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EMERITA MENDOZA RENGIFO

**Exploring the selectivity of metal ions in the active site of the
enzyme superoxide dismutase (SOD) using site-directed
mutagenesis**

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With love,
to my parents (Genaro
and Zenaida) and my
brothers (Anibal,
Samuel and Genaro)

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ABSTRACT

MENDOZA RENGIFO, E. **Exploring the selectivity of metal ions in the active site of the enzyme superoxide dismutase (SOD) using site-directed mutagenesis.** 2016. 155 p. Dissertation (Master of Science) – Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, 2016.

Iron/Manganese superoxide dismutases (Fe/Mn-SODs) are metalloenzymes with highly conserved protein folds, active sites, and dimer interfaces. They protect cells against oxidative stress by catalyzing the conversion of the cytotoxic free radical superoxide to molecular oxygen and hydrogen peroxide. The majority are highly specific for the type of metal (iron or manganese) present within the active site. However, there are many key aspects of metal specificity and catalytic activity that lack a structural explanation. Computational analyses suggested that several residues are important for fine-tuning the redox potential of the metal in the active site and thereby the catalytic activity. The main objective of this thesis is to evaluate the influence of several point mutations (M27V, G73A, H75I, L80F, D150G and Q172D) and one double mutation (Q149G+G74Q)) in terms of metal specificity, catalytic activity and three-dimensional structure using the superoxide dismutase from *Trichoderma reesei* (TrSOD) as a model system. The corresponding genes were cloned, expressed and the resulting proteins characterized by X-ray crystallography, electron paramagnetic resonance (EPR), atomic absorption spectroscopy (AAS), dynamic light scattering (DLS) and their enzymatic activity determined. The native protein was shown to be able to use either Mn or Fe (5000 units/mg and 500 units/mg, respectively) for catalysis suggesting it to be properly classified as cambialistic. Structures for native TrSOD and the Mn-G73A, Fe-H75I, Mn-L80F, Fe-D150G and Fe-M27V, Mn-M27V mutants were solved at 2.3 Å, 2.0 Å, 2.03 Å, 2.0 Å, 1.85 Å, 1.4 Å and 1.6 Å resolution, respectively. The H75I, L80F and M27V mutations are easily accommodated by small local structural changes to the three-dimensional structure. On the other hand, the G73A mutation destabilize one of the dimer-dimer interfaces of the tetramer making it possible for two distorted tetramers to interact forming an octamer. This enzyme also lost all catalytic activity probably due to resulting exposure of the active site consistent with the observation of a sixth ligand (solvent molecule) bound to the metal in one subunit. The D150G mutant remained tetrameric but with reduced symmetry related to the rearrangement of the last helix (H9). Our results show that a large impact on activity and oligomerization of TrSOD can be

generated by a single amino acids substitution in some cases and provide some insights into our understanding of the structural details associated with the metal ion specificity and oligomerization in superoxide dismutases.

Keywords: Superoxide dismutase. Crystallography. Enzymatic activity. Catalytic metals.

RESUMO

MENDOZA RENGIFO, E. **Explorando a seletividade por íons metálicos no sítio ativo da enzima superóxido dismutase (SOD) usando mutagênese sítio dirigida**. 2016. 155 p. Dissertação (Mestrado em Ciências) – Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, 2016.

Superóxido dismutases de ferro e manganês (Fe/Mn-SODs) são metaloenzimas com enovelamentos, sítios ativos e interfaces diméricas altamente conservados. Estas enzimas protegem as células contra o estresse oxidativo pela conversão do ânion superóxido em oxigênio molecular e peróxido de hidrogênio. A maioria são altamente específicas pelo tipo de metal (ferro ou manganês) presente no sítio ativo. Entretanto, existem vários aspectos críticos sobre a especificidade pelo metal e da atividade catalítica que ainda não foram explicados em termos estruturais. Análises computacionais sugerem que vários resíduos são importantes para o ajuste do potencial redox do metal no sítio ativo e, portanto, a atividade catalítica. O objetivo principal deste trabalho é avaliar a influência de mutações simples (TrSOD) (M27V, G73A, H75I, L80F, D150G e Q172D) e dupla (Q149G + G74Q) em superóxido dismutases de *Trichoderma reesei* em termos de especificidade pelo metal, atividade catalítica e estrutura. Os genes correspondentes foram clonados, expressos e as proteínas resultantes caracterizadas por cristalografia de raios-X, ressonância paramagnética eletrônica (EPR), espectroscopia de absorção atômica (AAS), dispersão de luz dinâmica (DLS), e a atividade enzimática foi determinada. Foi mostrado que a proteína nativa é capaz de usar tanto Mn quanto Fe (5000units/mg e 500units/mg, respectivamente) para catálise sugerindo que deveria ser classificada como enzima cambialística. Estruturas da enzima nativa e mutantes (Mn-G73A, Fe-H75I, Mn-L80F, Fe-D150G, Fe-M27V e Mn-M27V) foram resolvidas a resoluções de 2.3 Å, 2.0 Å, 2.03 Å, 2.0 Å, 1.85 Å, 1.4 Å e 1.6 Å respectivamente. As mutações H75I, L80F e M27V são acomodadas facilmente por reajustes locais na estrutura tridimensional. Por outro lado, a mutação G73A desestabiliza uma das interfaces dímero-dímero do tetrâmero levando à formação de um octâmero feito por dois tetrâmeros distorcidos. Esta enzima também perde atividade provavelmente devido a um aumento na acessibilidade do sítio ativo, coerente com a observação de um sexto ligante (molécula de solvente) coordenando o metal em uma das subunidades. O mutante D150G continuou tetramérica mas com simetria reduzida relacionado com o rearranjo da última

hélice (H9). Estes resultados mostram que, em alguns casos, uma mutação simples pode ter um impacto significativo no estado oligomérico e atividade catalítica da proteína TrSOD e fornece conhecimentos para a nossa compreensão dos detalhes estruturais associados com a especificidade de íons metálicos e oligomerização em superóxido dismutases.

Palavras-chave: Superóxido dismutases. Cristalografia. Atividade enzimática. Metais catalíticas

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LIST OF ABBREVIATIONS AND ACRONYMS

α -helix	secondary structure element of proteins
β -sheet	secondary structure of proteins
AAS	Atomic absorption spectroscopy
D150G	Replacement of Asp150 with Gly
DO	Optic density
EPR	Electron paramagnetic resonance
G73A	Replacement of Gly73 with Ala
H75I	Replacement of His75 with Ile
IPTG	Isopropyl-B-D-thiogalactoside
L80F	Replacement of Leu80 with Phe
LB	Luria broth, media for bacterial growth
M	moles/liter, a measure of concentration
MR	molecular replacement
NCS	non-crystallographic symmetry
M27V	Replacement of Met27 with Val
PCR	polymerase chain reaction
PEG	polyethylene glycol
PDB	protein data bank
pH	activity of the hydrogen ion, measure of acidity.
PK	disassociation constant of proton or hydroxide.
Q172D	Replacement of Gln172 with Asp
Q149G+G74Q	Replacement of Gln149 with Gly + Gly74 with Gln
Redox	reduction/oxidation
RMSD	root-means square deviation

ROS	reactive oxygen species.
SOD	superoxide dismutase
SD	Standard Deviation
TLS	translation-libration-screw

LIST OF SYMBOLS

°C	Celsius degrees
Å	Angstrom
nm	Nanometer
L	Liter
kDa	Kilodalton
mV	Millivolts
RPM	revolutions per minute
mM	Milimolar
nm	Nanometer
µL	Microlitre

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1 INTRODUCTION

1.1 Reactive Oxygen Species

Reactive oxygen species (ROS) are inevitable bi-products of aerobic metabolism.^{1–3} ROS are highly reactive molecules that have been proposed to be responsible for oxidative damage to cells.^{4–6} These molecules have been correlated with various disease states such as diabetes, atherosclerosis, Parkinson's diseases, and Alzheimer's diseases as well as Amyotrophic Lateral Sclerosis.^{6–8} One possible explanation for this correlation is that ROS are able to oxidize DNA, RNA, proteins and lipids impeding a cell from performing its basic functions. As such, living organisms have developed various defenses to protect against ROS damage.⁹ Superoxide dismutase (SOD, IC 1.15.1.1) is one of the key enzymes in the cell that performs this function.^{10–15}

1.2 Superoxide Dismutases

Superoxide dismutases^{16–18} constitute the first line of cellular defense against oxidative stress arising from reactive oxygen species produced during aerobic respiration.^{19–22} In order to protect the cells, these metalloenzymes catalyze the conversion of superoxide radicals ($O_2^{\cdot-}$) to produce hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) through alternate oxidation and reduction of their metal cofactor (M).²³ The overall dismutation reaction of superoxide is shown in equations (1) and (2) and the mechanism of SOD has been studied for several decades.^{9,11,24–25} The rate constant for these enzymes corresponds to that of the diffusion limit ($k_{cat} = 0.3-5.3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) determined by Löffler.²⁶

Based on the catalytic metal ion, sequence homology and protein fold, SODs are classified²⁷ into three groups: copper/zinc SOD (Cu/ZnSOD),²⁸ nickel SOD (NiSOD)^{29–31} and manganese/iron SODs (MnSOD/FeSOD). Within the last of these groups, there are two subgroups: enzymes that are metal specific (i.e., only work with one metal – e.g., MnSOD^{32–34} which is inactive when Fe and occupies the metal center

FeSOD³⁵⁻³⁷ which is inactive when Mn occupies the metal center) and the so-called cambialistic³⁸⁻³⁹ SODs that are able to function with either Fe or Mn ions according to environmental availability. These cambialistic enzymes remain catalytically active when binding either metal.

Table 1 - Distribution, location and metal coordination of superoxide dismutases.

Enzyme	Organisms	Metal oxidation states	Metal coordination environment
FeSOD	Archaea: cytosol (tetramer);	Fe^{III}	5-coordinate, trigonal bipyramidal, 3 His, 1 Asp, 1 OH ⁻
Fe	Bacteria: cytosol (dimer or tetramer) and Eukarya: cytosol, glycosomes, mitochondria (tetramer)	Fe^{II}	5-coordinate, trigonal bipyramidal, 3 His, 1 Asp, and 1 H ₂ O
MnSOD	Archaea: cytosol (tetramer);	Mn^{III}	5-coordinate, trigonal bipyramidal, 3 His, 1 Asp, 1 OH ⁻
Mn	Bacteria: cytosol (dimer or tetramer) and Eukarya: mitochondrial matrix (tetramer) cytosol (dimer) peroxisomes (tetramer) chloroplasts	Mn^{II}	5-coordinate, trigonal bipyramidal, 3 His, 1 Asp, 1 H ₂ O
Cu/Zn SOD	Bacteria: periplasm-(monomer or dimer) and Eukarya: cytosol (dimer)	Cu^{II}	4-coordinate, distorted square planar, 4 His
Cu	mitochondrial IMS, nucleus (dimer)	Cu^I	3-coordinate, trigonal planar, 3 His
Zn	chloroplasts, peroxisomes (dimer) extracellular space (tetramer)	Zn^{II}	4-coordinate, distorted tetrahedral, 3 His and 1 Asp
NiSOD	Bacteria: cytosol (hexamer) and	Ni^{III}	5-coordinate, square pyramidal, 1 His, 2 Cys, backbone amide, N-terminal -NH ₂
Ni	Eukarya: cytosol (unknown)	Ni^{II}	4-coordinate, square planar, 2 Cys, backbone amide, N-terminal -NH ₂

Source: By the author.

