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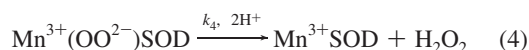
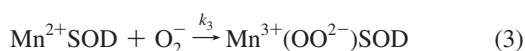
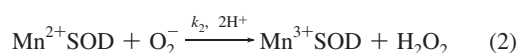
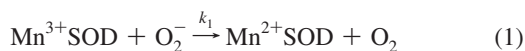
Investigation of the Highly Active Manganese Superoxide Dismutase from *Saccharomyces cerevisiae*Kevin Barnese,<sup>†,‡,§</sup> Yuewei Sheng,<sup>†,‡</sup> Troy A. Stich,<sup>||</sup> Edith B. Gralla,<sup>‡</sup> R. David Britt,<sup>||</sup> Diane E. Cabelli,<sup>\*,†,‡</sup> and Joan Selverstone Valentine<sup>\*,‡,§</sup>

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**Abstract:** Manganese superoxide dismutase (MnSOD) from different species differs in its efficiency in removing high concentrations of superoxide ( $O_2^-$ ), due to different levels of product inhibition. Human MnSOD exhibits a substantially higher level of product inhibition than the MnSODs from bacteria. In order to investigate the mechanism of product inhibition and whether it is a feature common to eukaryotic MnSODs, we purified MnSOD from *Saccharomyces cerevisiae* (ScMnSOD). It was a tetramer with 0.6 equiv of Mn per monomer. The catalytic activity of ScMnSOD was investigated by pulse radiolysis and compared with human and two bacterial (*Escherichia coli* and *Deinococcus radiodurans*) MnSODs. To our surprise, ScMnSOD most efficiently facilitates removal of high concentrations of  $O_2^-$  among these MnSODs. The gating value  $k_2/k_3$  that characterizes the level of product inhibition scales as ScMnSOD > *D. radiodurans* MnSOD > *E. coli* MnSOD > human MnSOD. While most MnSODs rest as the oxidized form, ScMnSOD was isolated in the  $Mn^{2+}$  oxidation state as revealed by its optical and electron paramagnetic resonance spectra. This finding poses the possibility of elucidating the origin of product inhibition by comparing human MnSOD with ScMnSOD.

Manganese superoxide dismutase (MnSOD) enzymes catalyze superoxide ( $O_2^-$ ) disproportionation by a mechanism that is more complex than those of the other SODs. In particular the reduction of superoxide can proceed via one of two pathways. One pathway dominates when the  $O_2^-$  concentration is low relative to the enzyme concentration (reaction 2), and the other pathway dominates when the ratio  $[O_2^-]/[MnSOD]$  is high (reactions 3 and 4). The paradoxical finding is that MnSOD is a less effective SOD catalyst when  $O_2^-$  levels are elevated.<sup>1</sup>



The depressed catalytic activity at high  $O_2^-$  concentrations is known to be due to the formation of a product-inhibited  $Mn^{3+}$ -

peroxo adduct resulting from the inner sphere oxidation of  $Mn^{2+}$ SOD by  $O_2^-$  (reaction 3). This pathway is considerably slower than the outer sphere oxidation and protonation pathway described in reaction 2. The relative levels of product inhibition are described kinetically by the value of  $k_2/k_3$  for different MnSODs (Table 1). The contribution from this slower pathway is particularly pronounced in human MnSOD.<sup>1</sup> Thus the  $O_2^-$  removal and  $H_2O_2$  production rates are dependent on the relative levels of MnSOD and  $O_2^-$  and the degree of product inhibition of the specific MnSOD present.

Table 1. Rate Constants for the Different MnSODs

Organism	$k_1$ (nM s <sup>-1</sup> )	$k_2$ (nM s <sup>-1</sup> )	$k_3$ (nM s <sup>-1</sup> )	$k_4$ (s <sup>-1</sup> )	$k_2/k_3$
Human <sup>8b</sup>	1.4	0.6	0.5	130	1.6
<i>E. coli</i> <sup>8c</sup>	1.1	0.9	0.17	60	5.3
<i>D. radiodurans</i> <sup>8a</sup>	1.2	1.1	0.07	30	16
<i>S. cerevisiae</i> (this work)	1.1–1.5	0.8	0.04–0.05	90–140	16–20

Superoxide concentrations are known to be variable in cells; for example, it has recently been shown that transient  $O_2^-$  bursts, termed “superoxide flashes”, are formed in human mitochondria,<sup>2</sup> creating the possibility of even greater variability in  $H_2O_2$  formation rates. However, a slower product-inhibited pathway in human MnSOD would allow for more constant  $H_2O_2$  formation even when  $O_2^-$  concentrations vary.

