Subcellular Distribution of Superoxide Dismutases in Rat Liver:

Cu,Zn-SOD in Mitochondria *

Running Title: Mitochondrial Cu,Zn-SOD

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ABSTRACT

Rat liver was homogenized in isotonic buffer, fractionated by differential centrifugation, and then subfractionated by equilibrium sedimentation in Nycodenz gradients. Fractions were assayed for both Cu,Zn-SOD and Mn-SOD by exploiting the cyanide-sensitivity of the former activity and by the use of specific antibodies. As expected, the cytosol and lysosomal fractions contained Cu,Zn-SOD; while the mitochondrial matrix contained Mn-SOD. In mitochondria, Cu,Zn-SOD was found in the intermembrane space and Mn-SOD in the matrix and also on the inner membrane. The Mn-SOD associated with the inner membrane was solubilized by 0.5 M NaCl. Surprisingly the intracellular membrane fraction (microsomes) contained bound Cu,Zn-SOD that could be solubilized with a detergent, and to lesser degree with 0.5 M NaCl. Both the cytosolic and mitochondrial Cu,Zn-SODs were isolated and compared. They have identical molecular mass, cyanide-sensitivity, SDS-sensitivity, heat stability, and chloroform + ethanol stability. Tissue from Cu,Zn-SOD knockout mice was entirely devoid of Cu,Zn-SOD; indicating that the cytosolic and the intermembrane space Cu,Zn-SODs are coded for by the same gene. The significance of this distribution of the SODs is discussed.

Keywords: Mitochondrial superoxide dismutases / Mn-SOD and Cu,Zn-SOD in mitochondria / Localization of SODs / SODs in organelles / SOD in lysosomes / SODs in liver /
INTRODUCTION

A small fraction of total biological reduction of oxygen occurs by a univalent pathway. Superoxide, the first intermediate encountered on this pathway, is capable of: initiating free radical chain oxidations; inactivating specific enzymes; and leading to the production of more powerful oxidants by liberating Fe (II) from the [4Fe-4S] clusters of dehydratases and by reacting with nitric oxide (1). The damaging potential of superoxide is muted by SODs, that catalyze its dismutation to oxygen plus hydrogen peroxide; as well as by superoxide reductases (SORs), that catalyze the reduction of superoxide to hydrogen peroxide (2).

When the subcellular distribution of SOD activity was first explored by fractionation of liver, the cytosol was found to contain a Cu,Zn-SOD and the mitochondrial matrix a Mn-SOD. At that time Cu,Zn-SOD was also noted in the intermembrane space of mitochondria, and in nuclei (3, 4). The cytosolic localization of Cu,Zn-SOD and the mitochondrial location of Mn-SOD was subsequently verified by means of colloidal gold immuno cytochemistry and electron microscopy (5, 6). However the presence of Cu,Zn-SOD in the intermembrane space of mitochondria was called into question and attributed to contamination of mitochondrial fractions with lysosomes and the presence of Cu,Zn-SOD in lysosomes (7).

We now report a painstaking reinvestigation of the distribution of SODs in rat liver and find that there is indeed a Cu,Zn-SOD in the intermembrane space of mitochondria that it is the same gene product as the cytosolic Cu,Zn-SOD. Culotta and her associates independently, and in parallel, investigated the situation in yeast and find as we do¹. We also find the Cu,Zn-SOD strongly associated with intracellular reticular membranes isolated as microsomes.
MATERIALS AND METHODS

Material

Nycodenz was from Invitrogen Life Technologies. Xanthine oxidase was from Roche Diagnostics Corporation. Rabbit anti-Mn-SOD antibody, sheep anti-Cu,Zn-SOD antibody, HRP conjugated anti-rabbit IgG, and HRP conjugated anti-sheep IgG were from Upstate Biotechnology. SP-sepharose, nitrocellulose membranes and the ECL kit were from Amersham Pharmacia Biotech. Cytochrome c was from Fluka Chemika, while AcA54, xanthine, NAD, L-lactic acid, L-malate, uric acid, p-nitrophenyl phosphate, benzylamine hydrochloride, and Lubrol PX were from Sigma. Broad range prestained SDS-PAGE standards were from Bio-Rad.

