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# Purification of a fully metal-depleted Cu, Zn superoxide dismutase from copper-deficient rat liver

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**A copper-deprived form of the enzyme Cu, Zn superoxide dismutase was identified in the liver of rats made copper-deficient by dietary restriction. In homogenates of such livers Cu, Zn superoxide dismutase presents a dis-homogeneous electrophoretic profile with respect to the native enzyme. When rat liver extracts were treated with exogenous copper an electrophoretic pattern resembling the native one was observed. Enzyme purified by chromatography on DE-52 resin shows two major components, one corresponding to genuine, native enzyme and another one, eluting at higher ionic strength. The latter protein (Fraction II) consists of several isoforms which show the same characteristics of the native superoxide dismutase as far as immunoreactivity and molecular weight are concerned, but with decreased contents of copper and zinc. Its catalytic constant, referring to copper content, was 15 times lower than that obtained for the native enzyme. Moreover, the catalytic power of purified Fraction II was not regained upon incubation with copper. The occurrence of a superoxide dismutase void of metals confirms the hypothesis that this protein plays a dual physiological role: in metal metabolism and in superoxide anion dismutation.**

**Keywords:** copper-deficiency, rat liver, superoxide dismutase, purification

## Introduction

Superoxide dismutases are ubiquitous metalloenzymes, containing either copper, manganese or iron at the catalytic site (Bannister *et al.* 1987, Fridovich 1995). They catalyse the dismutation of superoxide anion to hydrogen peroxide and oxygen. This function may have an important role *in vivo* in the defence of organisms against partially reduced forms of oxygen, which are strongly oxidant and therefore potentially noxious to biomolecules. While the manganese enzyme is strongly dependent on the presence of oxygen and the occurrence of oxidative stress (Autor 1982, Harris 1992), thus confirming its role as an antioxidative enzyme, the same cannot be stated for the copper-containing enzyme. This is a homodimer consisting of two identical subunits, each bearing, beside the catalytically important atom of copper, an atom of zinc per subunit (Cu, Zn SOD). Recently, the physiological role of this enzyme has been questioned, since its intracellular level is not directly influenced by oxygen but is responsive to copper content. A role in intracellular metal traffic has therefore been proposed: the protein may act as a chelator of transition metals, protecting cells against their toxic effects. In fact, in lower eukaryotes such as the yeast *Saccharomyces cerevisiae* (Carrì *et al.* 1991, Galiazzo *et al.* 1991, Culotta *et al.* 1995) regulation of the expression of this enzyme by copper has been demonstrated. It has been shown that the transcription factor ACE1, which responds to copper in regulating the gene expression of the metal-buffering protein metallothionein, co-regulates

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Cu, Zn SOD. Furthermore, both in *S. cerevisiae* and in mammalian cultured cells upon differentiation (Steinkuhler *et al.* 1991), regulation at the post-transcriptional level has been shown, with evidence of the presence of an inactive apo-form of the enzyme, to be reactivated upon exposure to copper. In K562 cells (Steinkuhler *et al.* 1991) this protein has been purified and demonstrated to have a lower catalytic constant, related to a lower copper content in the active site. Recently, an apo-Cu, Zn SOD has also been identified in human lymphoblasts (Petrovic *et al.* 1996).

A more intriguing regulative pattern has been proposed in the *in vivo* system represented by the rat with a copper-deficiency induced upon dietary restriction (Rossi *et al.* 1994). In this case, an organ-specific decrease of copper content was accompanied by a decrease in the activity of Cu, Zn SOD, which also appeared to be differently regulated in the various organs. The presence of an apo-form of the enzyme has been found in the liver, thus validating the hypothesis that SOD may act as a copper-binding protein. The delivery of copper to a constantly expressed apo-SOD may represent a mechanism for intracellular copper handling and defence against its toxicity by liver cells.

