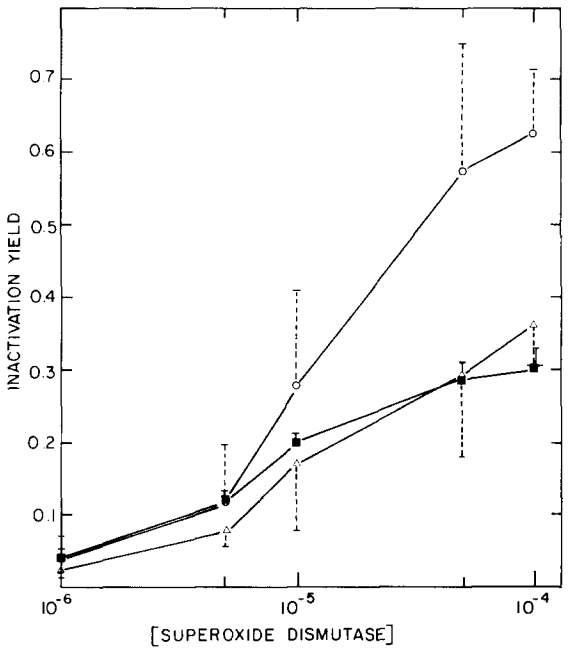

Fig. 2. Dependence of inactivation yield on superoxide dismutase concentration (M) following exposure to Co^{60} γ rays in air (■), N_2 (△) and N_2O (○) at room temperature. Error bars equal ± a standard deviation from four experiments.

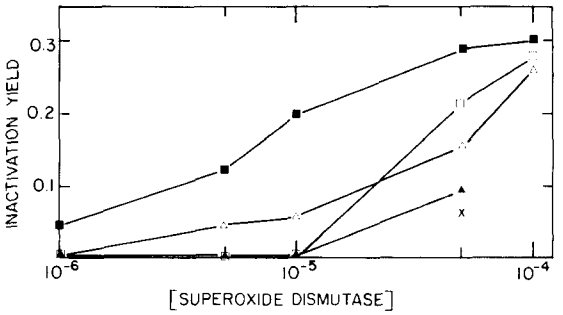

Fig. 3. Dependence of inactivation yield on superoxide dismutase concentration (M) of aerated solutions following exposure to Co^{60} γ rays: Control (■), 100 μM EDTA (□), 100 μg/ml catalase (△), 100 μM EDTA plus 100 μg/ml catalase (▲) and 10 mM EDTA plus 100 μg/ml catalase (X). Control curve is from Fig. 1.

plateaus to a limiting value [24]. Fig. 2 shows that inactivation increases with superoxide dismutase concentration, irrespective of whether the irradiated solutions are saturated with air, N_2 or N_2O. These data are best-fitted by exponential relationships (Table II). The correlation parameters reflect the fact that the highest yields, especially at high enzyme concentrations, occurred with N_2O, whereas the lowest yields, particularly at low enzyme concentrations, were seen in N_2-saturated solutions. At 10 μM, the N_2 and N_2O results are in good agreement with the yields given by Roberts et al. for the same concentration [13].

The exponential dependence of the inactivation yield on the initial enzyme concentration implies first of all that the active enzyme competes with reactions involving radiation chemical species formed radiolytically from water or possibly from itself. In terms of competition kinetics, the principal potential inactivating species in aerated aqueous solutions of the enzyme are $^{\cdot}OH$ and H_2O_2 (Ref. 13, Table II). As expected [11,12,13,27], scavenging the H_2O_2 with catalase (100 μg/ml) reduces the yield by 50%

TABLE II

Exponential increase in radiation-induced inactivation of superoxide dismutase (Y) with the enzyme's initial concentration (X) according to $Y = a\, e^{bx}$ and based on data of Fig. 2. Y is in number of inactivated molecules per 100 eV absorbed and X is in μM.

Irradiation condition	Radiation chemical species *	$Y = a\, e_{bx}$		
		a	b	Correlation coefficient
Air	O_2^-(2.8), $^{\cdot}OH$(3.25), H_2O_2(0.71)	0.102	0.013	0.73
N_2	e^-_{aq}(2.7), $^{\cdot}OH$(2.8), $H^{\cdot}$(0.55), H_2O_2(0.71)	0.066	0.020	0.78
N_2O	$^{\cdot}OH$(5.5), $H^{\cdot}$(0.55), H_2O_2(0.71)	0.109	0.021	0.79

* Values in parentheses refer to the yield per 100 eV of absorbed energy [25,26].