Fractionation of Liver

Sprague-Dawley rats were sacrificed and the livers were promptly removed and placed in ice cold homogenization buffer (200 mM mannitol, 50 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl) at pH 7.4. After mincing with scissors and washing to remove blood, the liver was homogenized in a Potter-Elvejhem homogenizer with a teflon piston, using 10 ml of the homogenization buffer per 2.5 mg of tissue. Centrifugation at successively higher speeds yielded the following fractions: crude nuclear fraction at 1,000 x g for 10 min; heavy mitochondria at 3,000 x g for 10 min; light mitochondria at 20,000 x g for 20 min; and microsomes at 144,000 x g for 90 min. The final supernatant was the cytosolic fraction. Each successive pellet was thrice washed with the homogenization buffer. The centrifuges used were the Avanti J-25 centrifuge and the Optima XL-100K ultracentrifuge, both from Beckman.

Nycodenz Density Gradient Fractionation

The procedures recommended by Nycomed Pharma and Invitrogen Life Technologies were followed (www.nycomed-diagnostics.com and www.invitrogen.com). Nycodenz was dissolved to 50 % (w/v) in buffer containing 5 mM Tris-HCl and 1 mM EDTA at pH 7.4. This stock solution
was diluted with buffer containing 0.25 M sucrose, 5 mM Tris-HCl and 1 mM EDTA at pH 7.4. The nuclear pellet was suspended in 25 % Nycodenz and 7.5 ml was then overlaid onto the following discontinuous Nycodenz gradient: 3 ml 50 %, 3 ml 40 %, 4.5 ml 34 %, and 6 ml 30 %. The sample on this gradient was topped off with 6 ml 20 % Nycodenz before sealing the tube. The heavy and light mitochondrial pellets suspended in 10 ml of 25 % Nycodenz were placed on the following discontinuous Nycodenz gradients: 4 ml 34 % and 7 ml 30 % and this was topped off with 7 ml 23 % and finally 2 ml 20 %. The sealed tubes were centrifuged for 90 min at 52,000 x g at 4 °C. The bands of particles seen after centrifugation have been identified by Nycomed Pharma and Invitrogen life technologies as follows: nuclei at the 40/50 % interface; peroxisomes at the 34/40 % interface; mitochondria at the 25/30 % interface, lysosomes at the 15/20 % interface, and Golgi membranes at the 10/15 % interface.

**Enzyme Assays**

SOD activity was assayed by the xanthine oxidase / cytochrome c method (8). Mn-SOD was distinguished from Cu,Zn-SOD by assaying in the presence of 50 µM and then 2 mM NaCN (9). Under our conditions the Cu,Zn-SOD was 24 % inhibited by 50 µM cyanide and 93 % inhibited at 2 mM cyanide. Mn-SOD was not inhibited at these levels of cyanide. Hemoglobin, as an erythrocyte marker, was measured by absorbance at 409 nm (7); acid phosphatase, as a lysosomal marker, by the hydrolysis of p-nitrophenyl phosphate (10); urate oxidase, as a peroxisomal marker, at 290 nm (11); lactate dehydrogenase (LDH), as a cytosolic marker, at 340 nm (12); fumarase, as a mitochondrial matrix marker, at 250 nm (13); cytochrome c oxidase, as a mitochondrial inner membrane marker, at 550 nm (14); sulfite oxidase, as a mitochondrial intermembrane space marker, at 550 nm (15); and monoamine oxidase, as a mitochondrial outer membrane marker, by the oxidation of benzylamine (16). All assays were repeated 5 times. All membrane fractions and samples for cytochrome c oxidase assay were treated with 0.1 % Lubrol.
for enzyme assays.

**Immuno Assays**

Samples were subjected to 15 % SDS-PAGE, and were then blotted onto nitrocellulose membranes under semi-dry conditions using the Bio-Rad Trans-Blot. After blocking by soaking in 5 % dry milk proteins in 10 mM Tris-HCl, 0.15 M NaCl, and 0.05 % Tween 20, at pH 7.4 and room temperature for 1h; the membranes were soaked with 1:500 sheep anti Cu,Zn-SOD or 1:1000 rabbit anti Mn-SOD for 1 h at room temperature. The membranes were then washed and soaked with HRP-conjugated anti sheep IgG (1:2000) or with HRP-conjugated anti rabbit IgG (1:5000) for 40 min at room temperature. The membranes were finally washed four times and then the HRP-containing bands were detected by luminol chemiluminescence with the ECL kit.