In the present work we purified and characterized the Cu, Zn SOD from liver of copper-deficient rats. It consists of two components: one corresponding to genuine rat enzyme, and another one, Fraction II, with an impaired dismutating power due to its lower content of copper. The activity of the enzyme is not regained when exposed to copper, probably because of a contemporaneous lack of zinc.

## Materials and Methods

*Animals and diets*

Male Wistar rats (Charles River, Calco, Italy), 70 g body weight (about four weeks of age), were fed with a copper-deficient diet (ICN Biomedicals Inc., Costa Mesa, CA, USA) containing 0.25 mg of copper per kg of diet. Paired controls were fed with the same diet supplemented with 20 mg of copper as $CuCO_3$ per kg of diet. Both groups were drinking double distilled water and had free access to diet and drinking water. After eight weeks, rats were injected with chloral hydrate and heparin and sacrificed.

Aliquots of total homogenate of small parts of livers (1:6, in phosphate buffered saline pH 7.4) were used for determination of copper content. Supernatants (obtained after 23 000 *g* centrifugation) of homogenates were assayed for SOD activity.

Proteins were determined by the method of Lowry *et al.* (1951). Statistical analysis of data was performed by the Student's *t*-test for unpaired data; $P$ values $< 0.05$ were considered significant.

*Purification of Cu, Zn SOD from livers of copper-deficient rats*

All steps were carried out using glassware devoid of metals by treatment with nitric acid followed by washing with double distilled water equilibrated with a Chelex-100 resin (Bio-Rad, Hercules, CA, USA). All solutions were prepared in Chelex-100 treated water to prevent copper contamination of samples. Purification was performed from homogenates of rat liver by a modification of the procedure of Kumagai *et al.* (1994). This method was selected because it does not require the drastic step of extraction with the chloroform–ethanol mixture which is present in the classical methods of SOD purification. Such care may preserve the putative copper-deficient isoform, which can be more sensitive than the native enzyme. In detail, 20 livers, perfused *in situ* with saline (0.9% NaCl), were homogenized (1:5, w:v) in water containing 1 mM EDTA. Solid ammonium sulphate was then added under stirring, to make a 90% saturated solution. The resulting mixture was adjusted to pH 5 with acetic acid and an equal volume of methanol was added slowly with stirring. The mixture was then vigorously stirred for 15 min and subsequently centrifuged. The supernatant was collected and concentrated with a Minitan Ultrafiltration System (Millipore, Molsheim, France), with a tangential flow device on an ultrafiltration membrane (cutoff 10 000 Da). The concentrated sample was dialysed against potassium phosphate buffer (10 mM) pH 6.5, containing 0.01 mM EDTA. After dialysis the sample was chromatographed on a DE-52 resin (Whatman, Springfield Mill, Maidstone, Kent, UK), previously equilibrated with the same buffer. The column was then washed and the protein eluted with a linear gradient of KCl (0–0.2 M).

*Electrophoresis and immunoblotting*

Polyacrylamide gel electrophoresis (PAGE) was performed in a slab gel system from Bio-Rad. Under non-denaturing conditions (Laemmli 1970) gels were stained either with Coomassie Blue R-250 or for SOD activity (Beauchamp & Fridovich 1971). SDS–PAGE (Laemmli 1970) was performed on 12% acrylamide gels. The samples were previously denaturated by heating at 92ºC for 15 min. Cu, Zn SOD was identified by the molecular weight of its monomer (16 000 Da) by comparison with molecular weight standards (Bio-Rad), run under the same conditions. For immunological determination, the gels were blotted on nitrocellulose membrane with a Bio-Rad transblot apparatus according to the method of Towbin *et al.* (1979). Antisera against rat liver Cu, Zn SOD were raised in rabbits and purified according to standard procedures. After incubation with the secondary antibody (anti-rabbit, Sigma Immunochemicals, St Louis, MO, USA), conjugated with horseradish peroxidase, the blots were developed with 4-chloro napthol.