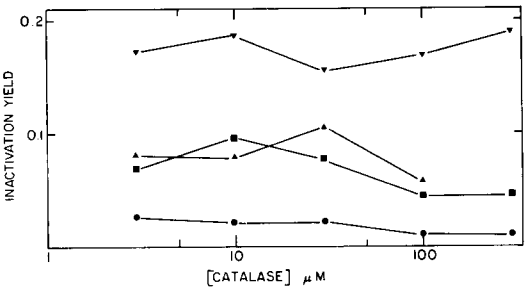

Fig. 4. Inactivation yield vs. the amount of catalase in aerated Co^{60} exposed superoxide dismutase solutions of 1 μM, (•) 5 μM (■), 10 μM (▲) and 50 μM (▲).

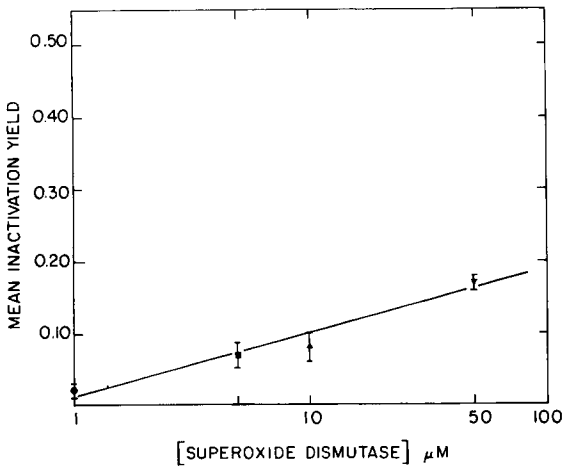

Fig. 5. Mean inactivation yield, as calculated from the data in Fig. 4, vs. the superoxide dismutase contcentration. Symbol connotation the same as in Fig. 4. Equation of line is $y = 0.04\ e^{0.03x}$ with a correlation of 0.80.

or more, except at 100 μM where the decrease was only about 14% (Fig. 3). Varying the amount of catalase within the 3–300 μg/ml range did not change the yield in a consistent pattern at a fixed concentration of superoxide dismutase (Fig. 4). However, the mean inactivation yield, calculated for each line in Fig. 4, increased exponentially with the superoxide dismutase concentration (Fig. 5), thus suggesting a competitive role for superoxide dismutase in reactions involving the remaining radiation chemical species.

In consideration of the reaction rates of H_2O_2 toward superoxide dismutase and catalase (Table III), 100 μg/ml catalase is sufficient to protect superoxide dismutase from the radiolytic quantity of H_2O_2. Therefore, the remaining potential inactivating species in aerated solutions are the 'OH and O_2^-. Although the efficiency of inactivation by O_2^- per se is small [5], the possibility exists that the radical may be converted to 'OH by adventitious transition metal ion complexes [28]. However, 'OH reacts with EDTA at a near diffusion controlled rate (Table III) so that the radical thus formed is unlikely to escape to react with neighbouring enzyme molecules. Moreover, EDTA in sufficiently high concentration will compete with superoxide dismutase for 'OH generated radiolytically (Table III). The expected protection of the enzyme by EDTA is clearly shown in Fig. 3 in which the combined effect of EDTA and catalase is also included. At 50 μM superoxide dismutase, the two radioprotective agents together lowered the yield by about 70% from 0.29 to 0.09. This decrease is equal to the sum of the inhibitory effects of EDTA and catalase taken separately and suggests independent

TABLE III

Some published rate constants relevant to present work ($M^{-1} \cdot s^{-1}$). Numbers in parentheses refer to references in bibliography.

Species	Radical scavenger		
	Catalase	EDTA	Superoxide dismutase
e^-_{aq}	$2.2 \cdot 10^{11}$ [29]	$1.5 \cdot 10^6$ [29]	$1.3 \cdot 10^{10}$ * [13]
'OH	$2.6 \cdot 10^{11}$ [29]	$3.0–4.2 \cdot 10^9$ [30,31]	$5.3 \cdot 10^{10}$ [13]
H'	relatively unimportant [35]	$6.5 \cdot 10^7$ [32]	$2.1 \cdot 10^{10}$ [13]
H_2O_2	$3.5 \cdot 10^6$ [33]	slow [34]	3.1 [24]

* Principle effect is reduction of copper, not enzyme inactivation.

action for each. The protective effect of their concerted action is enhanced at 10 mM EDTA (Fig. 3), a concentration at which the reactivity of 'OH toward EDTA and 50 μM superoxide dismutase is approx. 16-times in favour of the chelating agent (Table III). This dropped the yield to 0.06 or to about 20% of the control value in the absence of added reagents. The decrease is less than expected from the relative reactivities, a result also observed with N_2O-saturated solutions.

