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REPLACEMENT OF NATIVE COPPER IN SUPEROXIDE DISMUTASE WITH COPPER-64 WITHOUT MUCH LOSS IN ITS ENZYMATIC ACTIVITY

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Superoxide dismutase, containing copper and zinc, has been labeled with copper-64 by incubating the prepared apoenzyme with cupric(65) chloride at room temperature. No significant loss in the enzymatic activity was observed after labeling. The incorporation of copper-64 was ascertained by starch gel electrophoresis and high performance liquid chromatography. The labeling efficiency was found to be >95%.

Introduction

Copper in whole blood is distributed in several fractions between erythrocytes and plasma. About 60% of copper in red cells is associated with superoxide dismutase (SOD) containing copper and zinc.[1] The Cu – Zn SOD is composed of two identical subunits, each containing one atom of copper. It catalyzes the dismutation of superoxide via the following reactions:[2–4]

$$SOD - Cu^{2+} + O_2^- + H^+ \longrightarrow SOD - Cu^+ + O_2 \text{ (reductive)}$$

$$SOD - Cu^+ + O_2^- + H^+ \longrightarrow SOD - Cu^{2+} + H_2O_2 \text{ (oxidative)}$$

$$\text{Net: } 2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2.$$

Only the copper atoms undergo oxidation-reduction cycling during the dismutation of O_2^-. The zinc atoms play a strucctural role.

Bovine liver SOD, Orgateine,[5] which contains copper and zinc has been marketed for human use for the treatment of rheumatoid arthritis.[6] Its effectiveness has been

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compared to gold compounds and penicillamine. As far as its pharmacology is concerned, much remains to be investigated. We are of the opinion that preparation of ^{64}Cu-labeled SOD and subsequent study of its distribution in body will be of considerable help in this regard. It has been demonstrated[7] that native copper of the protein, SOD, can be removed and reincorporated without much loss in its activity. By making use of this property, the native copper of SOD is replaced with copper-64 in the present work.

Experimental

Materials

All chemicals were reagent grade and were used without further purification. Bovine SOD from bovine erythrocytes, M. W. 34000, was purchased from Sigma Chemical Co. $^{64}CuCl_2$ was prepared in this laboratory by the following method: Copper turnings (5 mg) were irradiated at a neutron flux of $2 \cdot 10^{13}$ n $\cdot$ cm^{-2} $\cdot$ s^{-1} at full power in the Pakistan Research Reactor (PARR) for 5 minutes. The activity thus obtained was 363 μCi. The irradiated turnings were dissolved in sufficient quantity of aqua regia. The resulting solution was evaporated to almost dryness. To the residue concentrated hydrochloric acid (a few drops) was added and evaporation was repeated followed by addition of distilled water to obtain $^{64}CuCl_2$ solution.

Methods

Preparation of apoprotein and ^{64}Cu-labeled protein: The apoprotein from SOD was obtained according to ROTILIO et al.[8] by reducing the native copper with an excess of potassium ferrocyanide and dialyzing for 18 hours at 4 °C against 0.05M Tris – HCl buffer, containing 0.05M KCN at pH 8. This method ensures removal of copper with no loss of zinc. The apoprotein (10^{-6}M) thus obtained was incubated with $^{64}CuCl_2$ (10^{-4}M) at room temperature for 30 minutes to obtain ^{64}Cu-labeled SOD.

Electrophoresis: Although acrylamide gel[9] and cellulose acetate[10] techniques are available, starch gel electrophoresis was considered suitable as it provides a clear picture of both holo- and apoprotein.[8] This was carried out according to POULIK.[11] After staining, the bands were cut and counted for radioactivity using a well-type gamma-counter.

Chromatography: High performance liquid chromatography (HPLC) was carried out by Varian 3600 chromatograph using Zorbax reversed phase (octadecylsilane bonded to 5 μm spherical silica particles), 4.6 mm × 250 mm, and Wescan cation-exchange (polymer supported), 2.1 mm × 250 mm, columns and UV-100 programmable variable wavelength detector. The chracteristic λ_{max} 258 nm of SOD was used for these measurements. The eluent used was 0.0025M phosphate buffer (pH 7.8) with a flow-rate of 1 ml $\cdot$ min^{-1}.

Analysis of copper and zinc: Copper and zinc in the holo- and apoprotein was determined by the use of Hitachi Z-8000 atomic absorption spectrophotometer with electrothermal atomization and Zeeman background correction. The instrument coditions used were those recommended by the manufacturer.

Enzymatic activity measurement: The dismutase activity was measured according to McCORD and FRIDOVICH.[7]

Results and discussion

The results of copper and zinc analysis for the protein and apoprotein are given in Table 1. It is seen that about 98% copper has been removed without any significant loss of zinc in the preparation of apoprotein by the method used. It has been reported[7]

Table 1
Analysis of copper and zinc before and after dialysis of SOD

Treatment	Copper, g-atom · mol^{-1}	Zinc, g-atom · mol^{-1}
Before dialysis	1.95	2.10
After dialysis	0.04	2.00

Table 2
Specific activity of native and labeled SOD

Compound	Specific activity, units · mg^{-1}
Native SOD	3000
Apoprotein	<1
Labeled SOD	2395

that 80% activity is resumed during the replacement of native copper with the external copper. The activity measurements before and after labeling show (Table 2) that about 80% of the enzyme activity is resumed suggesting apparently no loss during the process of radio-labeling of the enzyme which could be possible due to a radiation effect. However, at very high doses of radiation as compared to this, up to 20% losses of activity have been reported.[12] The incorporation of ^{64}Cu was ascertained by starch gel electrophoresis. The electrophoretic behavior of the protein, the apoprotein and the

radio-labeled protein is shown in Fig. 1. There were two types of radioactivity found in the electrophoretograms, i.e., the bound and free activity. The band representing the radio-labeled protein contained over 95% of the bound radioactivity. The chromatograms obtained through HPLC also confirm the reconstitution of the protein

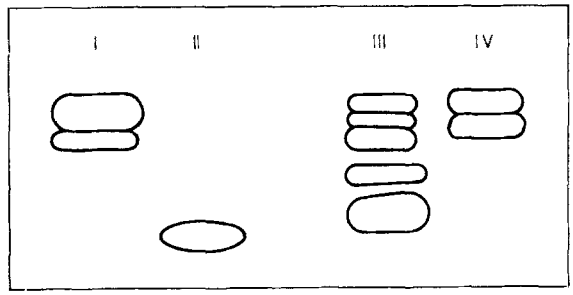

Fig. 1. Electrophoresis pattern of I – holoprotein, II – unlabeled radioactivity (no zinc removed), III – holoprotein, IV – labeled protein

as the peak for the labeled SOD appears at the same time (t_R = 0.58 on Wescan column) as required for normal SOD. This technique appears to be more efficient for quality control but due to non-compatibility of our radioactivity counters with the HPLC equipment we could not evaluate it properly.

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