Subcellular Distribution of Superoxide Dismutases (SOD) in Rat Liver

Cu,Zn-SOD IN MITOCHONDRIA

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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-superoxide dismutase (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 mitochondrial 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 intermembrane space Cu,Zn-SODs are coded for by the same gene. The significance of this distribution of the SODs is discussed.

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, 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 immunocytochemistry 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 as the cytosolic Cu,Zn-SOD. Culotta and associates independently, and in parallel, investigated the situation in yeast and have the same findings. We also find the Cu,Zn-SOD strongly associated with intracellular reticular membranes isolated as microsomes.

EXPERIMENTAL PROCEDURES

Materials—Nycodenz was from Invitrogen Life Technologies. Xanthine oxidase was from Roche Molecular Diagnostics Corp. 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-Elvehjem homogenizer with a Teflon piston, using 10 ml of the homogenization buffer per 2.5 g of tissue. Centrifugation at successively higher speeds yielded the following fractions: crude nuclear fraction at 1,000 × g for 10 min; heavy mitochondria at 3,000 × g for 10 min; light mitochondria at 20,000 × g for 20 min; and microsomes at 144,000 × g for 90 min. The final supernatant was the cytosolic fraction. Each successive pellet was washed three times with the homogenization buffer. The centrifugates 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 mM 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 at 50%, 3 ml at 40%, 4.5 ml at 34%, and 6 ml at 30%. The sample on this gradient was centrifuged at 110,000 × g for 90 min at 4°C.

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with PBS to remove soluble proteins derived from the matrix or inter-
membrane spaces. In our conditions the Cu,Zn-SOD was 24% inhibited by 50 
\(\mu\)M cyanide and 93% inhibited at 2 mM cyanide. Mn-SOD was not inhibited at these

- **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 \(\mu\)M and then 2 mM NaCN (9). Under
our conditions the Cu,Zn-SOD was 24% inhibited by 50 \(\mu\)M 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, as a
cytoplasmic 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 frac-
tions and samples for cytochrome c oxidase assay were treated with

- **Immunoassays**—Samples were subjected to 15% SDS-PAGE, and
then blotted onto nitrocellulose membranes under semi-dry condi-
tions 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,

**RESULTS**

**SOD Activities in Liver Fractions**—When rat liver was ho-
genized in isotonic buffer and fractionated, as described
under “Experimental Procedures,” the soluble cytosolic fraction
(144,000 \(\times\) g supernatant) contained abundant Cu,Zn-SOD and
detectable Mn-SOD while the heavy and the light mito-
ochondrial fractions contained both SODs with Mn-SOD pre-
dominating. 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 immunoblots, are shown in Fig. 1, A and B.

**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 under “Experimental Procedures.” The data in Fig. 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 Fig. 2A, were assayed for SOD activity using cyanide to distinguish Cu,Zn-SOD from Mn-SOD and this data is shown in Fig. 2B. The heavy mitochondrial fraction 25/30, that had been found free of erythrocyte, lysosomal, and peroxi-

somal 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 Fig. 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. Fig. 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 × g for 30 min, the 0.5 M NaCl and 0.1% Lubrol supernatants from the inner membrane showed about 3 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-mitochondrial markers, and the 144,000 × g supernatant fraction was taken to be cytosol. The results of these isolation procedures are given in Tables I and II and Fig. 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 Tables I and II. It should be noted that the specific activities of the Cu,Zn-SOD was ~4,700 units/mg whether isolated from cytosol or mitochondria. Fig. 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 Fig. 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 \( \mu \text{M} \) in both cases. Fig. 6, A and B, make the same point with regard to inactivation at 70 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).

**TABLE I**

| Preparation stage | Volume | Protein Total protein | Total activity | Specific activity | Yield | Fold purification |
|---|---|---|---|---|---|---|
| Cytosol (144,000 supernatant) | 500 | 22.4 11,200 | 1,176,000 | 105 | 100 | 1 |
| EtOH/CHCl₃ | 600 | 8.4 5,040 | 1,038,240 | 206 | 88 | 2 |
| Acetone precipitation | 4 | 35.5 142 | 161,738 | 1139 | 14 | 11 |
| AcA54 | 1.0 | 28.4 28 | 108,488 | 3820 | 9 | 36 |
| SP-Sepharose | 0.6 | 26.6 16 | 75,347 | 4721 | 6 | 45 |

**TABLE II**

| Preparation stage | Volume | Protein Total protein | Total activity | Specific activity | Yield | Fold purification |
|---|---|---|---|---|---|---|
| Mitochondria (HM25/30) | 165 | 58.3 9,620 | 28,859 | 3 | 100 | 1 |
| EtOH/CHCl₃ | 200 | 8.6 1,720 | 24,080 | 14 | 83 | 5 |
| Acetone precipitation | 1.0 | 3.4 3.4 | 4,420 | 1300 | 15 | 433 |
| AcA54 | 0.2 | 3.8 0.8 | 3,547 | 4667 | 12 | 1556 |

**Mitochondrial Cu,Zn-SOD**

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 III.

**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 overexpress Cu,Zn-SOD, and knockout mice that do not express Cu,Zn-SOD. The results in Fig. 7 show that the overexpressing 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 (Tables I and II), prior to assaying them...
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 units/mg of 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 units/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 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 enclo sure 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 hydroper oxyl radical (HO2-) is a stronger oxidant of unsaturated lipids
Mitochondrial Cu,Zn-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 dismutated 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 immunoelectron microscopy 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.2 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. Polymeric macromolecules or polymeric 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 polymeric macromolecule (27, 28). The pKa of the hydroperoxyl radical (HO2•−) is ~−4.8 (29) and HO2•− is a much stronger oxidant than superoxide (30, 31). It follows that superoxide diffusing forwards the polymeric surface of the membrane could, if allowed to closely approach that surface, protonate to HO2− and then oxidize membrane components, such as polyunsaturated lipids. SOD bound to the membrane could prevent this by scavenging superoxide prior to its protonation.

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