The existence of multiple SODs is essential to protect the multiple cell compartments against oxidative stress. Interestingly, SOD speciation appears to correlate with the Earth's evolution.⁴⁰ FeSODs are the most ancient of the SODs and probably first appeared when Fe was relatively abundant near the beginning of the formation of the earth. MnSODs and NiSODs appeared later when the levels of O₂ in the environment increased and the amount of Fe(II) in the environment decreased.⁴⁰⁻

The last enzyme to appear was CuZnSOD which arose later after copper became bioavailable (in the Cu^{2+} form) in the more oxidizing environment the Earth's atmosphere.⁴¹ SODs are ubiquitous with a widespread distribution in multiple forms within a single organism or cell, as shown in Table 1. Although the FeSODs and MnSODs are closely related, sharing a high degree of amino acid sequence and structural identities,^{11,37,42} they do not have any structural similarity with the Cu/ZnSODs or NiSODs (represented in the Figure 1).

Figure 1 - A comparison of the enzyme structures for the four SODs. (A) *E. coli* MnSOD (PDB ref.: 1VEW), (B) *E. coli* FeSOD (PDB ref.: 1ISA), (C) human Cu/ZnSOD (PDB ref.: 1PU0) and (D) *Streptomyces coelicolor* NiSOD (PDB ref.: 1T6U). The metal ion is colored in red. The figures were generated using the PyMOL Molecular Graphics System.

Source: By the author.

1.3 Fe and Mn Superoxide Dismutases

Our research interest is primarily the Fe/MnSOD family, which through minor variations in amino-acid sequence achieves a wide variation in metal specificity, catalytic activity, inhibitor specificity and oligomerization states.

These enzymes are typically found as homodimers or homotetramers comprised of ~23 kDa identical subunits. Each subunit is composed of two domains: an α -helical

N-terminal domain and a mixed α/β C-terminal domain.^{43–46} The subunits have a single metal ion in the active site. The metal center is bound by equivalent residues, coordinated in trigonal bipyramidal geometry; two histidines and an aspartate residue occupy the equatorial plane with a histidine residue and a solvent molecule (water or hydroxide) occupying the axial positions.⁴³

Even though Mn and Fe SODs have a high similarity^{37,47–49} in the primary, secondary and tertiary structure (Figure 2), most of these enzymes cannot function without the correct metal present at the active site.⁵⁰

Figure 2 – (A) Overlay of the protein subunits of *E. coli* FeSOD (yellow) and MnSOD (green). B) Overlay of the active site structures of FeSOD (yellow) and MnSOD (green). The metal ion is colored in red. Structures were taken from PDB files 1ISA and 1VEW for FeSOD and MnSOD, respectively and pictures were created with PyMOL.

Source: By the author.

The active sites of both FeSOD and MnSOD are capable of binding both Fe and Mn (by erroneous incorporation during folding or by concentration dependent ion exchange)⁵¹ with very little change in either coordination geometries or electronic structures; however, in most of the known cases, incorporation of the wrong metal results in loss of catalytic activity indicating a high selectivity for the associated metal.^{50,52–53} Several different explanations for this extraordinary specificity have been proposed to arise from different phenomena: active site distortions upon binding of the non-native metal ion, increased anion affinity of Fe-substituted MnSOD and the tuning of the metal ion redox potential.^{54–55} (In what follows, Mn(Fe)SOD indicates Mn-substituted FeSOD and Fe(Mn)SOD indicates Fe-substituted MnSOD).

1.4 Redox Potential tuning in Fe- and MnSODs

The ideal reduction midpoint potential (E_m) for a SOD is near 360 mV, which is half-way between the E_m of SOD's two half-reactions.⁵⁶ However, catalytic activity is still retained within a reasonably wide range around the ideal value. The aqueous metal ions which bind SOD each have their own E_m shown in equations (3) and (4). High spin $Fe^{3+/2+}$ has a considerably lower reduction midpoint than does $Mn^{3+/2+}$.^{57–60} Consequently, the FeSOD protein does not need to lower the midpoint potential of its bound metal ion as much as the MnSOD protein. E_m 's of FeSOD (~100 mV)⁶¹ and MnSOD (~300 mV)⁶² are both intermediate between the E_m 's of SOD's two half-reactions allowing these enzymes to have catalytic activity (shown in Figure 3).

Miller⁵⁴ (Figure 3) found that Fe(Mn)SOD has a much lower reduction midpoint potential (E_m -250 mV at pH 7.8) than Mn(Fe)SOD (E_m > 960 mV at pH 7.8) from *E. coli*, these are too low and too high, respectively to be catalytically active.^{54, 62} This suggests that the lack of SOD activity of the metal-substituted enzymes stems from their E_m 's lying outside the range required for catalyzing both half-reactions.^{58,63-64} Unlike these enzymes, cambialistic SODs could exhibit intermediate redox tuning, which would enable either metal ion to function. These large differences in tuning effect of both enzymes may correspond to differences in the residues present in the second coordination sphere and may therefore play a role in maintaining the integrity of the metal specificity of SODs.^{53, 64}

Figure 3 - Reduction potentials of Fe- specific SOD protein (blue box) and the Mn-specific SOD protein (orange box) upon Fe (blue star) and Mn (orange star). Because of the higher reduction potential of Mn^{+3} than Fe^{+3} , the Mn-SOD protein should depress the reduction potentials of its bound metal much more than Fe-SOD.

Source: Adapted from MILLER.^{53,64}

For example, the residues Gln69 and Ala141 (FeSOD of *E. coli*) are described as specific for enzymes that incorporate Fe. For MnSODs, the corresponding residues are Gly and Gln, respectively.⁶⁵ In both cases, the Gln residue (occasionally His) is responsible for hydrogen bonding to the coordinated solvent molecule in the active site and also plays a crucial role in optimally tuning the redox potential⁶⁶ while also preventing dissociation of coordinated solvent upon binding of substrate (analogues), possibly avoiding the detrimental formation of long-lived reaction intermediates such as side-on bound peroxo species.⁶⁷

In FeSODs, the glutamine is at position 69 and arises from the N-terminal half of the molecule; the analogous glutamine of MnSODs is at position 146 and arises from the C-terminal region (numbering is based on the FeSOD and MnSOD of *E. coli*).⁶⁸⁻⁶⁹ This difference in the positioning of this residue has a profound effect on the electronic structure of the metal center.⁷⁰⁻⁷¹

Mutational studies^{72–77} were performed on residues that are responsible for fine tuning the redox potential of the active site of SODs. Edwards⁶⁹ presented a conversion of the metal-specific activity of *E. coli* Mn-SOD into a metal-tolerant type by the mutation of Glu146 to His146, although the enzyme activity for both metals was less than one tenth that of the wild-type Mn-SOD. They also found that mutation of Glu146 caused a rearrangement of the hydrogen-bonding network of the active site.⁷⁸ Further evidence comes from site-directed mutagenesis studies of the cambialistic SOD from *Porphyromonas gingivalis* in which the dual metal specificity was converted to Fe-specificity by substitution of Gly155 by Thr.⁷⁹ This residue, which is located away from the active site, is an essential residue for maintaining the hydrogen-bonding network necessary for Mn-based activity.

1.5 Computational analysis

There are three computational^{63, 80–81} methods that have been used to identify residues and characterize metal specificity and/or oligomerization in the Fe/MnSOD family as a whole.

The first method, which was developed by Wintjens^{82–83} is based on residue fingerprints, consisting of residues conserved in SOD sequences or typical of SOD subgroups, and of interaction fingerprints, containing residue pairs that are in contact in SOD structures. The identified residues were derived from a set of aligned SOD sequences and structures. The second method applied by Bleicher⁸⁴ is based on correlated mutations and network analyses to calculate and analyze groups of amino acids which are related to functional classes in protein families. The third method, (Statistical Coupling Analysis) performed by Bachega^{85–86} based on a large multiple sequence alignment, provides a means to examine coevolving and structurally coupled residues which might be characteristic for a given metal, and necessary for the fine-tuning of the active site.

In the first method, “residue fingerprints” correspond to a selected ensemble of positions. Using these “residue fingerprints”, an online tool is used to predict whether or not a sequence is a Fe/Mn SOD; if a sequence is determined to be a Fe/Mn SOD, the program then predicts the metal ion specificity and oligomeric state. The numbering of *Escherichia coli* SOD corresponding to protein data bank code 1ISA³⁷ was used as the basis for the alignments and these residues are presented in Table 2.

Table 2 - Strongly conserved and specific residues found in SODs. These residues are classified as residue pair interactions that have contact with other important residues in SOD proteins and are presented in Fe- and Mn-specific SODs.

Oligomeric state	Position *1ISA	metal ion	Position *1ISA	Metal ion and oligomeric state	Position *1ISA
Dimeric	T22	Fe	D165	Fe_Dimers	Q69, A141, F64, A68 and F75
	F118 N65 P144			Fe_Tetramers	L72 and H141
Tetrameric	Q165 I22 F65	Mn	Q141, G68, D142, M23 and V180	Mn_Dimers Mn_Tetramers	D19, S137 and R64 P143 and V169

Source: By the author.

The result of the second method, correlation analyses applied to Fe/MnSODs, is represented by seven communities (five groups and two individual residues). Community 1 consists of six residues found in MnSODs, while community 3 consists of five residues found in FeSODs.

Each of those communities has specific residues with high internal correlation within the community and high anti-correlation between communities because the residues are specific to each type of enzyme. Community 2 consists of dimeric SODs and does not present correlation or anticorrelation with other communities, demonstrating that metal selectivity and oligomeric state are independent properties⁸⁴ (Table 3). Communities 4 and 5 report contact pairs and the isolated residues 6 and 7 were reported as related to communities 1 and 3 but they lacked positive links to these two communities for the thresholds used.

Table 3 - Position of residues in SODs that are highly conserved and are representative of the seven communities according to the results presented by Bleicher⁸⁵

Communities	Position 3ESF	Features
1	H73, G73, G71, Q145, M25 and D146	Mn- binding the active site
2	T142, F121, P148, N144, T24 and N68	Dimeric SODs
3	D170, Q72, F78, A71 and F67	Fe- binding the active site
4	F109 and F113	Contact pairs
5	R175 and V162	Contact pairs
6	D21	Present anti-correlation
7	G89	

Source: By the author.

Statistical Coupling Analysis⁸⁷ performed on results from Winkjens, et al.⁸³ and Bleicher et al.⁸⁴ yielded other specific amino acids which tend to be present

simultaneously. This method was applied to a family of Fe/MnSODs in order to find the possible relationship between statistically coupled sequence positions of two sites (i and j). The degree to which i and j were statistically coupled was defined as the degree to which amino acid frequencies at site i change in response to a perturbation of frequencies at site, j.⁸⁵ Based on this analysis, three distinct clusters of residues related to positions were identified. Importantly, these three clusters had already been described as determinants of metal selectivity (shown in Table 4) which serves to validate the approach.

Table 4 - Statistical Coupling Analysis (SCA) in a protein family Fe/MnSOD. Group 1 corresponds to residues associated with the dimeric state, group 2-Mn with MnSODs and group 3-Fe with FeSODs. (Position of amino acids based on 3ESF)

Groups/ association	Cluster 1	Cluster 2	Cluster 3
Oligomerization state: dimers Fe-SOD	E56, N68, T24, F121 and P148	A70, W74, F78, A71, A145, F67, Q72, D170	
Mn-SOD			Q145, G72, D146, M25, A43, G71 and G159

Source: Adapted from BACHEGA.⁸⁴

Comparing the residues reported by the three methods (Table 4) we observe an overall good agreement among them. These results revealed that MnSODs and the oligomeric state (dimeric SODs) have four specific residues which are characteristic for each group while FeSODs have five.

Table 5 - Comparison of the results given by the three different methods. Residues that matched on the reported methods are highlighted in red.