**Submitochondrial Fractionation**

Mitochondria isolated on Nycodenz gradients were separated into outer membrane, intermembrane space, inner membrane, and matrix fractions according to Greenawalt (17) with the following modifications: the mitoplast fraction was subjected to 3 cycles of freezing and thawing and was then centrifuged at 144,000 x g for 90 min. The membrane fractions were washed 3 times with PBS to remove soluble proteins derived from the matrix or intermembrane spaces.

**Isolation of Cu,Zn-SODs from Cytosol and Mitochondria**

The procedure developed for isolation of Cu,Zn-SOD from bovine heart (18) was appliable, with minor modifications. Thus Ion exchange chromatography was performed on SP-sepharose and gel filtration on AcA54. The protein content of purified Cu,Zn-SOD was determined by the Biuret method (19) and protein mixtures were assayed by the Bradford method (20) using the Bio-Rad reagent. When assaying liver from Cu,Zn-SOD knockout mice, the tissue extracts were purified through the acetone precipitation step before being assayed. This was done with and without an internal standard of rat liver mitochondrial Cu,Zn-SOD to ensure our ability to recover
small amounts of activity.

Molecular Weight and Mass

Native molecular weights were determined by sedimentation equilibrium centrifugation in a model XL-A Beckman analytical ultracentrifuge as previously described (21). Mass Spectrometry measurements were made on a Micromass Quattro LC (Altrincham, UK) triple quadrupole mass spectrometer equipped with a pneumatically assisted electrostatic ion source operating at atmospheric pressure and in a positive ion mode. Apo-protein SOD samples in 50% aqueous acetonitrile containing 1% formic acid were analyzed by loop injection into a stream of 50% aqueous acetonitrile flowing at 10 µl/ min. Native SOD samples were electrosprayed in 0.01 M ammonium acetate. Spectra were acquired in the multi-channel analyzer (MCA) mode from m/z 600-1400 (scan time 5 sec). The mass scale was calibrated using the multiply charged envelope of myoglobin (MW 16951.48 Da). The raw mass spectra were transformed to a molecular mass scale using a maximum entropy based method (Max Ent) which uses the MemSys5 program (Max Ent Solutions Ltd., Cambridge UK) and is part of the Micromass Mass Lynx software suite.
RESULTS

SOD Activities in Liver Fractions

When rat liver was homogenized in isotonic buffer and fractionated, as described in materials and methods, the soluble cytosolic fraction (144,000 x g supernatant) contained abundant Cu,Zn-SOD and no detectable Mn-SOD while the heavy and the light mitochondrial fractions contained both SODs with Mn-SOD predominating. Surprisingly, the microsomal fraction contained Cu,Zn-SOD. Lactate dehydrogenase was followed as a cytosol marker and cytochrome c oxidase as a mitochondrial marker. Lactate dehydrogenase was found mostly in the cytosol, with a small amount in the microsomes; while cytochrome c oxidase was found only in the mitochondrial fractions. These results, based on both activity assays and on immunoassays, are shown in figures 1A and 1B.

SOD and Marker Assays of Nycodenz Fractions

The particulate fractions, obtained by differential centrifugation of the liver homogenates, were subfractionated in Nycodenz gradients and assayed for the SODs and the various marker proteins, as described in materials and methods. The data in figure 2A demonstrate that: the nuclear fraction was contaminated with erythrocytes and some lysosomes; the heavy mitochondrial fraction contained some erythrocytes and lysosomes at the 20/23 % and at the 23/25 % Nycodenz boundaries; but not at the 25/30 % boundary. The 30/34 % boundary of the heavy mitochondria showed contamination with peroxisomes. The light mitochondrial fraction contained lysosomes at the 20/23 and at the 23/25 % boundary and both lysosomes and peroxisomes at the 25/30 % boundary; while the 30/34 % boundary was almost all peroxisomes.

