Title
Highly Active Yeast MnSOD has a Novel Mechanism Involving Six-coordinate Mn(3+) Species

Permalink
https://escholarship.org/uc/item/2t44h65b

Author
Sheng, Yuewei

Publication Date
2012

Peer reviewed|Thesis/dissertation
Highly Active Yeast MnSOD has a Novel Mechanism
Involving Six-coordinate Mn(3+) Species

A dissertation submitted in partial satisfaction of the
Requirements for the degree Doctor of Philosophy
in Chemistry

by

Yuewei Sheng

2012
ABSTRACT OF THE DISSERTATION

Highly Active Yeast MnSOD has a Novel Mechanism
Involving Six-coordinate Mn(3+) Species

by

Yuewei Sheng
Doctor of Philosophy in Chemistry
University of California, Los Angeles, 2012
Professor Joan Selverstone Valentine, Chair

Manganese-bound superoxide dismutase (MnSOD) is a very important antioxidant enzyme. The mechanism by which MnSOD removes $O_2^-$ involves product inhibition, that is, reduction of $O_2^-$ occurs through either a “prompt protonation” pathway, or an “inner-sphere” pathway, with the latter leading to formation of an observable Mn-peroxo complex. Human MnSOD is more gated toward the “inner-sphere” pathway than bacterial enzymes.

To study whether product inhibition is a common feature to eukaryotic MnSODs, we studied a mitochondrial MnSOD from the eukaryote model organism Saccharomyces cerevisiae (ScMnSOD). To our surprise, ScMnSOD was found to display the highest catalytic efficiency at high levels of $O_2^-$ among MnSODs that had been characterized. To understand further the mechanism of product inhibition, we compared ScMnSOD with another yeast MnSOD, the cytosolic MnSOD from Candida albicans (CaMnSODc). CaMnSODc, like ScMnSOD, is less inhibited than human and bacterial MnSODs. Although the active site of yeast MnSODs closely
resembles that of MnSODs from other organisms, spectroscopic studies suggest the presence of a six-coordinate Mn$^{3+}$ species in oxidized yeast MnSODs.

To explore further the origin of the fast catalysis by yeast MnSODs, the Y34F (a strictly conserved second-sphere residue) form of ScMnSOD was created. Y34F ScMnSOD has a novel catalytic mechanism, in which protonation of the Mn-peroxo complex occurs through a fast pathway at neutral pH, leading to a putative six-coordinate Mn$^{3+}$ species, which actively oxidizes O$_2^-$ in the catalytic cycle. Because wild-type and the mutant yeast MnSOD both rest in the 2+ state and become six-coordinate when oxidized up from Mn$^{2+}$, six-coordinate Mn$^{3+}$ species could also actively function in the mechanism of wild-type yeast MnSODs.

ScMnSOD is a tetramer, while CaMnSODc is a dimer or loose tetramer, even though they are similar in many ways. Investigations of their crystal structures suggest that when CaMnSODc is in the dimeric form, its N-terminal regions are highly disordered, hindering it from forming a tetramer in solution. To further investigate the physiological significance of the tetramer structure, we mutated two residues (Lys182/Ala183 in ScMnSOD, Lys184/Leu185 in CaMnSODc) at the dimer interface in the two yeast MnSODs. We find that the dimer interface, which is critical for MnSOD activity, is reinforced by tetramer formation.
The dissertation of Yuewei Sheng is approved.

_____________________________________

James U. Bowie

_____________________________________

Robert P. Gunsalus

_____________________________________

Joan Selverstone Valentine, Committee Chair

University of California, Los Angeles

2012
# Table of Contents

Acknowledgements.................................................................................................................. xiii
Vita.................................................................................................................................................. xv
Publications..................................................................................................................................... xvi

Chapter 1: Introduction................................................................................................................. 1
  Superoxide and Superoxide Dismutase (SOD) ........................................................................... 2
  Biological Importance of Manganese-containing SOD (MnSOD) ............................................. 5
  MnSOD has a Unique Mechanism.............................................................................................. 7
  Product Inhibition in MnSOD and H₂O₂ Signaling................................................................. 10
  Tetramer and Dimer Interface in MnSOD.................................................................................... 11
  Yeast MnSOD and Its Maturation............................................................................................. 14
  References.................................................................................................................................. 16

Chapter 2: Investigation of the Highly Active Manganese Superoxide Dismutase from *Saccharomyces cerevisiae* .................................................................................................................. 21
  Introduction.............................................................................................................................. 22
  Materials and Methods............................................................................................................. 25
  Results and Discussion............................................................................................................ 28
  References.................................................................................................................................. 35

Chapter 3: Comparison of Two Yeast MnSODs: Mitochondrial *Saccharomyces cerevisiae* versus Cytosolic *Candida albicans* .................................................................................................................. 37
Discussion

Tyr34 is critical for “Prompt Protonation” Pathway

Putative Six-coordinate Int2 is Associated with the Additional Second-sphere Water

Proposed Mechanisms for Y34F ScMnSOD in which Six-coordinate Int2 is the Active Species

In Y34F ScMnSOD the Gating between Two Proton Transfer Pathways is Controlled by pH

A Six-coordinate Mn3+ could be An Active Species in Wild-type Yeast MnSOD

References

Chapter 5: Tetramerization in Yeast MnSOD and its Physicochemical Significance

Introduction

Materials and Methods

Results

The N-terminal Helixes are Highly Disordered in Dimeric CaMnSODc

RP-CaMnSODc is Susceptible to Dimer Dissociation

RP-Mutant Yeast MnSODs Catalyze the Dismutation of O2 like Wild-type Enzymes

RP-CaMnSODc is Inactivated by Heat, while RP-ScMnSOD is not

Wild-type and RP-mutant CaMnSODc are More Susceptible to Denaturant-Induced Protein Unfolding than Wild-type and RP-mutant ScMnSOD

Wild-type and RP-mutant CaMnSODc are Significantly Less Thermal Stable than Wild-type and RP-mutant ScMnSOD

Discussion

Tetramerization Stabilizes the Highly Disordered N-terminus of Dimeric CaMnSODc

Physicochemical Significance of the Tetramer Structure
List of Figures

Figure 1.1: The enzyme structures of four classes of SOD..................................................3
Figure 1.2: The active site structures of four classes of SOD............................................4
Figure 1.3: The monomer structure of MnSOD..................................................................13
Figure 1.4: The structure of the double glutamate bridge at MnSOD dimer interface.........13
Figure 2.1: ScMnSOD is a more effective catalyst than the other MnSODs studied to date.................................................................30
Figure 2.2: ScMnSOD was isolated in the reduced Mn$^{2+}$ oxidation state.....................30
Figure 2.3: Oxidation of ScMn$^{3+}$SOD in pulse radiolysis.................................................33
Figure 2.4: Parallel-mode CW-EPR spectrum of as-isolated ScMnSOD...............................34
Figure 3.1: The HPLC-SEC profiles of CaMnSODc and ScMnSOD.................................46
Figure 3.2: Decay of low and high concentrations of O$_2^-$ by MnSODs from different organisms..................................................................................46
Figure 3.3: Yeast MnSODs are sensitive to pH.....................................................................49
Figure 3.4: Dependence of the level of product inhibition of ScMnSOD on pH.................49
Figure 3.5: The oxidation state of ScMnSOD.......................................................................52
Figure 3.6: The Mn$^{3+}$SOD spectrum of CaMnSODc obtained from pulse radiolysis........56
Figure 3.7: Parallel-mode CW EPR spectrum of CaMn$^{3+}$SODc and ScMn$^{3+}$SOD........56
Figure 3.8: Active site structure of ScMnSOD.................................................................59
Figure 3.9: Superposition of the first and second coordination sphere of ScMnSOD to that of human MnSOD and EcMnSOD..............................61
Figure S3.1: ESI-MS of methylated ScMnSOD.................................................................71
Figure S3.2: SDS-PAGE gel of purified CaMnSODc.........................................................71
Figure S3.3: CaMn$^{3+}$SODc trace generated in pulse radiolysis........................................72
Figure S3.4: Comparison of kinetics of CaMnSODc and ScCuZnSOD..............................72
Figure S3.5: Product inhibition of CaMnSODc at different pH........................................73
Figure S3.6: Oxidation state of CaMnSODc.................................................................74
Figure S3.7: The deconvolution of the 390 nm absorption intensity..................................75
Figure S3.8: Optical spectra of anion-bound ScMnSOD....................................................75
Figure S3.9: Oxidation of ScMnSOD by O$_2^-$ at long timescales......................................76
Figure S3.10: The Mn$^{3+}$ spectra of yeast MnSODs at different pH as determined by pulse radiolysis.................................................................76
Figure S3.11: EPR spectra of as-isolated ScMnSOD and CaMnSODc..............................77
Figure S3.12: Temperature dependence of parallel-mode EPR spectra...........................78
Figure S3.13: The quaternary structure of ScMnSOD and CaMnSODc.............................79
Figure S3.14: Superposition of the active site of ScMnSOD and CaMnSODc…79
Figure 4.1: Active site structure and oxidation state of Y34F ScMnSOD…94
Figure 4.2: Comparison of the catalytic efficiency of Y34F ScMnSOD to wild-type MnSODs from different organisms…97
Figure 4.3: Mn3+ species observed from oxidation of Y34F ScMn2+SODc by O2- in pulse radiolysis…97
Figure 4.4: Protonation of the inner-sphere peroxo complex as a function of pH in Y34F ScMnSOD…102
Figure 4.5: A suggestive end-on model of the inner-sphere Mn-peroxo complex…108
Figure S4.1: Electrospray-ionization mass spectra of methylated Y34F ScMnSOD…115
Figure S4.2: Superimposition of Y34F ScMnSOD tetramer onto wild-type ScMnSOD tetramer…116
Figure S4.3: The difference absorption band of Y34F ScMn3+SOD as measured over time…116
Figure S4.4: Fitting the disappearance of O2- catalyzed by Y34F ScMnSOD at pH 7 into a mechanism in which Int2 is the active species…117
Figure S4.5: The dependence of decomposition of the product-inhibited complex on pH in Y34F ScMnSOD…118
Figure S4.6: Reduction of wild-type ScMn3+SODc (A) and CaMn3+SODc (B) by O2- in Pulse radiolysis…118
Figure S4.7: The water chain extending from active site to dimer interface in Y34F ScMnSOD…119
Figure S4.8: Mn-peroxo complexes in the crystal structure of cyrotrapped peroxide-soaked EcMnSOD…119
Figure 5.1: The N-terminal helixes are highly disordered, when CaMnSODc is in the dimeric form…134
Figure 5.2: Oligomer state of wild-type and RP-mutant ScMnSOD and CaMnSODc…136
Figure 5.3: RP-CaMnSODc is more subject to inactivation by pH than the wild type…138
Figure 5.4: RP-CaMnSODc is inactivation by heat like EcMnSOD…140
Figure 5.5: RP-CaMnSODc is more subject to GdHCl-induced unfolding than the wild type…141
Figure 5.6: Thermostability of wild-type and RP-mutant ScMnSOD and CaMnSODc…144
Figure 5.7: Tetramer interface of wild-type ScMnSOD and CaMnSODc…147
Figure S5.1: Electrospray-ionization mass spectra of methylated RP-mutant ScMnSOD and wild-type CaMnSODc…153
Figure S5.2: SDS-PAGE analysis of the purity of wild-type and RP-mutant ScMnSOD and CaMnSODc…154
Figure S5.3: Superimposition of the subunit of ScMnSOD over that of human and E. coli
Figure S5.4: Comparison of crystal structures between wild-type yeast MnSODs and their RP-mutant proteins

Figure S5.5: Decay of high concentrations of $O_2^-$ by wild-type and RP-mutant yeast MnSODs

Figure S5.6: CD spectra of wild-type and RP-mutant yeast MnSODs at increased concentrations of GdHCl
Lists of Tables

Table 2.1: Rate constants for the different MnSODs..........................................................24
Table S3.1: X-ray data collection and refinement statistics for wild-type yeast MnSODs…..80
Table S3.2: Active site crystallographic distances of yeast, human and bacterial MnSODs..........................................................................................................................81
Table 4.1: X-ray data collection and refinement statistics for Y34F ScMnSOD...............93
Table 4.2: Rate constants for wild-type MnSODs from S. cerevisiae and human and their Y34 mutant proteins.........................................................................................99
Table 5.1: Thermodynamic Parameters for Unfolding of Yeast MnSODs.........................143
Table S5.1: Interactions of Subunits at Dimer and Tetramer Interfaces in MnSODs from Different Organisms..................................................158
Table S5.2: Calculation of $K_d$ for K184R, L185P CaMnSODc......................................159
Acknowledgements

I would like to express my sincere gratitude to my supervisor Professor Joan Selverstone Valentine. I studied Chemistry when I was in college back in China, and I had very little experience in biochemistry when I came to UCLA five years ago. From the beginning, my supervisor had her confidence in me, and she took me as her last graduate student in her long, successful career. She opened the door toward an entirely novel and challenging science world that I had never experienced before. She passed her passion and insight toward science to me, and inspired me to explore the unknowns bravely and deeply. She is always very encouraging and optimistic. Without her constant support, I would not have gone through the stress and difficulties from life and research.

I would also like to thank Dr. Edith B. Gralla for her support and her aid in addressing every of my questions in biology. Edie is always very patient and willing to offer her help. Her enthusiasm and broad interests not only make me a better scientist, but also inspire me to have a better life. I would like to thank Prof. Julian Whitelegge for his supervision on the hydroxyl radical footprinting project.

I appreciate greatly the supervision from Prof. Diane E. Cabelli (Brookhaven National Laboratories) on my dissertation projects. Diane not only has spent tremendous amounts of time and effort on helping me with the pulse radiolysis experiments, but also took great care of me every time I travelled to Brookhaven. I would like to thank Dr. Duilio Cascio (Department of Energy-Institute for Genomics and Proteomics, UCLA) for teaching me and giving me tremendous help in X-ray crystallography. I would like to thank Dr. Chris Ryan (Department of Psychiatry and Biobehavioral Sciences, UCLA) for training me on LC-MS and helping me address every difficulty I encountered when using mass spectrometer. I would like to thank Prof. R. David Britt and Dr. Troy A. Stich (University of California, Davis), for performing spectroscopic studies on my enzyme samples. I would like to thank Prof. Yi Tang, Dr. Zhen Gu and Dr. Yanran Li (Department of Chemical Engineering, UCLA) for our collaboration on several very interesting metalloenzymes that participate in the biosynthesis of natural products in fungi.
I also received a lot of help from my previous and present group members. I would like to particularly thank Dr. Kevin Barnese, who was my mentor when I joined the group. He gave me great guidance in how to express, purify and characterize proteins. What Kevin taught me has always been the fundamentals of my research in the following years. I would like to thank Mikhail Schumacher for helping me with protein purification. I would like to thank Dr. Armando Durazo for running my ICP-MS and LC-MS samples. I would like to thank Dr. Herman Lelie for training me on ICP-MS. I would like to thank Dr. Lindsay Kane and Dr. Jake Martins for helping me with cloning. I would like to thank Becky Chan for providing me protein samples for hydroxyl radical footprinting experiments.

Finally I would like especially to thank my dad, who raised me up and supports me from his heart all the time. I would like to thank my friends for their encouragement and support in the past five years.
VITA

2007
B. S., Chemistry, Nanjing University
Nanjing, China

2010
M. S., Chemistry, University of California, Los Angeles
Los Angeles, California

Work Experience
2008 – 2009 Teaching Assistant, UCLA
2009 – 2011 Teaching Associate, UCLA
2011 Grad Intern, Amgen Inc., Thousand Oaks, California

Awards
2011 – 2012 Dissertation Year Fellowship, UCLA
2011 John M. Jordan Memorial Award for Research in Biochemistry, UCLA
2012 Chinese Government Award for Outstanding Self-Financed Students Abroad
1. **Sheng, Yuewei**; Stich, Troy A.; Barnese, Kevin; Gralla, Edith B.; Britt, R. D.; Cascio, Duilio; Cabelli, Diane E.; Valentine, Joan S. Comparison of two yeast MnSODs: Mitochondrial *Saccharomyces cerevisiae* versus cytosolic *Candida albicans*. *J. Am. Chem. Soc.* 2011, 133, 20878-20889

2. **Sheng, Yuewei**; Gralla, Edith B.; Schumacher, Mikhail; Cascio, Duilio; Cabelli, Diane E.; Valentine, Joan S. Six-coordinate manganese(3+) in catalysis by yeast manganese superoxide dismutase. (submitted to PNAS)

3. **Sheng, Yuewei**; Schumacher, Mikhail; Armando Durazo, Gralla, Edith B.; Cascio, Duilio; Cabelli, Diane E.; Valentine, Joan S. Tetramerization in Yeast MnSOD and its Functional Significance (in preparation)


7. Barnese, Kevin; **Sheng, Yuewei** (contributing equally); Stich, Troy A.; Gralla, Edith B.; Britt, R. David; Cabelli, Diane E.; Valentine, Joan Selverstone. Investigation of the highly active manganese superoxide dismutase from *Saccharomyces cerevisiae*. *J. Am. Chem. Soc.* 2010, 132, 12525-12527


9. Wang, Yan; Huang, Yong-Qing; Liu, Guang-Xiang; Okamura, Taka-aki; Doi, Mototsugu; **Sheng, Yue-Wei**; Sun, Wei-Yin; Ueyama, Norikazu. New metal-organic frameworks with large cavities: selective sorption and desorption of solvent molecules. *Chem. Eur. J.* 2007, 13, 7523-7531

Chapter 1

Introduction
**Superoxide and Superoxide Dismutase**

Superoxide (O$_2^-$) is generated in various in vivo processes through the attachment of a single electron to dioxygen. Mitochondrion is recognized as the major source for O$_2^-$, a byproduct of the respiratory chain (1). The reduction potential for the conversion of dioxygen to O$_2^-$ is −0.16 V (2). Due to the highly reducing environment in the mitochondria, the one-electron reduction of dioxygen is thermodynamically favorable for numerous respiratory components. Even though O$_2^-$ is not a strong oxidant, it is a precursor of most other reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (’OH). Only several proteins containing [Fe–S] clusters, including aconitase, dehydrases, and fumarases, have been identified as targets for O$_2^-$ reactivity in vivo.

**Scheme 1.1. The Dismutase Mechanism**

\[ M^{n+} + O_2^- \rightarrow M^{(n-1)+} + O_2 \]
\[ M^{(n-1)+} + O_2^- + 2H^+ \rightarrow M^{n+} + H_2O_2 \]

Superoxide dismutases (SODs) are enzymes that function to convert the superoxide (O$_2^-$) anion to dioxygen and hydrogen peroxide (H$_2$O$_2$) catalytically via sequential reduction and oxidation of a metal cofactor at virtually diffusion-controlled rate constants (Scheme 1.1). Based on the active-site metal, SODs are classified into copper-zinc SOD (CuZnSOD), nickel SOD (NiSOD), manganese SOD (MnSOD), and iron SOD (FeSOD). NiSOD is only found in prokaryotes. In eukaryotes CuZnSOD is located in the cytosol, nucleus, peroxisomes, mitochondrial intermembrane space, and extracellular space (3), while MnSOD is in
Figure 1.1. The enzyme structures of four classes of SOD. (A) Homo sapien CuZnSOD (pdb code: 1PU0); (B) Streptomyces coelicolor NiSOD (pdb code: 1T6U); (C) Saccharomyces cerevisiae MnSOD (pdb code: 3LSU); (D) Anaplasma phagocytophilum FeSOD (pdb code: 3JS4).
Figure 1.2. The active site structures of four classes of SOD. (A) Homo sapien CuZnSOD (pdb code: 1PU0); (B) Streptomyces coelicolor NiSOD (pdb code: 1T6U); (C) Anaplasma phagocytophilum FeSOD (pdb code: 3JS4).
mitochondria. CuZnSOD, MnSOD, and FeSOD can be found in the cytosol of prokaryotes, and in chloroplasts of plants (4).

Although CuZnSOD, NiSOD, and Mn(Fe)SOD display similar reactivity toward O$_2^-$, their protein structures and active site structures differ from each other dramatically. CuZnSOD from eukaryotes is a homodimer rich in β-barrel (Figure 1.1-A). Its binuclear active site consists of a copper and a zinc ion in close enough proximity to share an imidazolate ligand (Figure 1.2-A). The copper ion in the 2+ state is coordinated by three other histidines and a water molecule. The zinc ion binds two other histidines and an aspartate in a pseudo tetrahedral geometry. NiSOD is the most recently discovered form in the SOD family. The enzyme is a homohexamer, and each subunit contains a 4-helix bundle (Figure 1.1-B). It has a mononuclear active site with the nickel coordinated by a histidine and two cysteines in an N$_2$S$_2$ square planar geometry (Figure 1.2-B). Mn- and FeSOD are structure homologs. FeSOD is always a homodimer, while MnSOD is either a homodimer or a homotetramer (Figure 1.1-C). The Mn or Fe ion is coordinated by three histidines, one aspartate and a water molecule in an approximately trigonal bipyramidal geometry (Figure 1.2-C).

**Biological Importance of Manganese-containing SOD (MnSOD)**

MnSOD is the major antioxidant in the mitochondria. Numerous studies on animal models with a MnSOD deficiency suggest that MnSOD is critical for life. MnSOD (−/−) homogeneous knockout mice, which exhibit extensive mitochondrial injury that causes severe neurodegeneration and dilated cardiomyopathy, have a greatly shortened survival of 10-20 days after birth (5,6). The activities of succinate dehydrogenase (complex II) and aconitase are
reduced significantly in the heart (7). MnSOD (+/−) heterogeneous knockout mice, with a partial deficiency of MnSOD activity, also show an increase in oxidative damage to mitochondria and altered mitochondrion function (8).

MnSOD is associated with oxidative stress-related apoptosis and aging. Overexpression of MnSOD protects mammalian cells from tumor necrosis factor (TNF)–α–induced apoptosis by increasing the production of H₂O₂ (9). Overexpression of MnSOD also prevents neural apoptosis induced by Fe²⁺, amyloid β-peptide (Aβ), and nitric oxide–generating agents (10). Mitochondria from MnSOD (+/−) heterogeneous knockout mice show evidence of age-related rapid accumulation of oxidative damage in relatively early life stages (11). MnSOD (+/−) mice show pronounced phenotypes, such as vascular dysfunction and mitochondrial DNA damage (12). Although several early studies suggested that overexpression of MnSOD increases the lifespan of animal models, more recent studies have shown that overexpression of major antioxidant enzymes, including CuZnSOD, MnSOD and catalase, have no effect on the lifespans of transgenic mice (13).

MnSOD is also implicated in several biomedical applications, including treatment of cancer. Although the role of MnSOD in cancer remains controversial, numerous cell line and animal model studies have shown the suppression of tumor formation and growth by increasing the expression or reactivity of MnSOD (14-17). Delivery of the MnSOD gene to the lungs of cancer mice prevents organ and tissue damage caused by ionizing irradiation (18). Increasing the MnSOD level in transgenic mice reduces ischemia/reperfusion injury by suppressing the basal level of oxidative stress in the brains (10,19).
Because MnSOD is such a critical antioxidant enzyme in vivo, it is important to investigate the mechanism and structure-function relationship of this enzyme, which is the main purpose of my project.

**MnSOD has a Unique Mechanism**

Like other SODs, MnSOD catalyzes the disproportionation of $O_2^-$ via sequential oxidation ($Mn^{3+}$) and reduction ($Mn^{2+}$) of the metal. MnSOD is unique in that it is fast when $[O_2^-]$ is low, and becomes slow when $[O_2^-]$ is high.

**Scheme 1.2. Catalytic Mechanism of MnSOD**

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

$$
\text{Mn}^{2+}\text{SOD} + O_2 \xrightarrow{k_{2,3}^{2H^+}} \text{Mn}^{3+}\text{SOD} + H_2O_2 \quad (2)
$$

$$
\text{Mn}^{2+}\text{SOD} + O_2 \xrightarrow{k_3} \text{Mn}^{3+}(\text{OO}^{2-})\text{SOD} \quad (3)
$$

$$
\text{Mn}^{3+}(\text{OO}^{2-})\text{SOD} \xrightarrow{k_{4,5}^{2H^+}} \text{Mn}^{3+}\text{SOD} + H_2O_2 \quad (4)
$$

The mechanism by which MnSOD removes $O_2^-$ involves product inhibition (4). Specifically, reduction of $O_2^-$ by $Mn^{2+}\text{SOD}$ (Scheme 1.2) occurs either through a pathway (called “prompt protonation” pathway here) (reaction 2), where protonation and dissociation of the peroxo moiety is instantaneous, or through an “inner-sphere” pathway (reaction 3), where a detectable intermediate is formed, which has been suggested to be a side-on Mn(III)-peroxo species (20). (We here refer to this intermediate as a Mn-peroxo complex instead of as a
“product-inhibited” complex, the latter term having been used frequently in previous reports.)

When protonation of the Mn-peroxo complex (reaction 4) is rate limiting, the “inner-sphere” pathway has the effect of slowing down MnSOD catalysis.

The level of product inhibition is described by $k_2/k_3$, the gating ratio of the two pathways. The phenomenon of product inhibition is the most prominent in the MnSOD from human, while the enzymes from bacteria are relatively less inhibited at high $\text{O}_2^-$ levels, as indicated by the $k_2/k_3$ in human, *E. coli*, *D. radiodurans* MnSOD as 1.6, 5.3, and 16, respectively (21). Although human and bacterial MnSODs display dramatically different catalytic efficiency at high $\text{O}_2^-$ levels, their monomer assembly and active site structure are highly conserved. Numerous studies have been focused on exploring the origin of the different extent of product inhibition in MnSODs from different organisms, through investigating the MnSOD mutant proteins with substitutions at the second sphere of the Mn ion.

Tyr34 is highly conserved and structurally similar in all known MnSODs, where it participates in a hydrogen-bonding network at the active site. Computational studies suggest that Tyr34 provides one of the two protons needed to form $\text{H}_2\text{O}_2$ in the catalytic cycle (22). Deprotonation of Tyr34 is responsible for the spectroscopic changes, including the blue shift and decrease in intensity of the optical absorption maximum, that occurs for Mn$^{3+}$SOD with increasing pH (23). Wild-type human MnSOD and its Y34F mutant have similar $k_{\text{cat}}/K_m$, but $k_{\text{cat}}$ is decreased by ~10 fold in the mutant (24). SOD activity, measured by the xanthine oxidase/cytochrome c assay, is only slightly reduced in *E. coli* Y34 mutants (25,26). Tyr34 also plays an interesting role in the “prompt protonation” pathway, as elucidated by the changes in the kinetics of human Tyr34 mutants (27).
Conserved in all Mn and FeSODs, Gln154 donates an H-bond to the metal-bound solvent molecule, and is the only contact of the metal-bound solvent molecule to the protein beyond the active site. Gln154 makes three hydrogen bonds to three highly conserved residues, Tyr34, Asn80 and Trp133 (numbering in Saccharomyces cerevisiae MnSOD (ScMnSOD)). Its hydrogen bonding potential is fully utilized and Gln154 seems firmly fixed in place. Gln154 together with Asp168 that is an H-bond acceptor from the metal-coordinated H2O or OH−, determine the position of the coordinated solvent molecule. Mutagenesis on Gln154 often causes dramatic depression of reactivity (26,28,29), indicating the key role of the NE2(Gln154)∙∙∙O(solv) bond.