Authors Metal ion/oligomeric state	Method 1 ^a 1ISA	Method 2 ^b 3ESF	Method 3 ^b 3ESF
MnSODs	Q141	Q145	Q145
	G68	G72	G72
	D142	D146	D146
	M23	M25	M25
	V180	---	---
	----	G71	G71
	----	H73	----
	----	----	A43
	---	---	G159
FeSODs	Q69	Q72	Q72
	A141	--	A145
	F64	F67	F67
	A68	A71	A71
	F75	F78	F78
	D165	D170	D170
	--	--	W74
	---	---	A70
Dimers	T22	T24	T24
	F118	F121	F121
	N65	N68	N68
	P144	P148	P148
	---	T142	---
	---	N144	---
	----	---	E56

^aThe 1ISA numbering is used corresponding to the SOD of *Escherichia coli*.

^bThe 3ESF numbering is used corresponding to the TbSODB2 of *Trypanosoma brucei*.

Source: By the author.

Some of these residues have already been mutated and characterized experimentally. For example, the point mutation Gly155Thr (Gly159 in the numbering scheme adopted on method 3), in the cambialistic MnSOD from *Porphyromonas gingivalis*^{39,79} converts it to an Fe-specific SOD, (mentioned previously) and it is part of cluster 3 (highlighted in purple) of Bachega's report (method 3). As the predictions agree with the experiments, it suggests that other members of this group may also play a role in determining metal ion specificity.

It is crucial to investigate the contribution of these residues to metal ion specificity in SODs in order to understand how the structure of the polypeptide chain influences the reduction potential and thereby the catalytic properties of the active site.

1.6 Manganese-Superoxide Dismutase (MnSOD) of *Trichoderma reesei*

Superoxide Dismutase from *T. reesei* was classified as a MnSOD⁸⁸ and is located in mitochondria, playing an important role in the antioxidant defense for the organelle. It has >80% residue identity to other SOD enzymes from filamentous fungi, and a high enzymatic activity (1000 U/mg) compared with other SODs. This enzyme was reported to be tetrameric and each monomer has an estimated molecular mass of 23 ± 0.5 kD. By homology it was inferred that the metal is bound by His30, His78, Asp163, and His167. This enzyme has been broadly studied and is very stable and retains biological activity at temperatures between 20 to 90°C for one hour at pH from 8 to 11.5 making it an ideal model for the Mn/FeSOD family.⁸⁸

Each monomer consists of 212 residues and the amino acids specific for MnSOD (M27, G73, G74, H75, Q149 and D150) reported in the previous methods, are shown in the Figure 4 (in the numbering scheme adopted on MnSOD from *Trichoderma reesei*).

1	ATGTCTGTCGGAACCTTTTCGCTGCCGGCTCTGCCGTATGCCTACGATGCCCTCGAGCCCAG	
	M S V G T F S L P A L P Y A Y D A L E P S	21
63	CATCTCGGCCAGATCATGAGCTTCACCACAGCAAGCACCACCAGACCTACGTGACCAAC	
	I S A Q I M E L H H S K H H Q T Y V T N	41
124	CTCAACAACGCCCTCAAGACCTATTCCACGGCCCTCGCCGCCAACGACGTGCCCTCCCAGA	
	L N N A L K T Y S T A L A A N D V P S Q	61
184	TCGCCCTCCAGGCCGCCATCAAATTCAACGGCGGCCGCCACATCAACCACTCCCTCTTCTG	
	I A L Q A A I K F N G G G H I N H S L F W	82
245	GGAGAACCTCTGCCCGCCTCCTCCCCGACGCGGACCCCGCCAGCGCGCCCGAGCTGAC	
	E N L C P A S S P D A D P A S A P E L T	102
305	GGCTGAGATTGCCAAGACCTGGGGCAGCCTGGACAAGTTCAAGGAGGCCATGGGCAAGGC	
	A E I A K T W G S L D K F K E A M G K A	122
365	CCTGCTCGGTATCCAGGGCAGTGGCTGGGGGTGGCTGGTCAAGGAGGGGAGTGGGTTGCG	
	L L G I Q G S G W G W L V K E G S G L R	142
425	GATCGTGACGACCAAGGATCAGGATCCGGTGGTTGGAGGAGAGGTGCCGGTTTTTGGCATT	
	I V T T K D Q D P V V G G E V P V F G I	162
486	GATATGTGGGAGCATGCCTATTACCTGCAGTATCTCAACGGCAAAGCTGCCTACGTGGACA	
	D M W E H A Y Y L Q Y L N G K A A Y V D	182
547	ACATCTGGAAGGTCATCAACTGGAAGACTGCTGAGCAGCGCTTCAAGGGCGACCGCGAGG	
	N I W K V I N W K T A E Q R F K G D R E	202
607	ACGCCTTCAAGATCCTCAAGGCTTCGCTGTAA	
	D A F K I L K A S L STOP	212

Figure 4 - Nucleotide and predicted amino acid sequences of MnSOD from *Trichoderma reesei* (TrMnSOD). Numbers on the left correspond to the nucleotide position relative to the adenine of the AUG start codon; amino acids are numbered the right. Amino acids specific for Mn-SODs are highlighted in red.

Source: By the author.

To further address the relationship between amino acid sequence and metal ion specificity, in the work described here we have chosen specific amino acid residues for substitution with the aim of experimentally investigating their effect on various aspects of SOD structure and activity. For this purpose, we have chosen TrMnSOD as a model system. The results of these studies form the basis of this dissertation.

1.7 Objectives

General:

1. To evaluate the influence of point and double mutations in superoxide dismutase from *Trichoderma reesei* (TrSOD) in terms of metal specificity, catalytic activity and structure.

Specific:

- 1) To construct point mutants (Q172D, D150G, G73A, H75I, L80F and M27V) and a double mutant (Q149A + G74Q) using Mn-SOD from *Trichoderma reesei*.
- 2) To express and purify the mutant enzymes.
- 3) To analyze the metal in the active site of the enzymes by Electron Paramagnetic Resonance (EPR).
- 4) To measure the catalytic activity of each enzyme.
- 5) To quantify the presence of metal in each enzyme by atomic absorption spectrometry
- 6) To crystallize the mutant proteins and perform diffraction X-ray experiments.
- 7) To solve and refine the structures.

2 MATERIALS AND METHODS

2.1 Rational for the selection of single and double mutants in superoxide dismutase from *T. reesei*, previously classified⁸⁸ as a Mn-SOD.

After further analysis of the three computational methods described in the previous section, we have selected eight residue positions (in good agreement with all three methods) for site-directed mutagenesis. Of the eight mutated positions, six (H75, G73, M27, D150, G74 and Q149) are highly conserved across Mn-SODs (Table 6) and two (F80 and D172) are highly conserved across Fe-SODs (Table 7). Mutations of these residues were performed on TrSOD and were chosen in attempt to elucidate the determinants of metal specificity with the initial intent being to convert TrSOD from a Mn-SOD to an Fe-SOD. For this reason, the substituted amino acid (final column, Table 6) is that which is typically observed in Fe-containing enzymes. To test the viability of a compensatory double mutant, both G74 and Q149 were simultaneously substituted so as to move a stabilizing glutamine residue from its position in a Mn-SOD to that of an Fe-SOD. This thesis stands as the initial work upon which further more complex analyses involving multiple substitutions will be performed.

Table 6 - Residue positions predicted to be specific for MnSOD and their chosen substitutions in superoxide dismutase from *Trichoderma reesei* which has previously been classified as a Mn-SOD.

Reported by ↓	Residues predicted as specific for MnSODs		Substituted amino acid
	Residue positions 1ISA (MnSOD <i>E. coli</i>) ^a	Homologous Residues (MnSOD <i>T. reesei</i>) ^b	
Method 3	H73	H75	I
Method 2 and 3	G71	G73	A
Method 1, 2 and 3	M25	M27	V
	D146	D150	G
	G72 + Q145	G74 + Q149	Q + G

^aThe 1ISA numbering is used corresponding to the SOD of *Escherichia coli*.

^bThe MnSOD from *Trichoderma reesei* is used.

Source: By the author.

Table 7 - Residue positions predicted to be specific for FeSOD and substituted amino acids by point mutations in superoxide dismutase from *Trichoderma reesei* previously classified as a Mn-SOD.

Reported by ↓	Residues predicted as specific for FeSODs	Homologous residues (MnSOD from <i>T. reesei</i>)	Substituted amino acid
	Residue positions 1ISA (FeSOD <i>E. coli</i>)		
Method 1,2 and 3	F78 D170	L80 Q172	F D

^aThe 1ISA numbering is used corresponding to the SOD of *Escherichia coli*.

^bThe MnSOD from *Trichoderma reesei* is used.

Source: By the author.

To visualize the eight positions with respect to the active site, we have highlighted the associated positions in the crystal structure of *Aspergillus fumigatus* (single monomer, PDB 1kkc) shown in Figure 5. Three mutations (G73A, H75I, L80F) are localized in helix 2 (H2) of the structure, the M27V mutation is in helix 1 (H1), D150G is localized in the loop that connects two β -stands and Q172D is found in the small helix 5 (H5) observed in one monomer of SOD from *A. fumigatus*. All selected residues are within 10 to 15 Å of the metal ion and at a linear distance of between one to five residues from a metal ion ligand.

Figure 5 - The spatial arrangement of the mutated residues (Met27, Gly73, Gly74, His75, Leu80, Gln172 and Asp150) relative to the active site, showing (A) two monomers (dimer interface) and (B) just the active site itself together with the chosen mutated residues mapped onto the structure of Mn-SOD from *Aspergillus fumigatus*. The eight mutant positions are marked in blue and green in (A) and in red in (B).

Source: By the author.

2.2 Construction of point (M27V, H75I, L80F, D150G and Q172D) mutations in Superoxide Dismutase from *Trichoderma reesei* (TrSOD) using site-directed mutagenesis.

The point mutations M27V, H75I, L80F, D150G and Q172D for TrSOD were generated by PCR-based site-directed mutagenesis. To generate these mutants, two overlapping mutant oligonucleotide primers based on the *T. reesei* SOD sequence were synthesized (Table 8).

Table 8 - Position of the mutated amino acids and oligonucleotides designed to perform site-directed mutagenesis. Nucleotides primers highlighted in red indicate the changes made to create the desired mutations.

Amino acid mutants from TrSOD	name of primers	Number of bases	Oligonucleotide primers Sequence 5'- 3'
Q172D	Q172D-F	24	GCC TAT TAC CTG GAC TAT CTC AAC
	Q172D-R	24	GTT GAG ATA GTC CAG GTA ATA GGC
D150G	D150G-F	23	CAA GGA TCA GGG TCC GGT GGT TG
	D150G-R	23	CAA CCA CCG GAC CCT GAT CCT TG
Q149G	Q149G-F	22	GAC CAA GGA TGG GGA TCC GGT G
	Q149G-R	22	CAC CGG ATC CCC ATC CTT GGT C
G74Q	G74Q-F	22	CAA CGG CGG CCA ACA CAT CAA C
	G74Q-R	22	GTT GAT GTG TTG GCC GCC GTT G
H75I	H75I-F	21	GGC GGC GGC ATC ATC AAC CAC
	H75I-R	21	GTG GTT GAT GAT GCC GCC GCC
L80F	L80F-F	22	CAA CCA CTC CTT CTT CTG GGA G
	L80F-R	22	CTC CCA GAA GAA GGA GTG GTT G
M27V	M27V-F	21	GCC CAG ATC GTG GAG CTT CAC
	M27V-R	21	GTG AAG CTC CAC GAT CTG GGC

Source: By the author.