In N_2O-saturated solutions, 100 μM EDTA were generally more effective than 100 μg/ml catalase in reducing the inactivation yield of superoxide dismutase (Fig. 6). This greater effectiveness of EDTA reflects the higher 'OH yield in N_2O-saturated solutions (Table II), the consequence of which is to also increase the inactivation yield proportionately (Fig. 2, Table II). The combined action of catalase and EDTA in N_2O gave the lowest inactivation yields (Fig. 6), especially with 10 mM EDTA when scavenging of 'OH by the chelator is favoured (Table III). However, the 81% reduction in yield from 0.58 to 0.11 (Fig. 6) is less than expected in terms of the relative reactivities. The 19% residual yield compares with the 20% left over in air-saturated solutions under otherwise identical conditions (Fig. 3). Thus, the almost doubling of the initial 'OH yield in N_2O vs. aerated solutions is reflected in the inactivation yield left over after protection by catalase and EDTA. The residual yield is presumably due to incomplete 'OH scavenging despite favourable competition kinetics. One possible reason may be that some of the 'OH radicals lie within the molecular space of superoxide dismutase where they would not be readily accessible to EDTA. Another may be that EDTA radicals, formed through 'OH attack, inactivated the enzyme. When N_2O saturated solutions of 0.1–10 mM EDTA were first irradiated to 380 Gy and then mixed with 50 μM superoxide dismutase, no inactivation of the enzyme occurred. However, if the EDTA radicals are too reactive and short-lived, a measurable effect would not be observed in such steady-state tests. A plot of the inactivation yield against EDTA concentration indicates a change to lower slope at 50 μM (Fig. 7). The biphasic curve suggests two kinetically distinct processes of which 'OH and H_2O_2 scavenging by low concentrations of EDTA is perforce the primary one. The less competitive process is either a relatively slow reaction or a combination of reactions with countervailing effects. These might include an inactivating role for EDTA radicals on superoxide dismutase. Catalase increases the EDTA concentration at which this change in kinetics occurs (Fig. 7).

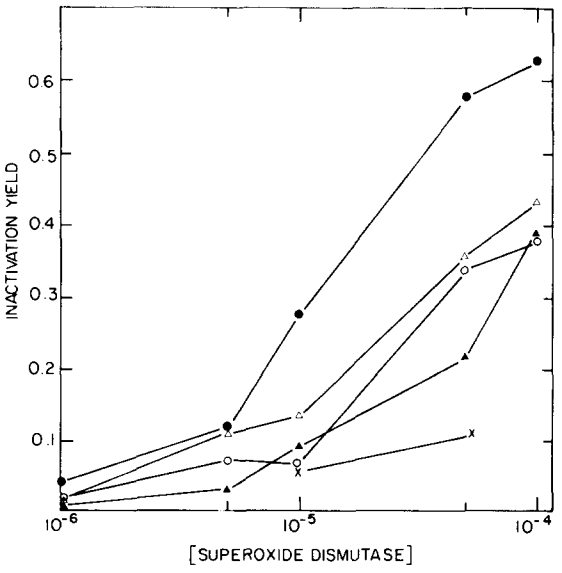

Fig. 6. Dependence of inactivation yield on superoxide dismutase concentration (M) of solutions exposed to Co^{60} γ rays under N_2O: Control (•), 100 μM EDTA (○), 100 μg/ml catalase (△), 100 μM EDTA plus 100 μg/ml catalase (▲) and 10 mM EDTA plus 100 g/ml catalase (X). Control curve is from Fig. 1.

