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Investigation of the Highly Active Manganese Superoxide Dismutase from *Saccharomyces cerevisiae*

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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* (*Sc*MnSOD). It was a tetramer with 0.6 equivalents of Mn per monomer. The catalytic activity of *Sc*MnSOD was investigated by pulse radiolysis and compared with human and two bacterial (*Escherichia coli* and *Deinococcus radiodurans*) MnSODs. To our surprise, *Sc*MnSOD 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 *Sc*MnSOD > *D. radiodurans* MnSOD > *E. coli* MnSOD > human MnSOD. While most MnSODs rest as the oxidized form, *Sc*MnSOD 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 *Sc*MnSOD

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.1

$$Mn^{3+}SOD + O_2^- \xrightarrow{k_1} Mn^{2+}SOD + O_2 \quad (1)$$

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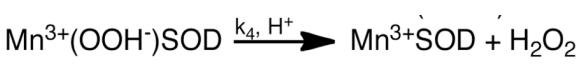
Supporting Information Available: *Sc*MnSOD isolation details, pulse radiolysis, complete ref 2, and parallel mode EPR. This information is available free of charge via the Internet at http://pubs.acs.org/.

(2)

(3)



(4)

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 I). The contribution from this slower pathway is particularly pronounced in human MnSOD.1 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.

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,2 creating the possibility of even greater variability in H_2O_2 formation rates. However, 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.3 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,1 to reduce H_2O_2 mediated oxidative damage and to optimize its signaling function.4 The k_2/k_3 values determined for human and bacterial MnSODs (Table I) 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.1

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.3,5 Both human MnSOD and *S. cerevisiae* MnSOD (*Sc*MnSOD) are tetramers6 and localized to the mitochondrial matrix,7 while most bacterial MnSODs are dimers. Moreover, human MnSOD shares greater sequence similarity with *Sc*MnSOD than with *Escherichia coli* or *Deinococcus radiodurans* MnSODs (62.2%, 52.9%, and 54.5% respectively).8 Published reports of the activity of *Sc*MnSOD, do not include a determination of the degree of product inhibition.6,9 We therefore turned our attention to characterizing the catalytic mechanism of *Sc*MnSOD 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 *Sc*MnSOD 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*.8a

The gene of *Sc*MnSOD,9 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).6 The protein was isolated as a tetramer with the leader sequence removed and contained 0.6 equivalents 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, *Sc*MnSOD was isolated in the reduced state, precluding direct measurement of k_1. k_1, k_3 and k_4 were determined by fitting the observed rate of O_2^- (ε_{260}=2000 $M^{-1}cm^{-1}$) loss at multiple enzyme concentrations (1–10 μM) and initial O_2^- concentrations (2–48 μM) using PRWIN, conditions that, while not physiological, are necessary to study kinetics by pulse radiolysis. 1,8 The rate constants are compared to those known for other MnSODs in Table I.

The ability of *Sc*MnSOD10 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 (Fig. 1A). However at high concentrations ($[O_2^-] >>$ [MnSOD]), the differences in activities are pronounced (Fig. 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,11 but our *Sc*MnSOD 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 *Sc*Mn^{3+}SOD was unusual relative to those of other MnSODs,9 but the spectrum of *Sc*Mn^{3+}SOD that we obtained by reaction of as-isolated *Sc*MnSOD with O_2^- using pulse radiolysis (Fig. 2A, dots) was very similar to those of other Mn^{3+}SODs, with an extinction coefficient of ~800 $M^{-1}cm^{-1}$. The small measured absorbance at 480 nm (Fig. 2A, line) corresponds to greater than 90% of the as-isolated enzyme being in the Mn^{2+} state.

The EPR spectrum of our as-isolated *Sc*MnSOD also indicates that the enzyme is reduced, since the perpendicular-mode EPR spectrum is similar to those of other Mn^{2+}SODs,12 with the usual six-line hyperfine splitting from the ^{55}Mn nucleus (I = 5/2) seen at g_{eff} = 6.0 (Fig 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).13

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.14 However, the factors that determine the resting oxidation state are unknown.

