The Paradigm That All Oxygen-Respiring Eukaryotes Have Cytosolic CuZn-Superoxide Dismutase and That Mn-Superoxide Dismutase Is Localized to the Mitochondria Does Not Apply to a Large Group of Marine Arthropods†

Marius Brouwer,*,‡ Thea Hoexum Brouwer,‡ Walter Grater,‡ Jan J. Enghild,§ and Ida B. Thogersen§

University of Southern Mississippi, Institute of Marine Sciences/Gulf Coast Research Laboratory, 703 East Beach Drive, Ocean Springs, Mississippi 39564, and Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710

Received May 6, 1997; Revised Manuscript Received August 26, 1997⊗

ABSTRACT: The enzyme superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide radical, is present in the cytosol and mitochondria of all oxygen-respiring eukaryotes. The cytosolic form contains copper and zinc (CuZnSOD), whereas the mitochondrial form contains manganese (MnSOD). The latter protein is synthesized in the cytosol as a MnSOD precursor, containing an N-terminal mitochondrial-targeting sequence. CuZnSOD is sensitive toward cyanide (CN) and hydrogen peroxide (H_2O_2), but MnSOD is not. Assays for SOD activity in cytosol from the hepatopancreas of the blue crab, *Callinectes sapidus*, showed the presence of a CN/H_2O_2-insensitive form of SOD. No CN/H_2O_2-sensitive CuZnSOD was found. This unexpected phenomenon was shown to occur in all decapod crustacea (crabs, lobsters, shrimp) examined. The cytosolic and mitochondrial SODs of *C. sapidus* were purified by means of ion-exchange, size-exclusion, and reverse-phase HPLC. The cytosolic SOD is a homodimeric protein, which exists in a monomer−dimer equilibrium (24 kDa ↔ 48 kDa). The protein contains approximately 1 Mn per subunit. No copper or zinc is present. Amino acid sequence analysis identified the novel cytosolic SOD as a MnSOD precursor with an abnormal mitochondrial-targeting sequence. The mitochondrial SOD of *C. sapidus* is similar to the MnSOD found in other eukaryotes. N-Terminal amino sequences of mitochondrial and cytosolic blue crab MnSOD differ in several positions. The MnSODs are thus encoded for by two different genes. The paradigm that all eukaryotes contain intracellular CuZnSOD and that MnSOD occurs exclusively in the mitochondria appears not to apply to a large group of marine arthropods.

Oxygen is essential for the survival of almost all eukaryotes. However, oxygen is also a precursor to formation of oxygen-derived free radicals which contribute to the processes of aging, the promotion of cancer, and several pathological disorders. Hence, oxygen is essential for life, but carries the risk of destroying the very life for which it serves as the source of useful energy (Keher, 1993; Yu, 1994).

Superoxide is the best known of all oxygen-derived free radicals, perhaps because it is the first intermediate in the sequential univalent reduction of O_2 that leads to the formation of H_2O. A major biological process leading to intracellular generation of superoxide is electron transport associated with mitochondrial membranes. Microsomal and nuclear membranes also contain electron transport systems, cytochromes P-450 and b_5, which may produce superoxide. In addition, several catalytic cytosolic enzymes contribute to the generation of superoxide and hydrogen peroxide. The superoxide radical is unique in that it leads to the formation of many other reactive species, including hydrogen peroxide. The iron-catalyzed reaction of superoxide with hydrogen peroxide produces the highly reactive hydroxyl radical that can oxidize practically any molecule contained in the cell (Halliwell & Gutteridge, 1990).

Cells contain a variety of antioxidant defenses that limit reactions brought about by activated oxygen species. The enzyme superoxide dismutase (SOD)[1] constitutes the first line of defense against oxidative damage by catalyzing the disproportionation of superoxide to give oxygen and hydrogen peroxide (Fridovich, 1989). Catalase and glutathione peroxidase remove hydrogen peroxide. Nonenzymic antioxidants, such as the lipid-soluble vitamin α-tocopherol, the water-soluble vitamin ascorbate, and the intracellular tripeptide glutathione, are essential in terminating chain reactions initiated in membrane lipids (Buettner, 1993) and in the removal of free radicals from the cytosol (Martensson & Meister, 1991; Reed, 1990).

Superoxide dismutases occur in virtually all O_2-respiring organisms. They are classified into three distinct groups depending on the metal content: FeSOD found in prokaryotes and in plants, MnSOD found in prokaryotes and in the mitochondria of eukaryotes, and CuZnSOD found in the cytosol and extracellular compartments of eukaryotes, and also in the periplasm of Gram-negative bacteria (Bordo et

† This work was supported by a grant from the National Science Foundation to M.B. (MCB-9696077).

‡ Institute of Marine Sciences/Gulf Coast Research Laboratory.

§ Duke University Medical Center.

⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

[1] Abbreviations: DEAE, diethylaminoethyl; DDC, diethyldithiocarbamate; DTPA, diethylenetriaminepentaacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; SOD, superoxide dismutase; TFA, trifluoroacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

S0006-2960(97)01052-0 CCC: $14.00

al., 1994; Kroll et al., 1995). MnSODs from higher organisms are nuclear-encoded, mitochondrial matrix enzymes composed of four identical 24 kDa subunits. A larger precursor form of the enzyme is made in the cytosol and transported into the mitochondria, with concomitant cleavage of an N-terminal leader peptide (Autor, 1982; Wisp et al., 1989; Neupert, 1997). Mitochondrial MnSOD is a highly-inducible enzyme that responds to a variety of stimuli, including bacterial endotoxins, interleukins, phorbol esters, and hydrogen peroxide (White et al., 1993). The amino acid sequences of all MnSODs, whether from animals, plants, or bacteria, are extremely similar to each other and unrelated to those of the CuZnSODs (Bordo et al., 1994; Fitch & Ayala, 1994).

