INHIBITION OF NITROBLUE TETRAZOLIUM REDUCTION BY CUPREIN (SUPEROXIDE DISMUTASE), $Cu(tyr)_2$ AND $Cu(lys)_2$

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1. Introduction

Although there are considerable doubts regarding the substrate specificity of superoxide dismutase for the superoxide anion, many workers concluded the participation of O_2^- in a reaction, whenever superoxide dismutase displayed an inhibitory effect. For example, such conclusions were drawn for the intestinal tryptophan 2,3-dioxygenase reaction [1] and for the autoxidation of epinephrine [2]. Otherwise, a reaction characteristic for superoxide and occurring in the same time (co-oxidation of epinephrine, co-reduction of oxidized cytochrome *c* or tetrazolium salts), which could be inhibited by superoxide dismutase was taken as evidence for the participation of the superoxide radical anion in the original reaction, e.g. the autoxidation of both hemoglobin [3] and ferredoxin [4]. Furthermore, the reaction of pig kidney diamine oxidase [5], the NADPH-cytochrome *c*-reductase reaction [6] and during the phagocytosis by leukocytes. [7,8].

However, since the discovery of McCord and Fridovich that cuprein exhibited a superoxide dismutase activity [9], many other Fe- and Mn-proteins and small transition metal complexes were found to exhibit a similar and even better superoxide dismutase activity [10–16]. The substrate specificity was better for reactions during which the generation of singlet oxygen and/or other excited oxygen species was inhibited by the cupreins [17–19].

Nevertheless, the inhibition of the reduction of nitroblue tetrazolium by superoxide dismutase was used as an assay for this activity [20–22]. Lately, however, it could be demonstrated that the inhibition of the formazan formation during the phagocytosis of latex particles by polymorphonuclear leukocytes was not specific. Apart from the known superoxide dismutases a great number of other proteins were able to inhibit the reduction of nitroblue tetrazolium [23].

In this context, it seemed of great interest to examine the specificity of this reaction compared to the small copper amino acid chelates which proved as good superoxide dismutases as the protein bound Cu(II) of native cuprein [13,14].

2. Materials and methods

Catalase, xanthine oxidase and xanthine were from Boehringer, Mannheim, potassium superoxide was from K & K, Hollywood, nitroblue tetrazolium was from Serva, Heidelberg, CuO, L-lys, L-tyr and dimethylsulphoxide were from Merck, Darmstadt. All chemicals employed were of analytical grade or better.

Cu, Zn-superoxide dismutase was isolated following the procedure of Weser et al. [24]. The copper chelates were prepared according to the method given in [13,14].

The activity tests were carried out in a modified form after Fried et al. [20] using a Unicam SP 1800 spectrophotometer.

2.1. *The xanthine–xanthine oxidase system*

A reagent mixture was prepared containing phosphate buffer, 0.1 M, pH 7.8 (11 parts), 1% gelatine solution in the phosphate buffer (5 parts), catalase, 6 units/ml (1 part) and nitroblue tetrazolium solution in the same buffer, 4 mg/ml (3 parts). To carry out

the assay 2 ml of the reagent mixture were pipetted in a 10 mm light path glass cuvette. 250 µl of a xanthine oxidase solution in the phosphate buffer (0.2 U/ml) and 250 µl of the test solution were added. After 30 sec the reaction was started by adding 500 µl of a xanthine solution, 1 mM in the phosphate buffer. The absorbance at 540 nm was followed spectrophotometrically until the reaction was completed.

2.2. *The KO_2 system*

In a 10 mm light path glass cuvette 2 ml of phosphate buffer, 0.1 M, pH 7.8, were pipetted; 100 µl of catalase solution, 6 U/ml were added followed by 100 µl of aquous nitroblue tetrazolium, 4 mg/ml and 200 µl of the solution to be examined. The reaction was started with 100 µl of saturated potassium superoxide solution in dimethylsulphoxide and monitored at 540 nm.

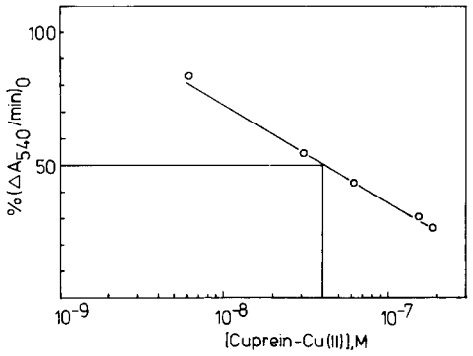

Fig.1. % Activity in the nitroblue tetrazolium assay of different concentrations of superoxide dismutase using the xanthine–xanthine oxidase system. Phosphate: 100 mM, gelatine: 1.4 mg/ml, nitroblue tetrazolium chloride: 342 µg/ml, xanthine: 0.14 mM, xanthine oxidase: 16.8 mU/ml, catalase: 2U/ml.

3. Results

The apparently established nitroblue tetrazolium assay for superoxide dismutase activity was examined regarding the specificity of cuprein bound Cu(II) and some low mol. wt. Cu(II) chelates.

3.1. *The xanthine–xanthine oxidase system*

Following the absorbance at 540 nm in the presence of different concentrations of either chelated Cu(II) linear curves could be observed which reached a certain end value. Both the slope and the end value depended upon the concentration of the inhibitor under investigation. The increase in the absorption at 540 nm per minute (ΔA_{540}/min) was determined in the absence and in the presence of different amounts of Cu(II). Considering ΔA_{540}/min in the absence of the chelates as the value for 100% activity, the activities in the presence of the chelates were calculated in percent of this value and plotted against the logarithm of the copper concentration. The plots were linear and allowed a simple determination of the Cu(II)-concentrations required to yield a 50% inhibition of the reaction (figs. 1 and 2, and table 1a).