Increased levels of MnSOD activity have been shown to slow down tumor growth in cultured human cells and in animal studies15, 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.1›8c As described above, human MnSOD already shares greater sequence similarity with *Sc*MnSOD than with the bacterial enzymes, and the two eukaryotic proteins are tetramers while the bacterial ones are dimers. Also, *Sc*MnSOD 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 *Sc*MnSOD 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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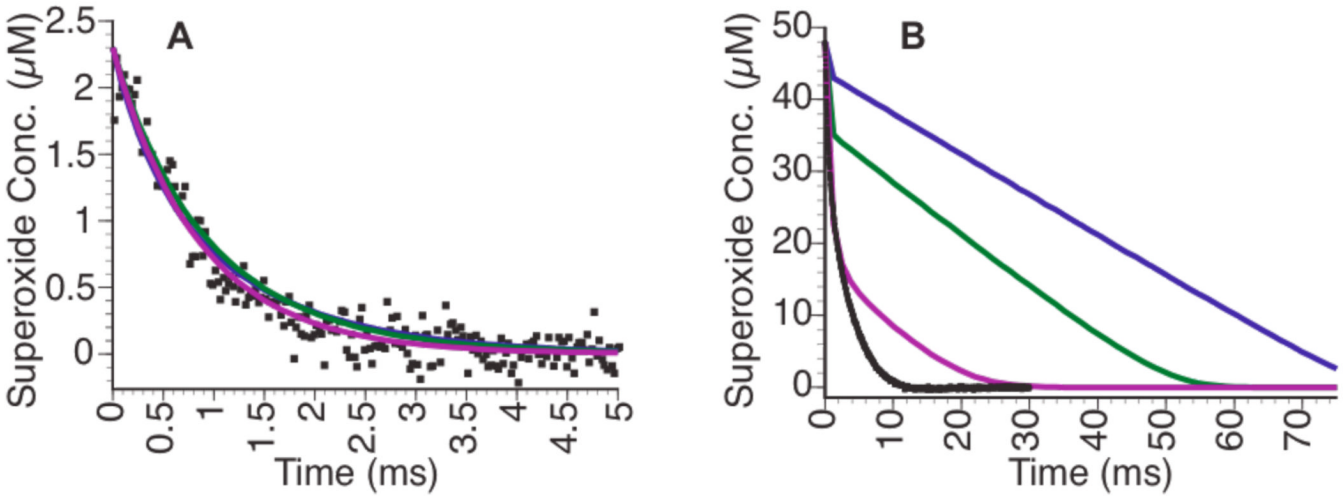

Figure 1.

The decay of 2.3-μM (A) and 48-μM (B) O_2^- concentrations with different types of MnSOD. *ScMnSOD* pulse radiolysis data are shown in black, while the lines are from Kintecus10 computer modeling using rate constants in Table I: MnSOD from *D. radiodurans* (purple); *E. coli* (green); and human (blue); all at 1 μM Mn.

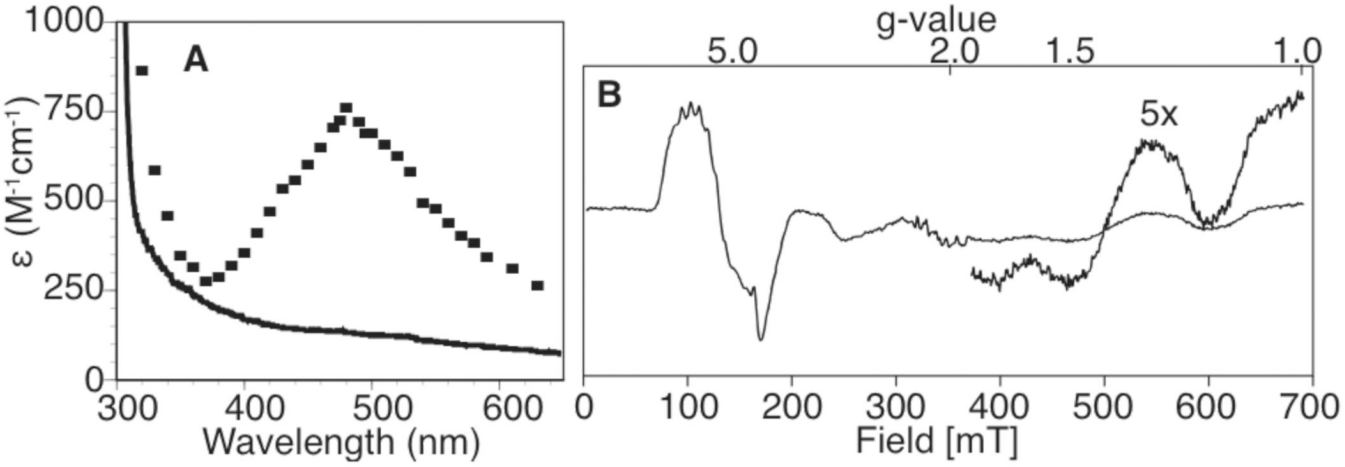

Figure 2.

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

Table I

Rate constants for the different MnSODs

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