Intracellular CuZnSODs are dimeric proteins, composed of two identical 16 kDa subunits. They are constitutively expressed, and are considered to be essential, "housekeeping" enzymes (Harris, 1992). The three-dimensional structure of bovine CuZnSOD has been determined (Tainer et al., 1982), and is conserved in the human enzyme (Getzoff et al., 1989). Amino acid sequences of cytosolic CuZnSODs from several fungi, plants, nematodes, insects, amphibians, fish, and mammals are all very similar, but those of extracellular mammalian CuZnSODs and periplasmic bacterial CuZnSODs diverge widely from the eukaryotic sequences (Bordo et al., 1994; Fitch & Ayala, 1994; Kroll et al., 1995). The virtually universal occurrence of intracellular CuZnSODs in eukaryotes, and the highly conserved structure of the protein, stresses the importance of the enzyme in cellular homeostasis. This is underscored by studies that show that structural alterations in the enzyme are linked to motor neuron (Lou Gehrig's) disease (Deng et al., 1993; Gurney et al., 1994), whereas down-regulation of CuZnSOD results in apoptotic death of neuronal cells (Troy & Shelanski, 1994; Rothstein et al., 1994). In contrast, overexpression of CuZnSOD in transgenic *Drosophilas* results in life-span extension (Orr & Sohal, 1994).

CuZnSOD activity is inhibited by cyanide anions (Mota de Freitas & Valentine, 1984), diethyldithiocarbamate (DDC)[1] (Heikkila, 1985), and hydrogen peroxide (Beauchamp & Fridovich, 1973; Yim et al., 1990). MnSOD is not affected by any of these components, which allows for easy differentiation between the two enzymes. Studies in our laboratory aimed at elucidating the mechanism of activation of copper-dependent proteins, using marine crustacea as experimental animals (Brouwer & Brouwer-Hoexum, 1992; Brouwer, 1996), have provided unexpected evidence that a large group of marine arthropods appear to lack cytosolic CuZnSOD. Instead, they have a CN/H_2O_2/DDC-resistant cytosolic SOD. In this paper, we report on the purification and characterization of the cytosolic and mitochondrial SOD from the blue crab, *Callinectes sapidus.* Our studies demonstrate that the blue crab has two MnSODs. One corresponds to the mitochondrial form found in other eukaryotes. The second is a novel, hitherto unknown, form of cytosolic MnSOD.

MATERIALS AND METHODS

Experimental Animals. (1) Decapod Crustacea. Blue crabs (*Callinectes sapidus*), Gulf mud fiddler crabs (*Uca longisignalis*), striped-legged hermit crabs (*Clibanarius vittatus*), and white shrimp (*Penaeus setiferus*) were collected locally in the Mississippi Sound/Davis Bayou. Ghost crabs (*Ocypode quadrata*) and stone crabs (*Menippe mercenaria*) were collected in the vicinity of the Duke University Marine Laboratory, Beaufort, NC. American lobsters (*Homarus americanus*) were purchased locally. To avoid potential effects of the molt cycle, all animals collected were in the intermolt stage.

(2) Mollusks. The common oyster (*Crassostrea virginica*) [class Bivalvia], the oyster drill (*Urosalpinx cinera*), and the tulip shell (*Fasciolaria tulipa)*[class Gastropoda], the brief squid (*Loliguncula brevis*), and the common octopus (*Octopus vulgaris*) [class Cephalopoda] were collected locally, or obtained from Beaufort, NC (*F. tulipa* and *O. vulgaris*).

Preparation of Mitochondria and Cytosol. Animals were anesthetized on ice, and the hepatopancreas was excised and homogenized in 2 volumes (w/w) of ice-cold buffer (5 mM Tris,[1] pH 7.4, + 200 mM mannitol + 83 mM sucrose + 0.1 mM PMSF[1]) using a Potter−Elvehjem tissue grinder with a Teflon pestle, using 2 vertical strokes over 30 s. Homogenates were diluted 5 times with Tris/sucrose/mannitol, centrifuged for 6 min at 700*g*, and filtered through four layers of cheesecloth. Mitochondria and cytosol were prepared by differential centrifugation (Chen & Lehninger, 1973). For SOD assays, the mitochondrial pellet was taken up in 50 mM potassium phosphate, pH 7.8, containing 0.244% Lubrol PX (500 μL/g of tissue). Mitochondrial extracts and cytosol can be stored at −70 °C without loss of SOD activity.

Enzyme Assays. SOD activity in solution was determined using the spectrophotometric cytochrome *c* reduction assay as described by McCord and Fridovich (1969) and modified by Spitz and Oberley (1989). Polyacrylamide gel electrophoresis (PAGE)[1] and staining for SOD activity were carried out as described before (Beauchamp & Fridovich, 1971; White et al., 1993). To distinguish between MnSOD and CuZnSOD, tissue homogenates were incubated with 3 mM KCN, whereas gels were soaked in 5 mM KCN, prior to measurement of SOD activity.

Protein Assay. Total protein concentration was measured using the Pierce biocinchoninic protein assay (Smith et al., 1985), with bovine serum albumin as the standard.

Purification of Cytosolic SOD. Cytosol was prepared from 140 g of hepatopancreas tissue obtained from 15 intermolt blue crabs, and dialyzed extensively against 50 mM Tris, pH 7.6, in the cold. The sample was pumped onto a 2.5 × 20 cm DEAE[1]-cellulose column at 50 mL/h at 4 °C. The column was washed with 100 mL of 50 mM Tris, pH 7.6, followed by a 1 L linear gradient from 50 → 500 mM Tris, pH 7.6, at a flow rate of 30 mL/h. Fractions of 6 mL were collected and screened for SOD activity using polyacrylamide gel electrophoresis and staining for SOD activity. SOD-containing fractions were collected, concentrated to 2 mL on an Amicon YM-10 membrane, dialyzed against 50 mM Tris, pH 7.6, and applied to a Q-Sepharose 26/10 Hi Load high-performance anion-exchange column connected to a Beckman Gold Nouveau HPLC system equipped with a photodiode array detector. The column was eluted with a linear 50 → 500 mM Tris, pH 7.6, gradient at a flow rate of 3 mL/min for 3 h. Absorbance was monitored at 280 nm. SOD-positive fractions were collected, concentrated to 2 mL, and chromatographed on a 16/60 Superdex 75 Hi Load size-exclusion column in 50 mM potassium phosphate, 0.1 mM DTPA,[1] pH 7.8, at a flow rate of 1 mL/min. Fractions of 2 mL were collected. SOD-containing fractions were concen-