The forward mutant primer paired with the T3 terminator universal reverse primer, and the reverse mutant primer paired with the T7 promoter universal forward primer, were used in two separate PCRs⁸⁹⁻⁹¹ using a 1.1-kb SUMO-SOD-containing fragment in pSMT3 as a template. The reaction conditions are shown in Table 9 and the amplification reaction graphically represented in Figure 6.

Table 9 - Amplification reaction conditions (PCR-1a and PCR-1b).

Amplification Reaction (PCR-1a)	Volume (μL)	Amplification Reaction (PCR-1b)	Volume (μL)
Phusion Buffer	10	Phusion Buffer	10
10mM dNTPs	1	10mM dNTPs	1
Forward T7 promotor (150ng/ μL)	1.5	Forward designed primer (150ng/ μL)	1.5
Reverse designed primer (150ng/ μL)	1.5	Reverse T3 terminator (150ng/ μL)	1.5
Template DNA	1.5	Template DNA	1.5
Phusion DNA polymerase	0.5	Phusion DNA polymerase	0.5
Mili-Q water (H_2O)	34	Mili-Q water (H_2O)	34
Final Volume	$V_f=50$	Final Volume	$V_f=50$

Source: By the author.

Figure 6 - Schematic representation of the amplification reaction condition (PCR1a and 1b) of the genes for mutant proteins.

Source: By the author

The two PCR⁹² products had partially overlapping sequences containing the desired mutation at the extremity. These PCR products were mixed and subjected to a second PCR (PCR-2), initially for 10 cycles, after which the universal T7 promoter and T3 terminator primers were added; the reaction was then allowed to continue for 30 cycles.⁹³ This is shown in Table 10 and the thermocycling conditions are schematized in Figure 7.

The PCR-2 product generates a larger DNA with the mutation in a more central location which was analyzed on a 1% agarose gel prepared with ethidium bromide (0.5 ug/ml) in 1X TAE buffer (8 mM Tris-acetate pH 8.0, 0.2 mM EDTA). The SUMO-SOD amplified fragment was purified from the agarose gel using the SV Gel Cleanup Kit Wizard® system (Promega) according to the manufacturer's instructions.

Table 10 - Amplification reaction conditions (PCR-2).

Amplification Reaction (PCR-2)	Volume (μL)
Phusion Buffer	10
10mM dNTPs	1
Forward primer (150ng/ μL)-(PCR1a product)	2
Reverse primer (150ng/ μL)-(PCR1b product)	2
Phusion DNA polymerase	0.5
Mili-Q water (H_2O)	34
Final Volume	$V_f=46$
After 10 cycles, it was added:	
T7 promotor (150ng/ μL)	2
T3 terminator (150ng/ μL)	2

Source: By the author.

Figure 7 - Schematic representation of the amplification reaction condition (PCR-2) of genes for the mutant proteins.

Source: By the author.

2.3 Purchased genes

Point mutation (G73A) and the double mutant (G74Q+Q149G) were purchased from Cellco Biotec do Brasil Ltda.

2.4 Cloning the mutant insert-TrSOD into the vector pSMT3

The pSMT3 vector includes a 6xHis-SUMO⁹⁴ gene product which increases the solubility⁹⁵ of the SOD during its expression. The cloning process started with a previous digestion of the purified PCR-2 product and the pSMT3 vector using

restriction enzymes as shown in Table 11.

Table 11 - Reagents used during the digestion of the cloning vector (digestion reaction A) and SOD genes (digestion reaction B).

Digestion reaction (A)	Volume (μL)	Digestion reaction (B)	Volume (μL)
Purified PCR-2 product	5	pSMT3 Vector	5
Sall	1	Sall	1
BglII	1	BamHI	1
enzymes buffer	2	enzymes buffer	2
MiliQ Water	11	MiliQ Water	11
Final volume	$V_f=20$	Final volume	$V_f=20$

Source: By the author

Digestion reactions (A and B of Table 11) were incubated at 37°C for 3 hours and then purified from an agarose gel as described by the manufacturer.

Table 12 - Reagents used during the ligation reaction of the desired insert with the pSMT3 vector.

Ligation reaction	Volume (μL)
Purified insert	6
pSMT3 vector	2
T4 DNA ligase	1
T4 DNA Ligase Reaction Buffer (10x) ^a	1
Final volume	10

^a50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT pH 7.5

Source: By the author.

The ligation reaction of these products (shown in Table 12) was incubated at 16°C overnight in a final volume of 10 μL . The result was a SUMO-SOD/pSMT3 construct. Furthermore, between the sequence encoded by the insert-SOD and the SUMO gene, is a recognition site for the SUMO-protease for subsequent cleavage of the SUMO protein, shown in Table 13. The desired mutation was confirmed by DNA sequencing.

Table 13 - Construction of 6His-tag-SUMO+TrSOD in pSMT3 (pET28a(+)). The amino acids recognized by ULP1-protease are highlighted in red. SUMO-protein is highlighted in blue.

Construction of 6His-tag-SUMO+TrSOD in pSMT3 (pET28a(+))	
6XHis-tag	SUMO-Protein
HHHHHHSSGLVPRGSH	MASMSDSEVQNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLR
	Cleavage site TrSOD-protein
	RLMEAFAKRQ GKEMDSLRF LYDGI RIQADQTPGD LDMGDN DIIGAHRGQIG/GSVGTFSLPALPYAY
	DALEPSIS AQIMELHHSKHHQTYVTNLNNA LKTYSTAL AANDVPSQIALQAAIKFNGGGHINHS LFW
	ENLCPASSPDADPASAPELTAEIAKTWGS LDKFKEAMGKALLGIQGS GWGWL VKEGSGLRIVTTKD
	QDPVVGGEVPVFGIDMWEHAYYLQYLN GKAAAYVDNIWKVINWKTAEQRFKGDREDAFKILKASL

Source: By the author

2.5 Theoretical analysis of the amino acid sequence of *T. reesei*

A theoretical analysis (physical and chemical) of TrSOD was performed using its amino acid sequence with the ProtParam program from the ExPASy⁹⁶ server. Alignments were performed with clustalW2⁹⁷ and with the ESPript 3.0⁹⁸ online server. The secondary structure assignments were performed using DSSP.⁹⁹

Table 14 - Parameters of the enzyme TrSOD and 6His-tag SUMO+TrSOD calculated by ProtParam server, where ϵ is the molar extinction coefficient, M.W molecular weight and pI is the isoelectric point of the chain.

Protein	ϵ ($M^{-1} \text{ cm}^{-1}$)	M.W (Da)	pI
TrSOD	50420	23235.3	5.95
6His-tag SUMO+TrSOD	51910	36277.9	6.36

Source: By the author.

2.6 Expression of proteins

For protein expression, ligation products were transformed into *E. coli* (DH5 α) and the resulting strains were inoculated in 5 mL of LB (with kanamycin) and incubated at 37°C overnight. Plasmid DNA was isolated using the GeneJET Plasmid Miniprep Kit.

The plasmid DNA was first transfected into *E. coli* BL21 (DE3) and then expressed either using Luria Bertani (Tryptone 10g, yeast extract 5g and NaCl 5g) or M9 minimal media (M9 salts, 2 mM MgSO₄, 0.1 mM CaCl₂ and 0.4% glucose) supplemented with 50 μ g/mL kanamycin. For M9 minimal media, when the OD_{600nm} reached 0.5, 0.1 mM MnCl₂, 0.1 mM FeSO₄ or both (0.1 mM MnCl₂ and 0.1 mM FeSO₄)

were added in order to aid in the formation of metal loaded SOD. Protein expression was induced with 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) and the culture left overnight. Bacterial pellets were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to check for protein induction. Protein products were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).¹⁰⁰

2.7 Purification by affinity chromatography and molecular exclusion

Cells were suspended in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.4) and lysed by sonication (8 min, 4°C, 30s pulse on, and 30s pulse off). The lysate was then centrifuged at 14,000 g for 40 min at 4°C. Both supernatant and pellet were analyzed by polyacrylamide gel electrophoresis (SDS-PAGE). The soluble protein (contained in the supernatant) was loaded onto a Ni-NTA agarose column and then eluted in steps using 10 mM, 50 mM, 100 mM, 250 mM and 500 mM of imidazole in PBS buffer pH 7.4. In order to remove the SUMO protein, ulp1 protease (final concentration 0,1 mg/mL) was added and the solution was then incubated at 4°C, overnight. The mixture containing 6xHis-SUMO, SOD and ulp1 protease was re-purified using a second affinity chromatography column, thus binding the his-tagged proteins (SUMO and ulp1) to the resin while allowing the SODs to pass into the eluant. The concentration of the SOD protein (~1mg/mL) was measured with a Nanodrop™ 1000 (Thermo Scientific) spectrophotometer on a 2 μ L sample reading at 280 nm (absorbance, A) using the theoretical molar extinction coefficient (ϵ) of the protein 50420 M⁻¹cm⁻¹, (see section 2.5) and the Beer-Lambert law:¹⁰¹

$$A(\lambda) = \log(I/I_0) = C\epsilon(\lambda)l \quad (5)$$

where L is the path length (cm), C the concentration (M), and ϵ is the molar extinction coefficient (M⁻¹ cm⁻¹).

Each recombinant protein was isolated from the rest of its eluant by molecular exclusion chromatography (Superdex 200 HR 10/300 (GE) column coupled to an AKTA Explorer10 (GE)) chromatographic system)¹⁰² previously equilibrated using 50mM of PBS buffer. The flow rate was 0.5 mL/min and protein peaks were collected by observing the absorbance at 280 nm. Molecular weights and hydrodynamic radius for the native TrSOD and mutants were calculated based on fits from known standards.

The eluant (protein purity) was assayed by 15% SDS-PAGE, stained with Coomassie blue and then the final samples were concentrated by ultrafiltration through a Millipore (cutoff 30kDa) membrane (4000 rpm at 4°C).

2.8 Electron Paramagnetic Resonance (EPR) measurements

EPR is a spectroscopy technique that can be used to study the structure and dynamics of metal complexes that have unpaired electrons.¹⁰³ An electron has both orbital magnetic moment and spin magnetic moment. The magnetic moment of the molecule is primarily contributed by unpaired electron's spin magnetic moment.

When a sample (containing molecules with an unpaired electron) is placed in a strong magnetic field, the magnetic moment (μ) will interact with this applied magnetic field, (B). The interaction between the magnetic moment (μ) and the magnetic field (B) is described by:

$$E = -\mu \cdot B \quad (6)$$

The spin for a single unpaired electron can align in two different ways creating two spin states, $m_s = \pm \frac{1}{2}$ (corresponding to two possible energy states). This effect is called Zeeman splitting.

$$E_{+1/2} = 1/2 g \mu B \beta \quad (7)$$

$$E_{-1/2} = -1/2 g \mu B \beta \quad (8)$$

Where: μ = magnetic moment, β = Bohr magneton ($9.274 \times 10^{-24} \text{ J T}^{-1}$), B = strength of the magnetic field (in Tesla), g = the proportionality factor (g-factor).

In the absence of an external magnetic field,

$$E_{+1/2} = E_{-1/2} = 0 \quad (9)$$

However, in the presence of an external magnetic field (Figure 8), the difference between the two energy states can be written as:

$$\Delta E = h\nu = g \mu B \beta \quad (10)$$

Where, h = Planck's constant ($6.626 \times 10^{-34} \text{ J s}^{-1}$), ν = the frequency of radiation.

This equation implies that the splitting of the energy levels is directly proportional to the magnetic field's strength, as shown in the Figure 8.

Figure 8 - The splitting of the energy levels for an electron spin ($M_s = \pm 1/2$) under an applied magnetic field B . When a molecule with an unpaired electron is placed in a strong magnetic field, the spin of the unpaired electron can align in two different ways creating two spin states, $m_s = \pm 1/2$.