Both Tyr34 and Gln154 are part of the hydrogen-bonding network extending from the active site to the dimer interface. This O(solvent ligand)∙∙∙NE2(Gln143)∙∙∙OH(Tyr34) ∙∙OH2(solvent)∙∙ND1(His30)∙∙OH(Tyr175) (numbering in ScMnSOD) hydrogen-bonding chain is conserved in all MnSODs and has been proposed to play an important role in proton transfer (30). Mutation of either His30 or Tyr174 in EcMnSOD reduces the enzymatic activity to 30–40% of that of the wild-type enzyme (31). The H30V variant of human MnSOD exhibits increased product inhibition by 1–2 orders of magnitude (32). Its H30N variant is significantly more efficient than the wild-type enzyme at high levels of O2− due to the increased rate of dissociation of the Mn-peroxo complex; this increased enzymatic efficiency causes anti-tumor effects in animal models (15). By constrast, substitution of Tyr166 in human MnSOD prolongs the lifetime of the Mn-peroxo complex (33). Nevertheless, these results have not yet elucidated the reason for MnSOD being so inhibited in the human enzyme but not the others.

The only success in weakening product inhibition in human MnSOD is through the substitutions of Phe66 at the dimer interface of the human enzyme (34). Therefore, the origin of the product inhibition became the major interest of my project. Yeast is an important single-cell
model for studying human and other eukaryotic cells. Like human cells, *S. cerevisiae* expresses a homotetrameric MnSOD in its mitochondria (ScMnSOD), which shares sequence similarity with human MnSOD. I have been trying to elucidate the mystery of the MnSOD mechanism through investigating the biochemical and biophysical properties of yeast MnSOD.

**Product Inhibition in MnSOD and H$_2$O$_2$ Signaling**

Efficient removal of high concentrations of O$_2^-$ will, by necessity, produce high local concentrations of H$_2$O$_2$. H$_2$O$_2$ is a biologically important class of reactive oxygen species (ROS). It can be reduced to hydroxyl radical (’OH) through a Fenton-type reaction with a reduced metal ($M^{(n-1)+} + H_2O_2 \rightarrow M^{n+} + OH^- + \cdot OH$), whose reaction with cellular components causes severe damage to lipids, proteins, and nucleotides. The oxidative damage caused both directly and indirectly by H$_2$O$_2$ is implicated in cancer and aging (35,36).

Human MnSOD is much less efficient at turning O$_2^-$ into H$_2$O$_2$ than MnSOD isolated from bacteria when O$_2^-$ levels are high. Mitochondria are recognized as the main source of O$_2^-$ with the production occurring through respiratory chain. H$_2$O$_2$ is generated in mitochondrial matrix via the dismutation reaction of O$_2^-$ catalyzed by MnSOD. The produced H$_2$O$_2$ then diffuses into cytosol. The roles of H$_2$O$_2$ are complex in higher organisms. In mammalian cells the upregulated expression of antioxidant enzymes is not a universal response to supraphysiologic levels of H$_2$O$_2$ (37). Instead, physiologic levels of H$_2$O$_2$ stimulate cell division, transformation, migration or apoptosis (37-40). This along with the fact that mammalian cells generate H$_2$O$_2$ in response to various stimuli supports that H$_2$O$_2$ is utilized as a signaling agent in numerous mammalian signaling pathways (37-43).
A well established H$_2$O$_2$-induced signaling is the modulation of the tyrosine phosphorylation of proteins by causing the oxidative inhibition of tyrosine phosphatases (43). Reversible inactivation of tyrosine phosphatases has been demonstrated to play a critical role in protein tyrosine phosphatase (PTP) and insulin signaling (44,45). Several mitochondrial signaling pathways are stimulated by increased H$_2$O$_2$, including tumor necrosis factor (TNF)-α-induced apoptosis (9) and c-Jun NH$_2$-terminal kinase (JNK)-induced apoptosis (41,46). The participation of H$_2$O$_2$ in signaling cascades may require tight regulation of its production in mitochondria.

A recent study demonstrates the production of large bursts of O$_2^-$, termed “superoxide flashes”, in human mitochondria (47). The flash events occur randomly in space and time, last for 20–30 seconds and are confined to tiny elliptical areas (47). Under the conditions that were tested human MnSOD in mitochondrial matrix was expected to eliminate superoxide flashes in a shorter time scale, considering that the reactivity of human MnSOD was thought to be diffusion controlled ($k = 2.3 \times 10^9$ M$^{-1}$ s$^{-1}$) and its intramitochondrial concentration is 10–40 μM (41,48). The observation of superoxide flashes indicates that human MnSOD is unable to maintain a steady-state concentration of O$_2^-$ in tiny elliptical areas in mitochondria. The most likely explanation for this phenomenon is that human MnSOD is significantly inhibited at these high concentrations of O$_2^-$. Under these conditions, bursts of O$_2^-$ are not translated into bursts of H$_2$O$_2$ that could result in aberrant oxidant-driven signaling in mitochondria.

**Tetramer and Dimer Interfaces in MnSOD**

MnSOD is either a homodimer or a homotetramer with a monomer size of ~23,000 Da. The enzymes located in eukaryote mitochondria (human (49), S. cerevisiae (50), Caenorhabditis
elegans (51), Aspergillus fumigatus (52)) were found to be homotetramers, while MnSODs isolated from bacteria (Thermus thermophilus (53), Escherichia coli (54), Deinococcus radiodurans (55)) are homodimers. In all MnSODs, each subunit is composed of two domains, a predominantly α-helical N-terminal domain and a mixed α/β C-terminal domain (Figure 1.3). The Mn ion is coordinated by four strictly conserved residues, two from the N-terminal domain and two from the C-terminal domain, and one solvent molecule.

The difference in the quaternary structure between human and bacterial MnSOD lies in the N-terminus of the subunit. In human MnSOD the N-terminus of each subunit folds into a hairpin structure holding two long α-helixes, while in bacterial MnSODs these α-helixes are much shorter. In human MnSOD, the two N-terminal regions form one of the two 4-helix bundles at the tetramer interface, and this configuration has been proposed to be critical for tetramer formation (49,53).

Scheme 1.3. Alignment of MnSOD C-terminal Sequence. Conserved residues and unconserved residues at the dimer interface are highlighted in bold and shadowed in gray, respectively.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaMnSODc</td>
<td>AI[DAWHAAYL][QY][N]KLDYFKAI[MN]V[IN]WAEAESRYSA------ 206</td>
</tr>
<tr>
<td>E. coli</td>
<td>G[LDWEHAYL][KF][QR]PDIKEFWN[VV]WDEAAARF[AA]KK---- 206</td>
</tr>
<tr>
<td>D. radiodurans</td>
<td>G[DVWEHAYL][NY][QR]PDLA[FW][V][VN]DEVSK[AA]KK---- 211</td>
</tr>
<tr>
<td>D. melanogaster</td>
<td>G[IVWEHAYL][QR]PYSYEAI[WD][I][AN]DDLSCR[EQ][AKKLGC 206</td>
</tr>
<tr>
<td>mouse</td>
<td>G[IVWEHAYL][QR]PVDYLKAIW[V][VIN]W[ENVENTERYTACKK--- 205</td>
</tr>
</tbody>
</table>

The MnSOD dimer is the basic unit for both dimeric and tetrameric MnSOD enzymes. A
**Figure 1.3.** The monomer structure of MnSOD (from *S. cerevisiae*, pdb code: 3LSU). The N- and C-terminal are colored in dark and light blue, respectively.

**Figure 1.4.** The structure of the double glutamate bridge at MnSOD dimer interface. The structure of *E. coli* MnSOD is shown (pdb code: 1VEW). The two subunit in the dimer are colored in red and green.
DXWEHXXYL motif is strictly conserved at the dimer interface of MnSOD (Scheme 1.3); it includes two metal-binding residues (Asp168 and His172, numbering in ScMnSOD) and a glutamate (Glu173, numbering in ScMnSOD) that forms hydrogen bond to His from the other subunit across the dimer interface (Figure 1.4).

Several studies suggest a significant role for the dimer interface in both catalysis and stability of MnSOD. Replacement of Glu170 in EcMnSOD, which spans the dimer interface and forms the double glutamate bridge results in dissociation of the dimer, complete loss of catalytic activity, and a change in metal specificity (25). Substitutions of its counterpart in human MnSOD, Glu162, reduce the catalytic activity to 5–25% of that of the wild-type enzyme (56). The Y166F mutant human MnSOD shows a significant decrease in catalytic activity and a major unfolding transition at a lower $T_m$ (33). Replacements of Phe66 at the dimer interface of human MnSOD reduced the extent of product inhibition in human MnSOD, and made it resemble EcMnSOD (34).

**Yeast MnSOD and Its Maturation**

Baker’s yeast, *S. cerevisiae*, expresses a CuZnSOD in the cytosol, and a MnSOD in the mitochondria (ScMnSOD). Yeast cells lacking CuZnSOD (*sod1Δ*) exhibit a broad range of phenotypes, including impaired growth in air, no growth in oxygen, sensitivity to temperature, specific amino acid auxotrophies, etc (57). In contrast, deletion of the MnSOD gene (*sod2Δ*) is relatively benign to *S. cerevisiae*. The only known phenotype for *sod2Δ* strain is low growth on non-fermentable carbon sources or in the presence of superoxide-producing paraquat.
Much is known about the pathway that leads to mature CuZnSOD in vivo. Copper is inserted to the active site of CuZnSOD by CCS (copper chaperone for superoxide dismutase) \(^{(58)}\). CCS delivers a copper to a SOD1 monomer through formation of a SOD1-CCS heterodimeric complex \(^{(59)}\). In contrast, the mechanism by which the activation of MnSOD occurs in vivo remains unclear. Two metal transporters are known to participate in the post-translational modification of ScMnSOD. The manganese trafficking to mitochondria requires the Nramp metal transporter, Smf2p, which is located in intracellular vesicles \(^{(60)}\). The second transporter, MTM1, is located in the mitochondrial matrix. Although MTM1 has no effects on manganese levels in mitochondria, it is nevertheless required for proper insertion of manganese into mitochondrial ScMnSOD \(^{(61)}\). A more recent study showed that ScMnSOD, if not imported into mitochondria, is largely apo and inactive in the cytosol, suggesting that a mitochondrial location is essential for manganese activation of ScMnSOD \(^{(62)}\). Manganese can only be inserted into a newly synthesized but not prefolded polypeptide of ScMnSOD \(^{(62)}\).
Reference


deficiency increase mitochondrial oxidative stress and aggravate age-dependent vascular

overexpression of major antioxidant enzymes does not extend the lifespan of mice. *Aging Cell* **8**, 73-75

 glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese
 superoxide dismutase. *Cancer Res* **60**, 3927-3939

15. Davis, C. A., Hearn, A. S., Fletcher, B., Bickford, J., Garcia, J. E., Leveque, V., Melendez, J. A.,
active site mutant of human manganese-superoxide dismutase. Evolutionary conservation of

formation by modulation of activator protein-1 signaling in a multistage skin carcinogenesis
model. *Cancer Res* **61**, 6082-6088

17. Church, S. L., Grant, J. W., Ridnour, L. A., Oberley, L. W., Swanson, P. E., Meltzer, P. S., and
Trent, J. M. (1993) Increased manganese superoxide dismutase expression suppresses the

18. Epperly, M., Bray, J., Kraeger, S., Zwacka, R., Engelhardt, J., Travis, E., and Greenberger, J.
(1998) Prevention of late effects of irradiation lung damage by manganese superoxide dismutase


and Mn-dependent superoxide dismutases: insights into second-sphere tuning of active site

Valentine, J. S. (2011) Comparison of two yeast MnSODs: mitochondrial Saccharomyces

and electronic structures of the low-temperature azide adduct and the product-inhibited form of
oxidized manganese superoxide dismutase. *Biochemistry* **44**, 1504-1520

23. Maliekal, J., Karapetian, A., Vance, C., Yikilmaz, E., Wu, Q., Jackson, T., Brunold, T. C., Spiro,
T. G., and Miller, A. F. (2002) Comparison and contrasts between the active site PKs of Mn-


61. Luk, E., Carroll, M., Baker, M., and Culotta, V. C. (2003) Manganese activation of superoxide dismutase 2 in Saccharomyces cerevisiae requires MTM1, a member of the mitochondrial carrier family. Proc Natl Acad Sci U S A 100, 10353-10357

Chapter 2

Investigation of the Highly Active Manganese Superoxide Dismutase from 

*Saccharomyces cerevisiae*

**Introduction**

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).

\[
\begin{align*}
\text{Mn}^{3+}\text{SOD} + \text{O}_2^- & \xrightarrow{k_1} \text{Mn}^{2+}\text{SOD} + \text{O}_2 \quad (1) \\
\text{Mn}^{2+}\text{SOD} + \text{O}_2^- & \xrightarrow{k_2, 2\text{H}^+} \text{Mn}^{3+}\text{SOD} + \text{H}_2\text{O}_2 \quad (2) \\
\text{Mn}^{2+}\text{SOD} + \text{O}_2^- & \xrightarrow{k_3} \text{Mn}^{3+}(\text{OO}_2^-)\text{SOD} \quad (3) \\
\text{Mn}^{3+}(\text{OO}_2^-)\text{SOD} & \xrightarrow{k_4, 2\text{H}^+} \text{Mn}^{3+}\text{SOD} + \text{H}_2\text{O}_2 \quad (4)
\end{align*}
\]

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 2.1). The contribution from this slower pathway is particularly pronounced in human MnSOD (1). Thus the O$_2^-$ removal and H$_2$O$_2$ 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 $\text{O}_2^-$ bursts, termed “superoxide flashes”, are formed in human mitochondria \(^{(2)}\), creating the possibility of even greater variability in $\text{H}_2\text{O}_2$ formation rates. However, a slower product-inhibited pathway in human MnSOD would allow for more constant $\text{H}_2\text{O}_2$ formation even when $\text{O}_2^-$ concentrations vary. Low levels of $\text{H}_2\text{O}_2$ 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 $\text{H}_2\text{O}_2$ levels,\(^{1}\) to reduce $\text{H}_2\text{O}_2$ mediated oxidative damage, and to optimize its signaling function \(^{(4)}\). The $k_2/k_3$ values determined for human and bacterial MnSODs (Table 2.1) 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 \(^{(1)}\).

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 $\text{H}_2\text{O}_2$ than human cells, and the only currently known $\text{H}_2\text{O}_2$ 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 (ScMnSOD) are tetramers \(^{(6)}\) and localized to the mitochondrial matrix \(^{(7)}\), while most bacterial MnSODs are dimers. 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) \(^{(8-10)}\). Published reports of the activity of ScMnSOD do not include a determination of the degree of product inhibition \(^{(6,11)}\). We therefore turned
Table 2.1. Rate constants for the different MnSODs

<table>
<thead>
<tr>
<th>Organism</th>
<th>$k_1$ (nMs$^{-1}$)</th>
<th>$k_2$ (nMs$^{-1}$)</th>
<th>$k_3$ (nMs$^{-1}$)</th>
<th>$k_4$ (S$^{-1}$)</th>
<th>$k_2/k_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (9)</td>
<td>1.4</td>
<td>0.6</td>
<td>0.5</td>
<td>130</td>
<td>1.6</td>
</tr>
<tr>
<td>E. coli (12)</td>
<td>1.1</td>
<td>0.9</td>
<td>0.17</td>
<td>60</td>
<td>5.3</td>
</tr>
<tr>
<td>D. radiodurans (8)</td>
<td>1.2</td>
<td>1.1</td>
<td>0.07</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>S. cerevisiae (this work)</td>
<td>1.1 - 1.5</td>
<td>0.8</td>
<td>0.04 -0.05</td>
<td>90 -140</td>
<td>16 - 20</td>
</tr>
</tbody>
</table>
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* (8).

**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 ScMnSOD 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 BamHI fragment containing the gene for
ScMnSOD) (13) 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<sub>600</sub>. The second overnight was used to inoculate 1 L cultures to 0.05 OD<sub>600</sub> in
pH 4 YPEG medium (1% yeast extract, 2% peptone, 2% ethanol, 2% glycerol) supplemented
with 500 µM Mn(II) acetate. Cells were grown for 3 days or until they reached an OD<sub>600</sub> ≥ 25.

We modified the purification from Ravindranath *et. al.* (6); 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 °C, Sorvall H6000A rotor), re-suspended in 250 mL of lysis buffer (50 mM pH 7.4 tris-Cl, 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)₂SO₄), 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 (14). 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 O₂⁻. 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 DEAE-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 myoglobin (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 (15). 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 (16). 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 O₂⁻ flux. The pH of the buffer was adjusted using sodium hydroxide and sulfuric acid.

**EPR Spectroscopy.** X-band perpendicular-polarization (B₀ ⊥ B₁) and parallel-polarization (B₀ || B₁) 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.

**Results and Discussion**

The gene of ScMnSOD (11), 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. (6). 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 (Figure 2.3), which has a characteristic absorption band near 480 nm. Unlike other MnSODs, ScMnSOD 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 $O_2^-$ loss ($\epsilon_{260} = 2000$ M$^{-1}$ cm$^{-1}$) at multiple enzyme concentrations (1-10 $\mu$M) and initial $O_2^-$ concentrations (2-48 $\mu$M) using PRWIN (16), conditions that, while not physiological, are necessary to study kinetics by pulse radiolysis. The rate constants are compared to those known for other MnSODs in Table 2.1.

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 $O_2^-$ ($[O_2^-] \approx [\text{MnSOD}]$), the observed rates of disappearance are similar for all the four enzymes (Figure 2.1A). However at high concentrations ($[O_2^-] >> [\text{MnSOD}]$), the differences in activities are pronounced (Figure 2.1B). At the highest concentrations of $O_2^-$ employed in pulse radiolysis, the order of the activities of the enzymes is $S. \text{cerevisiae} > D. \text{radiodurans} > E. \text{coli} > \text{human}$, 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 (17), but our ScMnSOD was consistently isolated as predominantly reduced Mn$^{2+}$SOD; the electronic absorption spectrum of as-isolated ScMnSOD lacked the visible absorption band with a maximum near 480 nm. It was reported previously that the optical absorption spectrum of ScMn$^{3+}$SOD was unusual relative to those of other MnSODs (6), but the spectrum of ScMn$^{3+}$SOD that we obtained by reaction of as-
**Figure 2.1.** ScMnSOD is a more effective catalyst than the other MnSODs studied to date. The first order decay of O$_2^-$ at 2.3 μM (A) and 41 μM (B) O$_2^-$ concentrations. Orange dots are data from pulse radiolysis, while the lines are from modeling using rate constants in Table I and the Kintecus computer-modeling program ([18]): MnSOD from *S. cerevisiae* (red); *D. radiodurans* (purple); *E. coli* (green); and human (blue); all at 1 μM Mn concentration.

**Figure 2.2.** ScMnSOD was isolated in the reduced Mn$^{2+}$ oxidation state. A) Electronic absorption spectra of as-isolated ScMnSOD (300 μM MnSOD, line), and that of Mn$^{3+}$SOD obtained after reaction with O$_2^-$ in pulse radiolysis (dots). B) EPR spectrum of as-isolated ScMnSOD. The instrumental parameters were: temperature, 4.7 K; microwave frequency, 9.685 GHz; microwave power, 1 mW; modulation amplitude, 5.01 G.
isolated ScMnSOD with $O_2^-$ using pulse radiolysis (Figure 2.2A, dots) was very similar to those of other Mn$^{3+}$SODs, with an extinction coefficient of $\sim 800$ M$^{-1}$ cm$^{-1}$. The small measured absorbance at 480 nm (Figure 2.2A, line) corresponds to greater than 90% of the as-isolated enzyme being in the Mn$^{2+}$ state.

The EPR spectrum of our as-isolated ScMnSOD also indicates that the enzyme is reduced, since the perpendicular-mode EPR spectrum is similar to those of other Mn$^{2+}$SODs (19,20), with the usual six-line hyperfine splitting from the $^{55}$Mn nucleus ($I = 5/2$) seen at $g_{\text{eff}} = 6.0$ (Figure 2.2B). 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 (Figure 2.4) (21).

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 (22). 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 (23,24), 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 (1,10). 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.
Oxidation of ScMn$^{2+}$SOD in pulse radiolysis (method a) is shown by the change of absorbance at 480 nm with time after 1.8 $\mu$M of $O_2^-$ was generated. Solutions contained 60 $\mu$M enzyme (in Mn), 10 mM potassium phosphate (pH 7.5), 10 mM sodium formate, and 10 $\mu$M EDTA. The enzyme had been reduced prior to the experiment with 120 $\mu$M $H_2O_2$. 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 linear fitting. In addition to MnSOD the solutions contained 10 mM potassium phosphate (pH 7.5), 10 mM sodium formate, and 10 $\mu$M EDTA. The enzyme had been reduced prior to the experiments with at least 200 $\mu$M $H_2O_2$. 

Figure 2.3.
Figure 2.4. Parallel-mode CW-EPR spectrum of as-isolated ScMnSOD. Instrument settings: $\nu = 9.3959$ GHz; microwave power = 32 mW; sweep rate = 1.81 mT/s; temp = 4.7 K.
References


Chapter 3

Comparison of Two Yeast MnSODs:

Mitochondrial *Saccharomyces cerevisiae* versus Cytosolic *Candida albicans*

**Introduction**

Superoxide dismutases (SODs) are enzymes that function to convert the superoxide (O$_2^-$) anion to dioxygen and hydrogen peroxide (H$_2$O$_2$) catalytically via sequential reduction and oxidation of a metal cofactor at virtually diffusion-controlled rate constants (Scheme 3.1).

**Scheme 3.1. The Dismutase Mechanism**

\[
M^{n+} + O_2^- \rightarrow M^{(n-1)+} + O_2 \\
M^{(n-1)+} + O_2^- + 2H^+ \rightarrow M^{n+} + H_2O_2
\]

Manganese-containing superoxide dismutases (MnSODs) are found in a wide variety of species and, in these species, it can be localized either in the cytosol or the mitochondria or both. In bacteria, MnSOD is cytosolic while in yeast both mitochondrial and cytosolic forms exist. In mammalian cells it is mitochondrial. MnSOD catalyzes the disproportionation of O$_2^-$ by a mechanism that distinguishes it from the other SODs in that it becomes a less effective antioxidant when O$_2^-$ levels are high relative to enzyme concentration (1). Specifically, human MnSOD (2) has been shown to be very inefficient at removal of high concentrations of O$_2^-$ whereas the MnSOD from bacteria (Escherichia coli (2) and Deinococcus radiodurans (3)) and yeast (Saccharomyces cerevisiae) (4) are more efficient under the same conditions. It is this feature of differential efficiency at O$_2^-$ removal that led to our interest in exploring the activity of MnSODs from different cellular sources and locations.

Efficient removal of high concentrations of O$_2^-$ will, by necessity, produce high local concentrations of H$_2$O$_2$. H$_2$O$_2$ is a biologically important class of reactive oxygen species (ROS). It can be reduced to hydroxyl radical (’OH) through a Fenton-type reaction with a reduced metal
(M^{n+} + H_2O_2 → M^{n+} + OH^- + •OH), whose reaction with cellular components causes severe damage to lipids, proteins, and nucleotides. The oxidative damage caused both directly and indirectly by H_2O_2 is implicated in cancer and aging (5,6). Besides its toxic effects, the roles of H_2O_2 in aiding phagocytes in the killing of ingested microorganisms as well as in some oxidative biosynthesis are well known (7). In addition in recent years there is rising evidence of H_2O_2 functioning as a signaling agent in higher organisms (7-11).

Scheme 3.2. Catalytic Mechanism of MnSOD

\[
\begin{align*}
Mn^{3+}\text{SOD} + O_2 & \xrightarrow{k_1} Mn^{2+}\text{SOD} + O_2 \quad (1) \\
Mn^{2+}\text{SOD} + O_2 & \xrightarrow{k_2, 2H^+} Mn^{3+}\text{SOD} + H_2O_2 \quad (2) \\
Mn^{2+}\text{SOD} + O_2 & \xrightarrow{k_3} Mn^{3+}(OO^2-)\text{SOD} \quad (3) \\
Mn^{3+}(OO^2-)\text{SOD} & \xrightarrow{k_4, 2H^+} Mn^{3+}\text{SOD} + H_2O_2 \quad (4)
\end{align*}
\]

The mechanism by which MnSOD removes high O_2^- concentrations less efficiently than other SODs involves product inhibition. The overall reduction of O_2^- by an oxidized metal (Scheme 3.2), when carried out by Mn^{2+}\text{SOD}, occurs either through a pathway (reaction 2) where delivery of protons to the bound peroxo moiety is effectively instantaneous (an “outer-sphere” pathway), or through a pathway (reaction 3) where an identifiable transient is formed (an “inner-sphere” pathway), defined here as a “product-inhibited” complex that has been described as a side-on Mn(III)-peroxo species (12). The inner-sphere pathway can become significant at high O_2^- concentrations and, if the dissociation of peroxide (k_4) is relatively slow, this can lead to reduced catalytic efficiency of MnSOD. The level of product inhibition is described by \(k_2/k_3\), the gating ratio of the two pathways.
*S. cerevisiae* is an important single-cell model for studying human and other eukaryotic cells. Like human cells, it expresses a homotetrameric MnSOD in its mitochondria (ScMnSOD), which shares sequence similarity with human MnSOD. To our surprise, our previous study showed ScMnSOD to be the most efficient at removing high O$_2^-$ concentrations, among MnSODs that have been characterized to date (4). However, the origins of the low level of product inhibition are unclear.

*S. cerevisiae* and humans have a common cellular localization of their SODs. They both have MnSODs only in their mitochondrion and copper-zinc SODs (CuZnSOD) in their cytosol. In contrast, a closely related yeast *Candida albicans* expresses a MnSOD that lacks the mitochondrial leader sequence and is active in the cytosol (CaMnSODc) in addition to its mitochondrial CaMnSOD and cytosolic CaCuZnSOD. CaMnSODc shares 70% sequence similarity with ScMnSOD and has been proposed to be important for survival of *C. albicans* in nutrient-limiting conditions (13). Overexpression of CaMnSODc has been shown to rescue the phenotypes of *S. cerevisiae* strain lacking the cytosolic CuZnSOD (13).

In order to understand further the mechanism of product inhibition in MnSOD and its implication in H$_2$O$_2$ signaling in higher organisms, we isolated and characterized ScMnSOD and CaMnSODc. Herein we report the first comparison of the catalytic activity of the two yeast MnSODs, the crystal structures of their metal-binding sites, and their spectroscopic characterization. We discuss how these factors relate to the function of yeast MnSODs; we

---

*We have chosen for the sake of convenience to distinguish between cytosolic and mitochondrial MnSODs by adding a “c” to the MnSOD to indicate that it is a cytosolic enzyme. This distinction becomes important in those organisms (*C. albicans*) where MnSOD is found in both locations. This distinction is not relevant to human and *S. cerevisiae* as all of the MnSOD is mitochondrial.*
further compare human, bacterial, and yeast MnSODs; and we explore the implications with respect to the evolution of H$_2$O$_2$ signaling.

**Materials and Methods**

*DNA Manipulation of CaMnSODc and Transformation into S. cerevisiae.* The pVT102U-CaMnSODc (with URA3 and AMP marker) vector containing the complete coding sequence of CaMnSODc was generously provided by Prof. Bourbonnais [13]. Sequencing shows that it is an I169V mutant of the wild-type protein accidentally created during cloning. The primers 5’-GTTCCAATTATGCCCATGCTTTGG-GAA-C-3’ and 5’-GTTCCCAAGCATCGATGGC-AATAA-TTGA-AC-3’ were used to create the wild-type cDNA (mutations are in bold type). The PCR products were transformed into *E. coli* DH5α strain and screened by ampicillin selection. The purified pVT102U-CaMnSODc vector was transformed into *S. cerevisiae* sod2Δ strain (EG110; *Mat-alpha, ura3, leu2, trp1, his3, sod2::TRP1*).