trated to 2 mL and reapplied to Superdex 75 HiLoad. SOD-containing fractions were concentrated to 1 mL. A 100 μL sample was applied to a 4.6 mm × 20 cm Synchropack RP-4 reverse phase column in 0.1% TFA[1] (buffer A). The column was eluted with a stepwise gradient generated from buffers A and B (0.1% TFA in 100% acetonitrile) at a flow rate of 0.5 mL/min, using the following time course: buffer A (5 min), 0 → 40% B (10 min), 40 → 60% B (60 min) and 60 → 100% B (5 min). The absorbance of the column eluate was monitored at 220 nm. Fractions, 0.25 mL, corresponding to absorbance peaks were dried at 50 °C using a Mini Gel/Vac apparatus and analyzed by SDS[1] gel electrophoresis. The absorbance peak with a retention time of 32.8 min corresponded to a 24−25 kDa protein. In four subsequent runs, this peak was collected and used for amino acid sequence analysis. All HPLC procedures were carried out at room temperature.

Purification of Mitochondrial SOD. Mitochondria were prepared from 170 g of hepatopancreas tissue obtained from 20 intermolt crabs. Pellets were taken up and homogenized in 25 mL of 50 mM Tris, pH 7.6, with 0.1 mM PMSF, dialyzed against the same buffer (without PMSF), and applied to a 2.5 × 20 cm DEAE-cellulose column. The column was developed with a linear Tris-HCl gradient as described above. SOD-containing fractions were purified on Q-Sepharose, Superdex 75, and Synchropack RP-4 as detailed in the previous paragraph.

Molecular Weight Determinations. The molecular weight of intact, undissociated, SOD was determined by size-exclusion chromatography on a Superdex 75 HiLoad 16/60 column in 50 mM potassium phosphate, 0.1 mM DTPA, pH 7.8. The column was calibrated with cytochrome *c* (12.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), and an ovalbumin dimer (86 kDa). The subunit molecular weight of SOD was determined by SDS gel electrophoresis on a 12% PAA gel using Laemmli's buffer system (Laemmli, 1970). The calibration line was composed of phosphorylase *b* (94 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Protein bands were visualized with Coomasie Blue or silver stain.

Amino Acid Sequence Analysis. Automated Edman degradation was carried out using an Applied Biosystems Model 477A sequencer with on-line phenylthiohydantoin analysis by HPLC (Applied Biosystems 120A). The samples were applied to Porton protein sample support disks and sequenced using the modified cycles, PI-BGN and PI-1, recommended by Porton Instruments (Enghild et al., 1989).

Atomic Absorption Spectroscopy. Copper, zinc, manganese, and iron in purified SOD were measured by standard flame atomic absorption spectroscopy using a Perkin Elmer Model 3110 atomic absorption spectrometer.

RESULTS

Electrophoretic Characterization of Cytosolic and Mitochondrial SODs from Marine Decapod Crustacea and Mollusks. The results presented in Figure 1 show that no CN-sensitive CuZnSOD was observed in the cytosolic fraction of the hepatopancreas of all species of decapod crustacea examined. For some of the crustacea (blue crab, stone crab, ghost crab, and fiddler crab), the cytosolic and mitochondrial SODs had the same electrophoretic mobility on polyacrylamide gels. For others (lobster, hermit crab, and shrimp), the two SODs had different mobilities (results not shown). To examine if the lack of CuZnSOD might be correlated with the copper-dependent synthesis of hemocyanin which takes place in blue crab hepatopancreas (Rainer & Brouwer, 1993; Engel & Brouwer, 1991, 1993), we analyzed hepatopancreas tissues from hemocyanin-containing mollusks for SOD. In contrast to the crustacea, the mollusks analyzed contain large amounts of CuZnSOD (Figure 1). The apparent lack of intracellular CuZnSOD in decapod crustacea will be further addressed under Discussion.

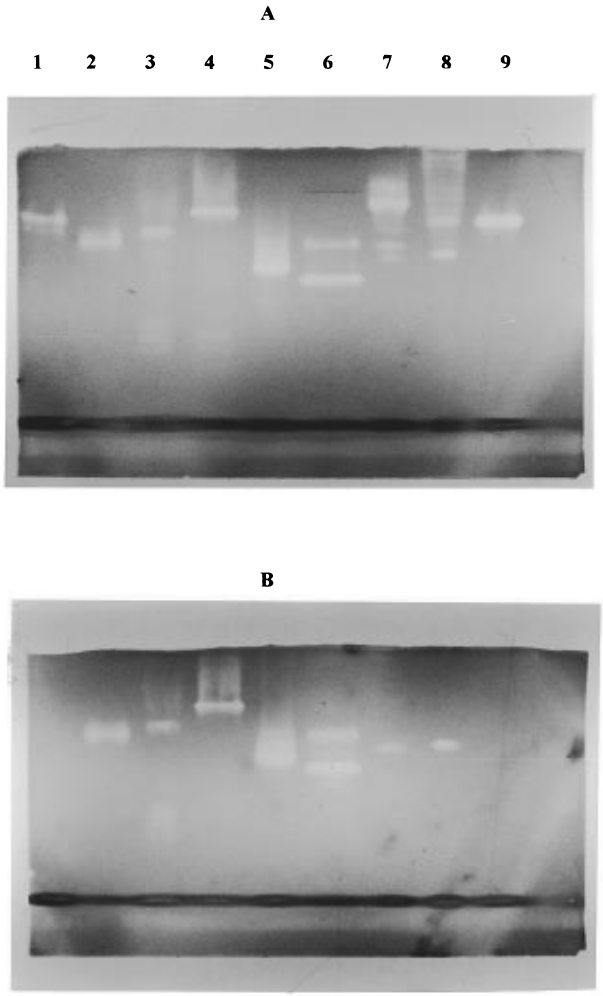

FIGURE 1: PAGE gels of hepatopancreas cytosol from five decapod crustacea and three mollusks. (A) Gel stained for SOD without KCN. lane 1, bovine CuZnSOD; lane 2, blue crab; lane 3, fiddler crab; lane 4, hermit crab; lane 5, lobster; lane 6, white shrimp; lane 7, squid; lane 8, oyster drill; lane 9, oyster. (B) Duplicate of gel A, but KCN was included in the SOD stain solution. Shrimp (*P. setiferus*) samples were prepared using hepatopancreas tissue from 14 animals. Ghost crabs and stone crabs had only CN-insensitive SOD, whereas the tulip shell and octopus had both CN-sensitive and CN-insensitive SOD (results not shown).