Source: Adapted from LAWRENCE.¹⁰³

In an EPR experiment typically the field of the spectrometer magnet is swept linearly to excite some of the electrons in the lower energy level to the upper energy level while the samples (in our case SODs) were exposed to a fixed microwave irradiation ($\nu = \sim 9.5\text{GHz}$). The condition where the magnetic field and the microwave frequency were “just right” to produce an EPR resonance (or absorption) is known as the resonance condition which was detected by the spectrometer.

Our EPR measurements were performed in two different laboratories: (1) In the Penner Hahn’ group of the chemistry building at the University of Michigan, United States using the Bruker EMX electron spin resonance spectrometer which is equipped with dual microwave bridges, and can acquire spectra in the X-band range. These measurements were performed with the research faculty Aniruddha Deb and a PhD student Andrew Crawford. (2) At the São Carlos Institute of Physics of the university of São Paulo using the Bruker Elexsys E-580 spectrometer which is equipped with the dielectric cavity ER 4118X-MD5, operating at X-band range (9,5 GHz). These measurements were performed with the Postdoctoral fellow Luis Basso.

The spectra were recorded under the following conditions: microwave frequency, 9.5 GHz; microwave power, 5 dB; modulation frequency, 10 kHz; and modulation amplitude, 4.00 G. Samples were dialyzed, concentrated and diluted in 10 mM PBS

buffer at pH 7.4. Final protein concentrations were 5mg/mL for native superoxide dismutase from *Trichoderma reesei* (TrSOD) (and the same concentration was likewise used for all mutants). Solutions were mounted in standard 4 mm quartz EPR tubes with no addition of cryoprotectant. Temperature-dependent intensities of the observed transitions were collected within the temperature range of 9-11 K. As the linewidth does not appear to broaden appreciably in the temperature range used, the EPR intensity was measured by the amplitude of specific signals. Non-power-saturation conditions were checked at the lowest temperature. Temperatures were carefully calibrated with a rhodium- nickel thermocouple sensor located in the EPR cavity at the sample position.

2.9 Crystallization assays

Initial crystallization assays were automatically performed using the native SOD (8 mg/mL) using a HoneyBee 931 (Genomin Solutions Inc.) crystallization robot. Three different kits were used (index HT, Crystal screen HT, and Morpheus® HT-96) with each one having 96 different conditions. The best crystals were obtained using 25% polyethylene glycol (PEG) 3350, 0.1 mM Bis-Tris, pH 6.5. These conditions were further refined to optimize crystal formation for both native and mutant SODs by varying the percentage PEG (23% to 30%, intervals of 1%), Bis-Tris concentration (0.05 to 0.5 mM, intervals of 0.01), and pH (5.5 to 7, intervals of 0.1). The protein solutions were first concentrated to 5-10 mg/mL and crystallized using the hanging-drop vapor-diffusion method.¹⁰⁴ The “hanging-drops” were prepared by mixing 2 μ L of protein solution (10 mM PBS, pH 7.4) with an equal volume of reservoir solution equilibrated with 1000 μ L of crystallization solution. These assays were prepared in Hampton plates (24 wells) at 18 °C. All the solutions used in crystallization were previously filtered through sterile 0.45 μ m filtration membranes (Millipore).

2.10 Data collection and processing

For data collection, the crystals were mounted in a fine rayon loop and cryoprotected by rapid soaking in solution containing mother liquor with the addition of 20% (v/v) polyethylene glycol, flash-frozen in liquid nitrogen, and measured at 100 K. X-ray data collection (for native Mn-TrSOD, H75I, L80F, G73A, D150G, and

Q172DSOD) was performed remotely using a Mar 300 (rayonix MX 300) S/N 025 at Argonne National Laboratories with the collaboration of Prof. Jeanne Stuckey from the Life Science Institute, University of Michigan-United States. The data were collected for native and mutant crystals then processed and scaled using the programs mosflm¹⁰⁵ and scala¹⁰⁶ from the CCP4 package.

For crystals of Fe-M27V and Mn-M27V the X-ray diffraction analyses were performed at the Diamond Light Source, UK using a Pilatus 6M-F detector. Data sets were processed and scaled using the XDS program by Humberto Pereira during data collection.

2.11 Molecular replacement

During the diffraction experiment all phase information is lost, and only the structure amplitudes can be extracted from the measured intensities (this is typically called “the crystallographic phase problem”). Initial phase approximation can be several techniques including: molecular replacement, direct methods, multiple isomorphous replacement, and anomalous dispersion (or a combination of these).¹⁰⁷ Molecular replacement requires the availability of a structurally similar model (normally a protein which shares more than 30% sequence identity) in order to determine the orientation and position of the molecules within the unit cell.

For the studies herein, the phase problem was solved using molecular replacement using the program PHENIX.¹⁰⁸⁻¹⁰⁹ For the structures of the mutant enzymes Fe-H75I TrSOD and Fe-D150G TrSOD, we used the crystal structure (single monomer) of *Aspergillus fumigatus* MnSOD (PDB code: 1KKC), which shares 63% of sequence identity with TrSOD. The monomer was rotated and translated in the unit cell until the solution with the best fit between calculated diffraction data from the replaced model and observed data from the TrSOD structure was obtained. The structure of native MnTrSOD and mutants (G73A, L80F, Fe-M27V and Mn-M27V) were solved using a monomer of the H75I mutant. The Matthews coefficient was used in order to estimate the probably number of molecules present in the asymmetric unit.

2.12 Refinement

Once sufficiently accurate phases have been obtained to yield an interpretable electron density map, an initial model is built. Refinement of the initial model aims at optimizing the agreement between the structure factor amplitudes calculated from the model parameters (F_{calc}) and the structure factor amplitudes observed in the experimental data (F_{obs}). Model parameters include atomic coordinates, atomic displacement parameters (ADP or B-factors) and scale factors. The number of the refinement cycles depends on how many iterations are needed to minimize the difference between the diffraction data and the model. Every cycle of this process leads to the improvement of the model fitting atomic positions and B-factors. Two parameters are used to evaluate the quality of the refinement: the residual factor (R-factor) and free R-factor (R_{free}). The R-factor measures the fit of the model to the diffraction data. In principle, the lower the R-factor, the better the model, so long as the model has not been over refined leading to distortions in the stereochemistry of the structure. The R factor is given in equation (11):

$$R = \frac{\sum |F_{\text{obs}} - F_{\text{calc}}|}{\sum |F_{\text{obs}}|} \quad (11)$$

R_{free} is computed according to the same formula but on a small, random sample of data that are set aside for the purpose and never included in the refinement (data omitted on the model). R_{free} is used to detect over-refinement of the structure and will normally be a few percentage points above R, although the absolute difference depends on many factors.

In the case of the SOD structures described in the present work, after molecular replacement, the initial models were refined using the phenix.refine program from the PHENIX package.¹¹⁰ The methodology adopted to refine all the structures is explained below.

These initial models were subjected to several cycles of refinement. For each cycle, the structure was subjected to energy minimization and geometric idealization with the use of isotropic temperature factors. TLS groups were defined automatically by phenix.refine and non-crystallographic symmetry (NCS) was used in each cycle. Each run was alternated with manual model building using coot.¹¹¹ At the end of each cycle, the stereochemistry of the model was monitored using Molprobit.¹¹² This

included an evaluation of Ramachandran outliers, rotamer outliers, the clashcore, Ramachandran favored, C β outliers, and overall Molprobity score). Subsequently water molecules were added automatically by phenix.refine and then each water molecule individually analyzed in coot. The analysis of Fourier difference maps, calculated with ($F_0 - F_c$) and ($2F_0 - F_c$) coefficients at the final stage of refinement, allowed the positioning of ions in the active sites and the correction of several water molecules. The R_{free} and R-factor were considered as the main quality criteria of the refinement protocols. The drawings were prepared with Pymol (<http://pymol.org>) or Chimera.¹¹³ The coordinates of the structures will be deposited in the Protein Data Bank.

2.13 Atomic Absorption Spectroscopy (AAS)

Metal analysis and quantification were performed via atomic absorption spectrometry using Fe and Mn lamps run at 248.3 nm and 279.5 nm, respectively. Samples of SODs were prepared with two solutions: 0.1% nitric acid and 0.1% hydrochloric acid. In order to decompose the protein and release the metal ion, 100 μ L of protein sample (5 mg/ml in 10mM PBS buffer) was mixed with both 100 μ L of 0.1% nitric acid and 100 μ L of 0.1% hydrochloric acid and exposed at 65 °C for 3 hours. This solution of 300 μ L was diluted to 1000 μ L using 0.1% nitric acid and kept at room temperature overnight. The solution was then mixed with 20mL of 0.1% nitric acid giving a final volume of 21mL and the metal content was then measured. AAS measurements were performed at the Chemical Analysis Center of the Institute of Chemistry of Sao Carlos-University of Sao Paulo. Calibration was achieved using standard Fe and Mn solutions spanning the range of 0.2-1.5 μ g/L.

2.14 Enzymatic activity

SOD activity was measured at 37°C in 10mM PBS buffer at pH 7.4 using an indirect colorimetric method (SOD Kit).¹¹⁴ This SOD kit utilizes a tetrazolium salt (WST-1) for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (Figure 9). Absorbance was measured at 450 nm. SOD activity in the experimental sample is measured as the percent inhibition of the rate of formazan dye formation, compared with that observed in the absence of SOD.

Figure 9 - SOD inhibition assay mechanism: The conversion of xanthine and O_2 to uric acid and H_2O_2 by XOD generates superoxide radicals. The superoxide anions reduce a tetrazolium salt (tetrazolium WST-1) to a colored formazan product (WST-1 formazan) that absorbs light at 450 nm. SOD scavenges superoxide anions, thereby reducing the rate of formazan dye formation.

Source: Adapted from RUPP.¹⁰⁷

The SOD kit contains: 20X WST-1 Solution: 1 mL, Xanthine oxidase solution (XO): 15 μ L, Assay Buffer: 20 mL, Xanthine oxidase Dilution Buffer: 10mL. (XO dilution Buffer), SOD Enzyme (from bovine liver): 30 μ L, 1 plate (96 well) and the adhesive Plate Cover:1. Sample control and a SOD standard curve are measured together with three controls described in Table 15 using the following protocol:

1. Firstly, 20 μ L of sample solution was added to each sample and control 2 well.
2. Then 20 μ L of Mili-Q water was added to each control 1 and control 3 well.
3. Then 200 μ L of 1X WST Working Solution was added to all the sample and control wells and mixed.
4. After that, 20 μ L of Xanthine Oxidase Dilution Buffer was added to each control 2 and control 3 well.
5. Consequently, 20 μ L of Enzyme Working Solution was added to each sample or SOD standard and control 1 well, and then mix thoroughly.
6. Finally, the plate was incubated at 37°C for 20 min and then was checked for absorbance using the microplate reader at 450 nm.

Table 15 - Solution volume (μL) added to each well.

	Sample or SOD standard curve	Control 1	Control 2	Control 3
Sample solution	20	----	20	----
Double distilled H ₂ O	----	20	----	20
WST 1X working Solution	200	200	200	200
XO working solution	20	20	----	----
XO dilution buffer	----	----	20	20

Source: By the author

In order to calculate the SOD activity (inhibition rate %), the following equation was used.

$$\text{SOD activity} = \{[(A_{\text{control 1}} - A_{\text{control 3}}) - (A_{\text{sample}} - A_{\text{control 2}})] / (A_{\text{control 1}} - A_{\text{control 3}})\} \times 100 \quad (12)$$

The same procedure was applied for all TrSODs.

