On the Action of Diethyldithiocarbamate as Inhibitor of Copper-Zinc Superoxide Dismutase

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Z. Naturforsch. **34 c,** 1292–1294 (1979);
received May 2/August 8, 1979

Superoxide Dismutase, Diethyldithiocarbamate, Inhibition, Copper Complex

The rate constants of the reactions between pulse radiolytically produced superoxide radicals and the Cu(II) chelate of diethyldithiocarbamate were determined at pH 7.0. It was found that diethyldithiocarbamate forms a copper complex, which has no dismutating activity. The removal of the protein bound copper in superoxide dismutase by diethyldithiocarbamate yields the same effect as coordination of the copper in the enzyme.

Until now three different types of superoxide dismutase (SOD) are found in living organisms: cyanide-sensitive copper-zinc enzymes in eukaryotes, cyanide-insensitive mangano enzymes mainly in eukaryotic algae and in mitochondria of eukaryotes, and cyanide-insensitive iron enzymes in prokaryotes [1]. All these enzymes catalyze the dismutation of superoxide radicals yielding hydrogen peroxide and molecular oxygen. In mammalian tissues the cytoplasmic copper containing enzyme makes up about 80% of the total SOD activity [2]. Besides cyanide, which is not applicable to *in vivo* experiments, certain copper chelating compounds act as inhibitors of the copper enzyme [3, 4], while others do not [3, 6]. Penicillamine with its high chelating activity caused only a small decrease in SOD activity of various rat tissues [5], but no effect was observed *in vitro* [5, 7]. The copper chelating agent diethyldithiocarbamate (DDC) has been shown to cause marked inactivation of SOD both *in vitro* and *in vivo* in mice [6, 7]. However, it remained open, why diethyldithiocarbamate is an inhibitor of copper containing SOD and penicillamine is not. The present report describes the results of a pulse radiolysis study comparing reactions of superoxide radicals with DDC and its copper chelate with published [8] results of reactions of Cu-penicillamine.

Abbreviations: SOD, superoxide dismutase (E. C. 1.15.1.1.); DDC, diethyldithiocarbamate.

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0341-0382/79/1200-1292 $ 01.00/0

Experimental

All chemicals used were of analytical grade purity. Diethyldithiocarbamate (DDC) was purchased as sodium salt from Sigma, Na-EDTA from Schuchardt (both in Munich). Pulse radiolysis experiments were carried out using a Febetron unit. A Xenon lamp, Osram XBO 450 W 4, served as light source. The optical detection system was composed of a Zeiss M4QIII monochromator, an EMI 9659 QB photomultiplier unit and a Datalab 905 transient recorder. The accelerator delivered electron pulses with an energy of 1.8 MeV and 40 nsec duration. Triply distilled and pyrolized water was used for the experiments. The pH was adjusted with NaOH and no buffers were used. The solutions contained 0.01 M sodium formate and were saturated with oxygen in order to convert all primary water radicals into superoxide radicals. The orders of the reactions were determined by regression analysis. The reactions were monitored by absorption measurements at 250 nm. All rate constants represent the constants of second order reactions. In the cases of spontaneous dismution evaluation of the decay kinetics of superoxide radicals resulted in second order type reactions directly. When substrates reacted with O_2^-, the reaction showed pseudo first order type kinetics, from which with regard to the concentration of substrate, the second order rate constants were determined. Further details are described in ref. [8].

Results and Discussion

The absorption spectra and decay kinetics of pulse radiolytically produced superoxide radicals were determined in the presence of DDC and its copper chelates at pH 7.0. The absorption spectra of O_2^- in the presence and absence of DDC were identical (data not shown), indicating that no absorbing reaction product was formed. For the spontaneous dismutation of O_2^- a rate constant of 1.3×10^5 M^{-1} sec^{-1} was determined. In the presence of DDC no significant increase of the rate constants could be detected. For the copper ion (10^{-5} M $CuSO_4$) catalyzed dismutation of O_2^-, a marked increase of the rate constant was found, which by addition of EDTA came down again to a value of the spontaneous decay of superoxide (Table I).

In the following set of experiments the concentration of $CuSO_4$ was 10^{-5} M throughout, while differ-

Table I. Second order rate constants of the reactions between superoxide, Cu(II), diethyldithiocarbamate (DDC), and the copper chelates of DDC and EDTA. The procedure of evaluation is described in Experimental.