Spectrophotometric Assays for SOD Activity in Cytosol and Mitochondria. The results of the electrophoretic analysis were confirmed by spectrophotometric assays for SOD activity. Cytosol derived from 1 g of hepatopancreas tissue of *C. sapidus* contained 121 ± 28 (SEM; $n = 9$) CN-insensitive SOD units, or 2.6 ± 0.8 units/mg of protein [units defined by and McCord and Fridovich (1969)]. No CN-sensitive SOD activity was detected. Mitochondria obtained

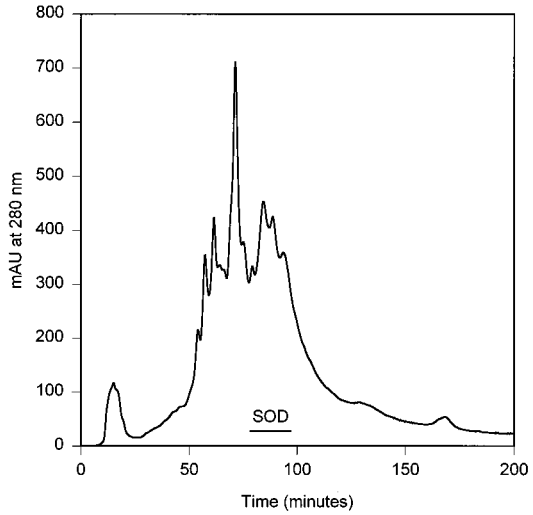

FIGURE 2: Q-Sepharose anion-exchange HPLC elution profile of pooled cytosolic SOD fractions obtained after DEAE-cellulose chromatography of blue crab, *C. sapidus*, hepatopancreas cytosol. The column was developed with a linear (50 → 500 mM) Tris, pH 7.6, gradient. SOD-containing fractions elute at a Tris concentration of 210 mM. Pooled fractions, indicated by the bar, were concentrated and applied to Superdex 75 (Figure 3). No mitochondrial SOD was present (see Figure 5).

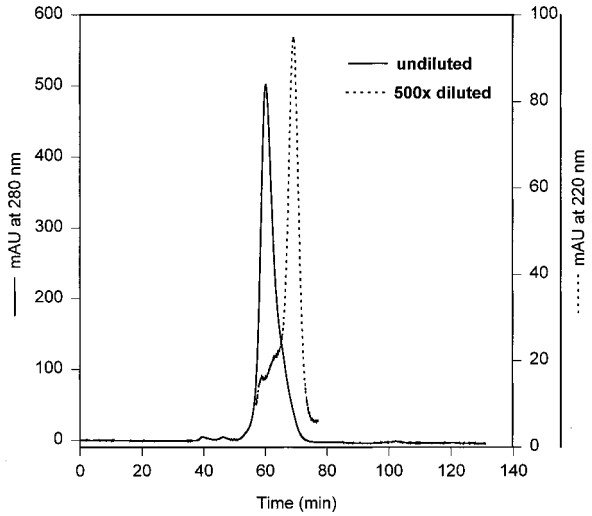

FIGURE 3: Size-exclusion HPLC elution profile of Q-Sepharose-purified cytosolic SOD (see Figure 2). The Superdex 75 HiLoad column was calibrated with molecular mass standards as described under Materials and Methods. The molecular mass of the undiluted SOD was 48 kDa. That of the 500×-diluted SOD sample was 25 kDa.

from 1 g tissue contained 16 ± 2 CN-insensitive SOD units, or 6.2 ± 0.6 units/mg of protein. The majority of the total cellular SOD activity thus resides in the cytosol. It should be emphasized that mitochondria remain intact during the homogenization procedure (see next paragraph).

Purification and Characterization of Cytosolic SOD. Cytosolic SOD elutes from the DEAE-cellulose column at a concentration of 150 mM Tris (results not shown). The elution profile of the DEAE-purified SOD on Q-Sepharose is shown in Figure 2. The retention time of SOD corresponds to a Tris concentration of 210 mM. No mitochondrial MnSOD, which elutes at 365 mM (Figure 5), is observed, demonstrating that the conditions used for preparation of the subcellular fractions preserve the structural integrity of the mitochondria. Chromatography on Superdex 75 HiLoad shows a single, slightly asymmetric, peak with a retention time corresponding to a molecular mass of 48 kDa (Figure 3). Gel electrophoresis of the purified SOD followed by SOD and protein staining showed one single band. The mobilities of the activity and protein band were the same. SDS-denaturing gel electrophoresis showed one band with a molecular mass of 24−25 kDa, and reverse phase chromatography revealed one major absorbance peak (Figure 4). This indicates that the protein is pure and suggests that the asymmetry of the peak on the size-exclusion column is not due to contamination with a low molecular weight protein, but possibly to dissociation of a dimeric SOD protein. To explore this possibility, the SOD solution was diluted 500× and reapplied to the Superdex column (Figure 3). The retention time shifted from 59 to 69 min, corresponding to a shift in molecular mass from 48 kDa to 25 kDa, corroborating our hypothesis. Atomic absorption spectroscopy and protein concentration measurement of the purified SOD showed the presence of manganese with a stochiometry of 0.82 Mn/SOD subunit. No zinc, copper, or iron was present. These data indicate that the cytosolic SOD is a MnSOD, which was unequivocally confirmed by amino acid sequence analysis (Table 1). The cytosolic SOD is a "normal" mitochondrial MnSOD, with an "abnormal" leader sequence (see Discussion).