2.15 Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) analyses are routinely used to detect aggregates in macromolecular solutions and to determine the size of proteins. DLS measures fluctuations in scattered light due to Brownian motion of particles within the sample and relates this to their size. Particle motion is inversely related to particle size, such that larger particles move slowly and smaller particles move quickly.¹¹⁵ Brownian motion in the absence of an applied force is the origin of the diffusion or random movement of particles and is entropically driven.

To characterize this motion, laser light is focused into the solution, and a single photon counting detector is positioned at an angle with respect to the laser. As the particles move through the suspension, they pass through the laser beam, which causes some of the light to scatter. The velocity of the Brownian motion is defined by a property known as the translational diffusion coefficient (usually given the symbol, D) which is used to calculate the hydrodynamic radius (R_H). R_H gives us an estimative of the particle size and is calculated from the translational diffusion coefficient by using the Einstein equation:¹¹⁶

$$R_H = K_B T / 6 \pi \eta D \quad (13)$$

where K_B is the Boltzmann constant (1.381×10^{-23} J/K), T the temperature (25 °C in our case), and η the viscosity of the solvent (PBS, 0.899 Pa.s). Information about the light-scattering spectrum can be obtained from the autocorrelation function $G(t)$ of the light-scattering intensity.

$$G(t) = \langle I(t_0) \rangle \langle I(t_0 + \Delta t) \rangle \quad (14)$$

Where $I(t_0)$ is the scattering intensity at time t and $I(t_0 + \Delta t)$ is the scattering intensity a very small time later. Each monodisperse population of particle sizes produces its own unique autocorrelation $G(t)$ function which is given by:

$$G(t) = Ae^{-2\Gamma t} + B \quad (15)$$

Where A and B are constants and Γ is the constant decay of the exponential curve:

$$\Gamma = Dq^2 \quad (16)$$

where q is the scattering vector given by:

$$q = (4\pi n / \lambda) [\sin(\theta/2)] \quad (17)$$

n is the refractive index of the medium, λ the laser wavelength, and θ the scattering angle.

Particle size is given in terms of R_H . DLS measurements were carried out to evaluate TrSOD proteins at 25 °C employing Zetasizer μV equipment using software Zetasizer 6.32. Firstly, purified samples (2 μ L of 2-6 mg/mL) were centrifuged for 10 minutes at 10000g and then introduced into a quartz cuvette to perform the measurement.

3 RESULTS AND DISCUSSION

3.1 Cloning, expression, and purification of native TrSOD and mutant proteins

Figure 10 shows two separate PCR reactions performed using the oligonucleotide primers given in section 2. These two PCRs were performed for each mutant as explained in Methods.

Figure 10 - Forward amplification (PCR-1) and Reverse amplification (PCR-2) of Mn-TrSOD proteins. Results were analyzed in 1.5% agarose gel, TAE 1X. Lane 1 and 10: Molecular weight marker Gene ruler 1Kb ladder plus; lane 2: M27V-F; lane 3: G73A-F; lane 4: G74Q-F; lane 5: H75I-F; lane 6: L80F-F; lane 7: Q149G-F; lane 8: D150G-F; lane 9: Q172D-F; lane 11 and 12: M27V-R; lane 13: G73A-R; lane 14: G74Q-R; lane 15: H75I-R; lane 16: L80F-R; lane 17: Q149G-R; lane 18: D150G-R and lane 19: Q172D-R.

Source: By the author

Figure 11 shows the products of a second PCR (performed with the previous PCR products, as explained in methods). These products contain the desired mutations in the sequence of the TrSOD.

Figure 11 - PCR products. Results were analyzed in 1.5% agarose gel, TAE 1X. Lane 1: Molecular weight marker Gene ruler 1Kb ladder plus; lane 2: SUMO-M27VTrSOD; lane 3: SUMO-G73ATrSOD; lane 4: SUMO-G74QTrSOD; lane 5: SUMO-H75ITrSOD; lane 6: SUMO-L80FTrSOD; lane 7: SUMO-Q149GTrSOD; lane 8: SUMO-D150GTrSOD and lane 9: SUMO-Q172DTrSOD.

Source: By the author.

Sequencing results showed the presence of the mutant SUMO-M27VTrSOD, SUMO-H75ITrSOD, SUMO-L80FTrSOD, SUMO-D150GTrSOD and SUMO-Q172DTrSOD. Metal exchange could be obtained by denaturing the isolated protein, extracting the miscellaneous metals and refolding in the presence of the desired metal. However, in this study we used an alternative approach and prepared FeSOD, MnSOD and Fe/MnSOD by growing cultures using M9 minimal media to which an excess of Fe, or Mn, or both Fe and Mn had been added. Under these circumstances the metal cofactor is dependent upon the metal supply. Either a depletion of Mn or an excess of Fe in the bacterial culture medium may induce the production of FeSOD as suggested by Whittaker.⁵³

After centrifugation of the lysed cells, the enzymes were isolated from the soluble fraction using affinity chromatography, performed on Nickel resin. The protein concentrations from the eluted fractions obtained using 50mM, 100mM and 250mM imidazole, were ~ 0.5 - 3.0 mg/mL and gave a total sample volume of in 10 mL. The eluted samples were then characterized by electrophoresis. Shown in Figure 12 are the results of characterization for the native protein. This SDS-PAGE demonstrates the successful overexpression of SUMO-TrSOD induced when IPTG was added to the cells. The bands around 35 KDa represent the TrSOD together with the SUMO protein.

Figure 12 - SDS-PAGE-15%; analysis of Mn-TrSOD. 1: Molecular mass marker; lane 2: elution with 50mM imidazole; lane 3: elution with 100mM imidazole; lane 4: elution with 250mM imidazole.

Source: By the author.

Dialysis and subsequent ultrafiltration increased the concentration to ~5.0 - 20 mg/mL. After the cleavage of the His-tag-SUMO with the ULP-1 protease, the protein was subjected to two consecutive purification steps, first by affinity chromatography and then by molecular exclusion. The final SOD proteins presented a characteristic color, a consequence of the incorporated metal ions. The Fe- and Mn- containing samples showed a yellow and purple color respectively. Figure 13 shows the SDS-PAGE-15% analysis of the elution of the native protein after cleavage and subsequent affinity chromatography followed by molecular exclusion.

Figure 13 - SDS-PAGE-15%; analysis of Mn-TrSOD. Lane 1: Molecular mass marker; lane 2: elution with 50mM imidazole; lane 3 and 4: elution with 100mM imidazole; lane 5: elution with 250mM imidazole after the second affinity chromatography and lanes 6,7and 8: elution after gel filtration chromatography (tubes 13mL,14mL and 15mL eluted pure protein)

Source: By the author.

In Figure 13 a band of about 24 KDa is visible in all lanes, and this is approximately what was expected based on of computational analysis (shown in section 2.5). Lanes 2 and 3 show another band around 35 kDa which could be residual uncleaved SUMO-TrSOD. After the last purification step using size exclusion we obtained the pure proteins shown in lanes 6, 7 and 8.

3.2 Estimation of molecular weight and hydrodynamic radius by size exclusion chromatography

The oligomeric state of native TrSOD and mutants were verified through gel filtration chromatography with molecular weight standards (MW) described in Table 16.

Six proteins were chosen according to their molecular weight, categorized in 3 different groups (mixture of two reference proteins per group: Aldolase with carbonic anhydrase, catalase with ovoalbumine and ferritin with albumin bovine) and injected in the SUPERDEX 200 HR 10/30. The experimental results of the elution volume (V_e) for each standard group's protein are shown in Figure 14. The calibration curve for both, molecular weight and hydrodynamic radius is showed in Figure 16.

Table 16 - Calibration standards SUPERDEX 200 HR 10/30 and respective experimental elution volumes.

Standards	MM(KDa)	R_H (nm)	V_e (mL)	K_{av}^*
Blue dextran	2000	-----	8.92	-----
Aldolase from rabbit muscle	158	4.8	13.55	0.3070
Carbonic Anhydrase Bovin	29	2.3	17.36	0.5596
Ovoalbumine	43	3.0	15.87	0.4608
Ferritin (equine spleen)	440	6.7	11.15	0.1478
Bovin Serum Albumin	66	3.5	14.76	0.3872
Native-TrSOD (86 μ M)	92	3.9	14.50	0.3680
Native-TrSOD (8.6 μ M)	48	2.9	15.88	0.4610

$$* K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

Source: By the author.

Figure 14 - Elution profiles for SUPERDEX 200 HR 10/30 standard proteins and native TrSOD at 86 μ M concentration.

Source: By the author.

Figure 15 - Elution profiles for SUPERDEX 200 HR 10/30 standard proteins and native TrSOD at 8.6 μM concentration.

Source: By the author

The total volume (V_t) of the column was 24 mL and the dead volume is given by the volume of blue dextran. The protein concentration with respect to the native TrSOD was varied from 86 μM to 8.6 μM . The elution profiles of TrSOD revealed two peaks corresponding to the protein concentrations of 86 μM and 8.6 μM . The elution peak of 14.50 mL at 86 μM (Figure 14), corresponds to a molecular mass of 92 ± 47 kDa with a hydrodynamic radius of 39 ± 10 Å and the elution peak of 15.88 mL at 8.6 μM (Figure 15) corresponds to a molecular mass of 48 ± 27 kDa with a hydrodynamic radius of 29 ± 10 Å.

The elution profiles of TrSOD revealed two oligomeric forms, dimers and tetramers. At low concentration (8.6 μM), the protein eluted with the molecular mass compatible with dimers but on increasing the concentration (86 μM), the protein appeared to elute as tetramers. A dynamic equilibrium between the two states during time course of the experiment may lead to imperfect estimates of the molecular mass. It is possible that tetrameric TrSOD at low concentrations can reversibly dissociate into dimers without affecting activity. Such a phenomenon has been previously reported, for example, in the case of the Mn-SOD from the cytosol of *Candida albicans*.¹¹⁷

The SDS-PAGE estimated a molecular mass of approximately 24,000 Da for each monomer which is in good agreement with computational analysis and with the

experimental reports of Chambergo.⁸⁸

Figure 16 - Calibration Curves for SUPERDEX 200TM 10/300 (A) molecular mass and (B) hydrodynamic radius.

Source: By the author.

All mutants gave the same elution volume (14.5 mL) at about ~80 μM indicating that the mutant proteins behave as tetramers at this concentration.

Figures 17-21 show (A) the elution of the mutant proteins from size-exclusion column chromatography (Superdex 200 HR 10/30) and (B) SDS-PAGE gels of the eluants from the affinity chromatography and size exclusion purifications.

Figure 17 - A. Chromatogram for Mn-M27VTrSOD obtained by gel filtration as in Figure 3.2.1 B. 15% SDS-PAGE for mutant, M27V-MnTrSOD; lane 1: Molecular mass marker; lane 2: elution with 50mM imidazole-SUMO-M27VTrSOD for nickel affinity column; lane 3: elution with 500mM imidazole; lane 4: SOD, after removing the SUMO protein; lanes 5, 6, 7 and 8: elution volume of 13 mL, 14mL, 15mL and 16mL, respectively after gel filtration chromatography.

Source: By the author.

Figure 18 - A. Chromatogram for Mn-H75ITrSOD obtained by gel filtration as in Figure 3.2.1. B. 15% SDS-PAGE for mutant Mn-H75ITrSOD; lane 1: Molecular mass marker; lane 2: elution with 50mM imidazole-with ULP1-protease; lane 3: elution with 500mM imidazole- with ULP1 protease; lane 4: SOD, after removing the SUMO protein (for nickel affinity column) lanes 5, 6, 7 and 8: elution volume of 13 mL, 14mL, 15mL and 16mL, respectively after gel filtration chromatography.