Selected bands of particles from Nycodenz density gradients, identified by arrows in figure 2A, were assayed for SOD activity using cyanide to distinguish Cu,Zn-SOD from Mn-SOD and this data is shown in figure 2B. The heavy mitochondrial fraction 25/30, that had been found free
of erythrocyte, lysosomal and peroxisomal markers, contained both Cu,Zn-SOD and Mn-SOD in
the activity ratio 1:5. The light mitochondrial fraction 20/23, that had been found to contain
abundant lysosomal marker activity, was rich in Cu,Zn-SOD but contained only traces of Mn-SOD.
The light mitochondrial fractions 25/30 and 30/34 contained progressively less Cu,Zn-SOD and
small amounts of Mn-SOD.

The identities of the SODs were further probed immunochemically by immunoblotting and
these results are shown in figure 2C. In agreement with the results of activity assays using
cyanide the heavy mitochondrial fraction 25/30 contained abundant Mn-SOD and much less of
Cu,Zn-SOD. This is the fraction which was free of lysosomal and peroxisomal markers and is
therefore taken to be pure mitochondria. The light mitochondrial fraction 25/30 contained both
SODs, with Cu,Zn-SOD predominant, in keeping with its content of lysosomal marker activity.
The light mitochondrial fraction 30/34 exhibited a trace of Cu,Zn-SOD and Mn-SOD.

Submitochondrial Fractions

The heavy mitochondrial fraction 25/30, which was found free of non-mitochondrial markers,
was subfractionated and assayed for several marker activities and for SODs. Figure 3A illustrates
the result of the marker assays. The matrix fraction contained primarily the matrix marker
fumarase, while the inner membrane fraction contained primarily cytochrome c oxidase, as
expected, but also a substantial amount of the outer membrane marker monoamine oxidase. The
intermembrane space contained abundant sulfite oxidase and was practically free of other markers
and the outer membrane fraction showed mostly monoamine oxidase. Immunoblotting
demonstrated that the intermembrane space contained Cu,Zn-SOD, but not Mn-SOD; while the
matrix and the inner membrane contained Mn-SOD, but no Cu,Zn-SOD. The Mn-SOD that was
associated with the inner membrane persisted despite three washings with PBS. After incubation
with PBS, PBS + 0.5 M NaCl, or PBS + 0.1 % Lubrol for 30 min on ice, followed by centrifugation
at 144,000 x g for 30 min, the 0.5 M NaCl and 0.1 % Lubrol supernatants from the inner membrane showed about 3 times and 8 times higher Mn-SOD activity respectively than did the PBS supernatant (Data not shown).

**Isolation of Cu,Zn-SOD**

The Cu,Zn-SOD was isolated from both the cytosolic and the mitochondrial fractions of rat liver. The heavy mitochondrial Nycodenz fraction 25/30 was used, since it was demonstrably free of non-mitocondrial markers, and the 144,000 x g supernatant fraction was taken to be cytosol. The results of these isolation procedures are given in tables IA and IB and in figure 4, the tables record a 45-fold purification of the Cu,Zn-SOD from cytosol and a 1,556-fold purification of Cu,Zn-SOD from mitochondria. We can thus estimate that Cu,Zn-SOD constitutes ~2 % of the total protein of the cytosolic fraction and only ~0.06 % of the total protein of mitochondria. Since only ~6 % of total liver mitochondrial protein is in the intermembrane space (22) we can correct the estimated 0.06% by multiplying it by 16.7 to get ~1 %, thus the concentration of Cu,Zn-SOD in the intermembrane space (~1 %) is not very different from its concentration in the cytosol (2 %). We can also estimate that mitochondrial Cu,Zn-SOD constitutes ~2.8 % of the total Cu,Zn-SOD from table I. It should be noted that the specific activities of the Cu,Zn-SOD was ~4,700 U/mg whether isolated from cytosol or mitochondria. Figure 4 shows that the mobility on 15 % SDS-PAGE was identical for the Cu,Zn-SODs from both sources.