$CuSO_4$ [M]	DDC [M]	EDTA [M]	k_2 [M^{-1} sec^{-1}]
---	---	---	$1.3 \pm 0.2 \times 10^5$
---	9.1×10^{-7}	---	$2.8 \pm 0.2 \times 10^5$
---	4.8×10^{-5}	---	$3.4 \pm 0.9 \times 10^5$
10^{-5}	---	---	$1.3 \pm 0.1 \times 10^9$
10^{-5}	---	9.1×10^{-6}	$1.1 \pm 0.2 \times 10^8$
10^{-5}	---	4.8×10^{-5}	$1.8 \pm 0.3 \times 10^5$
10^{-5}	9.1×10^{-6}	---	$4.3 \pm 0.5 \times 10^8$
10^{-5}	4.8×10^{-5}	---	$5.7 \pm 2.2 \times 10^6$
10^{-5}	9.1×10^{-5}	---	$5.0 \pm 2.3 \times 10^5$

ent amounts of DDC were added. The addition of DDC to the $CuSO_4$ solution immediately resulted in the formation of a yellow complex. When the concentration of $CuSO_4$ was just a little higher than that of DDC, the rate constant of superoxide dismutation reaction was $k_2 = 4.3 \times 10^8$ M^{-1} sec^{-1}. This indicates that a certain amount of copper ions was no longer available for catalysis. Increasing the concentration of DDC resulted in decreasing of the rate constants down to values in the range of that of the spontaneous dismutation of O_2^-.

In the experiments described by Heikkila and Cohen, the incubation of SOD with DDC led to the formation of an intense yellow color, which was not extractable with organic solvents, with a concomitant loss of enzymic activity [7]. The yellow color of the dialyzed DDC-inactivated enzyme became extractable after incubation with $CuSO_4$, releasing a fully active superoxide dismutase. The authors suggested that DDC binds to the copper without removing it from the protein. In the same study penicillamine and other compounds had no inhibitory effect on *in vitro* SOD activity. *In vivo* administration of DDC to mice resulted in a large decrease in SOD activity of mouse brain, liver and blood.

The copper complexes of a number of chelating agents have been found to exert the same catalytic activity as the copper containing superoxide dismutase. Besides the aquocomplex of the copper ion, this applies to the copper chelates of some amino acids [9, 10] and salycilates [11]. However, these complexes show less resistance to the treatment with strong chelators as EDTA than the natural enzyme. In this respect the Cu-penicillamine complex behaves exceptional. In a pulse radiolysis study [8] it was found that in pure aqueous systems Cu-penicillamine (10^{-6} M) catalyzes the superoxide dismutation with a rate constant of $k_2 = 4.5 \times 10^8$ M^{-1} sec^{-1}. When EDTA ($10^{-8} - 10^{-5}$ M) was present, Cu-penicillamine (9.1×10^{-7} M) catalyzed the dismutation reaction also with rate constants higher than $k_2 = 10^8$ M^{-1} sec^{-1}. In the same study Cu-penicillamine exhibited high resistance against cyanide, which is known to be a potent inhibitor of Cu-Zn superoxide dismutase. Excessive cyanide did not inhibit the dismutative action of Cu-penicillamine in a study, were carotene bleaching by oxygen radicals, produced by the xanthine/xanthine oxidase system, was investigated [12].

The results of the present study show that neither the Cu-EDTA nor the Cu-DDC complex have relevant dismutating activities. Referring to the efficacy of these two strong chelators, EDTA brings about the same effect with lower concentrations than DDC. It is known that in the Cu(II)-EDTA complex the copper can be six-fold coordinated [13] preventing, as we assume, the course of the redox cycle necessary for dismutation. From the results presented here it is suggested that more than one molecule of DDC is necessary for six-fold coordination, which on the other hand seems to be possible for DDC. For the action of an inhibitor of the copper containing superoxide dismutase the additional coordination of the protein bound copper has the same effect as the removal of copper from the protein by the chelator, as long as the resulting copper complex has no dismutative activity. This is apparently the case for DDC, but not for penicillamine, which forms a stable copper complex with a dismutative activity comparable to the natural enzyme. DDC can be given to experimental animals in large doses [14]. Its inhibitory effect on copper containing superoxide dismutase makes it helpful for the investigation of the biological significance of this enzyme.

Acknowledgement

Special thanks go to Mrs. Ch. Fuchs for expert technical assistance.

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