Source: By the author.

Figure 19 - A. Chromatogram for Fe-Q172DTrSOD obtained by gel filtration as in Figure 3.2.1. B. 15% SDS-PAGE for mutant Mn-Q172DTrSOD; lane 1: Molecular mass marker; lane 2: elution with 50mM imidazole-with ULP1-protease; lane 3: elution with 100mM imidazole- with ULP1 protease; lane 4: elution with 500mM imidazole-with ULP1-protease; lane 5: Q172D-TrMnSOD after removing the SUMO protein (for nickel affinity column); lines 6, 7, 8 and 9: elution volume of 13mL, 14mL, 15mL and 16mL, respectively after gel filtration chromatography.

Source: By the author.

Figure 20 - A. Chromatogram for Mn-L80FTrSOD obtained by gel filtration as in Figure 3.2.1. B. 15% SDS-PAGE for mutant Mn-L80FTrSOD and Fe-L80FTrSOD; lane 1: Molecular mass marker; lane 2,3,4 and 5: elution volume of 13, 14, 15 and 16, respectively after gel filtration chromatography for Mn-L80FTrSOD; lanes 6, 7, 8 and 9: elution volume of 13mL, 14mL, 15mL and 16mL, respectively after gel filtration chromatography for Fe-L80FTrSOD.

Source: by the author

Figure 21 - A. Chromatogram for D150G-Mn-TrSOD obtained by gel filtration as in Figure 3.2.1. B. 15% SDS-PAGE for mutant Mn-D150GTrSOD and Fe-D150GTrSOD; lane 1: Molecular mass marker; lane 2,3,4 and 5: elution volume of 13, 14, 15 and 16, respectively after gel filtration for Mn-D150GTrSOD; lanes 6, 7, 8 and 9: elution volume of 13mL, 14mL, 15mL and 16mL, respectively after gel filtration chromatography for Fe-D150GTrSOD.

Source: By the author.

Based on these results we decided to use DLS as an independent method to estimate the molecular mass of the enzymes.

3.3 Analysis with Dynamic Light Scattering (DLS)

DLS was used to verify the molecular mass together with the homogeneity of native and mutants TrSODs in solution. Figure 22 gives examples of the size distribution for the native protein and the G73A mutant, showing the presence of a homogenous single peak. Similar results were obtained for the remainder of the samples. Table 17 shows the resulting molecular weights (KDa) and corresponding hydrodynamic radii (Å).

Table 17 - DLS analyses of TrSODs: Molecular weight and hydrodynamic radius of each protein.

Samples	(Å)	MW (KDa)
Native TrSOD (0.5 mg/mL)	29 ± 10	50 ± 8
Native TrSOD (1 mg/mL)	29 ± 10	58 ± 10
Native TrSOD (2 mg/mL)	39 ± 10	75 ± 10
M27VTrSOD (2 mg/mL)	39 ± 10	82 ± 12
H75ITrSOD (2 mg/mL)	39 ± 10	82 ± 11
L80FTrSOD (2 mg/mL)	39 ± 7	78 ± 8
G73ATrSOD (2 mg/mL)	48 ± 5	135 ± 17
D150GTrSOD (2 mg/mL)	37 ± 10	79 ± 12
Q172DTrSOD (2 mg/mL)	39 ± 10	82 ± 11
Double mutant-TrSOD (2 mg/mL)	39 ± 10	82 ± 12

Source: By the author.

According to DLS, the native protein at 0.5 mg/mL has a molecular mass of 50 ± 8 KDa corresponding to the dimeric form; while at 2 mg/mL, this same protein has a molecular mass of 75 ± 10 KDa, which is closer to that expected for a tetramer. However, the latter result is lower than that expected theoretically and it seems likely that at 2 mg/ml the sample contains a mixture of both forms (dimers and tetramers). Other SODs (e.g., the cambialistic SOD from *Corynebacterium glutamicum*)¹¹⁸ have also been analyzed by DLS and have given a similar molecular weight around 80 KDa, despite being characterized as tetramer. Since, the shape of the TrSOD is not a perfect sphere this could also be expected to contribute to imprecision in the estimated molecular mass.

By definition, the DLS measured radius is the radius of a hypothetical hard sphere that diffuses with the same speed as the particle under examination.¹¹⁵ In practice, however, macromolecules in solution are hydrated and non-spherical. As such, the radius calculated from the diffusional properties of the particle is only indicative of the apparent size of the dynamic hydrated/solvated particle but not precisely determinative.

In summary, it seems most likely that there is a dynamic exchange of dimers and tetramers in solution and DLS reports on the mean weight mean of both forms. This is consistent with the observation that at higher concentrations the tetramer becomes more evident.

All the SODs showed a similar molecular mass except for the G73ATrSOD mutant. DLS results show that mutant G73ATrSOD has a molecular mass of 135 ± 17 KDa which is different from the other mutants suggesting that its oligomeric state is larger than the native TrSOD. Figure 22 shows the differences in the hydrodynamic diameter for both, native and G73ATrSOD mutant.

Figure 22 – Hydrodynamic diameter from Native TrSOD (left) and G73ATrSOD (right).
Source: By the author.

3.4 Enzymatic activity of SODs

3.4.1 Calibration Curve of Fe-TrSOD and Mn-TrSOD

The SOD activity for native Mn-TrSOD and Fe-TrSOD are shown in Table 18. As expected, the activity is a function of SOD concentration. Table 18 shows that almost 100% activity is reached at 2mg/mL of each enzyme (Fe-TrSOD and Mn-TrSOD) under the conditions used.

Table 18 - SOD activity (at different protein concentrations) for Fe-TrSOD and Mn-TrSOD.

Native Fe-TrSOD (mg/mL)	(SOD activity $\pm$ SD*)	Native Mn-TrSOD (mg/mL)	(SOD activity $\pm$ SD*)
2	95 (+/- 0)	2	99 (+/- 0)
1	92 (+/- 1)	1	99 (+/- 0)
0.9	89 (+/- 1)	0.8	96 (+/- 1)
0.8	81 (+/- 2)	0.5	90 (+/- 1)
0.7	74 (+/- 2)	0.2	88 (+/- 3)
0.5	64 (+/- 1)	0.1	86 (+/- 1)
0.1	54 (+/- 2)	0.03	84 (+/- 1)
0.09	50 (+/- 1)	0.01	48 (+/- 3)
0.07	32 (+/- 1)	0.005	42 (+/- 3)
0.05	24 (+/- 3)	0.001	35 (+/- 2)

*Standard deviation

Source: By the author.

For the enzyme, assay employed here one unit of SOD activity is defined as the

amount of enzyme needed to reduce the production of WST-1 formazan to 50%. Figure 23 shows the calibration curve for both Fe-TrSOD and Mn-TrSOD used to determine one unit of both enzymes.

Figure 23 - Calibration Curves for A. Fe-TrSOD and B. Mn-TrSOD used to calculate one unit of the Fe-TrSOD and Mn-TrSOD enzymes.

Source: By the author

From the calibration curve we found that 0.01 mg/mL of native Mn-TrSOD exhibits 50% maximum activity which corresponds to 5000 units/mg. In the case of Fe-TrSOD the equivalent values are 0.1 mg/mL or 500 units/mg (shown in Table 19). The data reported here show that the enzymatic activity of native TrSOD is very much metal dependent since Mn-TrSOD seems to be 10-fold more catalytically active than Fe-TrSOD.

Table 19 - Concentration of native Mn-TrSOD and Fe-TrSOD that have given 50% of SOD activity.

Native SOD proteins	Concentration protein mg/mL (50% of SOD activity)	SOD (units/mg)
Mn-TrSOD	0,01	5000
Fe-TrSOD	0,1	500

Source: By the author

Subsequently, SOD activity was measured for all the native and mutant proteins, after purification with each metal ion (Fe-enzyme, Mn-enzyme and Fe/Mn-enzyme).

3.4.2 Activity of Fe-TrSOD, Mn-TrSOD and Fe/Mn-TrSOD proteins (native and mutants)

Table 20 shows the activity of all tested Fe-proteins at 0.1 mg/mL (this corresponds to 1 Unit/mg in the native Fe-TrSOD). It was not expected that the native Fe-TrSOD protein would present activity (because TrSOD was classified as Mn-dependent SOD) but surprisingly it does, (Table 20). However, this is not surprising as a similar trend was already reported in cambialistic SODs¹¹⁹ where incorporation of either metal (Fe or Mn) into the active site yielded a catalytically active enzyme), both metals being compatible with catalytic activity. This result for native TrSOD points to the cambialistic nature of the enzyme with possible regulation of its activity by the type of metal bound. A more detailed discussion of this finding is given in section 3.7 together with the AAS and EPR analyses.

Into Fe-TrSODs, the mutation G73A showed no activity at all in the presence of Fe. These results may relate to the change in oligomeric state mentioned above and will be discussed later when describing its crystal structure. Furthermore, when compared to the native Fe-TrSOD ($54 \pm 3 \%$), mutant Fe-Q172D had higher specific activity ($94 \pm 0,4 \%$) and the catalytic activity of other mutants differs from ($37 \pm 1 \%$) to ($66 \pm 1 \%$) which can be explained by the difference in the metal content.

Table 20 - SOD activity at 0.1mg/mL protein concentration for native and mutant proteins expressed in the presence of Fe. (In native Fe-TrSOD, 0.1 mg/mL represents 1 unit of protein).

Protein (Grown in the presence of excess Fe)	(SOD activity $\pm$ SD*)
Native -SOD	54 (+/- 3)
M27V-SOD	37 (+/- 1)
H75I-SOD	53 (+/- 5)
L80F-SOD	66 (+/- 1)
D150G-SOD	61 (+/- 2)
Double-SOD	38 (+/- 4)
Q172D-SOD	94 (+/- 1)
G73A-SOD	0

*Standard Deviation

Source: By the author.

Table 21 shows the activity of all the Mn-TrSOD proteins and Fe/Mn-TrSOD proteins at 0.05 mg/mL (for the native Mn-TrSOD protein, 0.05 mg/mL corresponds to 5 units of protein). In Mn-bound proteins, all Mn-mutants presented a slightly reduced activity when compared to native Mn-TrSOD with the exception being the Mn-L80FTrSOD mutant which showed very similar activity. Almost no activity ($5 \pm 2.5\%$) was presented by Mn-G73ATrSOD mutant suggesting that this point mutation radically affected the function of the enzyme. A similar trend was observed in the case of Fe/Mn-SOD, i.e., almost all Fe/Mn-TrSOD mutants presented lower activity compared to native Fe/Mn-TrSOD with the exception being the Fe/Mn-Q172D mutant which showed very similar activity to the native Fe/Mn-SOD and G73A mutant that showed no activity.

Table 21 - Mn-TrSOD and Mn/Fe-SOD activity at 0.05mg/mL of protein concentration for native and mutant proteins.

Proteins	Mn-SODs (SOD activity $\pm$ SD*)	Fe/Mn-TrSOD (SOD activity $\pm$ SD*)
Native-SOD	90 (+/- 1)	87 (+/- 1)
M27V-SOD	76 (+/- 2)	55 (+/- 1)
H75I-SOD	80 (+/- 3)	73 (+/- 2)
L80F-SOD	89 (+/- 1)	80 (+/- 2)
G73A-SOD	5 (+/- 3)	0
D150GSOD	71 (+/- 1)	71 (+/- 2)
Doble-SOD	52 (+/- 2)	39 (+/- 1)
Q172DSOD	67 (+/- 2)	86 (+/- 1)

*Standard Deviation

Source: By the author.