*Expression and Purification of ScMnSOD and CaMnSODc.* Yeast cells carrying Yep352-ScMnSOD [4] were grown in YPEG media (1% yeast extract, 2% peptone, 3% glycerol, 2% ethanol, pH 4) supplemented with 0.5 mM Mn(II) sulfate at 30 °C to O.D. > 20, and then harvested by centrifugation at 12,000 × g for 10 min. ScMnSOD was isolated as previously described [4].

Yeast cells carrying pVT102U-CaMnSODc were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose, pH 4) media supplemented with 0.5 mM Mn(II) sulfate at 30 °C to O.D. > 10, and then harvested by centrifugation at 12,000 × g for 10 min. The procedures for isolation of CaMnSODc and ScMnSOD were the same [4] except that the procedures of the DE52 column were different. CaMnSODc does not bind tightly to the DE52 resin while most other proteins in
the crude extract do. The active fractions from the hydrophobic interaction column were pooled and dialyzed against water and then 5 mM potassium phosphate, pH 8 at 4 °C. The protein then was loaded on a DE52 column, washed with 2 column volumes of 5 mM potassium phosphate, pH 8. CaMnSODc fell into the load and wash during the chromatography. The load and wash were combined, concentrated, and loaded onto a G300 gel filtration column.

Pulse Radiolysis. The pulse radiolysis experiments were carried out using the 2 MeV Van de Graaff accelerator at Brookhaven National Laboratory. Upon irradiation of water with a pulse of energetic electrons, hydrated electrons (e_{aq}^-), hydroxyl radicals (⋅OH) and, in lesser yield, hydrogen atoms (H\(^+\)) are the primary radicals produced. Superoxide radical is then generated in air-saturated aqueous solution containing sodium formate through the following reactions:

\[
\begin{align*}
\text{\textit{OH} + HCO}_2^- & \rightarrow \text{H}_2\text{O} + \text{CO}_2^- , \\
\text{O}_2 + \text{CO}_2^- & \rightarrow \text{O}_2^- + \text{CO}_2 , \\
\text{e}_{aq}^- + \text{O}_2 & \rightarrow \text{O}_2^\cdot , \\
\text{H}^+ + \text{O}_2 & \rightarrow \text{HO}_2^\cdot .
\end{align*}
\]

The experiments to measure catalytic rates were carried out using two methodologies. One set of studies involved oxidizing the resting enzyme with substoichiometric quantities of O\(_2^-\) and following the appearance of Mn\(^{3+}\)SOD which has an absorbance band at around 480 nm (extinction coefficient \(\approx 800 \text{ M}^{-1}\text{cm}^{-1}\)). The rate of reaction 2 \((k_2)\) was determined by fitting the resultant time trace of Mn\(^{3+}\) growth to a first-order reaction. In this set of experiments, 2:1 [H\(_2\)O\(_2\)]:[MnSOD] was added as previous studies had shown that H\(_2\)O\(_2\) effectively reduces Mn\(^{3+}\)SOD to Mn\(^{2+}\)SOD. The other method involved following the decay of various concentrations of O\(_2^-\) at 260 nm using a 1:1 to 1:50 ratio of [MnSOD]:[O\(_2^-\)]. Here, all the rate constants except \(k_2\) were calculated by fitting the data obtained to a mechanism described in Scheme 3.2 using the Chemical Kinetics program in PRWIN (14).

All pulse radiolysis samples were prepared in 10 mM potassium phosphate, 10 mM sodium formate and 10 or 100 μM EDTA at 25 °C. All MnSOD concentrations were taken as the
ICP-measured concentration of manganese in the sample. As all of the manganese concentrations were less than the corresponding protein concentrations, we assumed that all of the manganese was bound. This had the advantage of allowing direct comparison of activity of a tetrameric and a dimeric enzyme as everything is normalized to a monomeric unit. However, it also assumes no cooperativity between metal sites. The pH of the buffer was adjusted using ultrapure (Baker Ultrex) sodium hydroxide and sulphuric acid as needed.

**EPR Spectroscopy.** X-band (9.47 GHz) perpendicular-polarization ($B_0 \perp B_1$) continuous-wave electron paramagnetic resonance (CW EPR) spectra were recorded using a Bruker ECS106 spectrometer equipped with a TE102 cavity (ER3902ST). Parallel-polarization ($B_0 \parallel B_1$) data were acquired using a dual-mode cavity (ER 4116DM) operating at 9.39 GHz. 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. Spectral simulations were performed in MatLab using EasySpin 3.1 (15,16).

**Crystallization of ScMnSOD and CaMnSODc.** The apoprotein of ScMnSOD aggregates after the native protein is heated at 70 °C in MOPS, while the metallated ScMnSOD stays folded in solution. Fully metallated ScMnSOD was obtained by heating as-isolated protein (20 mM MOPS, pH 7) at 70 °C for 1 hr and removing aggregated protein by centrifugation. The reductive methylation of lysine residues of ScMnSOD was carried out as described, in order to improve the diffraction of protein crystals (17). The activity of native and methylated ScMnSOD is similar in SOD activity assay (18). All the free amino groups of lysine residues and the N-terminal were methylated as confirmed by mass spectrometry (Figure S3.1).

Methylated ScMnSOD was crystallized by hanging-drop vapor diffusion at 4 °C against a well solution of 0.2 M sodium malonate (pH 7) in 20% (w/v) polyethylene glycol 3,350 with a
protein concentration of 7 mg/mL. Native CaMnSODc was crystallized by hanging-drop vapor diffusion at 4 °C against a solution of 0.1 M magnesium chloride, 0.1 M sodium chloride and 0.1 M tri-sodium citrate (pH 5.5) in 30% (w/v) polyethylene glycol 400 with a protein concentration of 7 mg/mL. ScMnSOD and CaMnSODc crystals were cryo-protected in mother liquor solution containing 30% glycerol and flash frozen in liquid nitrogen prior to data collection.

Crystallography: Data Collection and Refinement. All data was collected at 100K at the UCLA X-ray diffraction facility, using a Rigaku FRE+ generator and a Rigaku HTC detector. All data was processed using DENZO and SCALEPACK (19). ScMnSOD was phased by molecular replacement using human MnSOD Q143N mutant (1QNM). CaMnSODc was phased by molecular replacement using ScMnSOD (3LSU). All the molecular replacement was done using PHASER (20). The models were built using COOT (21). All model refinement was done using REFMAC (22) and PHENIX (23).

Coordinates and structure factors have been deposited in the PDB with accession numbers 3LSU for the ScMnSOD structure and 3QVN for the CaMnSODc structure.

Miscellaneous Methods. The metal contents of the purified proteins were determined by ICP-MS (Agilent 7500 series). UV-Visible absorption spectra were collected on a Shimadzu UV-2501 PC spectrophotometer.

The mass weight of the protein subunit was determined by an electrospray ionization mass spectrometry (ESI-MS) via a triple quadrupole instrument (API III, Applied Biosystems). An m/z 600–2,200 range was scanned (positive mode, orifice 90 V, 0.3 Da step size, 5.61 s/scan). Data were processed and analyzed using MacSpec 3.3, Hypermass and BioMultiview 1.3.1 software (Applied Biosystems).
The mass weight of the native protein was determined by a HPLC (Agilent 1200 series) fitted with a size exclusion column (Tosoh Bioscience, TSK gel G2000SW 7.5 mm ID X 30 cm) at a flow rate of 0.5 mL/min. The column was calibrated using five standards: bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B12 (1.35 kDa).

**Results**

*Tetramer versus Dimer.* CaMnSODc was overexpressed and purified from S. cerevisiae, with 0.6 equiv of manganese per subunit as determined by ICP-MS. The subunit mass weight is 22.7 kDa as determined by ESI-MS. The purity was confirmed by SDS-PAGE gel (Figure S3.2). CaMnSODc has been reported to be a homotetramer (13), but our investigation of the native protein using gel filtration chromatography indicates that its molecular weight in solution under our conditions is ~46 kDa, corresponding to a homodimer (Figure 3.1). It should be noted, however, that we found that CaMnSODc crystallized as a tetramer under other conditions (see below). ScMnSOD, in contrast to CaMnSODc and analogous to the human MnSOD, is a homotetramer under our conditions, although the two yeast enzymes share great sequence similarity.

*Kinetic Studies.* Solutions containing 1 μM MnSOD at pH 7 were pulse irradiated and the disappearance of $O_2^-$ was followed at 260 nm; see Figure 3.2. The traces we collected for both CaMnSODc and ScMnSOD were plotted along with previous data collected under identical conditions for MnSOD from human, *E. coli* and *D. radiodurans* (2,3). When the $O_2^-$ concentration was seven-fold excess over the enzyme, human MnSOD already showed a depressed activity, yet the $O_2^-$ disappearance as a function of time was similar for the other four
Figure 3.1. The HPLC-SEC profiles of CaMnSODc (a) and ScMnSOD (b). Inset: The plot of the molecular weight of the five standards (black, circles), ScMnSOD (green, triangle) and CaMnSODc (red, square) versus their retention time. The concentration of proteins loaded into the column is 6 mg/mL. The column was calibrated using five standards: 1) bovine thyroglobulin (670 kDa), 2) bovine γ-globulin (158 kDa), 3) ovalbumin (44 kDa), 4) horse myoglobin (17 kDa), and 5) vitamin B12 (1.35 kDa). The buffer contained 50 mM sodium phosphate (pH 6.7) and 50 mM sodium chloride.

Figure 3.2. Decay of 7 μM (A) and 41 μM (B) O$_2^-$ catalyzed by 1 μM (in Mn) human (a, black), E. coli (b, red), D. radiodurans (c, blue) MnSOD, ScMnSOD (d, green) and CaMnSODc (e, pink). The sample for pulse radiolysis contained 10 mM potassium phosphate (pH 7), 10 mM sodium formate and 10 μM EDTA. The O$_2^-$ concentration in these figures is calculated from the absorbance at 260 nm.
enzymes and could be fit to a first-order process (Figure 3.2A). When the ratio of [O$_2^-$]:MnSOD increased to 41, the five MnSODs displayed dramatically different efficiencies at removing O$_2^-$ (Figure 3.2B), which scale as CaMnSOD > ScMnSOD > D. radiodurans (DrMnSOD) > E. coli (EcMnSOD) > human MnSOD. For human and E. coli MnSOD, the O$_2^-$ decay was characterized by a fast decrease, followed by a slow almost zero-order phase. It is the biphasic nature of the trace that suggests dismutation proceeded by a fast catalytic pathway and a slower, concomitant product-inhibited pathway. The gating values for these two enzymes are 1 and 5 respectively (2). The trace obtained for the DrMnSOD showed a much less distinct plateau region and fitting the data yielded a gating value of 12 (3). Our earlier study of the reaction of ScMnSOD with high O$_2^-$ concentrations led to a calculated gating value $k_2/k_3$ to be 16-20, indicating that the fast protonation pathway dominates (4).

CaMnSODc removes high concentrations of O$_2^-$ even more efficiently than ScMnSOD. Here, $k_2$ of CaMnSODc was determined to be $1.0 \times 10^9$ M·s$^{-1}$ by following the appearance of Mn$^{3+}$SOD ($\lambda_{\text{max}} = 480$ nm; see method, Figure S3.3) as CaMn$^{2+}$SODc was oxidized by substoichiometric amounts of O$_2^-$. However, at physiological pH (6.5–7.5), CaMnSODc removed various concentrations of O$_2^-$ ([O$_2^-$]:[MnSOD] from 2–48:1) at similar rates, showing that only minimal product inhibition occurred under these conditions. Even at [O$_2^-$]:MnSOD ratios as high as 48, the activity of CaMnSODc was comparable to that of S. cerevisiae CuZnSOD, an enzyme that does not exhibit product inhibition (Figure S3.4). The disappearance of O$_2^-$ catalyzed by CaMnSODc could be fitted to a first-order process.

To achieve such a high efficiency, CaMnSODc would either carry such a high gating ratio ($k_2/k_3$) that the reaction of Mn$^{2+}$SOD with O$_2^-$ proceeds almost completely via the fast protonation (reaction 2), or it has such a high dissociation rate ($k_4$) that the product-inhibited
complex decomposes before it is detected during pulse radiolysis (reaction 4). Assuming that $k_4$ is $50 \text{ s}^{-1}$, which is close to that of bacterial dimeric MnSODs, the calculated $k_2/k_3$ is 65. Assuming that $k_2/k_3$ is 20, which is comparable to that for ScMnSOD, $k_4$ is calculated to be $210 \text{ s}^{-1}$. The latter scenario is more likely, because a dissociation rate of $210 \text{ s}^{-1}$ is not dramatically different from a $k_4$ of $130 \text{ s}^{-1}$ for human MnSOD and $90\text{-}140 \text{ s}^{-1}$ for ScMnSOD. In addition, dissociation rates as large as $1000 \text{ s}^{-1}$ have been reported in human Y34 mutants (24). A $k_2/k_3$ ratio of 65, however, is three times as large as the gating ratio of ScMnSOD, the least inhibited MnSOD that has been characterized to date. Therefore, the high efficiency of CaMnSODc is more likely due to the fast dissociation of the inhibited complex from the metal center.

Previous studies have shown that the rate of enzymatic catalysis as a function of pH is different for the human and bacterial MnSODs. Given that a major difference between the human and bacterial MnSODs is that the former is a tetramer while the latter are dimers, we compare here two yeast MnSODs, where one is a dimer and the other is a tetramer. In these studies we were initially interested only in the change in the fast catalytic cycle ($k_1$ and $k_2$), which was measured by following the disappearance of $\text{O}_2^-$ in the presence of ScMnSOD or CaMnSODc under conditions where the $[\text{O}_2^-]$:MnSOD ratio was between 1 and 3. The overall activity of both ScMnSOD and CaMnSODc decreased considerably as pH was increased (Figure 3.3). Both yeast enzymes resemble EcMnSOD, with a relatively constant activity up to pH 9, but are distinct from human MnSOD, where catalysis is relatively unchanged even up to pH 11. It is surprising that ScMnSOD and CaMnSODc are different in their quaternary structure, yet they exhibit such similar pH dependence. It is notable that the yeast MnSODs are even more sensitive to the change of pH than the bacterial MnSODs. The $pK$ is $\sim 8.5$ in two yeast MnSODs, $\sim 9.5$ in EcMnSOD and $\sim 10.5$ in human MnSOD. Adjusting the pH of yeast MnSOD sample solutions
**Figure 3.3.** Effects of pH on the overall activity of MnSODs. The rate constant was determined by fitting the disproportionation of low doses of $\text{O}_2^-$ ([O$_2^-$]:MnSOD from 1–3) to a first-order process. Under these [O$_2^-$]:MnSOD ratios, the reaction of Mn$^{2+}$SOD with O$_2^-$ proceeds predominantly through the outer-sphere pathway. This rate constant reflects a combination of $k_1$ and $k_2$. MnSOD is from human (black, diamonds), *E. coli* (red, rectangles), *S. cerevisiae* (green, circles) and *C. albicans* (pink, triangles). The data points marked with an arrow correspond to the rate constants measured for the yeast enzymes after adjusting the pH of sample solution from 9.5 to neutral.

**Figure 3.4.** Dependence of the level of product inhibition of ScMnSOD on pH. Decay of 48 μM O$_2^-$ catalyzed by 1 μM (in Mn) ScMnSOD at pH 7.5 (a) and 8.9 (b). Inset: Dependence of the values of $k_2/k_3$ (circles) and $k_4$ (triangles) of ScMnSOD on pH. The sample for pulse radiolysis contained 10 mM potassium phosphate (pH 7), 10 mM sodium formate and 10 μM EDTA.
from 9.5 back to 7 resulted in the restoration of their activity to ~50% of that of the intact enzymes (Figure 3.3).

pH also has an impact on the level of product inhibition exhibited by ScMnSOD. The slow almost zero-order phase, which is a sign of the saturation of MnSOD by O$_2^-$, was discernable from the reaction of high concentrations of O$_2^-$ ([O$_2^-$]:MnSOD = 48:1) with ScMnSOD at pH 8.9 (Figure 3.4). This feature was barely seen under the same conditions at pH 7.5. The gating ratio $k_2/k_3$ and the dissociation rate $k_4$ were calculated by fitting the O$_2^-$ decay into the four-reaction mechanism (Scheme 3.2; Figure 3.4, inset). The calculated $k_2/k_3$ is a constant value over the pH range of 7–9.5. The dissociation rate $k_4$, however, dramatically decreases when pH is raised to 8 and reaches a plateau at pH ~8.5 with a pK of ~8. Due to a depressed $k_4$, the dissociation of H$_2$O$_2$ from the active site may become the rate-limiting step at high pH, resulting in a more inhibited ScMnSOD. The values of $k_2/k_3$ and $k_4$ of ScMnSOD were difficult to calculate at pH > 9.5, at which point $k_1$ and $k_2$ corresponded to the rate-limiting steps of the reaction. In the case that $k_4$ stays constant at the plateau while $k_1$, $k_2$ and $k_3$ continue decreasing at pH >9.5, the dissociation of the inhibited complex may occur faster than its formation, leading to a decay of O$_2^-$ at pH >9.5 that appeared to be non-inhibited.

CaMnSODc, in contrast, did not show a pronounced occurrence of the slow zero-order phase even at elevated pH (Figure S3.5). The decay of O$_2^-$ over the pH range of 7–9.5 could still be fit to a first-order process ($R^2 = 1.5$). In this case it is difficult to calculate either the ratio of $k_2/k_3$ or the value of $k_4$ based on fitting.

Oxidation State. Our previous work showed that tetrameric ScMnSOD, unlike all other wild-type MnSODs studied thus far, is isolated predominantly in the 2+ oxidation state (4). Dimeric CaMnSODc, structurally similar to bacterial MnSODs, is also isolated in the Mn$^{2+}$ state.
(Figure S3.6-A). The two enzymes from yeast are also very similar with respect to the thermodynamics of oxidation and reduction. From this point on, if the results are very similar for the two yeast MnSODs, data from only one enzyme are shown.

The optical absorption spectrum of as-isolated ScMnSOD has a very weak visible absorption band around 480 nm (Figure 3.5A) that suggests that a small amount of Mn$^{3+}$SOD is present along with a significantly larger amount of Mn$^{2+}$SOD. The addition of sodium hyposulfite fully reduced the enzyme and completely quenched this absorption intensity (Figure 3.5A). Based on the difference in absorption between the fully reduced and the as-isolated enzyme (Figure 3.5A, insert) and using the extinction coefficient for Mn$^{3+}$SOD obtained from pulse radiolysis ($\varepsilon_{475} = 780$ M$^{-1}$cm$^{-1}$), ~5% of the as-isolated enzyme is oxidized. The oxidized enzymes found in both as-isolated ScMnSOD and CaMnSODc have spectra that differ from those of most reported Mn$^{3+}$SODs in that additional absorption intensity at ~390 nm is visible (Figure 3.5A, inset; Figure S3.6-A, inset). They are, however, similar to the spectrum of ScMn$^{3+}$SOD that was reported previously (25).

Oxidation of as-isolated ScMn$^{2+}$SOD or CaMn$^{2+}$SODc by 0.75 equivalents of potassium permanganate rapidly gave absorption spectra characteristic of 5-coordinate Mn$^{3+}$SOD with a maximum at 480 nm (Figure 3.5B, S6-B) with no residual absorbance of permanganate. Both yeast MnSODs remained oxidized in solution and retained full activity as determined by pulse radiolysis. Interestingly, an absorption intensity around 390 nm increased slowly over time (Figure 3.5B, S6-B). Based on the difference between the immediately scanned and the final spectrum (Figure S3.7), a Mn$^{3+}$-containing species different from the 5-coordinate Mn$^{3+}$SOD was formed over time. Further addition of 1 equivalent of sodium ascorbate selectively bleached the absorption of ScMn$^{3+}$SOD or CaMn$^{3+}$SODc around 390 nm, yielding the characteristic 5-
Figure 3.5. The oxidation state of ScMnSOD. (A) Optical spectra of as-isolated ScMnSOD (solid line) and ScMnSOD reduced by sodium hydrosulfite (dashed line). Inset: The difference spectrum between as-isolated and reduced ScMnSOD. (B) Optical spectra of ScMnSOD oxidized by potassium permanganate ([KMnO₄]:MnSOD = 0.75:1) at pH 7.4 as measured over time. Time 0 refers to the start of the first scan immediately after mixing KMnO₄ with the enzyme. (C) Reduction of ScMn³⁺SOD (solid line, oxidized by 0.75 equiv of KMnO₄ and allowed to equilibrate at room temperature for 2 hr) by one equiv of sodium ascorbate (dotted line). (D) Optical spectra of ScMn³⁺SOD at different pH. The sample solutions contained 190 μM (in Mn) enzyme in 25 mM potassium phosphate (pH 7.4).
coordinate Mn$^{3+}$SOD spectrum (Figure 3.5C, S6-C), which has been reported previously (25). Treatment of the yeast enzymes with 10-fold molar excess of ascorbate yielded the completely reduced enzymes. Therefore, the unknown species is kinetically more prone to reduction by ascorbate than the well-characterized 5-coordinate species. In contrast, 0.5 equivalent of H$_2$O$_2$ reduced the 5-coordinate Mn$^{3+}$ and the unknown species simultaneously in one equivalent of yeast Mn$^{3+}$SODs, giving optical spectra of full reduced MnSOD (data not shown). The slow formation of the 6-coordinate adduct and its susceptibility to reduction by ascorbate are very puzzling, and we will continue exploring the explanation in future.

One species known to absorb around 390 nm is the low-temperature EcMn$^{3+}$SOD:azide adduct. At room temperature, the 5-coordinate EcMn$^{3+}$SOD binds azide in such a fashion that the coordination number of the Mn ion does not change. At lower temperatures (pK = 200 K) this adduct transforms into an inner-sphere 6-coordinate azide-Mn$^{3+}$SOD complex (26). The room-temperature spectrum of azide-treated ScMn$^{3+}$SOD shows a shift of the absorption band similar to that observed in other azide-Mn$^{3+}$SODs (26) (Figure S3.8). However, there remains additional intensity at 390 nm that is reminiscent of the features present in the spectrum of the low-temperature azide adduct of EcMn$^{3+}$SOD where azide is directly bound to the Mn ion. This may indicate that the unknown species in ScMn$^{3+}$SOD is also 6-coordinate.

The measurement of the redox potential of MnSOD is notoriously difficult. Our electrochemical studies on ScMnSOD and CaMnSODc failed to achieve efficient electron transfer between the electrode and the enzymes and thus only yielded an approximation of their redox potential as ~0.3 V. This value is comparable to those determined for E. coli (0.3 V) (27) and human (0.4 V) (28) MnSOD, which are a reflection of the redox potential of the 5-coordinate
Mn$^{3+}$SOD species. The redox potentials of human and E. coli MnSOD are close to that of ascorbate$^-$, H$^+$/ascorbateH$^-$ ($E^\circ = 282$ mV) at neutral pH.

The Mn$^{3+}$SOD spectra obtained from pulse radiolysis, in which ScMn$^{2+}$SOD or CaMn$^{2+}$SODc was oxidized by substochiometric amount of O$_2$ (see method), merely showed formation of the well characterized 5-coordinate Mn$^{3+}$SOD (4) and were comparable to those of other Mn$^{3+}$SODs (3,29) (Figure 3.6). However, the time scale of each single measurement at each wavelength was shorter than 1 ms. Over 10 ms the reduction of yeast Mn$^{3+}$SODs by excess H$_2$O$_2$ was discernable. Therefore, the generation of the putative L-Mn$^{3+}$SOD species likely occurred more slowly under pulse radiolysis conditions than the reduction of the 5-coordinate Mn$^{3+}$ center by H$_2$O$_2$. Indeed the shifting of the spectrum around 390 nm was discernable in a much longer timescale (> 10 min) from the oxidation of as-isolated ScMnSOD using $^{60}$Co radiation as the source of O$_2^-$ (Figure S3.9).

MnSODs that are isolated in the Mn$^{3+}$ state possess absorption spectra that change modestly as pH is raised. For example, at pH > 9 the 478 nm absorption feature corresponding to EcMn$^{3+}$SOD begins to diminish slightly in intensity and shifts to shorter wavelength. This pK event has been ascribed to deprotonation of Tyr34 (numbering in ScMnSOD) (30). However, for ScMnSOD and CaMnSODc the absorption intensity around 390 nm increased as pH was increased (Figure 3.5D, S6-D). ScMnSOD and CaMnSODc may not have the same pK as other Mn$^{3+}$SODs, or the spectral change due to the pK may have been obscured by the absorption shifting around 390 nm. The Mn$^{3+}$ spectra of ScMnSOD obtained from pulse radiolysis at pH 7 and 10 were compared, and a roughly 20% decrease in intensity but no shift in peak for the Mn$^{3+}$SOD absorbance at 480 nm were observed (Figure S3.10). The spectral shift over pH
indicates that the sixth ligand is likely a hydroxide that with a higher concentration binds to Mn\(^{3+}\) more readily at high pH.

**EPR Spectra.** In agreement with the electronic absorption data shown above, results from perpendicular-mode X-band EPR experiments confirm that CaMnSODc is isolated predominately in the Mn\(^{2+}\) oxidation state (Figure S3.11-A). These spectra are similar to those obtained for EcMn\(^{2+}\)SOD with five separate electronic spin transitions observed due to zero-field splitting of the manifold of spin levels of the high-spin (\(S = 5/2\)) Mn\(^{2+}\) ion. These resonances are further split by the hyperfine interaction of the electron spin with the \(^{55}\)Mn nuclear spin (\(I = 5/2\)). The corresponding parallel-mode EPR spectrum for as-isolated CaMnSODc possesses little intensity (Figure S3.11-B). In contrast, the spectra of the pH 7.65 permanganate-oxidized samples have at least eight intense transitions centered at 80 mT (g = 8.4) (Figure 3.7A) that are somewhat similar to the six parallel-mode EPR transitions separated by \(\approx 10\) mT observed previously for EcMn\(^{3+}\)SOD (31). Campbell et. al. (31), showed that these signals arise from transitions within the \(m_S = |\pm 2\rangle\) manifolds of a high-spin (\(S = 2\)) Mn(III) system. Analysis of the temperature-dependence of the integrated signal intensity led to an evaluation of the magnitude and, importantly, sign of the zero-field splitting parameters—\(D = +2.10\) cm\(^{-1}\) and \(E = 0.24\) cm\(^{-1}\). (NOTE: These values are consistent with those determined by analysis of variable-temperature, variable-field magnetic circular dichroism saturation behavior (32).) That \(D > 0\) indicates that the lowest unoccupied metal-centered molecular orbital (MO) is 3d\(_z^2\)-based (33). These conclusions are borne out by the trigonal bipyramidal coordination geometry of the Mn\(^{3+}\) site determined by X-ray crystallography as well as results of numerous electronic structure calculations (34).
Figure 3.6. The Mn$^{3+}$SOD spectrum of CaMnSODc obtained from pulse radiolysis. The pulse radiolysis sample contained 60 μM (in Mn) CaMnSODc in 10 mM potassium phosphate (pH 7), 10 mM sodium formate and 100 μM EDTA. The enzyme was reduced prior to each pulse with 120 μM H$_2$O$_2$.