*Determination of Cu, Zn SOD activity*

Activity measurements were performed by a polarographic method (Rigo *et al.* 1975) with an AMEL polarographic analyser (Model 466). The determinations were carried out in borate buffer at pH 9.8, where the activity of the Cu, Zn isoform of SOD is selectively detected (Argese *et al.* 1983). Data were expressed in $\mu g\ mg^{-1}$ protein, with reference to purified rat liver Cu, Zn SOD.

*Analysis of metals*

Aliquots either from ground diet or from liver homogenates or from purified SOD were digested in 65% nitric acid. Copper and zinc were measured by an atomic absorption spectrophotometer (Perkin Elmer 3030), equipped with a graphite furnace. For determination of zinc, the samples were previously treated with 0.3% diphenylthiocarbazone in carbon tetrachloride to eliminate zinc contamination (Kumamaru *et al.* 1982).

*Reconstitution of copper-deprived SOD*

Incubation of supernatants with 1 mM $CuSO_4$ was performed for 3 hours at room temperature and was followed by extensive dialysis of the samples against 50 mM phosphate buffer pH 7.4, containing 0.1 mM EDTA.

Exposures of purified Fraction II to $CuSO_4$ or Cu(I)–glutathione complex were performed in 50 mM phosphate buffer pH 7.4, with a copper concentration equimolar to the copper available sites of the protein. Preparation of Cu(I)–glutathione complex was performed as described by Ciriolo *et al.* (1990).

## Results and Discussion

Supernatants of liver homogenates from rats fed with the copper-deficient diet show a copper content which accounts for 33% of that present in control samples; it reflects a loss of SOD activity of about 80% of the values found in the organs of the copper-adequate rats (Table 1).

When analysed by PAGE, SOD of copper-deficient rat liver homogenates is split into a multiplicity of bands, almost indistinguishable, with an increased anodic mobility, as shown in Figure 1.

We have previously demonstrated that, in the liver of copper-deficient animals, the loss of SOD activity is greater than the loss of immunoreactive protein (Rossi *et al.* 1994). This finding implies both the existence of an inactive, presumably copper-deprived, form of the enzyme and that copper exerts a control on Cu, Zn SOD gene expression. In fact, when supernatants from liver homogenates are incubated with exogenous copper, the activity of the enzyme significantly increases (128%, $P < 0.001$, $n = 6$) (Table 1), still being a half of that shown by control samples. Furthermore, upon incubation with copper, the electrophoretic pattern becomes more homogeneous, with the appearance of two distinguished bands, thus approaching the SOD profile of control samples (Figure 1).

**Table 1.** Copper content and Cu, Zn superoxide dismutase activity in 23 000 *g* supernatant of liver from copper-deficient rats

| | Copper (nmol $mg^{-1}$ protein) | Cu,Zn superoxide dismutase ($\mu g\ mg^{-1}$ protein) | |
|---|---|---|---|
| | | | + 1 mM $CuSO_4$ |
| Copper-adequate | 0.40 ± 0.03 | 3.4 ± 0.9 | 3.1 ±1.1 |
| Copper-deficient | 0.13 ±0.02** | 0.7 ±0.2* | 1.6 ±0.4** |

*$0.001 < P < 0.01$, with respect to copper-adequate; **$P < 0.001$, with respect to copper-deficient; $n = 6$.

During purification of Cu, Zn SOD from liver homogenates of copper-deficient animals, an altered chromatographic profile is obtained. In fact, after passage on DE-52 resin, two major peaks can be observed (Figure 2). The first protein component (Fraction I) elutes at 90 mM, an ionic strength similar to that necessary for eluting the native rat enzyme (Yano 1990), the second one (Fraction II) at higher ionic strength values (146 mM). Despite the fact that both fractions reveal multiple bands when electrophoresed under non-denaturing conditions (Figure 3A, B), when analysed under denaturing conditions, both fractions migrate as a single band with identical mobility, showing the same molecular