Similar results were obtained when the values for maximal absorption at 540 nm were compared (table 1b).

Scavengers of singlet oxygen (1,4-diazabicyclo-(2,2,2)-octane and 1,3-diphenylisobenzofuran) had not effect even in ^2H_2O on the reduction of nitroblue tetrazolium in the assay.

3.2. *The KO_2-system*

To avoid uncontrolled side reactions of the components required in the xanthine–xanthine oxidase system KO_2, a strictly inorganic system, was employed instead. The absorption of the formazan colour at 540 nm following the addition of a saturated solution of potassium superoxide in dimethylsulphoxide was measured in the absence and in the presence of different copper chelates. The Cu(II)-concentrations which yielded a 50% inhibition were determined

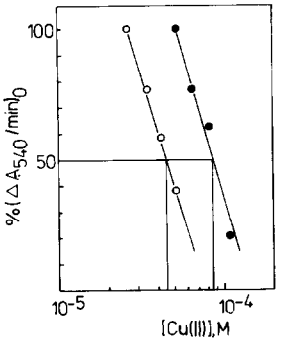

Fig.2. % Activity in the presence of different amounts of $Cu(tyr)_2$ (○ - ○ - ○), and $Cu(lys)_2$ (● - ● - ●). For experimental details see legend to fig.1.

graphically. It can be clearly seen that again the protein bound Cu(II) was of remarkable specificity compared to the copper chelated to tyrosine or lysine (table 2).

3.3. *Inhibition of the Cu(II)-activity*

Blocking of the Cu(II) by potent ligands such as cyanide should give some indication whether or not the metal ion is involved in the inhibition of the formazan formation. Thus, approximately equimolar concentrations of cyanide were added to the assay system. Cyanide itself had no inhibitory effect in the absence of Cu(II). By contrast, the Cu(II) mediated inhibition could be reversed almost completely regardless whatever Cu(II)-compound was used. Even in the xanthine–xanthine oxidase system the action of the cuprein bound copper could be blocked by cyanide, which itself did not influence the xanthine oxidase.

Table 1
Concentrations of the chelated Cu(II) which yield a 50% inhibition of the generation of formazan per min using the xanthine–xanthine oxidase system

Part a

Inhibitor	Concentration of chelated Cu(II) required to yield 50% inhibition (μM)	Ratio Cu^{2+} (cuprein): Cu^{2+} (chelate)
Cuprein	0.04	–
$Cu(tyr)_2$	45	1 : 1125
$Cu(lys)_2$	86	1 : 2150

Part b

Inhibition of the nitroblue tetrazolium reduction by the xanthine–xanthine oxidase system using Cu(II) chelates; the values for maximal absorption at 540 nm were taken as basis of comparison

Inhibitor	Concentration of chelated Cu(II) required to yield 50% inhibition (μM)	Ratio Cu^{2+} (cuprein): Cu^{2+} (chelate)
Cuprein	0.04	–
$Cu(tyr)_2$	33	1 : 825
$Cu(lys)_2$	75	1 : 1875

Experimental conditions as in the caption to fig.1.

Table 2
Concentrations of the Cu(II) chelates which yield a 50% inhibition of the reduction of nitroblue tetrazolium by a saturated solution of potassium superoxide in dimethylsulphoxide

Inhibitor	Concentration of chelated Cu(II) required to yield 50% inhibition (μM)	Ratio Cu^{2+} (cuprein): Cu^{2+} (chelate)
Cuprein	0.06	–
$Cu(tyr)_2$	22	1 : 367
$Cu(lys)_2$	25	1 : 417

Phosphate: 100 mM, nitroblue tetrazolium chloride: 1.6 mg/ml, catalase 2.5 U/ml.

4. Discussion

Although $Cu(tyr)_2$ and $Cu(lys)_2$ showed nearly the same activity towards superoxide as the native superoxide dismutase in pulse radiolysis experiments even in the presence of the natural chelator serum albumin [13,14], their ability to inhibit the reduction of nitroblue tetrazolium was three orders of magnitude lower than the inhibitory effect of native erythrocuprein. This allows considerable doubts, that the generation of the blue formazan is exclusively due to the effect of the superoxide anion. Similar doubts were presented by Amano et al. [23], who observed that proteins other than the known superoxide dismutases were able to suppress the formazan generation during the phagocytosis of latex particles by polymorphonuclear leukocytes with the same effectiveness as the cupreins. Using the xanthine–xanthine oxidase system we observed a higher specificity for the inhibition of the nitroblue tetrazolium reduction by native erythrocuprein compared to the effect of the small copper chelates than in the case when potassium superoxide was used as the reducing factor. The reactivity of $Cu(tyr)_2$ was raised three-fold, that of $Cu(lys)_2$ even five-fold, when the tetrazolium salt was reduced by the KO_2-solution. In the case of the xanthine–xanthine oxidase system many more reactive species could be generated than in the case of KO_2. Massey has postulated, that the adducts of oxygen and flavins in flavoenzymes could yield different species depending on the surrounding protein moiety [25]. Even electrons might be released which could account for a part of the nitroblue tetrazolium reduction.

Anyway, although it is tempting to regard the inhibition of various reactions by superoxide dismutases as an evidence for the participation of the superoxide anion in these reactions, such conclusions should not be drawn until the substrate specificity of these enzymes is proved.

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