Figure 3.7. (A) Parallel-mode CW EPR spectrum of CaMn$^{3+}$SODc and ScMn$^{3+}$SOD oxidized by potassium permanganate ([ScMnSOD]:KMnO$_4$ = 1:0.75) and of ScMn$^{3+}$SOD treated with 1 equivalent of sodium ascorbate. (B) The deconvolution of the two spectra (top two spectra in panel A) of the permanganate-oxidized species shows two components, one assigned to a Mn$^{3+}$ center with a $5A_{1g}$ ground state and one with a $5B_{1g}$ ground state. Instrument settings: $\nu = 9.3959$ GHz; microwave power = 32 mW; sweep rate = 1.81 mT/s; temp = 4.9 K.
In the present case of ScMn\(^{3+}\)SOD and CaMn\(^{3+}\)SODc, more than six parallel-mode resonances are observed, indicating that there are at least two Mn\(^{3+}\)-containing species present in the sample. Subtraction of the EPR spectrum of EcMn\(^{3+}\)SOD from that of ScMn\(^{3+}\)SOD reveals an underlying sextet with interpeak spacings of approximately 4.5–5.0 mT (Figure 3.7B). Such a small effective \(^{55}\)Mn hyperfine coupling parameter \(A_{||}\) has been observed previously for several 6-coordinate Mn(III) centers \((33, 35, 36)\). There has also been a preliminary report that showed a fraction of 5-coordinate EcMn\(^{3+}\)SOD converts to a new species at pH 11.54 that is characterized by a parallel-mode EPR spectrum with \(A_{||}(^{55}\)Mn) = 3.3 mT \((31)\).

The dramatic difference in \(A_{||}\) for the two spectroscopically distinct ScMn\(^{3+}\)SOD species is likely resulted from a change in the electronic ground state for the Mn\(^{3+}\) ion—from 5\(A_{1g}\) to 5\(B_{1g}\) \((33)\). Respectively, these states correspond to d-electron configurations wherein the 3d\(_x^2\)-based and 3d\(_{x^2−y^2}\)-based MOs are empty. A simple explanation for this change would be a new, strong ligand binding in the equatorial plane to the Mn\(^{3+}\) ion. This event would raise the energy of the 3d\(_{x^2−y^2}\)-based MO allowing the fourth d-electron to occupy the 3d\(_z^2\) orbital instead.

The EPR transition observed for both electron configurations is between the \(m_S = \pm 2\) spin levels. For the 5\(B_{1g}\) state, the axial zero-field splitting interaction \(D\) will be negatively signed, putting the \(|\pm 2\rangle\) manifold lowest in energy. Thus this manifold will be populated at all temperatures and if the energy splitting \(\Delta\) of the \(m_S = |\pm 2\rangle\) spin levels at zero field is less than the energy of the incident microwave radiation \((h\nu = 0.319 \text{ cm}^{-1} \text{ for the } 9.396 \text{ GHz radiation employed here})\) a parallel-mode EPR transition will be observed. However, for the 5\(A_{1g}\) state, the sign of \(D\) is positive and the \(|\pm 2\rangle\) manifold will not be populated at low temperatures. For CaMn\(^{3+}\)SODc, increasing the temperature thermally populates this manifold and the EPR transition becomes apparent. By monitoring the temperature-dependent intensity of the peak at
58 mT we have determined that $D = +1.90 \text{ cm}^{-1}$ and $E = 0.20 \text{ cm}^{-1}$, values similar to those found for the 5-coordinate form of EcMn$^{3+}$SOD (see Figure S3.12).

Upon treatment of ScMn$^{3+}$SOD with 1 equivalent of ascorbate, the $5B_{1g}$ contribution to the parallel-mode EPR spectrum completely disappears (compare second and third traces in Figure 3.7A). This spectral change coincides with bleaching of the 390 nm feature in the electronic absorption spectrum (Figure 3.5C) upon addition of ascorbate, suggesting the narrowly split EPR signal and the 390 nm absorption intensity are spectral signatures of the same species.

**Crystal Structure.** The active site structure of the two yeast MnSODs was investigated by crystallography (Tabls S3.1). The crystals of both yeast MnSODs are colorless. The X-ray crystallography structure of ScMnSOD is always a tetramer (Figure S3.13-A). To our surprise, the quaternary structure of CaMnSODc varied with crystallization conditions. It is either a dimer (data not shown) or a tetramer (Figure S3.13-B). We believe that the quaternary structure of CaMnSODc depends on buffer condition (ion strength and viscosity) and protein concentration, and we will explore the factors that control its quaternary structure in a future publication.

ScMnSOD and CaMnSODc both maintain the 5-coordinate sphere of manganese conserved in all other MnSODs (Figure 3.8) (34,37-40). Their active sites are superimposable, giving a root mean square deviation (RMSD) of 0.20 Å (Figure S3.14). For both enzymes the observed manganese bond lengths are ~2.2 Å to each His NE2, 2.0 Å to Asp OD2, and 2.3 Å to OH2 (Tabls S3.2). These are only slightly longer than the corresponding distances in MnSODs that rest predominantly in the Mn$^{3+}$ state. Spectroscopic and computational evidence suggests that in the reduced state Mn is bound by a water molecule and in the oxidized state it is bound by a hydroxide (41). The distance of the Mn–O bond should be farther in the bound water as it is not
Figure 3.8. Active site structure of ScMnSOD, showing the coordination sphere of manganese (chain A). Some second coordination sphere residues are also shown. Coordination and hydrogen bonds are indicated as solid and dashed lines, respectively.
as strong a bond and closer in the bound hydroxide. The long Mn–O (solvent molecule) distances in both yeast MnSODs are consistent with Mn$^{2+}$–OH$_2$ moieties. Although these shifts are small and within possible experimental error, the observed longer coordination bond distances could reflect the 2+ oxidation state of ScMnSOD and CaMnSODc.

During X-ray crystallography data collections, metalloenzyme crystals are likely to be reduced by photo-electrons generated from the excitation of protein and solvent atoms by the incident X-ray beam (42). As ScMnSOD and CaMnSODc are both isolated in the reduced form as indicated by their optical and EPR spectra (Figure 3.5, S6 and S11), the X-ray structures are certainly those of the reduced (Mn$^{2+}$SOD) enzymes. For other MnSODs that are isolated in the Mn$^{3+}$ oxidation state, active site bond distances measured in their X-ray structures (Tabls S3.2) would suggest that the human and *Thermus thermophilus* crystals contained primarily Mn$^{3+}$SOD and the remaining MnSODs were likely primarily reduced during the data collection.

**Discussion**

*The Relationship of Product Inhibition to Active Site Structure.* Based on the structural and sequence similarity shared between ScMnSOD and human MnSOD, ScMnSOD was expected to exhibit a level of product inhibition similar to human MnSOD. However, we found that ScMnSOD is dramatically gated toward the fast protonation pathway. CaMnSODc resembles ScMnSOD in its efficiency of removing O$_2^-$ at high O$_2^-$ doses. The yeast MnSODs are more like bacterial MnSODs in terms of function. Our studies on yeast MnSODs place human MnSOD in a unique class that predominantly undergoes the product-inhibited pathway at high levels of O$_2^-$ among MnSODs that have been characterized to date.
Figure 3.9. Superposition of the first and second coordination sphere of ScMnSOD to that of human MnSOD (pink, A) and EcMnSOD (yellow, B).
Numerous studies on the structure-function relationship of MnSOD have been focused on the structure of second coordination sphere structure (24,43-47), which is highly conserved in MnSOD from different organisms (34,37-39). Superposition of all active site residues (within 6 Å of Mn) of ScMnSOD on those of human or E. coli MnSOD gives RMSD of 0.25 and 0.18 Å, respectively (Figure 3.9), indicating that in terms of second coordination sphere, ScMnSOD resembles EcMnSOD more than it does human MnSOD, in spite of the fact that ScMnSOD and human MnSOD are both tetramers and share greater sequence similarity. Of particular significance are highly conserved residues Tyr34 and His30, mutations of which usually cause changes in product inhibition (24,48). Their positions in ScMnSOD overlap more with those in EcMnSOD than with those in human MnSOD (Figure 3.9). One would expect subtle shifts of these residues in the second coordination sphere to cause dramatic differences in the degree of product inhibition among MnSOD from different species. The factors that control the level of product inhibition will be subjects of future investigations.

The Unusual Resting State of Yeast MnSODs and the Role of Gln154. In contrast to human and bacterial MnSODs that rest predominantly as Mn$^{3+}$SOD, the as-isolated proteins of yeast MnSODs are both ~95% reduced. Our preliminary electrochemical results indicate that the redox potentials of yeast MnSODs are comparable to those determined for human and bacterial MnSODs. However, previous studies on EcMnSOD have shown spontaneous oxidation of EcMn$^{2+}$SOD in air (29), which does not occur in yeast MnSODs. Therefore, the unusual resting state of the yeast enzymes may result from thermodynamically driven shifts in oxidation-reduction equilibria.

The fact that yeast MnSODs rest as Mn$^{2+}$SOD could be linked with the positioning of an outer-sphere residue, Gln154 (the numbering in ScMnSOD; CaMnSODc: Gln163; human:
Gln143; *E. coli*; Gln146). Conserved in all Mn and FeSODs, Gln154 donates an H-bond to the metal-bound solvent molecule, and is the only contact of the metal-bound solvent molecule to the protein beyond the active site. Gln154 makes three hydrogen bonds to three highly conserved residues, Tyr34, Asn80 and Trp133 (numbering in *Sc*MnSOD). Its hydrogen bonding potential is fully utilized and Gln154 seems firmly fixed in place. Gln154 together with Asp168 that is an H-bond acceptor from the metal-coordinated H$_2$O or OH$^-$, determine the position of the coordinated solvent molecule. Mutagenesis on Gln154 often causes dramatic depression of reactivity (43,44,46), indicating the key role of the NE2(Gln154)$\cdot\cdot\cdot$O(solv) bond.

The active site reduction midpoint potential ($E_m$) contributes directly to the driving forces of the two half reactions of O$_2^-$ dismutation. In Mn and FeSOD (structural homologs), Gln154 is critical in modulating $E_m$ and thus contributes to the ion specificity as well as determining the resting state of Mn and FeSOD (12,49). The second coordination sphere of Mn and FeSOD is similar except that the Gln154 amide nitrogen is $\sim$0.7 Å further from the metal in FeSOD. DFT calculations on the structure of Mn and FeSOD suggest the reason of MnSOD resting predominantly in the Mn$^{3+}$ state as the destabilization of coordinated H$_2$O vs. OH$^-$ by the closely placed glutamine side chain (12,50). In Mn-substituted *Ec*FeSOD in which Mn is placed away from Gln69, the $E_m$ is elevated by 670 mV and the enzyme completely rests as the reduced form (51). Mutation of Gln143 to asparagine in human MnSOD also causes the enzyme to rest as Mn$^{2+}$SOD (43). Either *Ec*Mn(Fe)SOD or human Q143 mutants is only able to catalyze one half reaction, the oxidation of O$_2^-$ (43,51).

Most MnSODs with solved structure conserve a NE2(Gln154)$\cdot\cdot\cdot$O(solv) H-bond of 2.85–2.95 Å (Tabs S3.2), and, as indicated by previous studies, the further the Gln154 amide nitrogen is away from Mn, the more the 2+ state is favored by the enzyme. Thus, the observation that the
distance of Gln154 to Mn is slightly longer in the two yeast MnSODs (4.79 and 4.73 Å in ScMnSOD and CaMnSODc, respectively) than in the MnSODs that predominantly rest in the oxidized form (Human: 4.65 Å; E. coli: 4.64 Å; D. radiodurans: 4.54 Å; Caenorhabditis elegans: 4.54 Å) may be related to fact that the yeast MnSODs rest in the reduced state.

The Relationship of Product Inhibition of Human MnSOD to H$_2$O$_2$ Signaling in Mammals. Human MnSOD is much less efficient at turning O$_2^-$ into H$_2$O$_2$ than ScMnSOD when O$_2^-$ levels are high, although the two enzymes share great sequence similarity and are both located in mitochondria. Mitochondria are recognized as the main source of O$_2^-$ with the production occurring through respiratory chain. H$_2$O$_2$ is generated in mitochondrial matrix via the dismutation reaction of O$_2^-$ catalyzed by MnSOD. The produced H$_2$O$_2$ then diffuses into cytosol. The roles of H$_2$O$_2$ are complex in higher organisms. In mammalian cells the upregulated expression of antioxidant enzymes is not a universal response to supraphysiologic levels of H$_2$O$_2$ (7). Instead, physiologic levels of H$_2$O$_2$ stimulate cell division, transformation, migration or apoptosis (7,8,10,11). This along with the fact that mammalian cells generate H$_2$O$_2$ in response to various stimuli supports that H$_2$O$_2$ is utilized as a signaling agent in numerous mammalian signaling pathways (7-11,52,53). A well established H$_2$O$_2$-induced signaling is the modulation of the tyrosine phosphorylation of proteins by causing the oxidative inhibition of tyrosine phosphatases (9). Reversible inactivation of tyrosine phosphatases has been demonstrated to play a critical role in protein tyrosine phosphatase (PTP) and insulin signaling (54,55). Several mitochondrial signaling pathways are stimulated by increased H$_2$O$_2$, including tumor necrosis factor (TNF)-α-induced apoptosis (56) and c-Jun NH$_2$-terminal kinase (JNK)-induced apoptosis (52,57). The participation of H$_2$O$_2$ in signaling cascades may require tight regulation of its production in mitochondria.
A recent study demonstrates the production of large bursts of $O_2^-$, termed “superoxide flashes”, in human mitochondria (58). The flash events occur randomly in space and time, last for 20–30 seconds and are confined to tiny elliptical areas (58). Under the conditions that were tested human MnSOD in mitochondrial matrix was expected to eliminate superoxide flashes in a shorter time scale, considering that the reactivity of human MnSOD was thought to be diffusion controlled ($k = 2.3 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$) and its intramitochondrial concentration is 10–40 μM (52,59). The observation of superoxide flashes indicates that human MnSOD is unable to maintain a steady-state concentration of $O_2^-$ in tiny elliptical areas in mitochondria. The most likely explanation for this phenomenon is that human MnSOD is significantly inhibited at these high concentrations of $O_2^-$. Under these conditions, bursts of $O_2^-$ are not translated into bursts of $H_2O_2$ that could result in aberrant oxidant-driven signaling in mitochondria.

Mitochondria are the main source of $H_2O_2$ and its decomposition in the mitochondrial matrix is catalyzed by glutathione peroxidase and catalase. The rate constants of mammalian glutathione peroxidase and catalase are $5 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$ and $4.6 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$, respectively, and their intramitochondrial concentration is ~10% that of MnSOD (59). These antioxidants maintain a steady-state intramitochondrial $H_2O_2$ concentration of ~0.5 × 10^-8 M (59). As no comparison of $H_2O_2$ concentration in areas with or without superoxide flashes has been reported, it is unclear whether superoxide flashes cause “$H_2O_2$ flashes” in tiny confined areas in mitochondria.

In contrast, neither superoxide flashes nor evidence of $H_2O_2$ signaling in yeast have been reported. The different levels of product inhibition found in human and ScMnSOD may well be explained by the necessity for constrained generation of $H_2O_2$ in human mitochondria, especially under the conditions associated with “superoxide flashes”.

65
**Binding of a Sixth Ligand and the Proposed Mechanism of Yeast MnSODs.** Native ScMn$^{3+}$SOD and CaMn$^{3+}$SODc display optical absorption spectra that are different from other Mn$^{3+}$SODs. When the yeast enzymes were chemically oxidized, the spectra changed over time from characteristic 5-coordinate Mn$^{3+}$ spectra to spectra with additional absorption intensity around 390 nm. The growth of this absorption intensity can be ascribed to the binding of a sixth ligand, L, leading to formation of an L-Mn$^{3+}$SOD complex, and since its formation is favored at high pH the sixth ligand is highly likely to be a hydroxide. EPR spectra suggest the presence of at least two Mn$^{3+}$-containing species with distinct electronic ground states in yeast Mn$^{3+}$SODs. One of them is the well-characterized 5-coordinate Mn$^{3+}$SOD, and the other could be an L-Mn$^{3+}$SOD species with the sixth ligand bound in the equatorial plane.

A 6-coordinate MnSOD that binds two H$_2$O/OH$^-$ has been obtained in the past at cryogenic conditions or at high pH. The structure of cyro-trapped EcMnSOD crystallized at pH 8.5 shows a H$_2$O/OH$^-$ bound to the equatorial plane of Mn, giving an octahedral coordination center (60). Probing the Mn$^{2+}$ center of EcMnSOD using high-field EPR indicates transfer of a water molecule to the Mn center at 240–268 K (61). EcMn$^{3+}$SOD at pH 11.65 forms a putative 6-coordinate species, giving a heterogeneous EPR spectrum that is comparable to those reported here for ScMn$^{3+}$SOD and CaMn$^{3+}$SODc at pH 7.65 (31). Recently Porta et. al. have reported a 6-coordinate structure of EcMnSOD with peroxide bound as a side-on ligand to active site manganese (62). However, the existence of a 6-coordinate MnSOD binding two H$_2$O/OH$^-$ that is stable in solution at room temperature and neutral pH is novel.

Examination of the second coordination sphere of ScMnSOD shows two outer-sphere solvent molecules within 6 Å of the Mn center, in addition to the Mn-coordinated water. As discussed by Tabares et. al. (61), the sixth ligand is most likely the water that is hydrogen bonded
to Tyr34 and His30 (Figure 3.8). Tyr34 and His30 are highly conserved residues that are part of the proposed hydrogen-bonding network, which pumps protons from protein surface to active site (1,40,45,46). They are also involved in the gating between fast and slow protonation pathways as well as the dissociation of H$_2$O$_2$ from the active site (24,48). This water, however, only occupies 50% of the subunits in the structure of ScMnSOD or EcMnSOD and cannot be seen in the structure of human MnSOD or CaMnSODc.

The binding site of O$_2^-$ in the MnSOD active site has been investigated using small anions like fluoride and azide as O$_2^-$ analogues. Azide binds to the sixth ligand site in proximity of Try34 in the plane of His26, His81 and Asp168 at low temperatures (26). Fluoride also sits close to the same binding site and is likely hydrogen bonded to Tyr34 (63). Therefore, to react with the Mn ion O$_2^-$ is highly likely bound in the sixth ligand site in the vicinity to Tyr34. O$_2^-$ has also been proposed to form hydrogen bonds with Tyr34 and the Mn-coordinated H$_2$O but does not directly bind Mn (63). In both cases, the outer-sphere water, which has been proposed to become the sixth ligand, is located in the vicinity to the O$_2^-$ binding site.

**Scheme 3.3. Proposed Mechanism of the Reaction of Yeast Mn$^{2+}$SOD with O$_2^-$**

Herein we propose a mechanism based on the tendency of yeast Mn$^{3+}$SODs to bind two solvent molecules, which may explain their high efficiency at high O$_2^-$ doses (Scheme 3.3).
When $O_2^-$ binds to (or merely approaches) the sixth ligand binding site close to Tyr34, Mn$^{2+}$ transfers an electron to $O_2^-$ (which becomes peroxide) and is oxidized to Mn$^{3+}$. Since Mn$^{3+}$SOD tends to bind two solvent molecules, the water molecule that is hydrogen bonded to Tyr34 and His30 may travel to the Mn$^{3+}$ center to compete with the peroxide for the sixth ligand site (Scheme 3.3). This could cause steric effects at the metal binding site, which may destabilize the inner-sphere peroxo-Mn$^{3+}$SOD complex. This is consistent with the fact that ScMnSOD and CaMnSODc are significantly gated toward the outer-sphere fast protonation pathway, in which formation of the peroxo-Mn$^{3+}$SOD species is disfavored. The outer-sphere water may not necessarily bind Mn$^{3+}$ ion, which could be a slow process, but merely fluctuate between the Mn center and Tyr34/His30 to create steric effects at the active site. Deviations in the positioning of outer-sphere solvent molecules have been reported to be important in enhancing proton transfer in other metalloenzymes (64). In human carbonic anhydrase II (HACII), the positioning and structuring of water molecules in the active site cavity vary with the conformation of an outer-sphere residue, His64 (64).

The putative translocation of the outer-sphere solvent molecule in yeast MnSODs may also facilitate dissociation of the bound peroxide by enhancing proton transfer. Numerous previous studies show that MnSODs from various organisms exhibit a similar hydrogen isotope effect of ~2, suggesting that proton transfer is involved (65). Dissociation of H$_2$O$_2$ from MnSOD active site requires two protons transferred from the protein. Rapid proton transfer from the axial solvent ligand to peroxide is expected to occur in all MnSOD. In yeast MnSODs the other proton is likely donated by the outer-sphere H$_2$O that sits between Tyr34 and His30 upon translocation, which may highly enhance the proton transfer and facilitate the removal of the peroxide from the active site.
Indeed, the rate of peroxide release from active site \( (k_4) \) in \( ScMnSOD \) is dramatically depressed at increased pH with a \( pK \) of \(~8\), which causes the level of product inhibition to increase (Figure 3.4). This is significantly different from human MnSOD, which maintain a constant \( k_4 \) over a wide range of pH (6–10) (65). The depression of \( k_4 \), which is likely caused by slower proton transfer, could either be resulted from deprotonation of the axial \( H_2O \) or the outer-sphere \( H_2O \) that is hydrogen bonded to Tyr34 and His30, or from deprotonation of Tyr34 as proposed to be responsible for the spectroscopic \( pK \) of \( EcMnSOD \) (30). In the latter scenario, deprotonation of Tyr34 may destabilize the potential sixth ligand by eliminating its hydrogen bonding with Tyr34. Considering that the \( pK \) of \( k_4 \) for \( ScMnSOD \) is as low as 8 (Figure 3.4) while the \( pK \) of Tyr34 is 11.5, deprotonation of the solvent ligands is more likely. This may support the hypothesis that the outer-sphere solvent molecule approaches the sixth ligand site of the \( Mn^{3+} \) ion and transfers a proton to the peroxide to facilitate its leaving.

However, the binding of two \( H_2O/OH^- \) in \( ScMn^{3+}SOD \) and \( CaMn^{3+}SODc \) may not be relevant to their fast catalytic kinetics and low level of product inhibition, but merely a coincidence. We will therefore continue investigating the correlation between product inhibition and the L-Mn\(^{3+}\)SOD species by investigating yeast MnSOD mutants with mutations at certain second coordination sphere residues.

In conclusion, MnSODs from \( S. cerevisiae \) mitochondria and \( C. albicans \) cytosol both exhibit higher efficiencies of \( O_2^- \) dismutation at high [\( O_2^- \)] than human and bacterial MnSODs. The unusual resting state of yeast MnSODs could be attributed to the slightly longer distance between Gln154 and Mn. Both yeast Mn\(^{3+}\)SODs form a 6-coordinate species. The potential sixth ligand could be an outer-sphere water and its translocation to Mn ion in the catalytic cycle may facilitate the reaction of Mn\(^{2+}\)SOD with \( O_2^- \) to undergo through the non-inhibited pathway.
Among MnSODs that have been characterized to date, human MnSOD is the only enzyme that significantly undergoes the product-inhibited pathway at high $[O_2^-]$, which can be ascribed to complicated roles of $H_2O_2$ in mammalian cells, especially as a signaling agent.
**Figure S3.1.** Electrospray-ionization mass spectra (top panel) of methylated ScMnSOD and its reconstructed mass distribution profiles (bottom panel). Ordinate units of intensity are arbitrary and the abscissa units of average molecular mass are in Daltons. The expected mass is 23,560 Da.

**Figure S3.2.** The SDS-PAGE analysis of the purification of CaMnSODc: 1) purified ScMnSOD; 2) cell lysate; 3) supernatant of ammonium sulfate cut; 4) active HIC fractions; 5) DE52 load and wash; 6) 5 μg of purified CaMnSODc after G300 column.
Figure S3.3. Oxidation of CaMn$^{2+}$SODc by O$_2^-$ in pulse radiolysis. The formation of Mn$^{3+}$SOD is indicated by the change of absorbance at 480 nm over time upon generation of 2.2 μM O$_2^-$. The sample contained 60 μM (in Mn) CaMnSODc, 10 mM potassium phosphate (pH 7), 10 mM sodium formate, and 100 μM EDTA. The enzyme was reduced prior to each pulse with 120 μM H$_2$O$_2$.

Figure S3.4. Decay of 48 μM O$_2^-$ catalyzed by 1 μM (in Mn) CaMnSODc (a, black) and ScCuZnSOD (b, red). The sample of pulse radiolysis contained 10 mM potassium phosphate (pH 7), 10 mM sodium formate and 10 μM EDTA.
Figure S3.5. Dependence of the level of product inhibition of CaMnSODc on pH. Decay of 25 μM $\text{O}_2^-$ catalyzed by 1 μM (in Mn) CaMnSODc at pH 7.5 (a) and 9.0 (b). The sample of pulse radiolysis contained 10 mM potassium phosphate (pH 7), 10 mM sodium formate and 10 μM EDTA.
Figure S3.6. The oxidation state of CaMnSODc. (A) Optical spectra of as-isolated CaMnSODc (solid line) and CaMnSODc reduced by sodium hydrosulfite (dashed line). Inset: The difference spectrum between as-isolated and reduced CaMnSODc. (B) Optical spectra of CaMnSODc oxidized by potassium permanganate ([KMnO₄]:MnSOD = 0.75:1) as measured over time. (C) Reduction of CaMn³⁺SODc (solid line, oxidized by 0.75 equivalent of KMnO₄ and allowed to equilibrate at room temperature for 2 hr) by one equivalent of sodium ascorbate (dotted line). (D) Optical spectra of CaMn³⁺SODc at different pH. The sample solution contained 160 μM (in Mn) enzyme in 25 mM potassium phosphate (pH 7.4).
Figure S3.7. The absorption band of ScMn$^{3+}$SOD around 390 nm as measured over time. ScMnSOD oxidized by potassium permanganate ([KMnO$_4$]:MnSOD = 0.75:1) at pH 7.4 as measured over time. Time 0 refers to the start of the first scan immediately after mixing KMnO$_4$ with the enzyme.

Figure S3.8. Anion binding causes spectral shifts of ScMn$^{3+}$SOD. Solutions contained 190 μM (in Mn) ScMnSOD oxidized by 0.75 equivalent of KMnO$_4$ (thick solid, allowed to equilibrate at room temperature for 2 hr) in 25 mM potassium phosphate (pH 7.4) with 100 mM NaF (thin solid) and 100 mM NaN$_3$ (dashed).
**Figure S3.9.** Oxidation of as-isolated ScMnSOD by $O_2^-$ at long timescales. $O_2^-$ was generated by $^{60}$Co radiation source. The legend shows the doses of $O_2^-$ that reacted with ScMnSOD before spectra were taken. The sample solution contained 130 μM (in Mn) ScMnSOD in 25 mM potassium phosphate (pH 7.4) and 200 units/mL catalase.