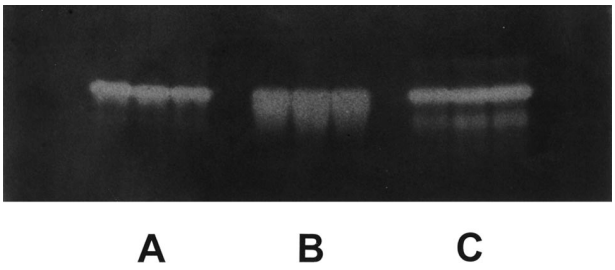


**Figure 1.** Electrophoretic pattern of Cu, Zn superoxide dismutase in homogenates of copper-adequate and copper-deficient rat livers. Non-denaturing PAGE (activity stained) of 23 000 *g* supernatants of homogenates from livers of: (A) copper-adequate; (B) copper-deficient; (C) same as in B, after incubation with 1 mM $CuSO_4$. Extracts from single livers were run on separate lanes. Each contains the same SOD activity.

mass (Figure 3C) and immunoreactivity (Figure 3D) as the native rat liver SOD. This indicates that both fractions are pure SOD. However, while under native conditions Fraction I shows an electrophoretic pattern similar to that of the native enzyme (Figure 3A), Fraction II splits into at least five bands (Figure 4), the first one corresponding to the native enzyme and the others having increased anodic mobility. The second band is apparently more abundant than the other two, but shows a lower dismutating power (Figures 3B and 4B). Furthermore, activity staining reveals that only the less anodic three bands are active. These data, together with the evidence that in SDS–PAGE Fraction II behaves as a single band, suggest that the more mobile isoforms are copper-deprived SOD. From the bulk of these results, we can conclude that SOD present in the liver of copper-deficient rats is made of two components: one corresponds to the genuine rat liver SOD (Fraction I), while the other one is a mixture of isoforms with variable $(Cu^{2+})$/(protein) ratios (Fraction II). In fact, isoforms of SOD containing different amounts of $Cu^{2+}$, from zero (apo-enzyme) up to two $Cu^{2+}$ ions per enzyme molecule, can be prepared *in vitro* either by removal of the metal from the holoprotein by treatment with $CN^-$ or by addition of $Cu^{2+}$ to the $Cu^{2+}$–free enzyme. Rigo *et al.* (1977) showed that electrophoresis of samples of the bovine enzyme processed by these methods presents an electrophoretic pattern with a maximum of three bands, corresponding to enzyme molecules with zero, one and two copper ions per molecule, with the electrophoretic mobility of the $Cu^{2+}$–free protein more anodic than the native one.

To better characterize Fraction II, we analysed its content of copper and zinc and its catalytic activity. As reported in Table 2, Fraction II shows a rather low content of both metals with respect to protein amount, when compared with native Cu, Zn SOD, for which a copper/protein ratio of 1.28 is obtained. In this connection, it should be remembered that native purified Cu, Zn SOD from rat liver is known to have a lower copper/protein ratio (Yano 1990) than SOD purified from bovine erythrocytes, for which a ratio close to 2 is reported. The catalytic constant obtained for Fraction II was $0.054 \times 10^9$ $M^{-1}$ $s^{-1}$ with respect to copper content, 15 times less active than the native rat SOD.

