Supporting Information to Accompany: Investigation of the Highly Active Manganese Superoxide Dismutase from *Saccharomyces cerevisiae*

Kevin Barnese, Yuewei Sheng, Troy A. Stich, Edith B. Gralla, R. David Britt, Diane E. Cabelli, Joan Selverstone Valentine

Materials and Methods

Isolation and Characterization of ScMnSOD. The MnSOD protein was grown in the native *S. cerevisiae* host. An over expression vector for the *Sc*MnSOD including the native mitochondrial matrix leader sequence (YEp352-SOD2, a yeast/*E. coli* shuttle vector containing the URA3 selectable marker and a 2-kb genomic *Bam*HI fragment containing the gene for *Sc*MnSOD) [1] was transformed in to wild type *S. cerevisiae* (EG103).

The strain was kept in frozen stocks at -80˚C. The yeast cells were streaked out on to SD-URA plates, which were then incubated at 30˚C for 3 days. A single colony was selected and used to inoculate a sterile test tube containing 4-mL SD-URA medium; the flask was then grown overnight. The first overnight was used to inoculate a second overnight (100 mL in 4- 125 mL flasks) to 0.05 OD_{600}. The second overnight was used to inoculate 1 L cultures to 0.05 OD_{600} in pH 4 YPEG medium (1% yeast extract, 2% peptone, 2% ethanol, 3% glycerol) supplemented with 500 μM Mn(II) acetate. Cells were grown for 3 days or until they reached an $OD_{600} \geq 25$.

We modified the purification from Ravindranath *et. al.* [2]; most notably we omitted the heat treatment, and we used a gentler ammonium sulfate cut.

After growth, the cells were spun down (3500 RPM for 10 minutes at 4 ^{o}C, Sorvall H6000A rotor), re-suspended in 250 mL of lysis buffer (50 mM pH 7.4 tris-HCl, 5 mM EDTA, 200 μM PMSF, and 1 μg/mL of leupeptin and pepstatin) and lysed by bead (.5 mm glass beads) beating (10 cycles at 2 minutes of beating and 2 minutes of chilling on ice). The resulting lysate was clarified by centrifugation (30 minutes at 11000 RPM, Beckman JA-14 rotor). The supernatant was adjusted to 50% ammonium sulfate by slowly adding ammonium sulfate (29.5 g of ammonium sulfate per 100 mL of supernatant) to the lysate. The solution was then stirred on ice for 30 minutes and clarified by centrifugation (45 minutes at 8000 RPM, Beckman JA-14 rotor).

The lysate was loaded on to a Hydrophobic Interaction column (Phenyl Sepharose 6 Fast Flow, high sub) with 230-250 mL of column media, washed with 2 column volumes of wash buffer (50 mM pH 7 sodium phosphate, 150 mM NaCl, 100 μM EDTA, and 2 M $(NH4)_2SO_4$), and then eluted with a gradient of elution buffer (50 mM pH 7 sodium phosphate, 150 mM NaCl, and 100 μM EDTA). The fractions with the highest SOD activities were pooled.

SOD activity was measured by a modification of the method of Quick and Dugan.[3] 20 µL of each of the fractions were aliquoted into a 96-well microtiter plate. Each well contained 162.5 µL of a PBS solution containing 200 µM hypoxanthine, 100 µM XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt), and 120 U/mL of bovine catalase. Then, 162.5 µL of a 6 x 10^{-3} U/mL solution of xanthine oxidase was added to each well. The plate was then monitored on a plate reader at 490 nm (orange color) for 30 minutes. The absence of color change indicates the inhibition of the reduction of XTT by O_2^-. Thus SOD activity inhibits the formation of the orange color.

The SOD active fractions were pooled and dialyzed twice against 4 L of water and then once again against 4 L of column buffer (25 mM potassium phosphate, pH 7.4). The dialyzed protein sample was then loaded on to an anion exchange DEAE column (150 mL of Whatman DE-52), washed with two column volumes of column buffer, and eluted with 4X column buffer (no gradient). Again the SOD activity of the fractions was measured, and the most active fractions were pooled.

For further purification the protein sample was concentrated to < 8 mL using a 10,000 Da MWCO Amicon filter, loaded on to a G300 (Sepharose HR300) gel filtration column, and eluted with column buffer. The SOD activity and protein purity (below) was measured, and the most pure fractions with the highest SOD activity were pooled.

The purity was analyzed by SDS poly acrylamide gel electrophoresis (PAGE) and HPLC (Agilent 1200 series) fitted with a size exclusion column (Tosoh Bioscience, TSK gel G2000SW 7.5 mm ID x 30 cm) with diode array spectrophotometer monitoring at 280 nm for detection of protein. The column was calibrated using five standard proteins: thyroglobulin (670 kDa), bovine g-globulin (158 kDa), ovalbumin (44 kDa), equine myoglobulin (17 kDa), and vitamin K (1.35 kDa). The running buffer was 25 mM sodium phosphate (pH 6.7) and 25 mM sodium chloride.