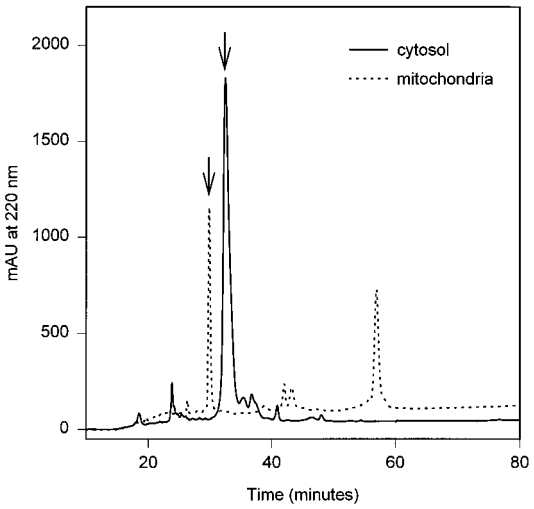

FIGURE 4: Elution profile of DEAE-cellulose/Q-Sepharose/Superdex-purified cytosolic and mitochondrial SOD on a Synchropack RP-4 reverse phase column developed with an acetonitrile gradient in 0.1% TFA (see Materials and Methods for details). The retention time of the mitochondrial and cytosolic SOD was 29.8 and 32.8 min, respectively.

Characterization of Mitochondrial SOD. Mitochondrial SOD eluted from DEAE-cellulose at a Tris concentration of 250 mM (results not shown), and from Q-Sepharose at 365 mM Tris (Figure 5). Even though the cytosolic and mitochondrial SODs cannot be separated by electrophoresis, they can be separated well by anion-exchange chromatography. The retention time of the purified SOD on Superdex was 52.3 min, corresponding to a molecular mass of 70 kDa (Figure 6). Subsequent analysis by gel electrophoresis showed the protein was not pure. Attempts to further purify the native protein by cation-exchange chromatography or reverse phase chromatography under nondenaturing conditions were unsuccessful. The final purification step was done under denaturing conditions in 0.1% TFA (Figure 4). The absorbance peak with a retention time of 29.8 min cor-

Table 1: N-Terminal Amino Acid Sequences of Mitochondrial and Cytosolic MnSOD from the Blue Crab, *Callinectes sapidus*, and Mitochondrial MnSOD from *Drosophila melanogaster* (Duttaroy et al., 1994)[a]

```
Crab Mt MnSOD       ----------------------XXTLPDLPYDYGALEPTISAEWMQLLHR
Drosophila          ------MFVARKISPNCKPG-VRGKHTLPKLPYDYAALEPIICREIMELHHQ
                                         ↑
Crab Cyt MnSOD      TGQVNPQVAAMFSHIKAELGEERGVHSLPPLGYDYGALEPHICTTIMQIHHT
                                        .** * *** **** *.   *.. *
                    1        10        20        30        40        50
```

[a] The identity of underlined residues could not be assigned with 100% certainty. The identity of X is unknown. The upward arrow indicates the cleavage site (between G and K) for removal of mitochondrial matrix-targeting sequence. The sequence of residues 1−40 of blue crab cytosolic MnSOD was determined by direct sequencing of the protein. Residues 41−50 were deduced from the cDNA sequence (Grater and Brouwer, unpublished results). Sequences were aligned using Clustal W version 1.6 (Thompson et al., 1994). Asterisks indicate identical residues. Periods indicate isofunctional residues.

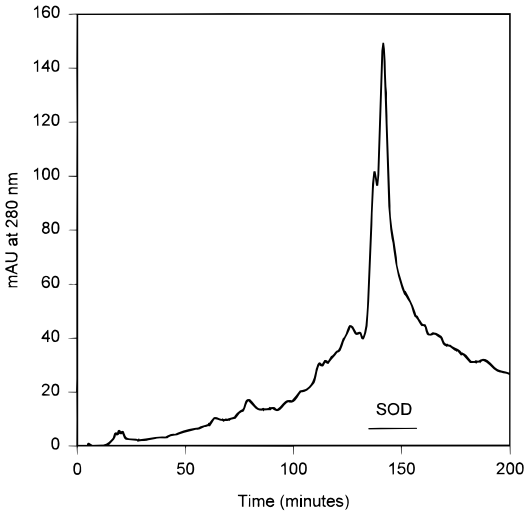

FIGURE 5: Q-Sepharose anion-exchange HPLC elution profile of pooled SOD fractions obtained after DEAE-cellulose chromatography of blue crab mitochondrial extracts prepared from the hepatopancreas. The column was developed with a linear (50 → 500 mM) Tris, pH 7.6, gradient. SOD-containing fractions elute at a Tris concentration of 365 mM. Pooled fractions, indicated by the bar, were concentrated and applied to Superdex 75 (Figure 6).

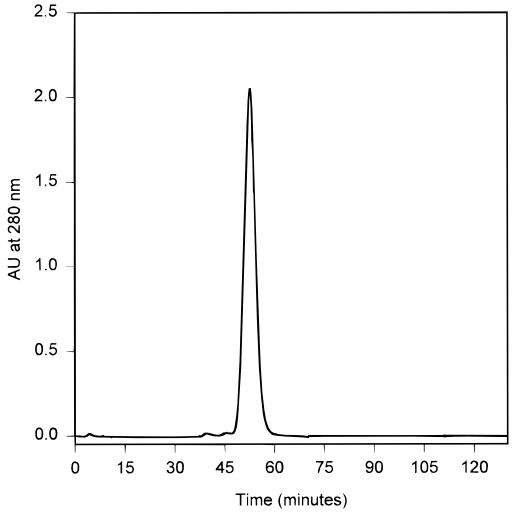

FIGURE 6: Superdex 75 size-exclusion HPLC elution profile of Q-Sepharose-purified mitochondrial SOD (see Figure 5). The molecular mass of the mitochondrial SOD was 70 kDa.

responded to a protein with a subunit molecular mass of 24−25 kDa. This protein was further analyzed by amino acid sequence analysis (Table 1), which indicated the mitochondrial SOD is a MnSOD (without leader sequence) similar to the MnSODs found in the mitochondria of other eukaryotes.