**Figure S3.10.** The Mn$^{3+}$ spectra of yeast MnSODs at different pH as determined by pulse radiolysis. Protein samples are: 1) 60 μM CaMnSODc at pH 7.5 (squares); 2) 40 μM ScMnSOD at pH 7.0 (open circles); 3) 40 μM ScMnSOD at pH 10.0 (closed circles).
Figure S3.11. These spectra are similar to those obtained for *EcMn*²⁺SOD. Five separate electron spin transitions are possible and these are shifted from $g = 2.0$ due to zero-field splitting of the manifold of spin levels of the high-spin ($S = 5/2$) Mn²⁺ ion. In some cases, these resonances are further split (e.g., the feature at $g = 5.8$) by the hyperfine interaction of the electron spin with the $^{55}$Mn nuclear spin ($I = 5/2$).
Figure S3.12. (Top panel) Temperature dependence of the parallel-mode EPR spectrum of permanganate-oxidized CaMnSODc. Except for temperature (given in legend), the spectrometer settings are the same as those given in the caption of Figure 3.7. Simulation of the $^5A_{1g}$ component is shown (thin blue line). (Bottom panel) Integrated intensity of feature centered at 58 mT (blue circles) as a function of the inverse of the temperature and corresponding simulated integrated intensity (green crosses) achieved using ZFS parameters $D = +1.90 \text{ cm}^{-1}$ and $E = 0.20 \text{ cm}^{-1}$.
**Figure S3.13.** The quaternary structure of ScMnSOD (A) and CaMnSODc (B) in crystal lattice. Crystallization condition for CaMnSODc: 0.1 M magnesium chloride, 0.1 M sodium chloride and 0.1 M tri-sodium citrate (pH 5.5) in 30% (w/v) polyethylene glycol 400 at 4 °C with a protein concentration of 7 mg/mL.

**Figure S3.14.** Superposition of the active site of ScMnSOD (green, chain A, PDB accession: 3LSU) and CaMnSODc (blue, PDB accession: 3QVN).
Table S3.1. X-ray Data Collection and Refinement Statistics $^a$.

<table>
<thead>
<tr>
<th></th>
<th>ScMnSOD</th>
<th>CaMnSODc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data Collection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-Ray Source</td>
<td>Rigaku FRE+</td>
<td>Rigaku FRE+</td>
</tr>
<tr>
<td>Detector</td>
<td>Rigaku HTC</td>
<td>Rigaku HTC</td>
</tr>
<tr>
<td><strong>Reflections observed</strong></td>
<td>197227</td>
<td>252317</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>59997</td>
<td>6933</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.5418</td>
<td>1.5418</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>1.90</td>
<td>2.60</td>
</tr>
<tr>
<td>Highest Resolution Shell (Å)</td>
<td>1.97-1.90</td>
<td>2.69-2.60</td>
</tr>
<tr>
<td><strong>Space group</strong></td>
<td>P1</td>
<td>P 6,22</td>
</tr>
<tr>
<td><strong>R$_{sym}$ (%)</strong> $^b$</td>
<td>8.3 (31.4)</td>
<td>17.9 (74.3)</td>
</tr>
<tr>
<td><strong>I/σ</strong></td>
<td>14.8 (3.7)</td>
<td>22.9 (8.1)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>89.5 (53.1)</td>
<td>99.0 (100.0)</td>
</tr>
<tr>
<td><strong>Unit cell dimensions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a (Å)</td>
<td>63.657</td>
<td>77.134</td>
</tr>
<tr>
<td>b (Å)</td>
<td>64.933</td>
<td>77.134</td>
</tr>
<tr>
<td>c (Å)</td>
<td>66.550</td>
<td>120.080</td>
</tr>
<tr>
<td>$α$ (°)</td>
<td>109.36</td>
<td>90.0</td>
</tr>
<tr>
<td>$β$ (°)</td>
<td>106.33</td>
<td>90.0</td>
</tr>
<tr>
<td>$γ$ (°)</td>
<td>109.68</td>
<td>120.0</td>
</tr>
<tr>
<td>Wilson B value (Å$^2$)</td>
<td>24.3</td>
<td>52.8</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>32.0 – 1.9</td>
<td>44.6 – 2.6</td>
</tr>
<tr>
<td>Reflections Used</td>
<td>59979</td>
<td>6626</td>
</tr>
<tr>
<td>$R_{work}$(%)</td>
<td>16.30 (16.50)</td>
<td>20.25 (27.42)</td>
</tr>
<tr>
<td>$R_{free}$ (%) $^c$</td>
<td>19.50 (19.20)</td>
<td>26.86 (40.48)</td>
</tr>
<tr>
<td>Protein Molecules in Asymmetric Unit</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td><strong>Number of non-H atoms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>6661</td>
<td>1593</td>
</tr>
<tr>
<td>Non-protein</td>
<td>450</td>
<td>23</td>
</tr>
<tr>
<td><strong>RMS deviations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.007</td>
<td>0.002</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.045</td>
<td>0.517</td>
</tr>
<tr>
<td><strong>Average B-factor (Å$^2$)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein atoms</td>
<td>24.52</td>
<td>52.85</td>
</tr>
<tr>
<td>Non-protein atoms</td>
<td>29.50</td>
<td>40.75</td>
</tr>
<tr>
<td>PDB accession code</td>
<td>3LSU</td>
<td>3QVN</td>
</tr>
</tbody>
</table>

$a.$ Highest resolution shell shown in parenthesis.

$b.$ $R_{sym} = \Sigma |I-I<|/\Sigma I$

$c.$ $R_{free}$ calculated using 5% of the data
Table S3.2. Active Site Crystallographic Distances

<table>
<thead>
<tr>
<th>Ligands</th>
<th>ScMnSOD (pdb: 3lsu)</th>
<th>CaMnSODc (pdb: 3qvn)</th>
<th>Human (pdb: 1n0j)</th>
<th>A. fumigatus (pdb: 1kkc)</th>
<th>C. elegans (pdb: 3dc5)</th>
<th>E. coli (pdb: 1vew)</th>
<th>T. Thermophilus (pdb: 3mds)</th>
<th>Drad (pdb: 2ce4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn-NE2 (His26)*</td>
<td>2.24 (0.05)</td>
<td>2.35</td>
<td>2.10 (0.01)</td>
<td>2.11 (0.09)</td>
<td>2.23 (0.01)</td>
<td>2.14 (0.04)</td>
<td>2.14 (0)</td>
<td>2.19 (0.05)</td>
</tr>
<tr>
<td>Mn-NE2 (His81)</td>
<td>2.22 (0.02)</td>
<td>2.27</td>
<td>2.10 (0.02)</td>
<td>2.19 (0.06)</td>
<td>2.19 (0.01)</td>
<td>2.21 (0.01)</td>
<td>2.10 (0.02)</td>
<td>2.10 (0.09)</td>
</tr>
<tr>
<td>Mn-NE2 (His172)</td>
<td>2.22 (0.02)</td>
<td>2.26</td>
<td>2.09 (0)</td>
<td>2.20 (0.08)</td>
<td>2.18 (0.07)</td>
<td>2.17 (0.01)</td>
<td>2.17 (0.02)</td>
<td>2.16 (0.08)</td>
</tr>
<tr>
<td>Mn-OD2 (Asp168)</td>
<td>2.05 (0.02)</td>
<td>2.11</td>
<td>1.94 (0)</td>
<td>1.99 (0.04)</td>
<td>2.06 (0.01)</td>
<td>2.02 (0.01)</td>
<td>1.79 (0.01)</td>
<td>1.93 (0.04)</td>
</tr>
<tr>
<td>Mn-O (coord solvent)</td>
<td>2.31 (0.04)</td>
<td>2.33</td>
<td>2.02 (0.01)</td>
<td>2.27 (0.06)</td>
<td>2.26 (0.01)</td>
<td>2.21 (0.05)</td>
<td>2.08 (0.01)</td>
<td>2.19 (0.01)</td>
</tr>
<tr>
<td>Coordinating Solvent Molecule</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-OD2 (Asp168)</td>
<td>2.89 (0.03)</td>
<td>3.08</td>
<td>2.95 (0.06)</td>
<td>2.89 (0.11)</td>
<td>2.78 (0)</td>
<td>2.78 (0.04)</td>
<td>2.78 (0.06)</td>
<td>2.83 (0.01)</td>
</tr>
<tr>
<td>O-ND (Gln154)</td>
<td>2.95 (0.02)</td>
<td>2.92</td>
<td>2.95 (0.01)</td>
<td>2.78 (0.13)</td>
<td>2.86 (0.01)</td>
<td>2.90 (0.08)</td>
<td>2.99 (0.03)</td>
<td>2.87 (0.06)</td>
</tr>
</tbody>
</table>

a. Numbering in ScMnSOD is use


84


Chapter 4

Six-coordinate Manganese(3+)

in Catalysis by Yeast Manganese Superoxide Dismutase

(This chapter was submitted to Proceedings of the National Academy of Sciences)
Introduction

Superoxide dismutase (SOD) with manganese at its active site (MnSOD) is remarkable in that it becomes less efficient when levels of its substrate \( \text{O}_2^- \) are high, while other subclasses of SOD catalyze the disproportionation of \( \text{O}_2^- \) at virtually diffusion-controlled rates independent of \( \text{O}_2^- \) level (1). The degree of depression of MnSOD activity at high \([\text{O}_2^-]\) is most prominent in human MnSOD, while the MnSODs from bacteria (Escherichia coli (2) and Deinococcus radiodurans (3)) and yeast (Saccharomyces cerevisiae (4,5) and Candida albicans (5)) are relatively more efficient under the same conditions. This unique property of human MnSOD could result from the need for stringent regulation of \( \text{H}_2\text{O}_2 \) production, due to the complicated roles of \( \text{H}_2\text{O}_2 \) in mammalian cells, especially as a signaling agent (5).

Scheme 4.1. Catalytic mechanism of MnSOD

\[
\begin{align*}
\text{Mn}^{3+}\text{SOD} + \text{O}_2 & \overset{k_1}{\rightarrow} \text{Mn}^{2+}\text{SOD} + \text{O}_2 \quad (1) \\
\text{Mn}^{2+}\text{SOD} + \text{O}_2 & \overset{k_2,2\text{H}^+}{\rightarrow} \text{Mn}^{3+}\text{SOD} + \text{H}_2\text{O}_2 \quad (2) \\
\text{Mn}^{2+}\text{SOD} + \text{O}_2 & \overset{k_3}{\rightarrow} \text{Mn}^{3+}\text{(OO}^{2-}\text{)}\text{SOD} \quad (3) \\
\text{Mn}^{3+}\text{(OO}^{2-}\text{)}\text{SOD} & \overset{k_4,2\text{H}^+}{\rightarrow} \text{Mn}^{3+}\text{SOD} + \text{H}_2\text{O}_2 \quad (4)
\end{align*}
\]

The mechanism by which MnSOD removes \( \text{O}_2^- \) involves product inhibition (1). Specifically, reduction of \( \text{O}_2^- \) by \( \text{Mn}^{2+}\text{SOD} \) (Scheme 4.1) occurs either through a pathway (called “prompt protonation” pathway here) (reaction 2), where protonation and dissociation of the peroxo moiety is instantaneous, or through an “inner-sphere” pathway (reaction 3), where a detectable intermediate is formed, which has been suggested to be a side-on Mn(III)-peroxo species (6). (We here refer to this intermediate as a Mn-peroxo complex instead of as a “product-
inhibited” complex, the latter term having been used frequently in previous reports.) When protonation of the Mn-peroxo complex (reaction 4) is rate limiting, the “inner-sphere” pathway has the effect of slowing down MnSOD catalysis.

Recently we have been investigating MnSODs from single-cellular eukaryotes in order to understand the mechanism of product inhibition. Our recent study showed that the two yeast MnSODs, one from *S. cerevisiae* mitochondria (*ScMnSOD*) and the other from *C. albicans* cytosol (*CaMnSODc*), are both very efficient at high levels of O$_2^–$, relative to other MnSODs that have been characterized to date (4,5). Spectroscopic analysis of the oxidized yeast enzymes suggests the presence of a six-coordinate Mn$^{3+}$ species, in addition to the well-characterized five-coordinate Mn$^{3+}$ (5). The sixth ligand is likely a hydroxide. Based on these findings, we earlier proposed, for the fast catalysis by yeast MnSODs, a mechanism that involves putative translocation of an outer-sphere solvent molecule (5). More recently we have been pursuing this mechanistic investigation by focusing on mutant yeast MnSOD with substitutions in the second coordination sphere.

Tyr34 is highly conserved and structurally similar in all known MnSODs, where it participates in a hydrogen-bonding network at the active site. Computational studies suggest that Tyr34 provides one of the two protons needed to form H$_2$O$_2$ in the catalytic cycle (7). Deprotonation of Tyr34 is responsible for the spectroscopic changes, including the blue shift and decrease in intensity of the optical absorption maximum, that occurs for Mn$^{3+}$SOD with increasing pH (8). Wild-type human MnSOD and its Y34F mutant protein have similar $k_{cat}/K_m$, but $k_{cat}$ is decreased by ~10 fold in the mutant protein (9). SOD activity, measured by the xanthine oxidase/cytochrome c assay, is only slightly reduced in *E. coli* Y34 mutant proteins.
Tyr34 also plays an interesting role in the “prompt protonation” pathway, as elucidated by the changes in the kinetics of Tyr34 mutant human MnSOD (12).

To test our previously proposed mechanism and to address questions regarding the role of Tyr34 in yeast MnSOD, we purified and characterized Y34F mutant ScMnSOD. To our surprise, we have found that catalysis by Y34F ScMnSOD is completely “inner-sphere”. More interestingly, our results reveal that Y34F ScMnSOD has a novel mechanism involving an active six-coordinate Mn$^{3+}$ species and gating between multiple proton transfer pathways, different from any previously characterized wild-type or mutant enzyme.

**Materials and Methods**

*DNA Manipulation of Y34F ScMnSOD and Transformation into S. cerevisiae.* Site-directed mutagenesis was carried out on an overexpression vector (YEp352-ScMnSOD) containing the URA3 selectable marker and a 2-kb genomic BamHI fragment containing the gene for ScMnSOD. The primers 5’-GCACCATCAAAACTTTTGTAACGGATTCAACAC-3’ and 5’-TGAATCCGTTC-AACAAGGTGGATGCTTGGG-3’ were used to create the wild-type cDNA. The PCR products were transformed into *E. coli* DH5α strain and screened by ampicillin selection. The purified vector was transformed into *S. cerevisiae* sod2Δ strain (EG110; *Mat*-alpha, *ura3*, *leu2*, *trp1*, *his3*, sod2::*TRP1*).

*Expression and Purification of Y34F ScMnSOD.* Y34F ScMnSOD was expressed and purified as described (5).

*Pulse Radiolysis.* The experiments were carried out using the 2 MeV Van de Graaff accelerator at Brookhaven National Laboratory. O$_2^-$ is generated in air-saturated aqueous solution
containing sodium formate through the following reactions: \( \cdot \text{OH} + \text{HCO}_2^- \rightarrow \text{H}_2\text{O} + \text{CO}_2^- \), \( \text{O}_2 + \text{CO}_2^- \rightarrow \text{O}_2^- + \text{CO}_2 \), \( \text{e}_{\text{aq}}^- + \text{O}_2 \rightarrow \text{O}_2^\cdot \), \( \text{H}^+ \text{O}_2 \rightarrow \text{HO}_2^\cdot \).

The experiments to measure catalytic rates were carried out using two methodologies. One set of studies involved either oxidizing the resting enzyme or reducing the oxidized enzyme with substoichiometric quantities of \( \text{O}_2^- \) and following the appearance or disappearance of \( \text{Mn}^{3+}\text{SOD} \), respectively, via following absorbance change in the visible range (370-550 nm). The other method involved following the decay of various \([\text{O}_2^-]\) at 260 nm using a 1:1 to 1:50 ratio of \([\text{MnSOD}] : [\text{O}_2^-]\). Here, rate constants were calculated by fitting the data obtained to a mechanism described in Scheme 4.1 using the Chemical Kinetics program in PRWIN (13).

All MnSOD concentrations were taken as the ICP-measured concentration of manganese in the sample. The pH of the buffer was adjusted using ultrapure (Baker Ultrex) sodium hydroxide and sulphuric acid.

**Crystallization of Y34F ScMnSOD.** Fully metallated Y34F ScMnSOD was obtained as described before (5). The reductive methylation of lysine residues of Y34F ScMnSOD was carried out as described, in order to improve the diffraction of protein crystals (14). The activity of native and methylated Y34F ScMnSOD is similar in SOD activity assay (15). All free amino groups of the lysine residues and the N-terminal were methylated as confirmed by mass spectrometry (Figure S1).

Methylated Y34F ScMnSOD was crystallized by hanging-drop vapor diffusion at 4 °C against a well solution of 0.2 M sodium malonate (pH 7) in 20% (w/v) polyethylene glycol 3,350 with a protein concentration of 7 mg/mL. Y34F ScMnSOD crystals were cryo-protected in mother liquor solution containing 30% glycerol and flash frozen in liquid nitrogen prior to data collection.
Crystallography: Data Collection and Refinement. All data was collected at 100K at the Synchrotron facility, using a APS BEAMLINE 24-ID-C generator and a ADSC QUANTUM 315 detector. All data was processed using DENZO and SCALEPACK (16). Y34F ScMnSOD was phased by molecular replacement using wild-type ScMnSOD (PDB code: 3LSU). All the molecular replacement was done using PHASER (17). The models were built using COOT (18). All model refinement was done using REFMAC (19) and PHENIX (20). Coordinates and structure factors have been deposited in the PDB with accession number 4E4E for the Y34F ScMnSOD structure.

Results

An Additional Water Sits in the Active Site of Y34F ScMnSOD. To aid in our investigation of the role of Tyr34, we determined the X-ray structure of Y34F ScMnSOD (Table 4.1). The subunit structure and the tetrameric assembly of wild-type and the mutant ScMnSOD closely resemble each other (Fig. S4.2). Superimposition of all backbone atoms of the mutant tetramer onto those of the wild-type protein gives a root-mean-square deviation (RMSD) of 0.22 Å. Y34F ScMnSOD maintains the strictly-conserved five-coordinate Mn at active site, and the substitution of Tyr34 causes only minimal changes to positioning and conformation of active-site residues (Fig. 4.1A). The Chi(1) and Chi(2) angles of Phe34 in Y34F ScMnSOD shift by 0.7 ° and 2.7 °, respectively, compared to those of Tyr34 in the wild-type enzyme.

An additional water molecule occupies the cavity created by the replacement of Tyr34 with phenylalanine in all four subunits in the unit cell of Y34F ScMnSOD. This water has substituted for the –OH of Tyr34 in the wild-type enzyme by forming hydrogen bonds to Gln154 and the water molecule hydrogen bonded to His30 (Fig. 4.1A). Its presence at that location
Table 4.1. X-ray data collection and refinement statistics a.

<table>
<thead>
<tr>
<th>Data Collection</th>
<th>Y34F SeMnSOD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>X-Ray Source</strong></td>
<td>APS BEAMLINE 24-ID-C</td>
</tr>
<tr>
<td><strong>Detector</strong></td>
<td>ADSC QUANTUM 315</td>
</tr>
<tr>
<td>Reflections observed</td>
<td>169216</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>59948</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.9641</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>1.88</td>
</tr>
<tr>
<td>Highest Resolution Shell (Å)</td>
<td>1.91–1.88</td>
</tr>
<tr>
<td>Space group</td>
<td>P1</td>
</tr>
<tr>
<td>$R_{sym}$ (%)</td>
<td>12.2 (54.7)</td>
</tr>
<tr>
<td>$I/\sigma$</td>
<td>12.1 (1.9)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>94.6 (54.0)</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td></td>
</tr>
<tr>
<td>a (Å)</td>
<td>61.139</td>
</tr>
<tr>
<td>b (Å)</td>
<td>63.022</td>
</tr>
<tr>
<td>c (Å)</td>
<td>63.943</td>
</tr>
<tr>
<td>α (°)</td>
<td>112.19</td>
</tr>
<tr>
<td>β (°)</td>
<td>106.78</td>
</tr>
<tr>
<td>γ (°)</td>
<td>103.88</td>
</tr>
<tr>
<td>Wilson B value (Å$^2$)</td>
<td>22.5</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>32.2 – 1.88</td>
</tr>
<tr>
<td>Reflections Used</td>
<td>59832</td>
</tr>
<tr>
<td>$R_{work}$ (%)</td>
<td>18.98</td>
</tr>
<tr>
<td>$R_{free}$ (%) c</td>
<td>23.13</td>
</tr>
<tr>
<td>Protein Molecules in Asymmetric Unit</td>
<td>4</td>
</tr>
<tr>
<td>Number of non-H atoms</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>6658</td>
</tr>
<tr>
<td>Non-protein</td>
<td>231</td>
</tr>
<tr>
<td>RMS deviations</td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.009</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.149</td>
</tr>
<tr>
<td>Average B-factor (Å$^2$)</td>
<td></td>
</tr>
<tr>
<td>Protein atoms</td>
<td>28.39</td>
</tr>
<tr>
<td>Non-protein atoms</td>
<td>29.51</td>
</tr>
<tr>
<td>PDB accession code</td>
<td>4E4E</td>
</tr>
</tbody>
</table>

a. Highest resolution shell shown in parenthesis.
b. $R_{sym} = \Sigma |I-I\langle I\rangle|/\Sigma I$
c. $R_{free}$ calculated using 5% of the data
**Figure 4.1.** Active site structure and oxidation state of Y34F ScMnSOD. (A) Superimposition of the active site of Y34F ScMnSOD (chain A, red) onto that of wild-type ScMnSOD (chain A, green). Coordination bonds are indicated as solid lines, and hydrogen bonds are shown as dashed lines in wild-type (black) and Y34F (gray) ScMnSOD, respectively. The graphic was generated using the PyMOL Molecular Graphics System. (B) (a) & (b) Optical spectra of as-isolated (solid line) and reduced (dotted line, by sodium hydrosulfite) Y34F ScMnSOD. Inset: The difference spectrum between as-isolated and reduced Y34F ScMnSOD. (c) Optical spectra of Y34F ScMnSOD oxidized by potassium permanganate ([KMnO₄]:[MnSOD] = 0.75:1) at pH 7.4 as measured over time. The spectra were recorded after mixing KMnO₄ with the enzyme for 0, 10, 20, 30, 45 and 60 min. (d) Reduction (dashed line) of Y34F ScMn³⁺SOD (gave the last spectrum in c) by one equivalent of sodium ascorbate. The sample solutions contained 200 μM (in Mn) enzyme in 25 mM potassium phosphate (pH 7.4).
supports the importance of the hydrogen-bonding chain involving Tyr34 (Gln154···Tyr34···H2O···His30) in the wild-type enzyme (21). This additional water molecule is also present in Y34 mutant (Y34A, Y34N, Y34H, and Y34V) human MnSOD (12). However, it was not observed in the crystal structure of human Y34F MnSOD, which had been carried out at a resolution similar to that of our study (1.9 Å) (9).

**Like the Wild-type Enzyme, Y34F ScMn$^{3+}$SOD Contains both Five- and Six-coordinate Mn$^{3+}$ Centers.** Wild-type MnSODs are usually isolated in the oxidized state, Mn$^{3+}$SOD (3,22,23). Yeast MnSODs appear to be an exception, since we showed previously that wild-type yeast MnSODs from *S. cerevisiae* and *C. albicans* rest predominantly in the reduced state, Mn$^{2+}$SOD (4,5). This preference to be reduced is unchanged in Y34F ScMnSOD (Fig. 4.1B-a). Also similar to wild-type ScMnSOD is the observation of a small amount of Mn$^{3+}$SOD in the as-isolated Y34F ScMnSOD, which absorbs below 400 nm in addition to the 480 nm band (Fig. 4.1B, inset).

As in the wild-type enzyme, the spectrum of Y34F ScMnSOD immediately after oxidation is characteristic of five-coordinate Mn$^{3+}$SOD (Fig. 4.1B-c). Additional absorption intensity appeared at lower wavelengths (<400 nm) over time (Fig. 4.1B-c), which was proposed to result from formation of a six-coordinate Mn$^{3+}$SOD, with hydroxide as the sixth ligand (5). The intensity is blueshifted from ∼390 nm in wild-type ScMn$^{3+}$SOD (5) to ∼370 nm in Y34F ScMn$^{3+}$SOD (Fig. S4.3). The time to reach the final spectrum was shorter in the Y34F mutant protein (∼45 min) (Fig. 4.1B-c) than in wild-type ScMnSOD (∼2 h) (5), indicating faster formation of the putative six-coordinate adduct in Y34F ScMn$^{3+}$SOD.

Y34F ScMn$^{3+}$SOD also resembles wild-type ScMn$^{3+}$SOD in that the six-coordinate adduct is more prone to reduction by ascorbate than the five-coordinate species (5), as revealed
by the fact that 1.0 equivalent of ascorbate selectively bleached the absorption of Y34F ScMn$^{3+}$SOD around 370 nm (Fig. 4.1B-d). This result suggests that the six-coordinate Mn$^{3+}$ has a higher reduction potential than the five-coordinate Mn$^{3+}$. Six-coordinate Mn$^{3+}$SOD, with a high-spin $d^4$ center, displays strong Jahn-Teller distortion, which favors the Mn$^{2+}$ over Mn$^{3+}$ state. The slow formation of the six-coordinate adduct and its high reduction potential in both wild-type and the mutant yeast MnSOD are still not understood in detail, and we will continue exploring their origins in future.

**Y34F ScMnSOD is Gated Exclusively toward “Inner-sphere” Pathway, yet still Displays Fast Catalysis.** To examine the impact of the Tyr34 on catalysis by ScMnSOD, we carried out pulse radiolysis studies on Y34F ScMnSOD. The disappearance of various concentrations (2-50 μM) of O$_2^-$ in solutions containing 1–5 μM Y34F ScMnSOD was followed at 260 nm, and the observed traces were compared to previous data collected under identical conditions for wild-type MnSOD from *S. cerevisiae*, *C. albicans*, human, *E. coli*, and *D. radiodurans* (Fig. 4.2). When [O$_2^-$] was in seven-fold excess over [MnSOD], a relatively uninhibiting condition, human MnSOD, the most product-inhibited wild-type MnSOD known, already showed a depressed activity, while the O$_2^-$ disappearance as a function of time was similar for each of the other four wild-type enzymes (Fig. 4.2A). Notably, substitution of Tyr34 caused ScMnSOD to remove O$_2^-$ more slowly under this condition than all of the wild-type enzymes, including wild-type human MnSOD (Fig. 4.2A).

We then increased the [O$_2^-$]:[MnSOD] ratio to 41, a condition where MnSOD from different organisms displays dramatically different efficiencies, due to different degrees of product inhibition (*3*). The fast decay of O$_2^-$ within a few milliseconds after its generation is the
Figure 4.2. Decay of 7 μM (A), and 41 μM (B) O$_2^-$ catalyzed by 1 μM (in Mn) human (a, black), *E. coli* (b, red), *D. radiodurans* (c, blue) MnSOD, ScMnSOD (d, green), CaMnSODc (e, pink) and Y34F ScMnSOD (f, gray). The samples contained 10 mM potassium phosphate (pH 7), 10 mM sodium formate and 10 μM EDTA. The O$_2^-$ concentrations in these figures are calculated from the absorbance at 260 nm.

Figure 4.3. Mn$^{3+}$ species observed from oxidation of Y34F ScMn$^{2+}$SODc by O$_2^-$ in pulse radiolysis. (A) The absorbance change at 420 nm within 2 (left) and 200 (right) ms after pulse radiolysis generated initial O$_2^-$ burst of 2.2 μM. Experimental data are shown in dots and fitted curves are shown in solid lines. (B) The spectra of the Mn$^{3+}$ species (Int$_1$: hollow circles; Int$_2$: open triangles; Int$_3$: solid circles). The sample solutions contained 60 μM (in Mn) Y34F ScMnSOD in 10 mM potassium phosphate (pH 7.4), 10 mM sodium formate, 120 μM H$_2$O$_2$, and 100 μM EDTA.
indicator of the “prompt protonation” pathway. Therefore, the absence of this fast decay in Y34F ScMnSOD (Fig. 4.2B) indicates that the mutant protein is gated predominantly toward the “inner-sphere” pathway. Nevertheless, to our surprise, Y34F ScMnSOD catalyzed the disappearance of $O_2^-$ more efficiently than human MnSOD (Fig. 4.2B), even though the former is gated exclusively toward the “inner-sphere” pathway ($k_2/k_3 < 0.01$, see below). The zero-order phase, characteristic of the presence of the Mn-peroxo complex, is controlled by the gating ratio $k_2/k_3$ and the dissociation rate of the bound peroxide ($k_4$). The fact that the zero-order phase was not as pronounced in Y34F ScMnSOD as in wild-type *E. coli* or human MnSOD (Fig. 4.2B) suggests faster peroxide dissociation steps in Y34F ScMnSOD (see below).