Pulse Radiolysis. The pulse radiolysis experiments were carried out using the 2 MeV Van de Graaf accelerator at Brookhaven National Laboratory. Superoxide (O_2^-) was generated in air-saturated aqueous solution containing sodium formate.[4] The experiments to measure catalytic rates were carried out in two methods: (a) oxidizing the resting enzyme with substoichiometric concentration of O_2^- and following the appearance of Mn(III)SOD which has an absorbance band around 480 nm; (b) following the decay of various concentrations of O_2^- at 260 nm. k_2 was determined by fitting the time trace of the generating Mn(III) to a first-order reaction. The other catalytic rates were determined by fitting the data obtained by using the Chemical Kinetics program in PRWIN.[5] All samples were analyzed in 10 mM potassium phosphate, 10 mM sodium formate, and 10 μM EDTA at 25 °C. Enzyme concentrations vary from 1 to 60 μM. In method a, hydrogen peroxide (2:1 ratio to enzyme) was added to sample buffer to ensure that the enzyme was fully reduced before each O_2^- flux. The pH of the buffer was adjusted using sodium hydroxide and sulfuric acid.

EPR Spectroscopy. X-band perpendicular-polarization ($B_0 \perp B_1$) and parallel-polarization ($B_0 \parallel B_1$) continuous-wave electron paramagnetic resonance (CW EPR) spectra were recorded using a Bruker ECS106 spectrometer equipped with a dual-mode cavity (ER 4116DM) operating at 9.69 and 9.39 GHz, respectively. Cryogenic temperatures were achieved and controlled using an Oxford Instruments ESR900 liquid helium cryostat in conjunction with an Oxford Instruments ITC503 temperature and gas flow controller. Spectra were acquired under slow-passage, non-saturating conditions. Additional spectrometer settings were: modulation frequency = 100 kHz; modulation amplitude = 0.5 mT.

References

(1) Vanloon, A. P. G. M.; Pesoldhurt, B.; Schatz, G. *Proc. Natl. Acad. Sci. U. S. A.* **1986**, *83*, 3820-3824.
(2) Ravindranath, S. D.; Fridovich, I. *J Biol Chem* **1975**, *250*, 6107-6112.
(3) Quick, K. L.; Hardt, J. I.; Dugan, L. L. *Journal of Neuroscience Methods* **2000**, *97*, 139-144.
(4) Fabrizio, P.; Liou, L. L.; Moy, V. N.; Diaspro, A.; Valentine, J. S.; Gralla, E. B.; Longo, V. D. *Genetics* **2003**, *163*, 35-46.
(5) Rabani, J.; Nielsen, S. O. *J. Phys. Chem.* **1969**, *73*, 3736-3744.
(6) Schwarz, H. BNL Pulse Radiolysis Program. Brookhaven National Laboratory.

Complete reference for reference #2:

Wang, W.; Fang, H. Q.; Groom, L.; Cheng, A. W.; Zhang, W. R.; Liu, J.; Wang, X. H.; Li, K. T.; Han, P. D.; Zheng, M.; Yin, J. H.; Wang, W. D.; Mattson, M. P.; Kao, J. P. Y.; Lakatta, E. G.; Sheu, S. S.; Ouyang, K. F.; Chen, J.; Dirksen, R. T.; Cheng, H. P. *Cell* **2008**, 134, 279-290.