DISCUSSION

Spectrophotometric solution assays for SOD and staining for SOD on PAGE gels showed enzymatically-active SOD in the cytosol prepared from the hepatopancreas of the blue crab, *Callinectes sapidus*. Contrary to what is observed for other eukaryotes, this cytosolic SOD could not be inhibited by CN or H_2O_2 or by dialysis against a buffer containing DDC, indicating that this form of SOD does not correspond to CuZnSOD. Since blue crabs synthesize the copper protein hemocyanin in their hepatopancreas (Rainer & Brouwer, 1993), we considered the possibility that the intracellular levels of copper might be insufficient to sustain the activation of apoSOD(Cu,Zn), as is found in copper-deficient animals (Harris, 1992). However, even after dietary exposure to copper, which leads to a large increase of copper in the hepatopancreas bound to glutathione and metallothionein (Schlenk & Brouwer, 1991; Brouwer et al., 1992; Brouwer & Brouwer-Hoexum, 1991), no CuZnSOD could be detected. The absence of CuZnSOD was not tissue specific, since the enzyme was also absent from the gill tissues (Brouwer and Hanly, unpublished results).

Addition of a cocktail of proteolytic enzyme inhibitors (EDTA, PMSF, aprotonin, pepstatin, leupeptin) to the homogenization buffer had no effect on SOD recovery, indicating that it was unlikely that the lack of CuZnSOD was due to proteolysis. CuZnSOD has been reported to be associated with catalase and the peroxisomal matrix (Keller et al., 1991), although later studies, using glutaraldehyde fixation to preserve normal distribution of the protein, indicate that CuZnSOD is a soluble cytosolic protein (Crapo et al., 1992). Homogenization of blue crab hepatopancreas tissues in 10 mM Tris, pH 7.6, with 0.1%Triton X-100 results in solubilization of peroxisomes and mitochondria as measured by catalase and cytochrome *c* oxidase activity (Brouwer & Hoexum Brouwer, 1996), yet no CuZnSOD was found in the homogenate. It seems therefore unlikely that the lack of CuZnSOD in hepatopancreas cytosol can be explained by trapping of the enzyme in a subcellular compartment removed during cytosol preparation.

ApoSOD(Cu,Zn) not only is found in copper-deficient animals (Harris, 1992), but also can be present under copper-adequate conditions (Petrovic et al., 1996). The apoenzyme can be activated *in vitro* and *in vivo* with a Cu(I)−glutathione complex (Ciriolo et al., 1990; Steinkuhler et al., 1992), or

with $Cu(NO_3)_2$ (Petrovic et al., 1996). To examine if an apoSOD pool exists in blue crab hepatopancreas, hepatopancreas cytosol and hepatopancreas tissue explants, which are capable of copper uptake and metallothionein synthesis for at least 24 h (Brouwer & Hoexum Brouwer, 1996), were incubated with 25−50 μM Cu(I)−glutathione for up to 24 h. Spectrophotometric assays for SOD in the copper-treated cytosol and tissues did not reveal any detectable CuZnSOD activity. Taken together, our studies indicate that blue crabs, and decapod crustacea in general, are devoid of cytosolic CuZnSOD and apoSOD(Cu,Zn), and seem to be incapable of synthesizing (an active form of) this enzyme that is such crucial importance in antioxidant defense in all other eukaryotes.

We hypothesized that organisms which use copper for oxygen transport may have developed an antioxidant defense system that is not copper-dependent. However, two observations argue against this hypothesis: (1) the hepatopancreas of mollusks, which are dependent on copper−hemocyanin for oxygen transport, contains cytosolic CuZnSOD; and (2) we have recently found a high molecular mass (130 kDa) *extracellular* CuZnSOD in blue crab hemolymph (Henderson et al., 1997). This suggests that the lack of *intracellular* CuZnSOD in decapod crustacea is not due to hemocyanin synthesis.

In contrast to mollusks, decapod crustacea molt. In the premolt stages, prior to ecdysis, blue crabs degrade their hemocyanin, which is resynthesized post-molt, during the papershell stage (Engel & Brouwer, 1991, 1993). The lack of cytosolic CuZnSOD in intermolt hard crabs may therefore be correlated with the profound changes in copper metabolism that occur during the molt cycle. Preliminary experiments show that blue crabs have a CN-sensitive SOD in hepatopancreas cytosol, when in the papershell stage. However, this form of SOD is different from the 32 kDa eukaryotic cytosolic CuZnSOD and corresponds to the 130 kDa extracellular form referred to in the preceding paragraph (Grater and Brouwer, unpublished results). Experiments to clone and sequence the multiple SOD genes of the blue crab and study their expression during the molt cycle are under way.

N-Terminal amino acid sequences of cytosolic MnSOD and mitochondrial MnSOD were compared against sequences deposited at the Swiss-Prot Protein Sequence Database. The search was performed using the National Center for Biotechnology Information's BLAST WWW Server (Basic Local Alignment Search Tool; Altschul et al., 1990). Results are presented in Table 1. Residues 1−23 of cytosolic MnSOD represent a unique amino acid sequence. There is no homology between residues 1−23 and the mitochondrial matrix-targeting sequences of precursor MnSODs which are rich in Lys, Arg, Ser, and Thr and devoid of Asp and Glu (Wispe et al., 1989; Shimoda-Matsubayashi et al., 1996), and are usually amphiphilic α-helices with positively charged and hydrophobic residues on opposite faces of the helix (Lemire et al., 1989; Roise et al., 1988; Neupert, 1997). To explore if such an arrangement is possible for residues 1−23 of the blue crab cytosolic MnSOD sequence, a secondary structure analysis of this sequence was conducted using the Protein Analysis Server maintained by the Biomolecular Engineering Center (BMERC) of Boston University, using the analysis algorithm described by Stulz (Stulz et al., 1993). Results showed that residues 9−20 (AAMFSHIKAELG) are likely to form an α-helix. Secondary structure analysis using the Self Optimized Prediction Method from Alignment (Geourjon & Deleage, 1994, 1995) corroborated these results. An axial projection of amino acid residues 9−20 on a helical wheel (Figure 7) showed that the helix lacked the amhiphilic structure characteristic of mitochondrial-targeting sequences. A number of hydrophobic residues (Met-11, Ile-15, Leu-19, Phe-12) are clustered on one side of the helix, but no positive charges are found on the opposite face of the helix. The only positive charge (Lys-16) is interspersed between two hydrophobic amino acid residues (Phe-12 and Ala-9).