**Scheme 4.2. Reduction of $O_2^-$ by Y34F ScMn$^{2+}$SOD**

To measure individual rate constants, 60 μM of Y34F ScMn$^{2+}$SOD was pulse irradiated to react with substoichiometric amounts (2-3 μM) of $O_2^-$, and the appearance of Mn$^{3+}$SOD was followed (Materials and Methods). The absorbance measured at 420 nm within 2 ms after generation of $O_2^-$ could be divided into two phases, a rapid emergence of absorption within 0.1 ms followed by a relatively slow increase that lasted for ~15 ms (Fig. 4.3A, left). Similar biphasic increases at 420 nm absorption have also been observed for several Y34 mutant human MnSOD (12). The 420 nm absorption of Y34F ScMnSOD slowly decreased at a longer time scale (Fig. 4.3A, right), leading to formation of a Mn$^{3+}$ species (Int$_3$) with an absorption
Table 4.2. Rate constants for wild-type MnSODs from *S. cerevisiae* and human and their Y34 mutant proteins

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_1$ (nM$^{-1}$s$^{-1}$)</th>
<th>$k_2$ (nM$^{-1}$s$^{-1}$)</th>
<th>$k_3$ (nM$^{-1}$s$^{-1}$)</th>
<th>$k_3^*$ (s$^{-1}$)</th>
<th>$k_4$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type ScMnSOD (4)</td>
<td>1.1-1.5</td>
<td>0.8</td>
<td>0.04–0.05</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Y34F ScMnSOD (this work)</td>
<td>*</td>
<td>&lt;0.01</td>
<td>0.70</td>
<td>310</td>
<td>20</td>
</tr>
<tr>
<td>wild-type human MnSOD (3)</td>
<td>1.5</td>
<td>0.6</td>
<td>0.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Y34F human MnSOD (12)</td>
<td>0.55</td>
<td>&lt;0.02</td>
<td>0.46</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Y34A human MnSOD (12)</td>
<td>0.25</td>
<td>&lt;0.02</td>
<td>0.38</td>
<td>1600</td>
<td>330</td>
</tr>
<tr>
<td>Y34N human MnSOD (12)</td>
<td>0.14</td>
<td>&lt;0.02</td>
<td>0.15</td>
<td>850</td>
<td>200</td>
</tr>
<tr>
<td>Y34H human MnSOD (12)</td>
<td>0.07</td>
<td>&lt;0.02</td>
<td>0.04</td>
<td>250</td>
<td>61</td>
</tr>
<tr>
<td>Y34V human MnSOD (12)</td>
<td>0.15</td>
<td>&lt;0.02</td>
<td>0.15</td>
<td>–</td>
<td>1000</td>
</tr>
</tbody>
</table>

* $k_1$ was not determined, because the reduction of ScMn$^{3+}$SOD by O$_2$ is not stoichiometric in a long timescale.
maximum at ~480 nm (Fig. 4.3B), consistent with the well-characterized five-coordinate Mn$^{3+}$SOD (2,3).

We attribute the rapid and slow absorption increase in Fig. 4.3A (left) to emergence of Intermediate 1 (Int$_1$) and 2 (Int$_2$), respectively (Scheme 4.2). The data were fitted into a two-exponential model, and the rate constants and the molar extinction coefficients were calculated. The resulting spectrum of Int$_1$, which has an absorption maximum at ~415 nm (Fig. 4.3B), is identical to those reported for the Mn-peroxo complexes in numerous wild-type and mutant MnSODs (2,3,12). Int$_2$ is an intermediate with broad absorption in the visible range and a maximum at ~425 nm. (Fig. 4.3B) The structure of this intermediate is unknown, but species with similar spectral features have been observed in similar studies of Y34A, Y34H, and Y34N human MnSOD (12).

The substitution of Tyr34 caused dramatic changes to the kinetics of ScMnSOD. The rate constant for the “prompt protonation” pathway, $k_2$ (Scheme 4.1), was too low to measure accurately. The rate constant for formation of the Mn-peroxo complex, $k_3$ (Scheme 4.1, 4.2), was dramatically elevated from 0.04–0.05 nM$^{-1}$s$^{-1}$ for wild-type ScMnSOD to 0.70 nM$^{-1}$s$^{-1}$ for the mutant protein (Table 4.2), such that the overall rate constant for reduction of O$_2^-$ by Mn$^{2+}$SOD, defined as a sum of $k_2$ and $k_3$, is similar in the wild type and the mutant protein. We define $k_3'$ the rate constant for formation of Int$_2$ from the Mn-peroxo complex (Scheme 4.2). It was determined to be 310 s$^{-1}$ for Y34F ScMnSOD, comparable to that of Y34H human MnSOD, but lower than those of Y34A and Y34N human MnSOD (Table 4.2). The rate constant for formation of the five-coordinate Mn$^{3+}$SOD from Int$_2$, $k_4'$ (Scheme 4.2), was determined to be 20 s$^{-1}$ for Y34F ScMnSOD, which is 3–16 fold lower than those determined for Y34 mutant human enzymes (Table 4.2).
**Int$_2$ is the Active Mn$^{3+}$ species in Y34F ScMnSOD.** As we have shown that the mutant yeast enzyme reacts completely through the Mn-peroxo complex, the question arises: what is the Mn$^{3+}$ species that oxidizes O$_2$? Is it Int$_2$ or the five-coordinate Mn$^{3+}$? Int$_2$ is a product from partial or full protonation of the Mn-peroxo complex. Formation of the five-coordinate Mn$^{3+}$ from Int$_2$ is so slow ($k_4' = 20$ s$^{-1}$) that if the former were the oxidant, Y34F ScMnSOD would be much slower than wild-type human MnSOD ($k_4 = 120$ s$^{-1}$) under inhibiting conditions. This contradicts our observation (Fig. 4.2B). Indeed, we were unable to fit our data to a mechanism in which formation of the five-coordinate Mn$^{3+}$ is the rate limiting step (data not shown). By contrast, the experimental data (Fig. 4.2A) with various enzyme and O$_2$ concentrations fit well to a mechanism in which Int$_2$ is the active Mn$^{3+}$ species (Fig. S4.4).

**Fast and Slow Proton Transfer Pathways in Y34F ScMnSOD.** Increased pH lowers the overall rate of protonation of the Mn-peroxo complex, which is a combination of $k_3'$, $k_4$, and $k_4'$ (see below), in Y34F ScMnSOD. At pH 7.0, the overall protonation rate equals to $k_3'$ (310 s$^{-1}$); at pH 8.5, it dropped by a factor of three to ~100 s$^{-1}$ (Fig. 4.4A). Also noticeably, the rapid absorption increase at 420 nm, resulting from formation of Int$_2$, gradually disappears at increased pH (Fig. 4.4B). These facts were not caused by depression of $k_3'$, which, calculated via fitting the data in Fig. 4.4B, stayed almost the same from pH 7.0–8.5 (Fig. 4.4A, inset). The most likely explanation is that Int$_2$ was no longer formed at increased pH. The same experiments recorded at 480 nm clearly indicated generation of the five-coordinate Mn$^{3+}$ directly from the peroxo complex at pH $\geq 8$ (Fig. S4.5). Protonation of the peroxo complex in Y34F ScMnSOD appears to partition into two pathways, a fast one at low pH leading to Int$_2$ and a slow one at high pH leading directly to the five-coordinate Mn$^{3+}$. The gating between the two pathways has a pK of
Figure 4.4. Protonation of the inner-sphere peroxo complex as a function of pH in Y34F ScMnSOD. (A) Decay of 41 μM O$_2$•$^-$ catalyzed by 5 μM (in Mn) Y34F ScMnSOD at pH 7.0 (a) and 8.5 (b). The solutions contained 10 mM potassium phosphate, 10 mM sodium formate, 1 mM Tris-Cl and 10 μM EDTA. Inset: rate constant $k_3$ (solid circles) and $k_4$ (hollow triangles) as functions of pH. (B) The absorbance change at 420 nm upon generation of 8.9 μM O$_2$•$^-$ at pH 7.5 (black), 8.0 (red), 8.5 (green), 9.0 (purple), and 9.5 (blue). Fitted curves generated from PRWIN (13) are shown. The solutions contained 80 μM (in Mn) Y34F ScMnSOD in 15 mM potassium phosphate, 10 mM sodium formate, 1 mM Tris-Cl, 160 μM H$_2$O$_2$, and 100 μM EDTA. Inset: percentage of the fast protonation pathway as a function of pH, calculated by dividing the highest 420 nm absorbance at each pH to that at pH 7.5, assuming that at pH 7.5 the enzyme reacts solely through the fast proton transfer pathway.
~8, and the enzyme reacts completely through the slow pathway at pH ≥ 9 (Fig. 4.4B, inset). The rate constant for the slow pathway ($k_4$) was calculated to be ~40 s$^{-1}$, by fitting the absorbance change at 480 nm to a first-order reaction (Fig. S4.5-A).

**Discussion**

*Tyr34 is critical for “Prompt Protonation” Pathway.* To examine the role of Tyr34 in catalysis by ScMnSOD, we replaced Tyr34 with phenylalanine. Wild-type ScMnSOD reacts predominantly through the “prompt protonation” pathway, with a gating ratio of 16–20 (Table 4.2) (4.5). As in Y34 mutant human enzymes (12), the “prompt protonation” pathway is completely disrupted in Y34F ScMnSOD. In Y34F ScMnSOD, $k_3$ is close in value (0.70 nM$^{-1}$s$^{-1}$) to $k_2$ of wild-type ScMnSOD (0.80 nM$^{-1}$s$^{-1}$) (Table 4.2), indicating that the “prompt protonation” pathway is substituted by the “inner-sphere” pathway in the mutant yeast enzyme.

**Putative Six-coordinate Int$_2$ is Associated with the Additional Second-sphere Water.** Spectroscopic analysis revealed that the resting state of wild-type yeast Mn$^{3+}$SODs consists of both five- and six-coordinate Mn$^{3+}$ centers (5). In wild-type yeast enzymes, the absorption intensities around 480 nm and <400 nm correlate with two effective $^{55}$Mn hyperfine couplings in the EPR spectra, consistent with five- and six-coordinate Mn$^{3+}$ centers, respectively (5). Like the wild-type enzyme, Y34F ScMn$^{3+}$SOD also absorbs at <400 nm in addition to the 480 nm band (Fig. 4.1B), suggesting that the oxidized mutant protein also contains both five- and six-coordinate Mn$^{3+}$.

Similar to several Y34 mutant human MnSOD (12), an intermediate (Int$_2$, Scheme 4.2) with an absorption maximum at 425 nm was observed when Y34F ScMn$^{2+}$SOD was oxidized by
O$_2^-$ (Fig. 4.3). Because $k_3^+$, the rate constant for formation of Int$_2$, is independent of ionic strength in Y34 mutant human MnSOD, generation of Int$_2$ is thought to involve an isomerization (12). Int$_2$ was proposed to be six-coordinate where the metal ion binds an additional water molecule (12).

The blue shifting of the Mn$^{3+}$SOD spectra could be ascribed to binding of a new, strong ligand in the equatorial plane of the Mn$^{3+}$ ion, causing the coordination geometry to switch from trigonal bipyramidal to distorted-octahedral and thus elevating the energies of the d-d transitions. Assuming that Int$_2$ observed in catalysis is also six-coordinate, it must differ from the six-coordinate species in the resting Mn$^{3+}$SOD, because the two have distinct absorption maxima (Fig. 4.1B, 3B). A simple explanation for the different extent of blue shifting is that the coordination geometry is slightly different in the six-coordinate species present under catalytic conditions from the non-catalytic form. The sixth ligand was proposed to be hydroxide in the resting wild-type ScMn$^{3+}$SOD (5). Because its binding to the Mn$^{3+}$ center is facile at neutral pH, the sixth ligand could also be a water molecule. Nevertheless, this conclusion does not necessarily hold for Int$_2$ in which H$_2$O, OH$,^-$, or OOH$^-$ are all potential candidates for the sixth ligand (see below).

All Y34 mutant proteins shown to react via Int$_2$ contain an additional second sphere water molecule occupying the cavity created by the replacement of Tyr34 (Fig. 4.1A) (12), suggesting that the observation of Int$_2$ and the presence of the additional water may be correlated. However, neither the additional water nor Int$_2$ are found in any Y34F mutant protein that has been studied previously (3,9), suggesting a particularly important role of Tyr34 in wild-type ScMnSOD. Previously we proposed that fast catalysis by yeast MnSOD could be related to fast proton transfer from an outer-sphere solvent molecule (5). Particularly in Y34 mutant proteins,
protonation of the peroxo complex is always fast so long as the additional water molecule is present. One would therefore expect that the additional second-sphere water in these Y34 mutant proteins donates one of the two protons to the bound peroxy group.

In order to explore the catalytic properties of the six-coordinate species in wild-type yeast MnSODs, we reduced ScMn$^{3+}$SOD or CaMn$^{3+}$SOD (40–80 μM) with substoichiometric amounts of O$_2^-$ and followed the absorbance change at 475 nm (Fig. S4.6). The resting form of oxidized yeast MnSODs is a mixture of five- and six-coordinate Mn$^{3+}$SOD. In the case of the six-coordinate Mn$^{3+}$SOD being a “dead-end” complex, $k_1$ would be significantly reduced due to inhibition by the sixth ligand. However, $k_1$ was determined to be $\sim$1.0–1.3 $\times$ 10$^9$ M$^{-1}$s$^{-1}$ for yeast wild-type MnSODs and is comparable to that of other wild-type MnSODs (Table 4.2), whose catalysis apparently only involves five-coordinate Mn$^{3+}$ species. Therefore, the six-coordinate Mn$^{3+}$ center with two solvent molecules bound is equally as catalytically competent as the well-characterized five-coordinate Mn$^{3+}$. This, along with the correlation between the additional second-sphere water molecule and fast proton transfer, suggests that the sixth ligand in Int$_2$ is most likely a solvent molecule. Unfortunately, it is difficult to confirm the structure of Int$_2$ using spectroscopic tools, due to short lifetime (~200 ms) of this intermediate.

*Proposed Mechanisms for Y34F ScMnSOD in which Six-coordinate Int$_2$ is the Active Species.* Y34F ScMnSOD is even more gated through the inner-sphere Mn-peroxo complex, Int$_1$, than is wild-type human MnSOD, as indicated by their gating ratios ($k_2/k_3$) of <0.01 and 1, respectively (Table 4.2). Nevertheless, to our surprise, the mutant ScMnSOD is faster than human MnSOD when O$_2^-$ levels are high (Fig. 4.2B). In the case of human MnSOD, high [O$_2^-$] causes the overall reaction to slow because of the relatively low rate of peroxide loss (120 s$^{-1}$ at
pH 7) upon protonation of Int$_1$. In the case of Y34F ScMnSOD, by contrast, protonation and loss of peroxide is much faster (310 s$^{-1}$ at pH 7). We conclude from curve fitting (Fig. S4.4) that Int$_2$ is the active Mn$^{3+}$ species that oxidizes O$_2^-$ in Y34F ScMnSOD. Although Int$_2$ has been observed during reaction of several Y34 mutant human enzymes, the active species that oxidizes O$_2^-$ in those systems remains the five-coordinate Mn$^{3+}$ (12). Since Int$_2$ is putatively six-coordinate, Y34F ScMnSOD represents the first MnSOD, whose catalysis may involve a six-coordinate Mn$^{3+}$.

**Scheme 4.3. Proposed mechanisms for Y34F ScMnSOD**

Here we propose a mechanism to describe the catalytic cycle of Y34F ScMnSOD (Mechanism A, Scheme 4.3, involving Int$_1$-A). It involves binding of O$_2^-$ to Mn$^{2+}$ in the sixth coordination position close to Tyr34 prior to inner-sphere electron transfer, as proposed in the “5–6–5” mechanism (24), referring to increase of coordination number from five to six upon binding of
O₂⁻, and decrease of coordination number from six to five upon dissociation of H₂O₂. The solvent ligand instantaneously transfers the first proton to the distal oxygen of the newly formed O₂²⁻ (25), leading to a six-coordinate end-on Mn³⁺-hydroperoxo species, which in this mechanism corresponds to the inner-sphere Mn-peroxo complex. The additional outer-sphere water molecule then transfers a second proton to the proximal oxygen of HOO⁻, leading to dissociation of the peroxide ligand. The vacant sixth ligand site is then occupied by a second-sphere solvent molecule. We formulate Int₂ as a six-coordinate Mn³⁺ center binding two solvent molecules. In the other half reaction, O₂⁻ first replaces the solvent molecule in the sixth ligand site (26), before it is oxidized by the Mn³⁺ ion.

Mechanism A is strongly supported by numerous experimental and computational studies. The “5–6–5” mechanism is supported by the fact that azide, an analog of O₂⁻, binds to the metal in the sixth coordination site in Fe and MnSOD (24). Cryotrapped EcMnSOD crystallized at pH 8.5 binds an additional water in the same sixth ligand site (27). These facts strongly corroborate binding of O₂⁻ in the sixth ligand site close to Tyr34. Several theoretical calculations suggest that the most stable conformation of a bound peroxyl is end-on (26,28). A density functional study by Carrasco et. al. indicates that the most probable candidate for the Mn-peroxo complex is an end-on Mn³⁺-hydroperoxo complex with HOO⁻ pointing toward the solvent ligand (28).

The structure of the Mn-peroxo complex is one of the most controversial topics in MnSOD research. To test the feasibility of Mechanism A, we modeled an end-on Mn³⁺-hydroperoxo species in the active site of Y34F ScMnSOD. The O1 of HOO⁻ in the model was placed at the same (x, y, z) coordinates as the N1 of azide in the six-coordinate azide-MnSOD (PDB code: 1MNG) (Fig. 4.5). HOO⁻, sitting in the sixth coordination site, does not have unfavorable steric interactions with the active-site residues. The only adjustment needed for a
Figure 4.5. A suggestive end-on model of the inner-sphere Mn-peroxo complex built from the crystal structure of Y34F ScMnSOD (chain A). The graphic was generated using the PyMOL Molecular Graphics System. Manganese ions are shown as purple spheres. Peroxyl groups are shown as red sticks with hydrogen atoms in gray. Existing and hypothetical solvent molecules are shown as red and cyan spheres, respectively.
good fit was a small displacement of the water molecule that has substituted for the –OH of Tyr34 to a position further away (~0.85 Å) from the Mn. The proposed new position for this shifted solvent is indicated in cyan in Fig. 4.5, which should be compared with Fig. 4.1A. When the Mn–O1(HOO⁻)–O2(HOO⁻) angle was modulated between 90 ° and 180 °, the distance between O2(HOO⁻) and the additional water molecule in Fig. 4.1A is always <2.0 Å, suggesting that this water molecule would be in close contact with the bound HOO⁻, and thus proton transfer from the former to the latter could be quite facile. This is consistent with the fast protonation rates observed for Y34F ScMnSOD (Fig. 4.2B, 4A) and several Y34 mutant human MnSOD (Table 4.2).

There is another mechanism (Mechanism B, Scheme 4.3, involving Int₁-B) that has been supported by many researchers. The major distinction between Mechanism A and B lies in the structure of the inner-sphere Mn-peroxo complex. In Mechanism B the reaction of O₂⁻ with Mn²⁺SOD initiates from displacement of the solvent ligand by O₂⁻ without occurrence of any proton transfer, and the peroxo complex is a six-coordinate Mn³⁺ binding a side-on O₂²⁻ (1,7). The displaced solvent molecule would become part of the water chain extending from active site to dimer interface (Fig. S4.7). The side-on peroxy then would need to accept two protons, either through the hydrogen bond chain extending from Gln154 or through direct hydrogen-bonding interactions with second-sphere water molecules. The structure of Int₂ and the process of oxidation of O₂⁻ are the same as in Mechanism A.

Formulation of the Mn-peroxo complex as a side-on Mn³⁺-peroxo species has been proposed based on computational and spectroscopic studies (6,7), and on the crystal structure of peroxyde-soaked EcMnSOD showing side-on binding of peroxyde to active-site Mn (Fig. S4.8-A) (29). The active site structure in Fig. S4.8-A, although determined under cryo conditions,
provides a potential pathway for protonation of the side-on peroxo complex through coordination of a water molecule in the sixth ligand site. An end-on Mn-hydroperoxo species is observed in two out of four chains in the same structure (Fig. S4.8-B), and it could be a product of the protonation implied in Fig. S4.8-A.

Here we favor Mechanism A over B, because, in the former, the reaction from the Mn-peroxo complex to Int$_2$ has a much simpler mechanism. This reaction in Mechanism A requires transfer of one proton and translocation of one solvent molecule, whereas in Mechanism B it requires transfer of two protons and translocation of two solvent molecules. Moreover, one would expect to observe additional intermediates between Int$_1$ and Int$_2$, if Mechanism B were in fact occurring.

The Y34 mutant MnSODs provide ideal systems for studying the reaction mechanism of product inhibition. Compared to wild-type MnSODs, in the Y34 mutant proteins, three instead of two Mn$^{3+}$ species are observed from the “inner-sphere” pathway, thus making it easier to break down the pathway into individual steps, including formation of the peroxo complex and transfer of each of the two protons. We will continue our exploration of the mechanism of product inhibition through theoretical calculations in the future.

In Y34F ScMnSOD the Gating between Two Proton Transfer Pathways is Controlled by pH. MnSOD is well known for the gating between the “prompt protonation” and “inner-sphere” pathways (Scheme 4.1), based on the likelihood of reduction of O$_2^-$ going through the Mn-peroxo complex. We report here that protonation of the peroxo complex in Y34F ScMnSOD is also gated between a fast and a slow pathway (Fig. 4.4), with the rate constant differing by ~7
fold. The fast pathway goes through the putative six-coordinate Int$_2$, while the slow pathway leads directly to the five-coordinate Mn$^{3+}$SOD and occurs in all MnSODs (Scheme 4.2).

Previous studies on bacterial reaction centers (RCs) elucidated mechanisms for electron transfer coupled with multiple proton transfer pathways, and the gating is controlled by conformational change of the protein (30). In Y34F ScMnSOD, by contrast, the gating is pH dependent. The fast pathway dominates at neutral pH, while the slow pathway dominates at increasing pH with a pK~8 (Fig. 4.4B, inset). Examples of “fast” and “slow” proton transfer being pH dependent and independent, respectively, have been reported for metalloenzymes (31). The pH effects could result from deprotonation of the active-site water molecules, especially the one that has substituted the –OH of Tyr34, at high pH. If the additional solvent molecule stays as a H$_2$O, it could donate a proton quickly to the Mn-peroxo complex. When it is deprotonated at increasing pH and forms a OH$^-$, the fast proton transfer is shut down, and the bound peroxy needs to get a proton through the slow pathway that occurs in all MnSODs.

Formation of the six-coordinate Int$_2$, which is coupled with the fast proton transfer pathway, no longer occurs at high pH. Therefore, at high pH, the five-coordinate Mn$^{3+}$ generated through the slow proton transfer pathway becomes the active species (Scheme 4.2, 4.3). Because we were unable to observe the six-coordinate species at high pH, either the sixth ligand is not a hydroxide, or binding of a hydroxide is a relatively slow process if not preceded by the proposed fast proton transfer. It is the first time for gating between multiple proton transfer pathways to be reported in any wild-type or mutant MnSOD.

A Six-coordinate Mn$^{3+}$ could be An Active Species in Wild-type Yeast MnSOD. Two unique biophysical properties distinguish yeast MnSODs from any previously characterized
MnSOD. First, yeast MnSODs rest predominantly in the reduced 2+ form (4,5), while all other MnSODs rest ≥ 50% in the oxidized 3+ form (3,22,23). Second, yeast Mn^{3+}SODs contain both five- and six-coordinate Mn^{3+} centers (5), while only the five-coordinate Mn^{3+} is present in oxidized MnSODs from other organisms (32). Y34F ScMnSOD resembles the wild-type enzyme in that it rests as Mn^{2+}SOD and is oxidized to a mixture of five- and six-coordinate Mn^{3+} centers (Fig. 4.1B).

The positioning of Gln154 has been proposed to control the resting oxidation state of MnSOD (6,33,34). Several theoretical studies suggest that the solvent ligand is a H_{2}O when the enzyme is reduced, and a OH\(^-\) when it is oxidized (35). Previously we revealed that the increased separation of the Gln154 side chain from the metal ion in ScMnSOD leads to a smaller stabilization of OH\(^-\) vs. H_{2}O and thus a relative stabilization of the 2+ state vs. 3+ state (5). On this basis, we would predict that the solvent ligands in wild-type and the mutant yeast Mn^{3+}SODs are less acidic than those in other Mn^{3+}SODs, due to stabilization of H_{2}O relative to OH\(^-\) in the yeast enzymes.

Electron-withdrawing/donating effects of ligands impact the coordination number of numerous metal complexes. The presence of electron-withdrawing substituents increases the propensity of a five-coordinate Mn^{3+} complex to bind a sixth ligand, via increasing the electrophilicity of the Mn (36). The presence of electron-donating methyl groups favors formation of an end-on over a side-on Mn^{3+}-peroxo (37). Because H_{2}O is a poorer electron donor than OH\(^-\), the Mn^{3+} centers of the yeast enzymes are predicted to have higher electrophilicity than those of other Mn^{3+}SODs, which could explain the tendency of yeast Mn^{3+}SODs to bind another solvent molecule.
Nevertheless, Fe$^{3+}$-substituted EcMnSOD, compared to wild-type EcFe$^{3+}$SOD, has a much higher affinity for OH$, even though the Gln residue in EcMnSOD is much closely placed to the metal ion (38). In addition, there is no experimental evidence for OH$^-$ binding to Mn$^{3+}$-substituted EcFeSOD at neutral pH, even though the Gln residue in EcFeSOD is very far away from the metal ion (33). We will thus continue exploring the origin of the six-coordinate Mn$^{3+}$ centers in yeast MnSODs in the future.

Although Y34F ScMnSOD and several Y34 mutant human enzymes share similar active sites and formation of the six-coordinate Int$_2$, only in the mutant yeast MnSOD does Int$_2$ react with O$_2^-$. This may result from the fact that Y34F ScMn$^{3+}$SOD is inclined to form a six-coordinate Mn$^{3+}$ adduct. Because wild-type and the mutant yeast Mn$^{3+}$SODs are both composed of five- and six-coordinate species (5), the active Mn$^{3+}$ species in wild-type yeast MnSODs could also be six-coordinate. Indeed, as discussed above, in wild-type yeast enzymes, the six-coordinate Mn$^{3+}$ with two solvent molecules bound is equally as catalytically active as the five-coordinate Mn$^{3+}$. Moreover, our recent work revealed that catalysis by wild-type yeast MnSODs may involve fast proton transfer (5). Compared to that of human MnSOD, the catalytic reactivity of wild-type yeast enzymes is significantly more decreased at high pH with indications of depression of the protonation rate (5). Therefore, the mechanism proposed for Y34F ScMnSOD could well apply to wild-type yeast enzymes. Decay of the Mn-peroxo complex and the six-coordinate Int$_2$ may be too fast to be detectible in catalysis by wild-type yeast enzymes under the conditions of our experiments.