Low levels of  $H_2O_2$  play an important role in signaling in mammalian cells, regulating numerous processes including rates of cell growth and division.<sup>3</sup> It has been proposed that the slower pathway for human MnSOD appeared in response to an evolutionary pressure to control more tightly intracellular  $H_2O_2$  levels,<sup>1</sup> to reduce  $H_2O_2$  mediated oxidative damage, and to optimize its signaling function.<sup>4</sup> The  $k_2/k_3$  values determined for human and bacterial MnSODs (Table 1) are consistent with this hypothesis. However, structural studies of different MnSODs, both wild type and mutant, have yet to reveal why  $k_2/k_3$  differs so dramatically for this enzyme.<sup>1</sup>

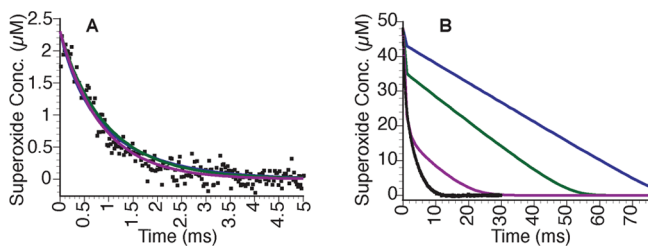
The budding yeast *Saccharomyces cerevisiae* is widely used as a single-cell model for higher eukaryotic organisms because it is remarkably similar to mammalian cells. *S. cerevisiae* also appears to be less sensitive to  $H_2O_2$  than human cells, and the only currently known  $H_2O_2$  sensing proteins in *S. cerevisiae* (YAP1p and Skn7p) are involved in regulating oxidative stress protection; a more general signaling role has yet to be found.<sup>3,5</sup> Both human MnSOD and *S. cerevisiae* MnSOD (ScMnSOD) are tetramers<sup>6</sup> and localized to the mitochondrial matrix,<sup>7</sup> while most bacterial MnSODs are dimers.

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Moreover, human MnSOD shares greater sequence similarity with *ScMnSOD* than with *Escherichia coli* or *Deinococcus radiodurans* MnSODs (62.2%, 52.9%, and 54.5% respectively).<sup>8</sup> Published reports of the activity of *ScMnSOD* do not include a determination of the degree of product inhibition.<sup>6,9</sup> We therefore turned our attention to characterizing the catalytic mechanism of *ScMnSOD* with the expectation that the high contribution from the product-inhibited pathway would prove to be a property common to eukaryotic MnSODs. Surprisingly, we found instead that *ScMnSOD* is even less product-inhibited than the bacterial MnSODs characterized to date, surpassing even the high activity of MnSOD from the radiation resistant bacterium *D. radiodurans*.<sup>8a</sup>

The gene of *ScMnSOD*,<sup>9</sup> which includes the mitochondrial targeting sequence, was inserted into the plasmid YEp352. The enzyme was overexpressed in *S. cerevisiae* and purified using a protocol modified from that of Fridovich et al. (Supporting Information).<sup>6</sup> The protein was isolated as a tetramer with the leader sequence removed and contained 0.6 equiv of Mn per monomer, as per size exclusion chromatography, mass spectrometry, and ICP-MS, respectively.

Although the fitting of the kinetic data reported here matches the experimental decay of pulsed  $O_2^-$ , out of the four rate constants, only  $k_2$  has been measured directly by pulse radiolysis.  $k_2$  was measured by oxidizing the resting enzyme with substoichiometric amounts of  $O_2^-$  and following the appearance of  $Mn^{3+}SOD$ , which has a characteristic absorption band near 480 nm (Supporting Information). Unlike other MnSODs, *ScMnSOD* was isolated in the reduced state, precluding direct measurement of  $k_1$ ,  $k_3$ , and  $k_4$  were determined by fitting the observed rate of  $O_2^-$  loss ( $\epsilon_{260} = 2000 \text{ M}^{-1} \text{ cm}^{-1}$ ) at multiple enzyme concentrations (1–10  $\mu\text{M}$ ) and initial  $O_2^-$  concentrations (2–48  $\mu\text{M}$ ) using PRWIN, conditions that, while not physiological, are necessary to study kinetics by pulse radiolysis.<sup>1,8</sup> The rate constants are compared to those known for other MnSODs in Table 1.

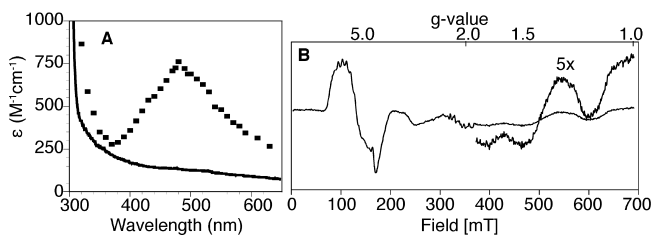


**Figure 1.** Decay of 2.3  $\mu\text{M}$  (A) and 48  $\mu\text{M}$  (B)  $O_2^-$  concentrations with MnSODs from different organisms (space permitting). *ScMnSOD* pulse radiolysis data are shown in black, while the lines are from Kintecus<sup>10</sup> computer modeling using rate constants in Table 1: MnSOD from *D. radiodurans* (purple); *E. coli* (green); and human (blue); all at 1  $\mu\text{M}$  Mn.