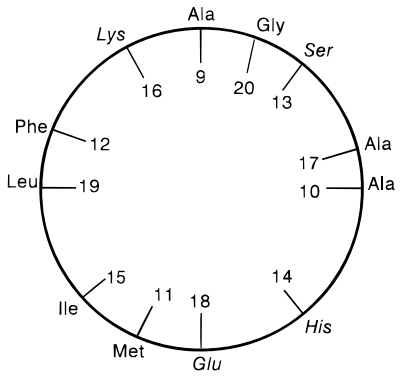

FIGURE 7: Helical wheel presentation of the α-helix formed, according to secondary structure analysis, by residues 9−20 of cytosolic MnSOD. Polar and charged residues are in italics. Apolar, hydrophobic residues are in normal letter type. This putative α-helix lacks the amphiphilic character which is typical of the mitochondrial-leader sequences of MnSODs.

Starting with residue 23, there is a strong match between blue crab cytosolic MnSOD and *Drosophila melanogaster* mature MnSOD (20/30 identities). It appears, therefore, that blue crab MnSOD found in the cytosol is a normal mitochondrial MnSOD with an abnormal leader sequence. It appears that the latter prevents the MnSOD from being imported into the mitochondria, since the cytosolic MnSOD, in either its precursor or its mature form, is not found in the mitochondria. In addition, incubation of total tissue homogenate followed by anion-exchange chromatography shows the presence of both the cytosolic precursor MnSOD and the mature mitochondrial MnSOD. This indicates that mitochondrial enzymes are incapable of removing the N-terminal leader sequence from the cytosolic MnSOD. It is of interest to note that amino acid substitutions in the MnSOD leader sequence and impaired mitochondrial import of MnSOD have been suggested to play a role in Progeria (a genetic disease of premature aging) and Parkinson's disease (Rosenblum et al., 1996; Shimoda-Matsubayashi et al., 1996).

Comparison of the N-terminal sequence of blue crab mitochondrial MnSOD against published sequences showed that the protein has greatest sequence homology (19/26 identities) with two plant MnSODs (*Triticum aestivum* and *Nicotiana plumbaginifolia)*. Sequence homology between *C. sapidus* and *D. melanogaster* mitochondrial MnSOD is 16/26. Mitochondrial and cytosolic blue crab MnSOD differ in 11 out of 26 positions (in addition to lack of leader sequence in mitochondrial MnSOD protein). Since proteins were purified from hepatopancreas tissues from at least 15 animals, the observed differences are not due to difference between animals. We conclude, therefore, that the cytosolic and mitochondrial MnSODs from *C. sapidus* are encoded

for by two different genes. This contrasts with all other organisms which have a single MnSOD gene, with one exception. Maize (*Zea mays*) has three MnSOD-encoding genes. However, unlike the blue crab MnSODs, all of the maize MnSODs are mitochondria-associated isozymes with conserved mitochondrial-targeting sequences (Zhu & Scandalios, 1993).

The cytosolic MnSOD is a homodimeric enzyme with a subunit molecular mass of 24−25 kDa. In this respect, the cytosolic MnSOD resembles prokaryotic MnSODs which are generally dimeric (Bordo et al., 1994). The molecular mass of the mitochondrial MnSOD (70 kDa) is less than that expected for a tetrameric enzyme with 24 kDa subunits. Similar discrepancies between subunit molecular mass and the molecular mass of native MnSOD determined by size-exclusion chromatography have been reported before (Meier et al., 1982, 1994).

Although tetrameric MnSOD in eukaryotes is always associated with mitochondria, the gene was not acquired as a result of endosymbiosis (Smith & Doolittle, 1992). The presence of archaebacterial sequences, intermediate between eukaryotes and eubacteria, emphasizes the conventional descent of MnSOD. This being the case, targeting sequences would have had to be provided in order that the protein could be translocated into the organelle (Smith & Doolittle, 1992). How MnSODs with an "abnormal" and "normal" leader sequence have evolved is unclear. Cloning and sequencing of the two MnSOD genes of the blue crab are currently under way to shed further light on their evolutionary origin.

In conclusion, our studies have shown that the paradigm that all oxygen-respiring eukaryotes have a cytosolic CuZn-SOD and that MnSOD occurs exclusively in the mitochondria does not apply to a large group of marine arthropods. These organisms use two distinct forms of MnSOD, one in the cytosol and the second in the mitochondria, in defense against potentially toxic superoxide. The functional and regulatory properties of this novel system, and its evolutionary origin, are under investigation.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990) *J. Mol. Biol. 215*, 403−410.

Autor, A. P. (1982) *J. Biol. Chem. 257*, 2713−2718.

Beauchamp, C., & Fridovich, I. (1971) *Anal. Biochem. 44*, 276−287.

Bordo, D., Djinovic, K., & Bolognesi, M. (1994) *J. Mol. Biol. 238*, 366−386.

Brouwer, M. (1996) *Adv. Inorg. Biochem. 11*, 235−260.

Brouwer, M., & Brouwer-Hoexum, T. (1991) *Arch. Biochem. Biophys. 290*, 207−213.

Brouwer, M., & Brouwer-Hoexum, T. (1992) *Biochemistry 31*, 4096−4102.

Brouwer, M., & Hoexum Brouwer, T. M. (1996) *Soc. Environ. Toxicol. Chem. Abstr. Book*, 260.

Brouwer, M., Schlenk, D., Brouwer-Hoexum, T., & Ringwood, A. H. (1992) *Arch. Biochem. Biophys. 294*, 461−468.

Buettner, G. R. (1993) *Arch. Biochem. Biophys. 300*, 535−543.

Chen, C. H., & Lehninger, A. L. (1973) *Arch. Biochem. Biophys. 154*, 449−459.

Ciriolo, M. R., Desideri, A., Paci, M., & Rotilio, G. (1990) *J. Biol. Chem. 265*, 11030−11034.