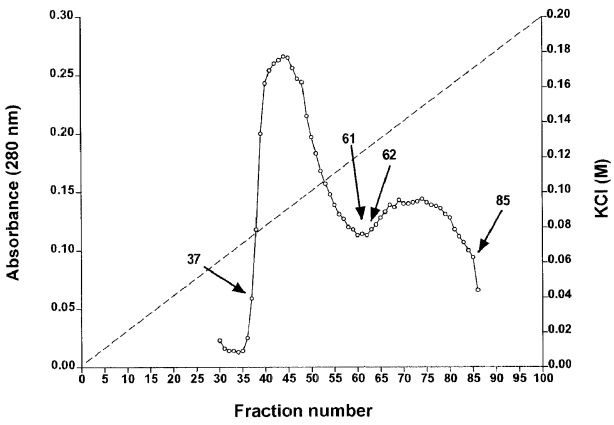


**Figure 2.** DE-52 cellulose chromatogram of Cu, Zn superoxide dismutase from livers of copper-deficient rats. Sample was applied on DE-52 resin, equlibrated with 10 mM potassium phosphate buffer, pH 6.5 and eluted with a linear gradient of KCl (0–0.02 M). Fraction I: aliquots 37–61; Fraction II: aliquots 62–85.

As far as the ability of Fraction II to be reconverted to a native, active form is concerned, all attempts failed. Incubation of the protein with $CuSO_4$ does not result in the recovery of dismutating activity. Furthermore, incubation of Fraction II with copper does not affect the electrophoretic behaviour of the protein (data not shown). This is a rather surprising finding, since this is the usual *in vitro* procedure for reconstitution of the copper-free form of native SOD and is in contrast to the results obtained upon incubation of rat liver supernatants with $CuSO_4$ (Figure 1 and Table 1). It is conceivable that during purification, a copper-binding molecule, which supplies copper to copper-free SOD in rat liver extracts, is lost. In fact, it has been demonstrated that several factors favour reconstitution of copper-free SOD. Monovalent copper complexed to the physiological antioxidant tripeptide glutathione has been demostrated to be delivered *in vitro* to copper-free SOD as far as the copper-binding protein metallothionein (Ciriolo *et al.* 1990, Ferreira

**Table 2.** Copper and zinc content and catalytic constant of Fraction II purified from copper-deficient rat liver

| | Cu/protein | Zn/protein | $K_{cat}$/Cu | $K_{cat}$ |
|---|---|---|---|---|
| | | | $(M^{-1} \times s^{-1})$ | |
| Rat liver Cu,Zn SOD | 1.28 | 3.38 | $0.82 \times 10^9$ | $0.98 \times 10^9$ |
| Fraction II | 0.08 | 0.06 | $0.054 \times 10^9$ | $0.004 \times 10^9$ |