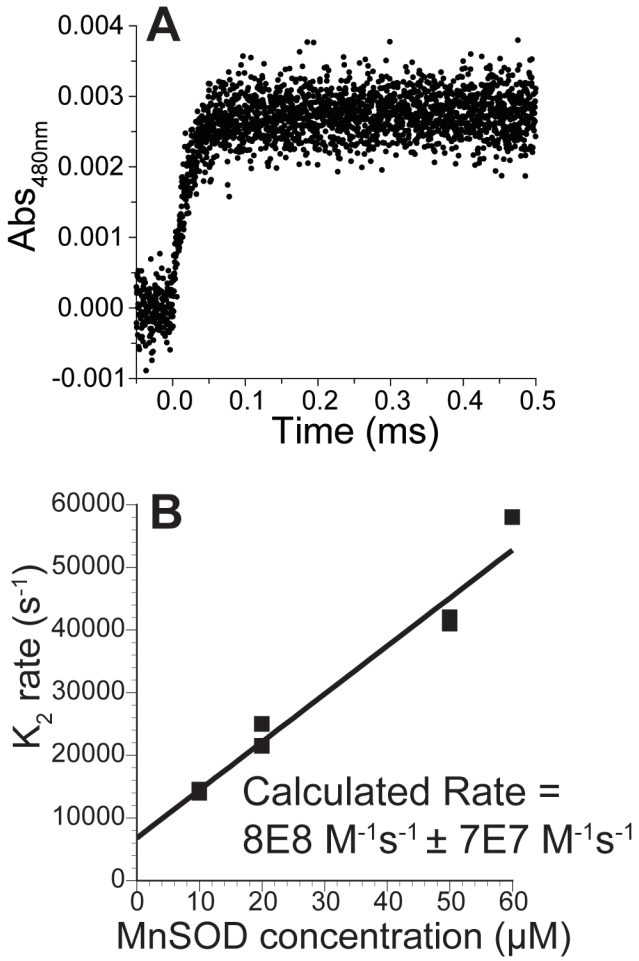

Figure S1. **A.** The oxidation of *Sc*Mn^{2+}SOD in pulse radiolysis (method a) is shown by the change of absorbance at 480 nm with time after 1.8 μM of O_2^- was generated. Solutions contained 60 μM enzyme (in Mn), 10 mM potassium phosphate (pH 7.5), 10 mM sodium formate, and 10 μM EDTA. The enzyme had been reduced prior to the experiment with 120 μM H_2O_2. **B.** The first order rate of the enzyme was determined at multiple different concentrations of enzyme. The rate was calculated by finding the slope of the least squares liner fitting. In addition to MnSOD the solutions contained 10 mM potassium phosphate (pH 7.5), 10 mM sodium formate, and 10 μM EDTA. The enzyme had been reduced prior to the experiments with at least 200 μM H_2O_2.

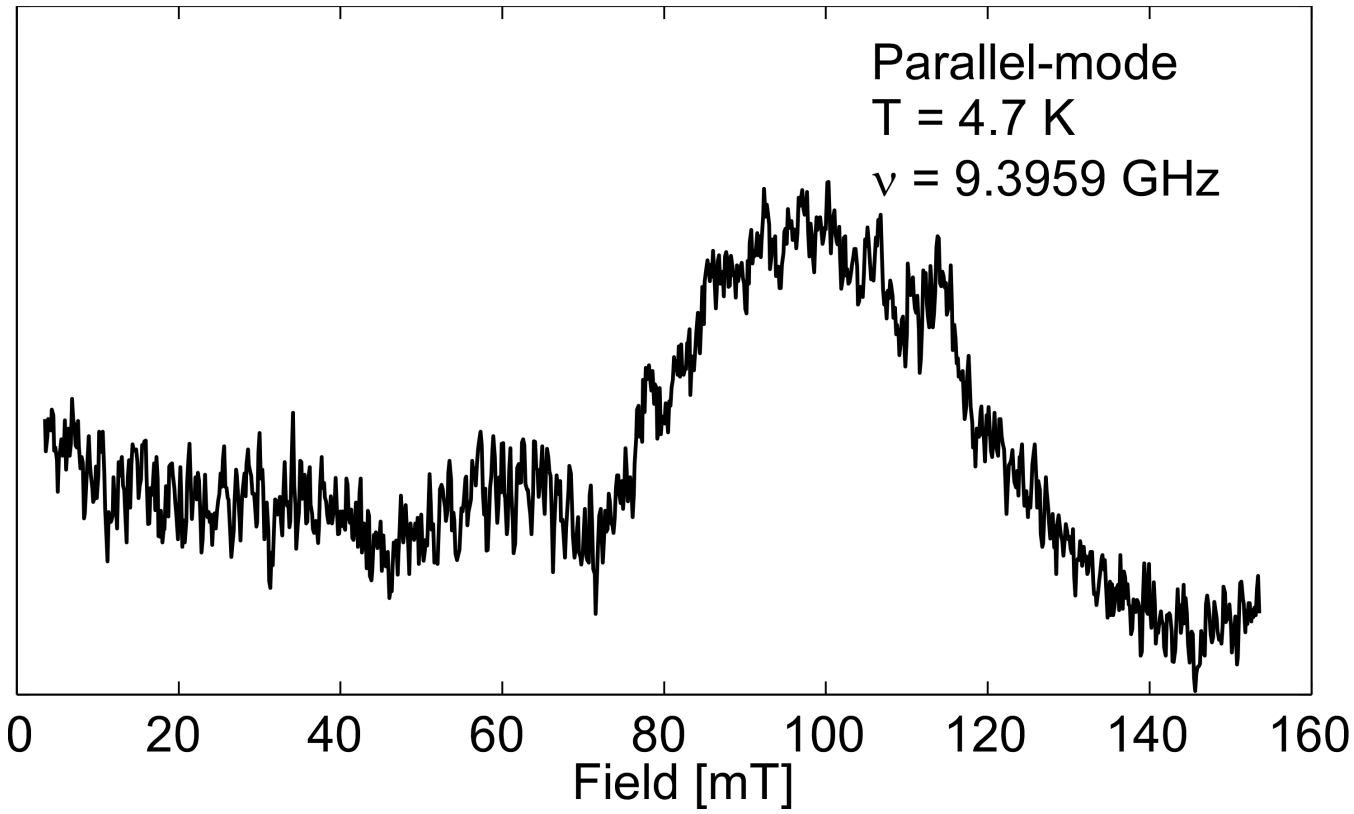

Figure S2. Parallel-mode CW-EPR spectrum of as-isolated *Sc*MnSOD. Instrument settings: ν = 9.3959 GHz; microwave power = 32 mW; sweep rate = 1.81 mT/s; temp = 4.7 K.