**Cyanide Inhibition and Thermal Stability**

The Cu,Zn-SODs derived from cytosolic and mitochondrial fractions were: isolatable by the same procedure; of identical specific activities; and indistinguishable on SDS-PAGE. Nevertheless it remained possible that they might differ in subtle ways that could be detected in terms of sensitivity to inhibition by the superoxide analogue cyanide, or in terms of thermal stability. The data in figure 5 demonstrate that Cu,Zn-SODs isolated from the cytosolic fraction,
or from the pure mitochondrial fraction, are indistinguishable in their sensitivities to cyanide inhibition. Thus the concentration of NaCN needed for 50% inhibition of cytosolic and mitochondrial Cu,Zn-SODs was 230 µM in both cases. Figure 6A and 6B make the same point with regard to inactivation at 70 °C and 80 °C. Cu,Zn-SOD is stable to SDS but Mn-SOD is not (9). We examined the SDS stability of cytosolic and mitochondrial Cu,Zn-SODs and both were stable for 24 h under the conditions of Geller and Winge (9) (Data not shown).

**Molecular Weights and Mass**

The native molecular weight of the Cu,Zn-SODs isolated from the cytosolic and the mitochondrial fractions were determined by analytical sedimentation equilibrium. They were found to be identical to within the limits of precision of the method *ie.* 31,800 and 31,400 Daltons. The Mass of the subunits were found by electrospray mass spectrometry to be 15,820 and 15,818 Daltons respectively. These results are given in Table II.

**Cu,Zn-SOD: One Gene Product**

The identical properties of the Cu,Zn-SODs isolated from cytosol and mitochondria suggested that they might be the product of one gene. This was established by examining liver from normal control mice, transgenic mice that over express Cu,Zn-SOD, and knockout mice that do not express Cu,Zn-SOD. The results in figure 7 show that the over expressing mice (OE1 and OE2) had 2.5 times more Cu,Zn-SOD than the controls (CT1 and CT2); while the knockout mice (KO1 and KO2) had no detectable Cu,Zn-SOD. Since the mitochondrial Cu,Zn-SOD accounted for only ~2.8% of the total Cu,Zn-SOD, we processed the extract of knockout mouse livers through the acetone precipitation step of the purification procedure (Table I), prior to assaying then for Cu,Zn-SOD activity. To ensure the validity of this procedure one aliquot of each liver extract was doped with an internal standard of rat liver mitochondrial Cu,Zn-SOD, added to 1.7 U/mg protein. This is the amount of Cu,Zn-SOD derived from mitochondria in wild type mouse liver extract. After the
partial purification, no Cu,Zn-SOD activity was found in the fractions from the KO1 and KO2 mouse livers; while the aliquots that had been doped with Cu,Zn-SOD yielded fractions containing 50 U/mg of Cu,Zn-SOD activity. This procedure would have easily detected SOD in the knockout extracts, had it been present at even 0.5% the amount expected to have derived from mitochondria. All expressed the same level of Mn-SOD. It thus appears that all the Cu,Zn-SOD in murine liver, and by extension in rat liver, is the product of one gene.
DISCUSSION

Fractionation of liver homogenate by differential sedimentation into nuclear, heavy and light mitochondria, microsomal and soluble fractions, followed by subfractionation on Nycodenz gradients; yielded fractions that were, on the basis of marker activities, pure mitochondria and nearly pure peroxisomes and lysosomes. The mitochondria contained both Mn-SOD and Cu,Zn-SOD, the lysosomes only Cu,Zn-SOD, and the peroxisomes traces of both SODs, the cytosolic fractions contained only Cu,Zn-SOD. Further fractionation of the pure mitochondria demonstrated that the Cu,Zn-SOD was in the intermembrane space while the Mn-SOD was both in the matrix and associated with the inner membrane. The presence of Mn-SOD in the inner membrane fraction and of Cu,Zn-SOD in the microsomal fraction, that could be solubilized with a detergent and also with 0.5 M NaCl, is reminiscent of the finding of lactate and malate dehydrogenases associated with chicken liver microsomes (23). Solubilization with detergent could signify enclosure of the SOD in membrane vesicles, but solubilization by NaCl indicates binding to the surface of the membranes by electrostatic forces. Since phospholipids impart a negative charge to biological membranes; cations, including protons, will be concentrated adjacent to these membranes. Hence the pH adjacent to the membranes will be lower than in the bulk solution and superoxide approaching the membranes would be more protonated than superoxide in the bulk. Since hydroperoxyl radical (HO$_2^-$) is a stronger oxidant of unsaturated lipids than superoxide (24) it may be important to have SOD bound to membrane to intercept incoming superoxide.