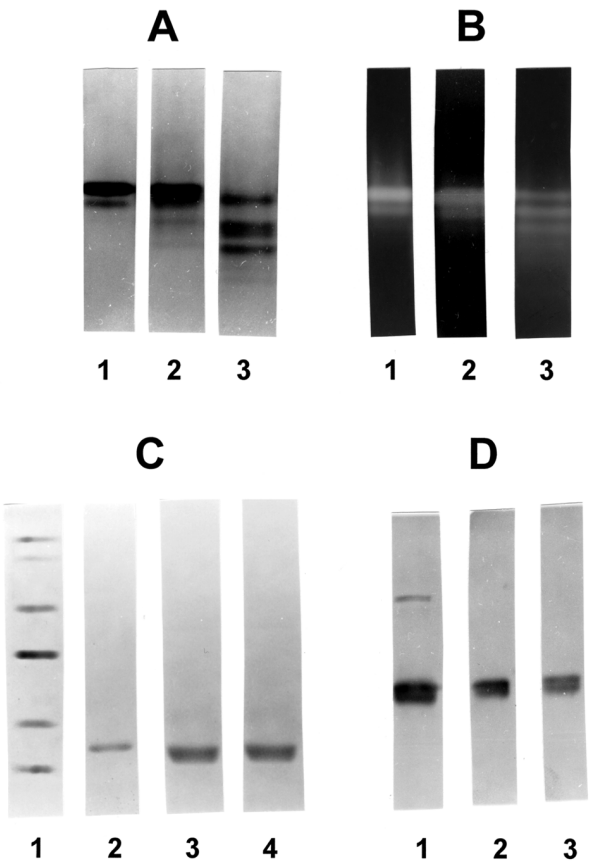


**Figure 3.** Electrophoretic characterization of purified Cu, Zn superoxide dismutase from livers of copper-deficient rats. (A) Non-denaturing PAGE, Coomassie stained. Lane 1: native rat liver Cu, Zn SOD (6 μg); lane 2: Fraction I (13 μg); lane 3: Fraction II (13 μg). (B) Non-denaturing PAGE, activity stained. Lane 1: native rat liver Cu, Zn SOD (1 μg); lane 2: Fraction I (3 μg); lane 3: Fraction II (50 μg). (C) SDS–PAGE, Coomassie stained. Lane 1: molecular weight markers; lane 2: native rat liver Cu, Zn SOD (4 μg); lane 3: Fraction I (20 μg); lane 4: Fraction II (20 μg). Molecular weight markers: rabbit muscle phosphorylase b (97 400 Da); bovine serum albumin (66 200 Da); hen egg white ovalbumin (45 000 Da); bovine carbonic anhydrase (31 000 Da); soybean trypsin inhibitor (21 500 Da); hen egg white lysozyme (14 400 Da). (D) Western blotting, peroxidase–activity stained. Lane 1: native rat liver Cu, Zn SOD (4 μg); lane 2: Fraction I (3 μg); lane 3: Fraction II (3 μg).

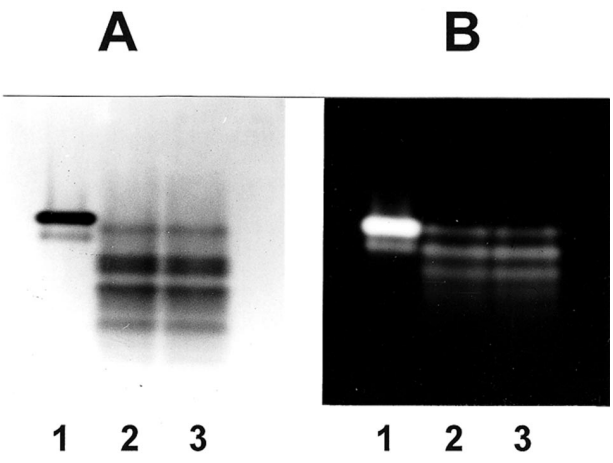


**Figure 4.** Lack of reconstitution of Fraction II by Cu(I)–glutathione complex. (A): Coomassie stained. Lane 1: native rat liver SOD (4 μg); lane 2: Fraction II (18 μg); lane 3: Fraction II (18 μg), after incubation with Cu(I)–glutathione complex. (B): Activity stained. Lane 1: native rat liver SOD (1 μg); lane 2: Fraction II (50 μg); lane 3: Fraction II (50 μg), after incubation with Cu(I)–glutathione complex.

*et al.* 1991). In the present study, however, neither increase of activity nor change in the electrophoretic pattern of Fraction II was obtained upon incubation with Cu(I)–glutathione complex (Figure 4). The evidence that Fraction II has a low zinc content may represent the explanation of this anomalous behaviour. In fact, metal-free SOD has been reported not to be able to recover SOD activity unless zinc is supplied before copper (Rotilio *et al.* 1972). This may also represent the clue to the results obtained by Petrovic *et al.* (1996) who succeeded in reconstituting apo-SOD only in intact human lymphoblasts but not in cell cytosol. In this case too, the availability of zinc may represent the limiting factor for protein reconstitution.

Altogether, the findings of this study confirm that copper regulates Cu, Zn SOD at the post-translational level, so that its restriction results in the *in vivo* occurrence of forms of the enzyme totally or partially lacking this metal. These isoforms are sufficiently stable to be purified as metal-free proteins. Great benefit may derive from the constant presence of a metal-uncharged SOD, since it may represent a metal-buffering system protecting cells from the toxicity induced by the redox-cycling of transition metals. The finding of systems holding such uncharged SOD (K562 cells or human lymphoblasts) seems to validate this theory.

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