In conclusion, the replacement of Tyr34 with phenylalanine (Y34F) causes ScMnSOD to react completely through the “inner-sphere” pathway. Y34F ScMnSOD has fast catalysis due to a novel mechanism in which a putative six-coordinate Mn$^{3+}$ species actively reacts with O$_2^-$ at
neutral pH. Formation of this six-coordinate species and occurrence of fast proton transfer could relate to the presence of an additional active-site solvent molecule. Protonation of the inner-sphere Mn-peroxo complex has two distinct pathways with the gating controlled by pH. Our studies on Y34F ScMnSOD provide novel perspectives on the mechanism of MnSOD involving active six-coordinate Mn$^{3+}$ species and gating between multiple proton transfer pathways. Because both wild-type and the Y34F mutant yeast Mn$^{3+}$SOD have the tendency to become six-coordinate, the active Mn$^{3+}$ species in the catalysis by wild-type yeast MnSODs could also contain a six-coordinate center.
**Figure S4.1.** Electrospray-ionization mass spectra (top panel) of methylated Y34F ScMnSOD and its reconstructed mass distribution profiles (bottom panel). Ordinate units of intensity are arbitrary and the abscissa units of average molecular mass are in Daltons. The calculated mass is 23,545 Da, and the expected mass is 23,546 Da.
**Figure S4.2.** Superimposition of Y34F ScMnSOD tetramer (red) onto wild-type ScMnSOD tetramer (green). The graphic was generated using the PyMOL Molecular Graphics System. Mn atoms are shown in purple spheres.

**Figure S4.3.** The difference absorption band of Y34F ScMn$^{3+}$SOD as measured over time. Y34F ScMnSOD was oxidized by potassium permanganate ([KMnO$_4$]:[MnSOD] = 0.75:1) at pH 7.4. Time 0 refers to the start of the first scan immediately after mixing KMnO$_4$ with the enzyme. The sample solution contained 200 μM (in Mn) enzyme in 25 mM potassium phosphate (pH 7.4).
Figure S4.4. Fitting the disappearance of $\text{O}_2^{-}$ catalyzed by Y34F $\text{ScMnSOD}$ at pH 7 into a mechanism in which $\text{Int}_2$ is the active species (A). The $\text{O}_2^{-}$ and enzyme concentrations are: (B) 1 μM MnSOD and 7 μM $\text{O}_2^{-}$; (C) 1 μM MnSOD and 41 μM $\text{O}_2^{-}$; (D) 5 μM MnSOD and 41 μM $\text{O}_2^{-}$. $k_3$ ($7 \times 10^8 \text{M}^{-1} \text{s}^{-1}$) and $k_3'$ (310 s$^{-1}$) determined individually from the oxidation of Mn$^{2+}$SOD by $\text{O}_2^{-}$ are used for the fitting. We assume that $k_2$ is negligible and $k_1$ is comparable to $k_3$. Experimental data are shown in black dots, and the fitted curves are shown in red lines. The sample contained 10 mM potassium phosphate (pH 7), 10 mM sodium formate and 10 μM EDTA. The $\text{O}_2^{-}$ concentrations are calculated from the absorbance at 260 nm.
Figure S4.5. Dependence of decomposition of the product-inhibited complex on pH in Y34F ScMnSOD. The absorbance change at 480 nm upon generation of 8.9 μM O$_2^-$ as a function of pH is shown. Fitted curves generated from PRWIN (13) are shown. The solutions contained 80 μM MnSOD (in Mn) in 15 mM potassium phosphate, 10 mM sodium formate, 1 mM Tris-Cl, 160 μM H$_2$O$_2$, and 100 μM EDTA.

Figure S4.6. Reduction of wild-type ScMn$^{3+}$SODc (A) and CaMn$^{3+}$SODc (B) by O$_2^-$ in pulse radiolysis. The formation of Mn$^{2+}$SOD is indicated by decrease of absorbance at 475 nm over time upon generation of 2.2 μM O$_2$. The sample solutions contained 40 μM (in Mn) enzyme, 10 mM potassium phosphate (pH 7), 10 mM sodium formate, and 100 μM EDTA. The oxidized enzymes were prepared by treating the as-isolated yeast enzymes with 0.75 equiv of KMnO$_4$, letting them sit at room temperature for 2 hr, and then buffer-exchanged to remove free metal ions.
**Figure S4.7.** The water chain extending from active site to dimer interface in Y34F ScMnSOD. Chain A and B are colored in red and green, respectively. The graphic was generated using the PyMOL Molecular Graphics System. Manganese ions are shown as purple spheres. Coordination and hydrogen bonds are indicated as solid and dashed lines, respectively.

**Figure S4.8.** Mn-peroxo complexes in the crystal structure of cyrotrapped peroxide-soaked EcMnSOD (PDB code: 3K9S). (A) A side-on Mn-peroxo complex (chain C); (B) An end-on Mn-peroxo complex (chain B). The graphic was generated using the PyMOL Molecular Graphics System. Manganese ions are shown as purple spheres. The peroxy group and solvent molecules are shown as red sticks and red spheres, respectively. Coordination and hydrogen bonds are indicated as solid and dashed lines, respectively.
References


13. Schwarz, H., BNL Pulse Radiolysis Program. Brookhaven National Laboratory


Chapter 5

Tetramerization in Yeast MnSOD and its Functional Significance

(This chapter is in preparation for submission to the Journal of Biological Chemistry)
Introduction

Manganese-containing superoxide dismutase (SOD) is an important intracellular antioxidant enzyme. It defends biological systems by catalyzing the disproportionation of superoxide (O$_2^-$) into dioxygen and hydrogen peroxide (H$_2$O$_2$). It is either a homodimer or a homotetramer with a monomer size of ~23,000 Da. The enzymes located in eukaryote mitochondria (human (1), Saccharomyces cerevisiae (2), Caenorhabditis elegans (3), Aspergillus fumigates (4)) were found to be homotetramers, while MnSODs isolated from bacteria (Thermus thermophilus (5), Escherichia coli (6), Deinococcus radiodurans (7)) are homodimers. In all MnSODs, each MnSOD subunit is composed of two domains, a predominantly α-helical N-terminal domain and a mixed α/β C-terminal domain. The Mn ion is coordinated by four strictly conserved residues, two from the N-terminal domain and two from the C-terminal domain, and one solvent molecule.

Assembly into oligomers has been proposed to confer several potential various advantages on proteins, including greater folding efficiency, high stability, specific morphological functions, amenability to allosteric regulation, and greater error control in synthesis (8-10). Tetrameric MnSODs are dimers of dimers, which display dihedral symmetry (D$_2$). However, being a tetramer does not grant MnSOD higher catalytic efficiencies. Dimeric bacterial MnSODs and tetrameric human MnSOD have similar efficiencies when O$_2^-$ levels are low, and bacterial MnSODs are faster than human MnSOD when O$_2^-$ levels are high (11,12).

In the budding yeast S. cerevisiae, there is only one MnSOD, and it is located to the mitochondria (13,14). The closely related yeast Candida albicans has two MnSODs, one in the mitochondria and the other in the cytosol (MnSODc) (15). ScMnSOD and CaMnSODc share 70% sequence similarity and are similar in enzyme kinetics, spectroscopy and redox chemistry (16).
However, CaMnSODc was found to exist as a dimer in solution but as a tetramer when it was crystallized, while ScMnSOD was found to be a tetramer under all conditions (16). Because ScMnSOD and CaMnSODc have most biochemical and biophysical properties in common and their dimer interface shares >90% similarity, they together provide an ideal system for elucidating the significance of the differences in MnSOD quaternary structures.

**Scheme 5.1.** Alignment of MnSOD C-terminal Sequence. Conserved residues and unconserved residues at the dimer interface are highlighted in bold and shadowed in gray, respectively. The RP-mutations in ScMnSOD and CaMnSODc are highlighted in black.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>GLDVWEHAYLEKRNPRPDYIKEFNVVVWDEAAARFAAKK------</td>
<td>206</td>
</tr>
<tr>
<td>D. radiodurans</td>
<td>GVDVWEHAYLYQNRPRPDYLAAFNVVVWDEGKRFAAAK------</td>
<td>211</td>
</tr>
<tr>
<td>D. melanogaster</td>
<td>GIDVWEHAYLYKHQPSYVEAIWDIANWDDICRSQEAKKLC-----</td>
<td>206</td>
</tr>
<tr>
<td>C. elegans</td>
<td>GIDVWEHAYLYKHQPRDVAIRKIAXWKNISERNARQ--------</td>
<td>200</td>
</tr>
<tr>
<td>mouse</td>
<td>GIDVWEHAYLYKHQPRDYLKAIWNVWENWERYTACKK--------</td>
<td>205</td>
</tr>
<tr>
<td>human</td>
<td>GIDVWEHAYLYQKNRPRDLAIWNVWENWERYYACXK--------</td>
<td>205</td>
</tr>
<tr>
<td>ScMnSOD</td>
<td>AIDAWEHAYLQYONKADYFKAIWNVNWKEASRFDAGK--------</td>
<td>207</td>
</tr>
<tr>
<td>CaMnSODc</td>
<td>AIDAWEHAYLQYONKADYFKAIWNVNWKEASRFDAGK--------</td>
<td>207</td>
</tr>
<tr>
<td>RP-ScMnSOD</td>
<td>AIDAWEHAYLQYONKADYFKAIWNVNWKEASRFDAGK--------</td>
<td>207</td>
</tr>
<tr>
<td>RP-CaMnSODc</td>
<td>AIDAWEHAYLQYONKADYFKAIWNVNWKEASRFDAGK--------</td>
<td>207</td>
</tr>
</tbody>
</table>

The dimer interface plays a crucial role in maintaining MnSOD activity. Most MnSODs have a conserved arginine close to the strictly conserved DXWEHXXYL motif, while in yeast MnSODs it is a lysine (Scheme 5.1, Lys182 in ScMnSOD and Lys184 in CaMnSODc). This lysine in yeast MnSOD and arginine in all other MnSODs, has been proposed to be critical for the catalytic activity of MnSODs (17). A more recent study suggested that the substitution of this lysine causes loss of stability in ScMnSOD (18).

The side chain of the lysine in yeast MnSODs has a different conformation compared to that of the arginine in MnSODs from other organisms. This difference derives from the residue
next to the lysine, which is an alanine or leucine in ScMnSOD and CaMnSODc, respectively, and a proline in all other MnSODs. To investigate whether modification of the dimer interface has the same effects on tetrameric and dimeric MnSOD, we engineered the two yeast MnSODs by mutating the lysine to arginine and changing the residue next to the lysine to proline (K182R, A183P ScMnSOD and K184R, L185P CaMnSODc) (Scheme 5.1), so that the conformation of the arginine is identical to that in other MnSODs. Here, we report the crystal structures of wild-type and RP-mutant (here we call the mutant proteins RP-mutant MnSOD or RP-MnSOD) yeast MnSODs and their biochemical and biophysical properties. We also discuss how the tetramer structure reinforces the dimer interface of MnSOD.

*We call the mutant proteins RP-mutant MnSOD or RP-MnSOD.

**Materials and Methods**

**Samples.** EcMnSOD was purchased from a commercial source (Sigma-Aldrich). The protein was resuspended in 25 mM potassium phosphate (pH 7.4), washed with 1 mM EDTA in the same phosphate buffer for several times, and then purified through a G200 size exclusion column.

**Construction of Plasmid for Expression of RP-mutant ScMnSOD and CaMnSODc.** Site-directed mutagenesis (19) was carried out on an overexpression vector (YEp352-ScMnSOD) containing the URA3 selectable marker and a 2-kb genomic BamHI fragment containing the gene for ScMnSOD. The primers 5’-CAGTACAAAAAACAGAGACCCGACTACTTCAAAGC-3’ and 5’-GCTTTGAAGTAGTCGGTTTCTCTTTGTTTGTACTG-3’ were used to create the cDNA for K182R, A183P ScMnSOD.
The pVT102U-CaMnSODc (with URA3 and AMP marker) vector containing the complete coding sequence of CaMnSODc was generously given by Prof. Bourbonnais (15). The primers 5’-CAAAATGTCAAGGCTGATTATTTCAAAAGCAATTTGGAA-CGTG-3’ and 5’-GAAATAATCAGGCCTGACATTITTGATATTGCAAGTAGTAC-GC-3’ were used to create the cDNA for K184R, L185P CaMnSODc.

The PCR products were transformed into E. coli DH5α strain and screened by ampicillin selection. The purified vectors were transformed into S. cerevisiae sod2Δ strain (EG110).

Expression and Purification of Wild-type and RP-mutant ScMnSOD and CaMnSODc. Yeast cells carrying Yep352-ScMnSOD (wild type or mutant) were grown in YPEG media (1% yeast extract, 2% peptone, 3% glycerol, 2% ethanol, pH 4) supplemented with 0.5 mM Mn(II) sulfate at 30 °C to O.D. > 20. Yeast cells carrying pVT102U-CaMnSODc (wild type or mutant) were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose, pH 4) media supplemented with 0.5 mM Mn(II) sulfate at 30 °C to O.D. > 10. Cells were harvested by centrifugation at 12,000 × g for 10 min. Isolation of wild-type and RP-mutant ScMnSOD and CaMnSODc was performed as previously described (16).

Size Exclusion Chromatography. The mass weight of native proteins was determined by a HPLC (Agilent 1200 series) fitted with a size exclusion column (Tosoh Bioscience, TSK gel G2000SW) at a flow rate of 0.25–0.5 mL/min. The column was calibrated using five standards: bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B12 (1.35 kDa).
HPLC-SEC measurements were used to determine the oligomer state of proteins. The equilibrium between dimers and monomers is shown as $D \Leftrightarrow 2M$ where $D$ represents dimer and $M$ represents monomer. The dimer dissociation constant ($K_d$) is calculated as

$$K_d = \frac{[M]^2}{[D]}$$

where $[M]$ and $[D]$ were calculated from area integrals of elution peaks (UV signal at 210 nm). The peak fitting was carried out in OriginPro 8.1 (OriginLab Corp.). The column buffer contained 10 mM potassium phosphate (pH 6.7). The protein concentration was varied from 10 µM to 200 nM.

**Crystallization of Wild type and RP-mutant ScMnSOD and CaMnSODc.** The crystallization of wild-type ScMnSOD and CaMnSODc was described before (16). Two conditions yielded protein crystals showing CaMnSODc dimers in the structure. First, Native CaMnSODc was crystallized by hanging-drop vapor diffusion at 4 °C against a solution of 0.2 M lithium sulfate and 0.1 M phosphate-citrate (pH 4.2) in 20% (w/v) polyethylene glycol 1,000 with a protein concentration of 7 mg/mL. Second, methylated CaMnSODc was crystallized by hanging-drop vapor diffusion at 4 °C against a solution of 0.2 M sodium nitrate and 0.1 M bis-tris propane (pH 7.5) in 20% (w/v) polyethylene glycol 3,350 with a protein concentration of 7 mg/mL. The reductive methylation of lysine residues of CaMnSODc was carried out using procedures described before.(20) All the free amino groups of lysine residues and N-terminal were methylated as confirmed by mass spectrometry (Figure S1-B). These two structures were not deposited to PDB bank, because they have clear breaks in the electron density in the N-terminal regions.
Reduction methylation of K182R, A183P ScMnSOD was carried out as described previously (20), in order to improve the diffraction of protein crystals. All free amino groups of the lysine residues and the N-terminus were methylated as confirmed by mass spectrometry (Figure S5.1-A), and the activity of the native and methylated forms of RP-mutant ScMnSOD was similar in SOD activity assay (21).

Methylated K182R, A183P ScMnSOD was crystallized by hanging-drop vapor diffusion at 4 °C against a well solution of 0.1 M tri-ammonium citrate (pH 7) in 20% (w/v) polyethylene glycol 3,350 with a protein concentration of 7 mg/mL. Native K184R, L185P CaMnSODc was crystallized by hanging-drop vapor diffusion at 4 °C against a solution of 2 M ammonium sulfate with a protein concentration of 7 mg/mL. The protein crystals were cryo-protected in mother liquor solution containing 30% glycerol and flash frozen in liquid nitrogen prior to data collection.

Crystallography: Data Collection and Refinement. All data were collected at 100K at the UCLA X-ray diffraction facility, using a Rigaku FRE+ generator and a Rigaku HTC detector. All data was processed using DENZO and SCALEPACK (22). The crystallography information for wild-type ScMnSOD and CaMnSODc was reported previously (16). RP-mutant ScMnSOD and CaMnSODc were phased by molecular replacement using wild-type ScMnSOD (PDB code: 3LSU) and CaMnSODc (PDB code: 3QVN), respectively. All the molecular replacement was done using PHASER (23). The models were built using COOT (24). All model refinements were performed using REFMAC (25) and PHENIX (26).

Analysis of interface contacts and estimation of changes in salvation energy were performed using the PISA server (PDBbePISA Protein Interfaces, Surfaces and Assemblies (27)).
**Other crystallization conditions for wild-type CaMnSODc.** Two conditions yielded wild-type CaMnSODc protein crystals showing CaMnSODc dimers in the structure. First, Native wild-type CaMnSODc was crystallized by hanging-drop vapor diffusion at 4 °C against a solution of 0.2 M lithium sulfate and 0.1 M phosphate-citrate (pH 4.2) in 20% (w/v) polyethylene glycol 1,000 with a protein concentration of 7 mg/mL. Second, methylated wild-type CaMnSODc was crystallized by hanging-drop vapor diffusion at 4 °C against a solution of 0.2 M sodium nitrate and 0.1 M bis-tris propane (pH 7.5) in 20% (w/v) polyethylene glycol 3,350 with a protein concentration of 7 mg/mL. The reductive methylation of lysine residues of CaMnSODc was carried out using procedures described before (20). All the free amino groups of lysine residues and N-terminus were methylated as confirmed by mass spectrometry (Figure S5.1-B).

These two structures were not adopted, because their resolutions were lower than those that give CaMnSODc tetramers.

**Pulse radiolysis.** Pulse radiolysis experiments were carried out using the 2 MeV Van de Graaff accelerator at Brookhaven National Laboratory. Upon irradiation of water with a pulse of energetic electrons, hydrated electrons (e_{aq}^-), hydroxyl radicals (\textsuperscript{·}OH) and, in lesser yield, hydrogen atoms (H\textsuperscript{+}) are the primary radicals produced. Superoxide radical is then generated in air-saturated aqueous solution containing sodium formate through the following reactions: \textsuperscript{·}OH + HCO_{2}^- → H_{2}O + CO_{2}^- \textsuperscript{·}, O_{2} + CO_{2}^- → O_{2}^- + CO_{2}, e_{aq}^- + O_{2} → O_{2}^- + H, H + O_{2} → HO_{2}^-.

The experiments to measure catalytic rates involved following the decay of various concentrations of O_{2}^- at 260 nm using 1:1 to 1:50 ratios of [MnSOD]:[O_{2}^-]. First-order rate
Constants were calculated by fitting the data using the Chemical Kinetics program in PRWIN (28).

The temperature deactivation measurements were performed by heating the pulse radiolysis cell holder to the desired temperature. The sample cell containing a protein solution was then inserted into the holder and allowed to equilibrate for 4 minutes prior to radiation of the sample solution.

All pulse radiolysis samples were prepared in 10 mM potassium phosphate, 10 mM sodium formate and 10 μM EDTA at 25 °C. All MnSOD concentrations were taken as the ICP-measured concentration of manganese in the sample. The pH of the buffer was adjusted using ultrapure (Baker Ultrex) sodium hydroxide and sulphuric acid as needed.

**Differential Scanning Calorimetry (DSC).** DSC scans were performed using a Nano II differential scanning calorimeter (Calorimetry Sciences Corp.). All buffers were degassed under vacuum and highly concentrated protein samples (~24 mg/mL) were diluted with degassed buffers to a concentration of 2 mg/mL (600 μL) right before the DSC run. All samples were run at a rate of 1 °C/min under 4 atm of pressure. A buffer (25 mM potassium phosphate, pH 7.4) base line was run prior to the analysis of protein samples with the same heating/cooling rates. Baseline subtraction was performed, and some of the peaks were fitted to non-two-state reversible transitions using Origin 8.0 (Microcal).

The other peaks were fitted to two-state irreversible transitions, obeying pseudo-first-order kinetics (29). The temperature dependence of the kinetic constant $k$ obeys the Arrhenius equation,
\[ k = A \exp \left( -\frac{E}{RT} \right) \]

where \( A \) is the preexponential factor and \( E \) is the activation energy of transition. The apparent excess heat capacity \( C_p(T) \) at a temperature \( T \) is given by Sánchez-Ruiz’s methods as

\[
C_p(T) = \left( \frac{\Delta HE}{RT^2} \right) \exp(u(T)) \exp[-\exp(u(T))]
\]

where \( u(T) = \frac{E}{R} \left( \frac{1}{T_m} - \frac{1}{T} \right) \), \( T_m \) is the temperature at which \( C_p \) reaches its maximum value. DSC data were fitted to equation (6) using SigmaPlot 11.0 (Systat Software).

**Miscellaneous Methods.** Metal contents of the purified proteins were determined by ICP-MS (Agilent 7500 series). UV-visible absorption spectra were collected on a Shimadzu UV-2501 PC spectrophotometer.

Circular dichroism (CD) measurements were performed on a JASCO J-715 spectropolarimeter at room temperature. The GdHCl concentration was varied from 0 to 5 M while the protein concentration was held constant at 0.2 mg/mL. Protein samples were mixed with GdHCl stock solutions and incubated at room temperature for 15 min prior to the scan.

Mass weight of the protein subunit was determined by an electrospray ionization mass spectrometry (ESI-MS) either via a triple quadrupole instrument (API III, Applied Biosystems) or via a hybrid linear ion-trap mass spectrometer (LTQ, Thermo Electron). Mass spectra were processed and analyzed using MacSpec 3.3, Hypermass and BioMultiview 1.3.1 software for data obtained from API III, and ProMass for Xcalibur 2.8 for data obtained from LTQ.
Results

The N-terminal Helixes are Highly Disordered in Dimeric CaMnSODc. Wild-type ScMnSOD and CaMnSODc and their RP-mutant proteins were overexpressed and purified from S. cerevisiae, and their purities were confirmed by SDS-PAGE gel (Figure S5.2). To solve the mystery of why ScMnSOD and CaMnSODc share high sequence similarity while one is a tetramer and the other is a dimer in solution, we determined their crystal structures (16). The crystal structure further confirmed that ScMnSOD is a homotetramer (Figure 5.1A). By contrast, the quaternary structure of CaMnSODc varies with crystallization conditions; it is either a homotetramer (Figure 5.1B) or a homodimer (Figure 5.1D).

As in other tetrameric MnSODs, the N-terminus of both yeast MnSODs folds into a hairpin structure holding two long α-helixes (Figure 5.1C), which are much shorter in dimeric MnSODs from bacteria (Figure S5.3-B). The N-terminal helical hairpins are considerably longer in ScMnSOD than in any previously characterized tetrameric MnSOD (Figure 5.1C, S5.3-A). This feature renders ScMnSOD a much larger tetramer interface of >1400 Å², as compared to 800–1000 Å² in other MnSOD tetramers (human, A. fumigates, and C. elegans) (Table S5.1).

A surprising observation for CaMnSODc is that the crystal structures showing CaMnSODc as tetramers have well-defined electron density throughout the entire sequence, while those showing CaMnSODc as dimers have clear electron density breaks in the region encompassing residues 45, 51–69, 89–97, and 156–163. While residues 89–97 and 156–163 belong to loop regions, which are expected to possess high structural disorder, residues 51–69 belong to the N-terminal helical region (Figure 5.1D), which holds two highly ordered α-helixes in the well-ordered structure of CaMnSODc (Figure 5.1C). 20 residues (45, 51–69) from the N-
Figure 5.1. The N-terminal helixes are highly disordered, when CaMnSODc is in the dimeric form. (A and B) Ribbon diagram of ScMnSOD (A) and CaMnSODc (B) tetramer. The four subunits are colored in: A, yellow; B, orange; C, green; D, cyan. Manganese ions are indicated as purple spheres. (C) Superimposition of the subunit of CaMnSODc (purple) in the tetrameric form over that of ScMnSOD (green). The N-terminal helical region (residues 1–91) of CaMnSODc is enlarged on the right. (D) Ribbon diagram of CaMnSODc in the dimeric form in the crystal lattice. The two subunits in the asymmetric unit are colored in: A, blue; B, yellow. Manganese ions are indicated as purple spheres. The N-terminal helical region (residues 1–88) is enlarged on the right.
terminal region are missing electron densities; therefore, the structure coordinates for a large fraction of the hairpin remote form the active site could not be built (Figure 5.1D). These findings suggest that the α-helix-based hairpin structure is highly disordered, when CaMnSODc is dimeric.

To investigate structural changes caused by the substitutions of Lys182 (Lys184) and Ala183 (Leu185), we solved the structures of the two RP-mutant proteins. The tetrameric assemblies of wild-type and RP-mutant yeast MnSODs closely resemble each other (Figure S5.4). Superimpositions of all backbone atoms of the mutant subunit onto those of the wild type subunit give root-mean-square deviations (RMSD) of 0.15 Å and 0.34 Å for ScMnSOD and CaMnSODc, respectively, suggesting that the substitutions cause greater structural perturbation to CaMnSODc than to ScMnSOD.

**RP-CaMnSODc is Susceptible to Dimer Dissociation.** The oligomeric states of the native proteins were investigated by HPLC-SEC. When the protein concentration was varied from 10 μM to 200 nM, wild-type and the RP-ScMnSOD both eluted solely as a tetramer (Figure 5.2A), while wild-type CaMnSODc eluted solely as a dimer (Figure 5.2B). By contrast, the elution profiles of RP-CaMnSODc revealed two peaks corresponding to dimeric and monomeric forms when the protein concentration was below 1 μM (Figure 5.2B). At 200 nM RP-CaMnSODc eluted solely as a monomer (Figure 5.2B). Based on the dimer-monomer equilibrium (Experimental Procedures), $K_d$ of RP-CaMnSODc was determined to be $2.0 \pm 0.1$ μM, with details of the calculation shown in Table S5.2.
Figure 5.2. Oligomer state of wild-type and RP-mutant ScMnSOD and CaMnSODc. (A) HPLC-SEC profiles of wild-type (solid line) and K182R, A183P (dashed line) ScMnSOD. Inset: The plot of the molecular weight of the five standards (square), ScMnSOD tetramer (circle) and CaMnSODc dimer (triangle down) and monomer (triangle up) versus their retention time. The column was calibrated using five standards: 1) bovine thyroglobulin (670 kDa), 2) bovine γ-globulin (158 kDa), 3) ovalbumin (44 kDa), 4) horse myoglobin (17 kDa), and 5) Vitamin B12 (1.35 kDa). (B) HPLC-SEC profiles of wild-type (solid lines) and K184R, L185P (dashed lines) CaMnSODc. Deconvoluted peaks are shown as grey lines. The protein concentration relative to monomer was 1 μM (a), 750 nM (b), 500 nM (c) and 200 nM (d). The elution buffer contained 10 mM potassium phosphate (pH 6.7).
**RP-Mutant Yeast MnSODs Catalyze the Dismutation of O$_2^-$ like Wild-type Enzymes.** The substitutions at the dimer interface make yeast RP-MnSODs structurally closer to human MnSOD. To explore whether these substitutions affect the catalysis of yeast MnSODs, wild-type and RP-mutant yeast enzymes were pulsed with various concentrations of O$_2^-$, and their dismutation efficiencies were compared. At neutral pH and room temperature, the wild types and the mutant proteins have similar kinetics. When [O$_2^-$] was high relative to enzyme concentration ([O$_2^-$]:[MnSOD] = 41), the decay curves for O$_2^-$ disappearance catalyzed by wild-type or RP-mutant yeast MnSODs were superimposable (Figure S5.4), suggesting that the mutant proteins resemble the wild types in displaying low degrees of product inhibition (16,30).