Parallels may be usefully drawn between the localization of SOD activities in mitochondria and in *Escherichia coli*; thus in the gram negative bacterium the inducible Mn-SOD associates with DNA and is most abundant in the central portion of the cell that contains the nucleoid (25); while the homologous and constitutive Fe-SOD is concentrated in the periphery of the cells, closest to the...
inner membrane. There is, in addition, a Cu,Zn-SOD found in the periplasmic space. The lessons to be derived from this distribution are that: there are multiple sites of superoxide generation; that superoxide does not readily cross membranes; and that superoxide cannot be allowed a long half life in cells and consequently must be dealt with by a SOD close to its site of generation. When applied to mitochondria these musings lead to the following deductions: superoxide made on the inner surface of the inner membrane will be largely dealt with by the Mn-SOD bound to that surface; superoxide made on the outer surface of the inner membrane, and hence entering the intermembrane space, will be scavenged by the Cu,Zn-SOD in that space; superoxide is probably also made in the matrix space and that will be dealt with by the matrix Mn-SOD.

Mitochondria are considered to be the major source of superoxide in eukaryotic cells because they consume most of the oxygen used by these cells and because respiring submitochondrial particles have been shown to convert 1-2% of the oxygen consumed into superoxide and hydrogen peroxide. The distribution of the SODs in mitochondria make it very unlikely, however, that superoxide made in intact mitochondria could escape to the cytosol. Thus any superoxide entering, or made in, the intermembrane space would be dismuted by the Cu,Zn-SOD or oxidized by the ferricytochrome c in that space.

Nishijima et al. (26) have reported on the distribution of Cu,Zn-SOD in gastric mucosa using immuno electronmicroscopy and noted this enzyme mainly in the cytoplasm but also associated with the outer membrane of mitochondria, and partially in the membranes of the endoplasmic reticulum and nuclei. Since they dealt with fixed sections it is understandable that the intermembrane Cu,Zn-SOD should have appeared to be associated with the outer mitochondrial membrane. We also detected the Cu,Zn-SOD in the microsomal fraction. This Cu,Zn-SOD could be solubilized with 0.5 M NaCl or more effectively by 0.1% Lubrol, for 30 min at 0 °C (Data not shown).
The question of how some Cu,Zn-SOD comes to reside in the intermembrane space of mitochondria while most of it remains in the cytosol has been answered by Culotta and associates †. Thus they found, in yeast, that the apoenzyme can cross the outer membrane and that it is trapped in the intermembrane space when metallated by the copper chaperone of superoxide dismutase in that space.

The significance of SODs bound to membranes requires further comment. Polyanionic macromolecules or polyanionic surfaces such as biological membranes will concentrate cations from the bulk solution. This applies to protons and the result has been calculated to be a ~2 pH unit drop in pH immediately adjacent to the polyanionic macromolecule (27, 28). The pKa of the hydroperoxyl radical (HO$_2^-$) is ~4.8 (29) and HO$_2^-$ is a much stronger oxidant than superoxide (30, 31). It follows that superoxide diffusing forwards the polyanionic surface of the membrane could, if allowed to closely approach that surface, protonate to HO$_2$· and then oxidize membrane components, such as polyunsaturated lipids. SOD bound to the membrane could prevent this by scavenging superoxide prior to its protonation.
REFERENCES


FOOTNOTES

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† Culotta, V. Personal communication

1 The abbreviations used are: SOD, superoxide dismutase; HRP, horseradish peroxidase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Fig. 1. SOD and marker enzyme activities and immunoblots of rat liver fractions.