Crapo, J. D., Oury, T., Rabouille, C., Slot, J. W., & Chang, L. Y. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10405−10409.

Deng, H. X., Hentati, A., Tainer, J. A., Iqbal, Z., et al. (1993) *Science 261*, 1047−1051.

Duttaroy, A., Meidinger, R., Kirby, K., Charmichael, S., Hilliker, A., & Phillips, J. (1994) *Gene 143*, 223−225.

Engel, D. W., & Brouwer, M. (1991) *Biol. Bull. 180*, 447−452.

Engel, D. W., & Brouwer, M. (1993) *Marine Environ. Res. 35*, 1−5.

Enghild, J. J., Thogersen, I. B., Pizzo, S. V., & Salvesen, G. (1989) *J. Biol. Chem. 264*, 15975−15981.

Fitch, W. M., & Ayala, F. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 6802−6807.

Fridovich, I. (1989) *J. Biol. Chem. 264*, 7761−7764.

Geourjon, G., & Deleage, G. (1994) *Prot. Eng. 7*, 157−162.

Geourjon, G., & Deleage, G. (1995) *Comput. Appl. Biosci. 11*, 681−684.

Getzoff, E. D., Tainer, J. A., Stempien, M. M., Bell, G. I., & Hallewell, R. A. (1989) *Proteins*: *Struct., Funct., Genet. 5*, 322−336.

Grater, W. D., Brouwer, T. H., & Brouwer, M. (1997) *Science in Mississippi 42*, 21.

Gurney, M. E., Pu, H., Chiu, A. Y., Dal Canto, M. C., Polchow, C. Y., et al. (1994) *Science 264*, 1772−1775.

Halliwell, B., & Gutteridge, J. M. C. (1990) *Methods Enzymol. 186*, 1−85.

Harris, E. D. (1992) *J. Nutr. 122*, 636−640.

Heikilla, R. E. (1985) in *CRC Handbook of Methods for Oxygen Radical Research* (Greenwald, R. A., Ed.) pp 387−389, CRC Press, Boca Raton, FL.

Henderson, T., Brouwer, T. H., & Brouwer, M. (1997) *Science in Mississippi 42*, 21.

Kehrer, J. P. (1993) *Crit. Rev. Toxicol. 23*, 21−48.

Keller, G. A., Warner, T. G., Steiner, K. S., & Hallewell, R. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7381−7385.

Kroll, J. S., Langford, P. R., Wilks, K. E., & Keil, A. D. (1995) *Microbiology 141*, 2271−2279.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Lemire, B. D., Fankhauser, C., Baker, A., & Schatz, G. (1989) *J. Biol. Chem. 264*, 20206−20215.

Martensson, J., & Meister, A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4656−4660.

McCord, J. M., & Fridovich, I. (1969) *J. Biol. Chem. 22*, 6049−6055.

Meier, B., Barra, D., Bossa, F., Calabrese, L., & Rotilio, G. (1982) *J. Biol. Chem. 257*, 13977−13980.

Meier, B., Sehn, A. P., Schinina, M. E., & Barra, D. (1994) *Eur. J. Biochem. 219*, 463−468.

Mota de Freitas, D., & Valentine, J. S. (1984) *Biochemistry 23*, 2079−2082.

Neupert, W. (1997) *Annu. Rev. Biochem. 66*, 863−917.

Orr, W. C., & Sohal, R. S. (1994) *Science 263*, 1128−1130.

Petrovic, N., Comi, A., & Ettinger, M. J. (1996) *J. Biol. Chem. 271*, 28331−28334.

Rainer, J., & Brouwer, M. (1993) *Comp. Biochem. Physiol. 104B*, 69−73.

Reed, D. J. (1990) *Annu. Rev. Pharmacol. Toxicol. 30*, 603−631.

Roise, D., Theiler, F., Horvath, S. J., Tomich, J. M., Richards, J. H., Allison, D. S., & Schatz, D. (1988) *EMBO J. 7*, 649−653.

Rosenblum, J. S., Gilula, N. B., & Lerner, R. A. (1996) *Proc. Natl. Acad. Sci U.S.A. 93*, 4471−4473.

Rothstein, J. D., Bristol, L. A., Hosler, B., Brown, R. H., & Kuncl, R. W. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4155−4159.

Schlenk, D, & Brouwer, M. (1991) *Aquatic Toxicol. 20*, 25−34.

Shimoda-Matsubayashi, S., Matsumine, H., Kobayashi, T., Nakagawa-Hattori, Y., Shimizu, Y., & Mizuno,Y. (1996) *Biochem. Biophys. Res. Commun. 226*, 561−565.

Smith, M. W., & Doolittle, R. F. (1992) *J. Mol. Evol. 34*, 175−184.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76−85.

Spitz, D. R., & Oberley, L. W. (1989) *Anal. Biochem. 179*, 8−18.

Steinkuhler, C., Sapora, O., Carri, M. T., Nagel, W., Marcocci, L., Ciriolo, M. R., Weser, U., & Rotilio, G. (1991) *J. Biol. Chem. 266*, 24580−24587.

Stulz, C. M., White, J. V., & Smith, T. F. (1993) *Protein Sci. 2,* 305−314.

Tainer, J. A., Getzoff, E. D., Beem, K. M., Richardson, J. S., & Richardson, D. C. (1982) *J. Mol. Biol. 160,* 81−217.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994) *Nucleic Acids Res. 22,* 4673−4680.

Troy, C. M., & Shelanski, M. L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91,* 6384−6387.

White, C. W., Nguyen, D. H., Suzuki, K., Taniguchi, N., Rusakow, L. S., Avraham, K. B., & Groner, Y. (1993) *Free Radical Biol. Med. 15,* 629−636.

Wispe, J. R., Clark, J. C., Burhans, M. S., Kropp, K. E., Korfhagen T. R., & Whitset, J. A. (1989) *Biochim. Biophys. Acta 994,* 30−36.

Yim, M. B., Chock, P. B., & Stadtman, E. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 5006−5010.

Yu, B. P. (1994) *Physiol. Rev. 74,* 139−161.

Zhu, D., & Scandalios, J. G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 9310−9314.

BI971052C