MnSOD enzymatic activity is represented by the first-order rate constant calculated at low O$_2^-$ doses ([O$_2^-$]:MnSOD = 1 – 3). We previously showed that inactivation by pH is significantly more prominent in yeast MnSODs than in human MnSOD, pK of 8.5 and 10.5, respectively (16). Here, although both yeast MnSODs were engineered to imitate human MnSOD, neither of the mutant proteins gain higher resistance to pH compared to the wild types (Figure 5.3).

The profile of RP-ScMnSOD activity as a function of pH closely resembles that of wild-type ScMnSOD (Figure 5.3). The same mutations on CaMnSODc, however, resulted in an enzyme even more sensitive to pH, with the pK decreasing from ~8.5 in the wild type to ~ 8 in the mutant protein (Figure 5.3). Loss of activity at high pH is reversible in wild-type yeast enzymes as well as in RP-ScMnSOD, with a restoration of ~50% of their original activity (Figure 5.3). By contrast, when the pH of the sample solution was adjusted from basic (≥ 9) to neutral, no restoration of activity was observed for RP-mutant CaMnSODc (Figure 5.3).
Figure 5.3. RP-CaMnSODc is more subject to inactivation by pH than the wild type. (left) Rate constants as a function of pH determined by fitting the disappearance of low doses of $O_2^-$ ($[O_2^-]:[\text{MnSOD}]$ from 1 – 3) to first-order processes. The enzymes were wild-type ScMnSOD (solid triangle), K182R, A183P ScMnSOD (hollow triangle), wild-type CaMnSODc (solid circle) and K184R, L185P CaMnSODc (hollow circle). (right) Rate constants of sample solutions with the pH adjusted from 9–9.5 to neutral. The sample solutions contained 1 μM (in Mn) MnSOD in 10 mM potassium phosphate (pH 7), 10 mM sodium formate and 10 μM EDTA.
RP-CaMnSODc is Inactivated by Heat, while RP-ScMnSOD is not. Yeast MnSODs, when treated with heat, do not exhibit loss of function. ScMnSOD retained its activity at full scale up to 75 °C, the highest temperature allowed in pulse radiolysis measurements. CaMnSODc, with a much lower thermostability than ScMnSOD (see below), retained its full activity as long as the enzyme stayed folded in solution. Protein aggregation was noticeable from decreased initial voltage V0, which quantifies the maximum intensity of UV light that can penetrate the sample cell. Melting of as-isolated CaMnSODc occurs at 50 °C (see below), and the enzyme stayed fully active up to 49 °C (Figure 5.4).

By contrast, the reactivity of EcMnSOD decreased by ~100 fold after the sample solution was allowed to equilibrate to 68 °C (Figure 5.4, see method). Because V0 stayed the same before and after the sample solution was equilibrated, the loss of activity in EcMnSOD resulted from dramatic inactivation by heat rather than aggregation of the enzyme.

The substitutions of Lys182 (Lys184) and Ala 183 (Leu185) at the dimer interface affect the temperature profile of ScMnSOD and CaMnSODc differently. Similar to wild-type ScMnSOD, RP-ScMnSOD retained full activity up to 73 °C (Figure 5.4). RP-CaMnSODc, however, differs from the wild type but resembles bacterial MnSODs in terms of inactivation by heat. When RP-CaMnSODc was heated at 41 °C, a loss of ~70% activity was observed (Figure 5.4). RP-CaMnSODc started aggregating at ~46 °C (see below), and stayed soluble in solution at 41 °C.

Wild-type and RP-mutant CaMnSODc are More Susceptible to Denaturant-Induced Protein Unfolding than Wild-type and RP-mutant ScMnSOD. In order to understand the impact of the quaternary structure and the dimer interface on MnSOD stability, we used CD
Figure 5.4. RP-CaMnSODc is inactivation by heat like EcMnSOD. The enzymes were EcMnSOD (grey rectangle), wild-type ScMnSOD (solid triangle), wild-type CaMnSODc (solid circle), K182R, A183P ScMnSOD (hollow triangle) and K184R, L185P CaMnSODc (hollow circle). The data points indicated with an arrow were obtained before the sample solution reached the desired temperature. All other data points were obtained after the sample solution was equilibrated to the desired temperature. The sample solutions contained 1 μM (in Mn) MnSOD in 10 mM potassium phosphate (pH 7), 10 mM sodium formate and 10 μM EDTA.
Figure 5.5. RP-CaMnSODc is more subject to GdHCl-induced unfolding than the wild type. The molar CD at 224 nm was used to monitor changes in α-helical structure content as a function of [GdHCl]. The enzymes were wild-type CaMnSODc (solid circles), K184R, L185P CaMnSODc (hollow circle), wild-type ScMnSOD (solid triangle) and K182R, A183P ScMnSOD (hollow triangle). The sample solutions contained 0.2 mg/mL (monomer concentration) MnSOD in 25 mM potassium phosphate (pH 7.4).
spectroscopy to monitor the denaturant-induced unfolding transitions of wild-type and RP-mutant yeast MnSODs. The molar CD at 224 nm was used to monitor changes in α-helical structure content as a function of [GdHCl] (Figure S5.6). In these experimental conditions, ScMnSOD is a tetramer while CaMnSODc is a dimer. The sharp decrease in helical structure content occurred at ~3.4 M GdHCl in ScMnSOD and ~1.6 M GdHCl in CaMnSODc (Figure 5.5). The unfolding of RP-CaMnSODc occurred at ~1.2 M GdHCl and proceeded considerably faster (pK ~ 1.7 M) than that of wild-type CaMnSODc (pK ~2.4 M) (Figure 5.5). By contrast, the unfolding profiles of wild-type and RP-mutant ScMnSOD are comparable to each other with a pK of ~4.2 and ~4.0 M, respectively (Figure 5.5).

**Wild-type and RP-mutant CaMnSODc are Significantly Less Thermal Stable than Wild-type and RP-mutant ScMnSOD.** To investigate the impact of the quaternary structure and the substitutions at the dimer interface on MnSOD thermostability, we monitored the unfolding transitions of wild-type and RP-mutant yeast MnSODs by DSC (Experimental Procedures). The wild-type and mutant proteins are both partially loaded with Mn and rest predominantly in the reduced (2+) state. The DSC profile of as-isolated ScMnSOD showed a single transition at 91 °C, corresponding to one irreversible process (Figure 5.6A). This suggests cooperativity in the melting of apo and metallated subunits in ScMnSOD. By contrast, three endotherms with much lower T_m's were observed upon heat treatment of as-isolated CaMnSODc, assigned as Therm 1, 2 and 3 (Figure 5.6B, Table 5.1). Therm 1 corresponded to the cooperative melting of apo- and Mn^{2+}-containing CaMnSODc. Due to the fact that Therm 3 was missing in the thermal stability profile of reduced CaMnSODc (Figure 5.6B), it correlated with the aggregation of Mn^{3+}-containing CaMnSODc. Therm 2 likely resulted from a small portion (~5%) of Fe-containing SOD.
Table 5.1. Thermodynamic Parameters for Unfolding of Yeast MnSODs

<table>
<thead>
<tr>
<th>Component Number</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H^a$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>As-isolated $Sc$MnSOD$^b$</td>
<td>91.0</td>
<td>705</td>
</tr>
<tr>
<td>Oxidized $Sc$MnSOD$^b$</td>
<td>91.5</td>
<td>659</td>
</tr>
<tr>
<td>As-isolated $Ca$MnSOD$^c$</td>
<td>1</td>
<td>60.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>66.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>69.7</td>
</tr>
<tr>
<td>Reduced $Ca$MnSOD$^c$</td>
<td>1</td>
<td>60.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>64.5</td>
</tr>
<tr>
<td>Oxidized $Ca$MnSOD$^c$</td>
<td>4</td>
<td>66.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>72.9</td>
</tr>
<tr>
<td>As-isolated $K182R, A183P$ $Sc$MnSOD$^c$</td>
<td>84.4</td>
<td>563</td>
</tr>
<tr>
<td>As-isolated $K184R, L185P$ $Ca$MnSOD$^c$</td>
<td>1</td>
<td>57.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>62.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>66.2</td>
</tr>
</tbody>
</table>

$^a$ $\Delta H$ are given per mole of tetramer or dimer. $^b$ The peaks of the DSC profile were deconvoluted using an irreversible two-state model. $^c$ The peaks of the DSC profile were deconvoluted using a reversible non-two-state model.
Figure 5.6. Thermostability of wild-type and RP-mutant ScMnSOD and CaMnSODc. (A) Unfolding transitions (black) of as-isolated (a) and oxidized (b) ScMnSOD and as-isolated K182R, A183P ScMnSOD (c). (B) Unfolding transitions (black) of as-isolated (a), reduced (b) and oxidized (c) CaMnSODc and as-isolated K184R, L185P CaMnSODc (d). The components (gray) were deconvoluted using a two-state irreversible model for wild-type ScMnSOD and a non-two-state reversible model for RP-ScMnSOD, and wild-type and RP-CaMnSODc. Reduced or oxidized enzymes were prepared by adding sodium hyposulfite or potassium permanganate to the sample solution prior to the DSC scan.
The stability profile of yeast MnSODs is affected by the protein oxidation state. Chemically oxidized (3+) CaMnSODc gave a wide therm (4) prior to the emergence of Therm 3. To explore the origin of Therm 4, oxidized CaMnSODc was heated at 65 °C for 20 min, and the metal content of the resulting supernatant was determined by ICP-MS. Because ~90% of the proteins in the supernatant contained Mn, Therm 4 was likely associated with the aggregation of apo subunits in oxidized CaMnSODc. The separation of apoprotein endotherm from holoprotein endotherm was subtle in oxidized ScMnSOD, although its main transition occurred at a lower temperature (~75 °C) than the as-isolated protein (~80 °C) and could well be deconvoluted to two irreversible processes (Figure 5.6A).

The as-isolated RP-ScMnSOD gave a single endotherm like the wild-type enzyme, with the $T_m$ lowered to 84 °C (Figure 5.6A). By contrast, the DSC profile of the as-isolated RP-CaMnSODc deviated more dramatically from that of wild-type CaMnSODc, in that the transition of the melting of apo-RP-CaMnSODc occurred at a lower $T_m$ (57 °C) and was separated from that of the melting of holo-RP-CaMnSODc (Figure 5.6B). The mutant protein maintained the two therms at 62 and 66 °C, corresponding to the aggregation of Mn$^{2+}$-containing and Fe-substituted CaMnSODc, respectively (Figure 5.6B, Table 5.1).

**Discussion**

_Tetramerization Stabilizes the Highly Disordered N-terminus of Dimeric CaMnSODc_– The two yeast MnSODs, one from _S. cerevisiae_ mitochondria and the other from _C. albicans_ cytosol, share ~70% sequence similarity. ScMnSOD is always a homotetramer like human MnSOD (2,16). To our surprise, CaMnSODc is a dimer in solution (Figure 5.2B), and it
is either a dimer or a tetramer when crystallized (Figure 5.1B, 5.1D). The only other experiment that suggests CaMnSODc as a tetrameric enzyme is analytical ultracentrifugation, when performed at 30,000 rpm for 24 hrs with a protein concentration of 2 mg/mL (data not shown). Tetrameric MnSODs are in equilibrium between dimers and tetramers, and this kind of equilibrium is usually dependent on factors such as ionic strength, temperature, pH, and concentration of denaturant (8,10). Our results suggest that the tetramer-dimer equilibrium lies toward dimers in CaMnSODc, while it lies toward tetramers in other tetrameric MnSODs.

The monomer of CaMnSODc and ScMnSOD resembles each other, in terms of the well-defined N-terminal α-helix-based hairpin (Figure 5.1C), which has been proposed a critical role in tetramer formation (1,5). However, electron density breaks were observed in the crystal structures of CaMnSODc in the dimeric form. They occur in two loop regions, and most dramatically in the N-terminal helixes, where 20 residues lack electron density (Figure 5.1D). These findings lead to the conclusion that the highly disordered N-terminal region hinders CaMnSODc from forming a tetramer in solution.

The N-terminal helixes are expected to be largely solvent accessible when CaMnSODc is dimeric; upon tetramer formation, these helixes are extensively involved in noncovalent interactions. Examination of the crystal structure of CaMnSODc in the tetrameric form reveals the stabilizing effects of the noncovalent forces at the tetramer interface. Like in ScMnSOD, the tetramerization domain in CaMnSODc contains several hydrogen bonds, between the two N-terminal helical hairpins, and between one helical hairpin and the loop connecting β2 and β3 (Figure 5.7). These hydrogen-bonding interactions, together with hydrophobic and salt bridge interactions, reduce the structural disorder of the N-terminus in tetrameric CaMnSODc, and
Figure 5.7. Tetramer interface of wild-type ScMnSOD (A) and CaMnSODc (B) with residues that form hydrogen bonds shown in sticks. The asymmetric unit of ScMnSOD has four chains, and here Chain B and C are colored in green and pink, respectively. The asymmetric unit of CaMnSODc has one chain (blue); the other chain (orange) in the figure was generated by a $C_2$ symmetry operation. Hydrogen bonds across the tetramer interface are shown in dashes.
likely restrain the overall mobility of the polypeptide, leading to the well-defined electron density throughout the entire sequence.

Because the helical hairpins contain two of the active site ligands, it was believed that the tetramer interface played a role in stabilizing the helical hairpins, and that only tetrameric enzymes would be active and stable (1). The I58T (a tetramer interface residue) variant of human MnSOD has a much shorter half life than the wild type at increased temperatures (31). Numerous other dihedral tetrameric proteins and enzymes, such as malic enzyme, chaperone SecB and the RUNX1/ETO fusion protein, display impaired function, when dissociating into dimers or monomers (32-34). CaMnSODc in our kinetics studies was in the dimeric form, since the compositions of the sample solutions in these studies were comparable to those used for determining the oligomer state of CaMnSODc (Figure 5.2B, Experimental Procedures). However, the catalytic properties of dimeric CaMnSODc resemble those of tetrameric ScMnSOD without any indication of activity loss (16). If the subunit of human and E. coli MnSOD is assigned as “tetrameric” and “dimeric” subunit, respectively, our studies presents the first MnSOD, which is comprised of “tetrameric” subunits while its functionality is not dependent on tetramer formation. This phenomenon has also been reported for Aristolochene Synthase from Aspergillus terreus, which functions as a dimer in solution, but is capable for tetramerization at high enzyme concentrations (35).

It is doubtful that tetrameric CaMnSODc significantly surpasses dimeric CaMnSODc in reactivity, because the latter is already near diffusion-controlled (16). Dimeric CaMnSODc could be the active form that functions in vivo. However, since it is difficult to compare the composition of C. albicans cytosol to the in vitro conditions, we are unable to elucidate the in vivo structure of CaMnSODc.
**Functional Significance of the Tetramer Structure**—Although CaMnSODc is indistinguishable from ScMnSOD in terms of enzyme kinetics, spectroscopy and redox properties (16), dimeric CaMnSODc is considerably less stable than tetrameric ScMnSOD. Compared to tetrameric ScMnSOD, the unfolding of dimeric CaMnSODc occurs at a much lower level of denaturant (GdHCl) (Figure 5), and the $T_m$ of the main components of dimeric CaMnSODc is lower by 30 °C. These facts suggest the important role of the tetramerization domain in enhancing the MnSOD enzyme’s resistance against unfolding in harsh environments.

Several studies suggest a significant role for the dimer interface in both catalysis and stability of MnSOD. Replacement of Glu170 in EcMnSOD, which spans the dimer interface and forms the double glutamate bridge, results in dissociation of the dimer, complete loss of catalytic activity, and a change in metal specificity (36). Substitutions at Glu162 in human MnSOD, the counterpart of Glu170 in EcMnSOD, reduce the catalytic activity to 5–25% of that of the wild-type enzyme (37). The Y166F mutant human MnSOD shows a significant decrease in catalytic activity and a major unfolding transition at a lower $T_m$ (38). Replacements of Phe66 at the dimer interface of human MnSOD reduces the degree of product inhibition in the human enzyme and makes it resemble EcMnSOD (12).

Here, two residues (Lys182 and Ala183 in ScMnSOD, Lys184 and Leu185 in CaMnSODc) were substituted at the dimer interface of the two yeast MnSODs. We show here that the two RP-mutant proteins resemble their wild-type enzymes in terms of SOD activity at room temperature and neutral pH (Figure S5.5), and the role of the two residues appear to be related to protein stability.
Even though one is a tetramer and the other is a dimer in solution, ScMnSOD and CaMnSODc, have >90% sequence similarity at the dimer interface and share most biochemical and biophysical characteristics. The mutations do not result in considerable perturbation in the subunit structure of the two yeast enzymes (Figure S5.4). Nevertheless, the dimer dissociation constant, $K_d$, which is too low to measure in wild-type CaMnSODc, significantly increases to 2.0 ± 0.1 μM in the mutant protein. RP-mutant CaMnSODc also becomes more sensitive to high pH than the wild type, and this inactivation by increased pH becomes completely irreversible (Figure 5.3). In contrast to wild-type CaMnSODc, the RP-mutant protein exhibits loss of activity at high temperatures (Figure 5.4), and it is more subject to unfolding by GdHCl, with the pK of the unfolding profile decreasing by 0.7 M (Figure 5.5).

The destabilization of the dimer interface in RP-mutant CaMnSODc is also suggested by the DSC protein stability data. The oxidized form of CaMnSODc has a higher thermal stability than the reduced form (Figure 5.6B). The dependence of protein stability on the oxidation state was also reported in EcMnSOD, because the enzyme has a larger affinity for Mn$^{3+}$ (39). Based on the DSC data in Figure 5.6B and Table 1, the molar energy required for aggregation of each enzyme species in wild-type CaMnSODc scales as Mn$^{3+}$-containing CaMnSODc > Fe-substituted CaMnSODc > Mn$^{2+}$-containing CaMnSODc ~ apo subunits. Since as-isolated wild-type and RP-mutant CaMnSODc contain ~0.6 and ~0.5 Mn per monomer, respectively, each CaMnSODc dimer contains one metal ion. As the 1Mn-CaMnSODc dimer is heated, the apo subunits melt at 57–60°C (Table 1). The remaining manganese-bound subunits then self-associate to create the 2Mn-CaMnSODc dimer, the melting of which occurs at 65–73°C, depending on the oxidation state of the manganese (Table 1). This phenomenon has been reported for another type of SOD, copper-zinc SOD loaded with one or two zinc ions (40). In
RP-CaMnSODc, the molar energy order changes to Fe-substituted CaMnSODc > Mn$^{2+}$-containing CaMnSODc > apo subunits (Figure 5.6B, Table 1). The modifications of the dimer interface lower the energy threshold for dimer dissociation, and thus make aggregation of apo subunits occur more readily in the mutant CaMnSODc.

By contrast, the same residue substitutions at the dimer interface have much milder effects on tetrameric ScMnSOD, although wild-type ScMnSOD and CaMnSODc are similar in many ways (16). RP-mutant ScMnSOD closely resembles the wild type in crystal structure, oligomer state, and resistance to pH, heat and denaturant (Figure 5.2–5.5, S5.4). The only sign of destabilization is the slightly lower thermal stability displayed by the mutant protein relative to the wild type (Figure 5.6A).

The tetramer interface has been reported to contribute to the thermal and pH-dependent stability of numerous dihedral tetrameric enzymes, including malate hydrogenase and serine hydroxymethyltransferase (41-43). Specifically in tetrameric MnSODs, each side of the protein is encircled by one of the two 4-helix bundles at opposite ends of the dimer, which acts as a clamp, holding the dimers in place. In addition to the contribution to protein stability, our results here suggest that the dimer interface, which is critical for MnSOD activity, is strongly reinforced through tetramer formation.

Comparison of ScMnSOD to EcMnSOD, which is a dimer, also suggests the reinforcement of the dimer interface in the former. Levy et al. reported recently that the disassembly of protein homooligomers usually leads to stable subcomplexes in which the larger interface is maintained (44). In the case of ScMnSOD, because the buried surface area at the tetramer interface (>1400 Å$^2$) is much larger than that at the dimer interface (>950 Å$^2$) (Table
S2), the dimer interface is expected to dissociate first. The oxidized form of EcMnSOD, which melts at 89.5 °C (39), resembles ScMnSOD ($T_m = 91.0$ °C, Table 1) in terms of thermostability. The solvation energy ($\Delta_i G$) at the dimer interface is higher for EcMnSOD (-11.9 kcal/mol) than for ScMnSOD (-7.8 kcal/mol) (Table S2), suggesting that dimerization is more favorable in EcMnSOD. Nevertheless, EcMnSOD is completely inactivated at <70 °C, while ScMnSOD stays fully active at 75 °C, the highest temperature allowed in the kinetics measurements (Figure 5.4). The deactivation of EcMnSOD at high temperatures likely results from dissociation of the dimer structure, which does not occur in ScMnSOD under the same conditions, due to the stabilizing effects of the tetramer structure.

In conclusion, the tetramer-dimer equilibrium of CaMnSODc lies toward dimers, although its subunit contains the well-defined N-terminal helical hairpin as in other tetrameric MnSODs. This finding is likely due to the highly disordered N-terminal region in CaMnSODc, while its functionality under physiological conditions does not rely on tetramerization. The significance of the tetramer structure lies in the stabilization of protein assembly against harsh environments (heat and denaturant). More importantly, we found that the tetramer structure strongly reinforces the dimer interface. As the dimer interface is critical for MnSOD activity, the reinforcement of the dimer interface through tetramerization could be one of the reasons for MnSOD being tetramers in higher organisms.
Figure S5.1. Electrospray-ionization mass spectra of methylated RP-mutant ScMnSOD (A) and wild-type CaMnSODc (B) and their reconstructed mass distribution profiles. Ordinate units of intensity are arbitrary and the abscissa units of average molecular mass are in Daltons. The theoretical mass weight of methylated RP-ScMnSOD and wild-type CaMnSODc is 23,589 and 23,239 Da, respectively.
**Figure S5.2.** SDS-PAGE analysis of the purity of wild-type and RP-mutant ScMnSOD and CaMnSODc: 1) protein ladder (NEB # p7702s); 2) purified wild-type ScMnSOD, 3) K182R, A183P ScMnSOD, 4) wild-type CaMnSODc, and 5) K184R, L185P CaMnSODc.

**Figure S5.3.** Superimposition of the subunit of ScMnSOD (green) over that of human (pink) and *E. coli* (orange) MnSOD. The two subunits are colored in: A, green; B, cyan.
**Figure S5.4.** Comparison of crystal structures between wild-type yeast MnSODs and their RP-mutant proteins. (A and B). Superimposition of the tetramer of K182R, A183P ScMnSOD (pink) and K184R, L185P CaMnSODc (blue) onto that of wild-type ScMnSOD (green) and CaMnSODc (orange), respectively. (C and D). Superimposition of the monomer of K182R, A183P ScMnSOD (pink) and K184R, L185P CaMnSODc (blue) onto that of wild-type ScMnSOD (green) and CaMnSODc (orange), respectively. Manganese atoms are shown as purple spheres.
Figure S5.5. Decay of 43 μM O$_2^-$ catalyzed by (A) wild-type ScMnSOD (solid, a), K182R, A183P ScMnSOD (dotted, b), (B) wild-type CaMnSODc (solid, a) and K184R, L185P CaMnSODc (dotted, b). The sample for pulse radiolysis contains 10 mM phosphate (pH 7), 10 mM sodium formate and 10 μM EDTA.
Figure S5.6. CD spectra of wild-type CaMnSODc (A), K184R, L185P CaMnSODc (B), wild-type ScMnSOD (C) and K182R, A183P ScMnSOD (D) at increased concentrations of GdHCl. The solutions contained 25 mM potassium phosphate (pH 7.4). The measurements were carried out at room temperature.
Table S5.1. Interactions of Subunits at Dimer and Tetramer Interfaces in MnSODs from Different Organisms

<table>
<thead>
<tr>
<th></th>
<th>ScMnSOD</th>
<th>CaMnSODc</th>
<th>human WT</th>
<th>A. fumigatus</th>
<th>C. elegans</th>
<th>E. coli WT</th>
<th>T. Thermophilus</th>
<th>Drad WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn•••Mn (A/B, dimer, Å)</td>
<td>18.2 (0.04)</td>
<td>18.3</td>
<td>18.3</td>
<td>18.1 (0.1)</td>
<td>18.3</td>
<td>18.4 (0.01)</td>
<td>18.4</td>
<td>18.3</td>
</tr>
<tr>
<td>Mn•••Mn (A/C, tetramer, Å)</td>
<td>39.7 (0.2)</td>
<td>38.4</td>
<td>42.0</td>
<td>39.8 (0.1)</td>
<td>41.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn•••Mn (A/D, tetramer, Å)</td>
<td>38.7 (0.04)</td>
<td>38.4</td>
<td>41.0</td>
<td>38.7 (0.2)</td>
<td>40.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimer interface area (Å²)</td>
<td>956</td>
<td>840</td>
<td>844</td>
<td>883</td>
<td>864</td>
<td>858</td>
<td>878</td>
<td>906</td>
</tr>
<tr>
<td>ΔG of dimer interface (kcal/mol)</td>
<td>-7.8</td>
<td>-6.2</td>
<td>-9.6</td>
<td>-8.8</td>
<td>-10.1</td>
<td>-11.9</td>
<td>-11.8</td>
<td>-10.5</td>
</tr>
<tr>
<td>Tetramer interface area (Å²)</td>
<td>1417</td>
<td>1254</td>
<td>961</td>
<td>797</td>
<td>800</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔG of tetramer interface (kcal/mol)</td>
<td>-25.0</td>
<td>-12.6</td>
<td>-13.8</td>
<td>-14.9</td>
<td>-14.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table S5.2.** Calculation of $K_d$ for K184R, L185P CaMnSODc

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>750 nM</th>
<th>500 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Area integral (mAU×s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dimer</td>
<td>1405</td>
<td>1519</td>
</tr>
<tr>
<td>monomer</td>
<td>2939</td>
<td>3216</td>
</tr>
<tr>
<td>Concentration of Species (nM)</td>
<td>[D]</td>
<td>[M]</td>
</tr>
<tr>
<td>[D]</td>
<td>121.3</td>
<td>120.3</td>
</tr>
<tr>
<td>[M]</td>
<td>507.5</td>
<td>509.4</td>
</tr>
<tr>
<td>$K_d$</td>
<td>2.1 μM</td>
<td>2.2 μM</td>
</tr>
<tr>
<td>Average $K_d$</td>
<td>2.0 ± 0.1 μM</td>
<td></td>
</tr>
</tbody>
</table>
References


28. Schwarz, H., BNL Pulse Radiolysis Program. Brookhaven National Laboratory


ion to one subunit of copper-zinc superoxide dismutase apoprotein substantially influences the structure and stability of the entire homodimeric protein. *J Am Chem Soc* **129**, 4575-4583