(A) SOD and marker enzyme activities of rat liver fractions. All enzyme activities were determined 5 times, and the mean and standard deviations are shown. Heavy mitochondria, light mitochondria, cytosol, and microsomes fractions were isolated as described in materials and methods. Membrane fractions were treated with 0.1 % Lubrol prior to assay for marker enzyme activities. We defined the activities of extract of the total liver as 100 %. (B) Immunoblots of Cu,Zn-SOD and Mn-SOD. Each lane contained 10 µg protein. The first lane contains the supernate (20,000 x g) of liver sonicated in PBS. Following SDS-PAGE on 15 % gels, probing with antibodies was done as described in materials and methods.

Fig. 2. Marker assays, SOD activities, and immunoblots of Nycodenz fractions.

(A) Marker assays. Hemoglobin, acid phosphatase and urate oxidase were assayed as markers of erythrocytes, lysosomes, and peroxisomes, respectively. All enzyme activities were determined 5 times. The arrows denote the fractions taken for activity assays (Fig. 2B) and for immunoblots (Fig. 2C). The numbers on the abscissa represent the Nycodenz concentration discontinuities at which bands formed. (B) SOD activities of Nycodenz fractions. All enzyme activities were determined 5 times, and the mean and standard deviations are shown. (C) Immunoblots of Cu,Zn-SOD and Mn-SOD. The conditions were as in the legend of figure 1B.

Fig. 3. Marker enzymes and immunoblots of mitochondrial fractions.

(A) Marker enzyme assays. Cytochrome c oxidase, sulfite oxidase, and monoamine oxidase were assayed as markers of inner membrane, intermembrane space, and outer membrane of mitochondria, respectively. (B) Immunoblots of Cu,Zn-SOD and Mn-SOD. The conditions were as in the legend of figure 1B.
Fig. 4.  **Purification of Cu,Zn-SODs followed by SDS-PAGE.**

Cytosolic Cu,Zn-SOD (C) and mitochondrial Cu,Zn-SOD (M) were purified. Samples from each purification stage were applied to and separated by 15 % SDS-PAGE and were then stained with Coomassie brilliant blue. Crude and ethanol/chloroform fractions 20 µg/lane; acetone fractions 10 µg/lane; and purified proteins 3 µg/lane.

Fig. 5.  **Inhibition of cytosolic and mitochondrial Cu,Zn-SODs by NaCN.**

All enzyme activities were determined 5 times, and the means are shown.

Fig. 6.  **Effect of heating on cytosolic and mitochondrial Cu,Zn-SOD.**

(A) *Heating at 70°C.*  (B) *Heating at 80°C.*  The purified cytosolic and mitochondrial Cu,Zn-SOD were incubated at 70 °C or 80°C in 2.5 mM potassium phosphate buffer (pH 7.4) and samples were taken at intervals for assay of residual activity. All enzyme activities were determined 5 times, and the mean and standard deviations are shown.

Fig. 7.  **SOD activities in normal, transgenic, and knockout mouse livers.**

We examined two mice of each normal controls (CT), Cu,Zn-SOD over expressing (OE), and knockout (KO) mice. We used partially purified protein of knockout mice liver for Cu,Zn-SOD assay. Another samples were used without purification. All enzyme activities were determined 5 times, and the mean and standard deviations are shown.
Table I-A  Purification of rat cytosolic Cu,Zn-SOD

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<tr>
<th>Preparation Stage</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Fold purification (U/mg)</th>
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Table I-B  Purification of rat mitochondrial Cu,Zn-SOD

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<th>Total activity (U)</th>
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Fig. 2 Ayako Okado-Matsumoto and Irwin Fridovich

Mitocondrial Cu,Zn-SOD
Fig. 3  Ayako Okado-Matsumoto and Irwin Fridovich

Miocondrial Cu,Zn-SOD

(A)

(B)
Fig. 5 Ayako Okado-Matsumoto and Irwin Fridovich

Mitochondrial Cu,Zn-SOD

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SOD activity (U)

NaCN (µM)

○ cytosolic Cu,Zn-SOD

× mitochondrial Cu,Zn-SOD
Fig. 7  Ayako Okado-Matsumoto and Irwin Fridovich  
Mitochondrial Cu,Zn-SOD

![Bar chart showing SOD activity (U/mg) for different samples.](http://www.jbc.org/)

- **Cu,Zn-SOD**
- **Mn-SOD**

**Samples:**
- CT1
- CT2
- OE1
- OE2
- KO1
- KO2