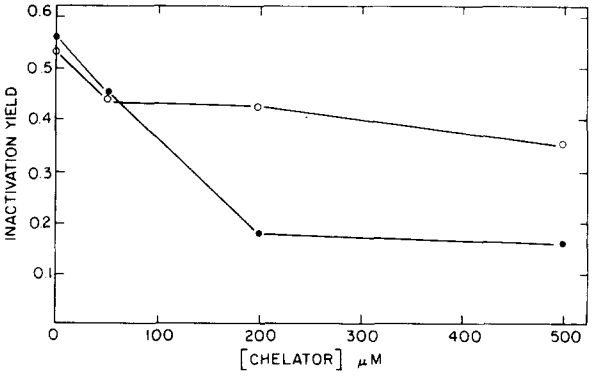

Fig. 7. Variation in inactivation yield vs. chelator concentration in 50 μM superoxide dismutase solutions exposed to Co^{60} γ rays under N_2O: (○) EDTA, (•) EDTA plus 30 μg/ml catalase.

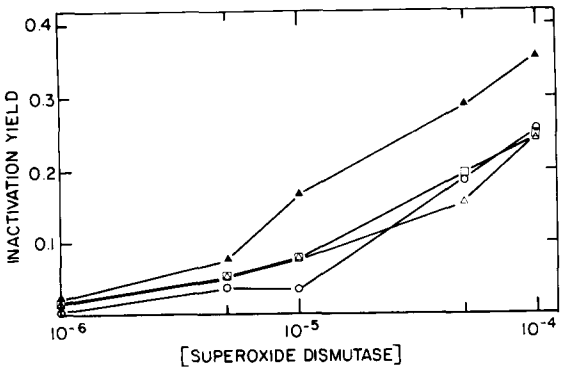

Fig. 8. Dependence of inactivation yield on superoxide dismutase concentration (M) of solutions exposed to Co^{60} γ rays under N_2: Control (▲), 100 μM EDTA (□), 100 μg/ml catalase (△) and 100 μM EDTA plus 100 μg/ml catalase (○). Control curve is from Fig. 1.

The result is not unexpected in view of the competitive 'OH scavenging potential of catalase and its catalytic destruction of H_2O_2 (Table III).

In N_2-saturated solutions, 100 μg/ml catalase reduced the inactivation yield of superoxide dismutase by 32–56% over the 1–100 μM range (Fig. 8). This reduction is the combined action of catalase on radiolytically generated H_2O_2 and its fast scavenging of e^-_{aq}, 'OH and H' (Table III). Amongst the latter, the effect of scavenging 'OH is the most important both in terms of relative reactivities and inactivation efficiency (Ref. 13, Table III). Protection of superoxide dismutase by EDTA at 100 μM was comparable to that by catalase and left a residual yield which increases with the superoxide dismutase concentration.

Discussion

The foregoing data consistently show that radiation-induced inactivation of superoxide dismutase is dependent on the initial enzyme concentration under conditions where several competitive reactions occur, involving dose-dependent yields of radiation chemical species. The fact that the concentration effect is reduced by as much as 80% through competitive free radical scavenging with catalase and EDTA strongly supports the application of competitive kinetics to any theoretical account of the inactivation process for this enzyme. In this respect, the inactivation of superoxide dismutase differs significantly from ribonuclease, where the inactivation yield is apparently constant [23], and from papain, where the theoretical analysis of the inactivation data assumes independence from the initial enzyme concentration [36].

Our use of competitive scavenging of radiation chemical species did not fully reduce the inactivation yield to a constant value, independent of the initial concentration. Several possible contributory factors for this result were suggested earlier, such as the formation of radicals within molecular (enzyme) spaces inaccessible to the scavengers used and the likelihood of reactions with EDTA radicals resulting in enzyme inactivation. The concentration effect may also indicate dissimilar rates of free radical reactions with active and inactive superoxide dismutase [36]. Such differences would arise from free radical attack on individual amino acid residues within the molecule, resulting in their chemical modification and possibly changes in the configuration of the enzyme [14,37,38]. Histidine and arginine are important constituents for superoxide dismutase activity [37–39] and their reactions with 'OH, in isolation at any rate, are among the fastest for amino acids generally [29]. Their reaction with 'OH may therefore affect the weighted average rate constant [36] of inactivated enzyme molecules toward the radical. In this connection, it was noted that the dose-response curves in N_2O-saturated solutions changed to lower slope at radiation doses resulting in more than 80% inactivation.

Interaction between inactivated and active enzyme, resulting in further loss of activity, is perhaps another factor contributing to the concentration effect. However, when 1 μM superoxide dismutase was irradiated to 460 Gy (>97% inactivation), mixed with 1 μM unirradiated enzyme for 1 h and then assayed, no decrease in activity of the latter was observed. The proposed interaction, if it exists at all, must therefore involve short-lived radicals of the enzyme protein and is not amenable for study by steady-state radiation exposures.

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