The ability of *ScMnSOD* to catalyze the dismutation of  $O_2^-$  was found to exceed that of the other characterized MnSODs. At low concentrations of  $O_2^-$  ( $[O_2^-] \approx [MnSOD]$ ), the observed rates of disappearance are similar for all the four enzymes (Figure 1A). However at high concentrations ( $[O_2^-] \gg [MnSOD]$ ), the differences in activities are pronounced (Figure 1B). At the highest concentrations of  $O_2^-$  employed in pulse radiolysis, the order of the activities of the enzymes is *S. cerevisiae* > *D. radiodurans* > *E. coli* > human, which is identical to the ordering of  $k_2/k_3$ , indicating that the deciding factor is the tendency to form the product-inhibited state.

Most known MnSODs rest in the  $Mn^{3+}$  oxidation state,<sup>11</sup> but our *ScMnSOD* was consistently isolated as predominantly reduced  $Mn^{2+}SOD$ ; the electronic absorption spectrum of as-isolated *Sc*-

MnSOD lacked the visible absorption band with a maximum near 480 nm. It was reported previously that the optical absorption spectrum of *ScMn<sup>3+</sup>SOD* was unusual relative to those of other MnSODs,<sup>6</sup> but the spectrum of *ScMn<sup>3+</sup>SOD* that we obtained by reaction of as-isolated *ScMnSOD* with  $O_2^-$  using pulse radiolysis (Figure 2A, dots) was very similar to those of other  $Mn^{3+}SOD$ s, with an extinction coefficient of  $\sim 800 \text{ M}^{-1} \text{ cm}^{-1}$ . The small measured absorbance at 480 nm (Figure 2A, line) corresponds to greater than 90% of the as-isolated enzyme being in the  $Mn^{2+}$  state.



**Figure 2.** *ScMnSOD* was isolated in the  $Mn^{2+}$  oxidation state. (A) Electronic absorption spectra of as-isolated *ScMnSOD* (line) and  $Mn^{3+}SOD$  obtained after reaction with  $O_2^-$  in pulse radiolysis (dots). (B) EPR spectrum of as-isolated *ScMnSOD*. Instrumental parameters: temperature, 4.7 K; microwave frequency, 9.685 GHz; microwave power, 0.2 mW; modulation amplitude, 0.8 mT.

The EPR spectrum of our as-isolated *ScMnSOD* also indicates that the enzyme is reduced, since the perpendicular-mode EPR spectrum is similar to those of other  $Mn^{2+}SOD$ s,<sup>12</sup> with the usual six-line hyperfine splitting from the  $^{55}\text{Mn}$  nucleus ( $I = 5/2$ ) seen at  $g_{\text{eff}} = 6.0$  (Figure 2B). We looked for evidence of the integer spin  $Mn^{3+}$  ( $S = 2$ ) by parallel-mode EPR, but our spectrum lacked the sextet hyperfine pattern typically displayed by  $Mn^{3+}SOD$  (Supporting Information).<sup>13</sup>

The only other MnSOD enzymes that have been isolated in the  $Mn^{2+}$  oxidation state are mutant MnSODs, most notably the Gln143 mutants of the human enzyme.<sup>14</sup> However, the factors that determine the resting oxidation state are unknown.

Increased levels of MnSOD activity have been shown to slow tumor growth in cultured human cells and in animal studies,<sup>15</sup> and it has been proposed that this effect is related to cellular  $H_2O_2$  levels. For that reason and to improve our understanding of the basis of the observed product inhibition, human MnSOD has been repeatedly mutated in attempts to make its activity resemble that of the bacterial proteins, but with limited success.<sup>1,8c</sup> As described above, human MnSOD already shares greater sequence similarity with *ScMnSOD* than with the bacterial enzymes, and the two eukaryotic proteins are tetramers while the bacterial ones are dimers. Also, *ScMnSOD* resembles the bacterial ones in that  $k_3$  is small but is similar to the human enzyme in that  $k_4$  is large. Thus, to improve our understanding of what causes the unusual kinetic properties of human MnSOD, it may be more productive to compare/contrast it with *ScMnSOD* than with the bacterial MnSODs. Investigation of the slight structural differences between the enzymes may provide a key to understanding the chemical mechanism of product inhibition. We will also continue to study the evolutionary significance of product inhibition by studying MnSOD from other organisms.

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Energy and supported by its Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences.

**Supporting Information Available:** ScMnSOD isolation details, pulse radiolysis, complete ref 2, and parallel mode EPR. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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