3.4.3 Comparison of Fe-TrSOD and Mn- TrSOD activity

Table 22 shows a comparison of SOD activity for Fe-TrSODs and Mn-TrSODs at 0.05mg/mL of protein. This table clearly reveals a lower activity presented by Fe-

bound enzymes with respect to Mn-bound enzymes.

Table 22 - SOD activity in native and mutant proteins that grew up in presence of either Fe or Mn.

SAMPLES	Fe-TrSOD activity $\pm$SD*	Mn-SOD activity $\pm$SD*
Native-SOD	22 (+/- 3)	90 (+/- 1)
M27V-SOD	23 (+/- 3)	76 (+/- 2)
H75I-SOD	14 (+/- 1)	80 (+/- 3)
L80F-SOD	27 (+/- 2)	89 (+/- 1)
G73A-SOD	0	5 (+/- 3)
D150GSOD	8 (+/- 1)	71 (+/- 1)
Doble-SOD	19 (+/- 1)	52 (+/- 2)
Q172DSOD	66 (+/- 1)	67 (+/- 2)

*Standard Deviation

Source: By the author.

Overall, there is a wide variation in activity: almost all Mn-bound enzymes exhibited higher activity compared to Fe- bound enzymes except for the 172DTrSOD mutant that showed similar activity with the two metals. G73ATrSOD presented low or no activity when bound either Fe or Mn. One explanation for this lack of activity could be the absence of metal in the active site. For instance, it is known that apo-proteins are catalytically inactive and therefore the variation in activity may be the result of variable metal content present in the active site. Indeed, variation in the metal content must be considered as a possible explanation for the variation in activity observed in all cases and not just that of the G73A mutant.

3.5 Metal content

3.5.1 Mn-SODs

Mn in the native TrSOD was clearly identified. When expressed in the presence of Mn we expected to find only Mn in the active site of the mutants but these enzymes also showed a significant amount of Fe (Table 23) presumably present in the PBS buffer at low concentrations suggesting that mutants were capable of taking up Fe even in the presence of greater concentrations of Mn. The calculated stoichiometries for Fe and Mn were 0.15 and 0.33 atoms/subunit, respectively showing that less than 50% of monomers were bound to metal. This unbalanced stoichiometry could be explained by the release of metal from the enzyme during purification. Additionally, the mutants also showed variation in the Fe/Mn ratio from one mutant to another with the

ratios varying from between 80% to 60% in all cases. This suggests an intrinsic ability of TrSOD to accept Fe in the active site and this was not greatly perturbed in the mutants.

Table 23 - Metal content in native and mutant proteins that grew in the presence of Mn.

SAMPLES (Grown in presence of Mn)	Saturation of TrSOD with metal (%)		Ratio Fe: Mn
	Monomer: Fe	Monomer: Mn	
Native-SOD	15	33	0.3:0.7
M27V-SOD	9	35	0.2:0.8
H75I-SOD	11	45	0.2:0.8
L80F-SOD	18	33	0.4:0.6
G73A-SOD	29	46	0.4:0.6
D150GSOD	16	36	0.3:0.7
Double-SOD	9	34	0.2:0.8
Q172DSOD	17	35	0.3:0.7

Source: By the author

3.5.2 Fe-SODs

The results of the metal content analysis for enzymes grown in the presence of Fe are shown in Table 24. Atomic absorption spectroscopy of most of the Fe-SOD samples showed a mean Fe occupancy at the active site of 40% with Mn occupancy being less than 3%. However, there was considerable variation and notably, the Q172D mutant presented similar Fe and Mn occupancy.

Table 24 - Metal content in native and mutant proteins that grew in the presence of Fe.

SAMPLES (grown in presence of Fe)	Saturation of Fe-TrSOD with metal (%)		Ratio Fe: Mn
	Monomer: Fe	Monomer: Mn	
Native -SOD	29	3	0.91:0.09
M27V-SOD	63	3	0.96:0.04
H75I-SOD	47	3	0.95:0.05
L80F-SOD	24	3	0.90:0.10
D150G-SOD	47	3	0.95:0.05
Double-SOD	29	3	0.91:0.09
Q172D-SOD	9	12	0.42:0.58
G73A-SOD	73	3	0.96:0.04

Source: by the author

3.5.3 Mn/Fe-SODs

Competitive binding experiments using a 1:1 mixture of Fe and Mn resulted in low Mn incorporation (Native TrSOD, M27V, L80F, G73A, D150G, and double-TrSOD) suggesting an unexpected preference of these enzymes for Fe (on average, 30% of Fe with respect to 12% of Mn). This indicates that in the presence of both metals, some proteins prefer to bind Fe(III). In contrast, Q172D presented similar Fe and Mn incorporation (see Table 25).

Table 25 - Metal content in native and mutant proteins that grew in excess of both Fe and Mn.

SAMPLES (50:50 mixture of Mn/Fe)	Saturation of Mn/Fe-TrSOD with metal content (%)		Ratio Fe: Mn
	Monomer: Fe	Monomer: Mn	
Native-SOD	26	11	0.70:0.30
M27V-SOD	16	10	0.62:0.38
H75I-SOD	3	9	0.23:0.77
L80F-SOD	36	9	0.80:0.20
G73A-SOD	38	3	0.93:0.07
D150GSOD	37	14	0.73:0.28
Double-SOD	42	16	0.72:0.27
Q172DSOD	18	21	0.47:0.54

Source: By the author

Fe in its ferrous form (Fe^{+2}) is similar to Mn^{+2} in size, valence and coordination geometry; and both compete to bind the active site of SODs. These competitions are known to occur in vivo and are related to the metal availability in the environment. Mn-SOD from *E. coli*, after grown in medium supplemented with Fe salts shows low selectivity for either Fe or Mn. In case of TrSOD, this enzyme tends to bind the metal which is present in excess in the media; however, if both metals are at the same concentration, Native TrSOD shows preferential binding for Fe.

In other SODs, e.g., Cu/Zn-SODs, metalation is aided by metallo-chaperone proteins. Metallo-chaperones capture a specific metal ion in spite of the absence of free intracellular metals¹²⁰⁻¹²¹ and directly transfer their cargo to metallo-protein targets (SODs).

Metal content in the active site of Fe/Mn-SODs influences the activity of these enzymes. An analysis of metal content, catalytic activity, and EPR results is given in section 3.7.

3.6 Electron Paramagnetic Resonance (EPR) measurements

3.6.1 EPR spectra of Fe-TrSOD (native and mutants)

EPR spectroscopy is particularly sensitive to subtle changes in the metal environment for SOD complexes. EPR spectra of native TrSOD grown in the presence of Fe is shown in Figure 24. These spectra reveal several intense signals in the 1500 G region with effective g values equal to 9.3, 4.6, 4.1, and 3.9 for Fe-TrSOD. The g values are consistent with those expected for high spin Fe(III) with an $S = 5/2$ ground state. The weak signals around $g \sim 2.1$ could represent Mn(II) in low concentrations. The spectra also display a signal at $g \sim 4.3$ which is very often observed in Fe proteins and which is very likely due to nonspecifically bound Fe(III).

Figure 24 - EPR spectra of Fe-TrSOD. Samples were buffered at pH 7.4 with 50 mM PBS.
Source: By the author.

In our results we can see that the ferric ion is found in two different possible environments:

Environment 1. nearly rhombic with EPR signals at $g \sim 4.8$, 3.9, and 3.6.

Environment 2. rhombic with an EPR signal at $g \sim 4.35$.

This is not unusual, as both types of signals, in various proportions, have been observed in SODs from other organisms.^{71, 122, 123}

In Fe-SOD, a more rhombic EPR signal ($g \sim 4.3$) was attributed to non-active site (NAS) Fe, whereas a less rhombic EPR signal ($g \sim 4.8$, 3.9 and 3.6) was attributed to Fe(III) in SODs active sites.

In order to assess the degree to which the active site is disrupted or deformed

as a consequence of the point mutations, we have compared the EPR spectra for native Fe-TrSOD with that of the mutant enzymes. Based on these comparisons, our data show that the mutants contain high-spin Fe(III), similar to native Fe-TrSOD.

The EPR spectrum of H75IFeSOD is the most similar to that of native Fe-SOD than that of other Fe-SOD mutants. This is based on the g values (Table 26) and the overall appearance of the spectrum, having environment_1 and environment_2 type sites, with the latter being in less intense (see Figure 25). These indicate that the coordination geometry and electronic state of the Fe, are not altered significantly by relocating the H75 side chain near the active site of Fe-SOD.

Figure 25 - EPR spectra of Fe-SOD mutants: Q172D, M27V, D150G, H75I, G73A and double mutant without background subtraction. Samples were buffered at pH 7.4 with 50 mM PBS.
Source: By the author.

Table 26 - g values of Fe(III) EPR spectra of SODs. Each spectrum varies widely with different contributions of Fe₁ and Fe₂ showing that the active site is sensitive to small perturbations caused by the mutations localized away from the active site.

Sample: Fe-TrSOD	g values				
Native -TrSOD	9.3	4.3	4.1	3.9	2.1
Q172D-TrSOD	4.9	4.3	4.1	4.0	
M27-TrVSOD	4.9	4.3	4.1	4.0	
H75I-TrSOD	9.3	4.7	4.3	4.2	3.9
Double-TrSOD	9.1	4.9	4.35	4.0	3.7 3.5
D150G-TrSOD	9.1	4.9	4.6	4.3	4.1 4.0
G73A-TrSOD	4.3	4.0			

Source: By the author

Although the spectrum of the double mutant at pH 7.4 consists of several iron states, the spectrum is dominated by anisotropic signals at $g = 3.7$, $g = 3.5$ and $g = 4.9$ which could be characterized as the absorption from the middle Kramer's doublet of a high-spin Fe(III) in a field of nearly rhombic symmetry. Similar signals were observed in the EPR spectra of M27V, H75I and D150G mutants ($g_x = 4.0$, and $g_y = 4.8$). The similarity of the Fe signals observed is not unexpected as they are presumably due to Fe coordinated to SOD in an identical fashion to that seen for the native protein when produced solely in the presence of Fe.

EPR signal of G73AFeSOD suggests a six-coordinate Fe(III), neglecting the somewhat larger contribution of rhombic non-active-site (NAS) (represented by $g \sim 4.34$). This signal is related to enzymes that do not present activity which is the case of G73ATrSOD mutant. A clearer explanation concerning the loss of activity will be presented along with the crystal structure.

3.6.2 EPR spectra of Mn-TrSOD (native and mutants)

The X-band EPR spectra for native TrSOD grown in the presence of Mn metal is shown in Figure 26. EPR spectra revealed g values of 2.16, 2.1, 2.07, 1.99, 1.95 and 1.89 corresponding to the presence of Mn(II). Mn(II) spontaneously oxidizes in air to give a mixture of Mn(II) and Mn(III) forms. Many of the Mn-bound mutants were heterogeneous with respect to the oxidation state of the metal.

Figure 26 - EPR spectra of Mn-TrSOD and close-up of the region centered on a g value of 2. Sample was buffered at pH 7.4

Source: by the author

The helium temperature EPR spectra of Mn-TrSOD mutants are shown in Figure 27. The spectra were recorded without reduction of Mn in the enzymes. Native Mn-TrSOD and mutants were purified predominantly in the Mn(III) state (because of the contact with the air) which is EPR silent.^{122,124} Because of the mixtures of oxidation states the EPR spectra of the native and mutants displayed a very weak spectra characteristic of Mn-SOD in the Mn(II) state (typical spectra shown in Figure 26) and the Mn(III) is also present making the typical deformation of the spectra. (e.g. spectra of M27V, D150G and double mutant) which is characteristic of Mn (shown in the Figure 25 D, E and F)).

There are a number of clear differences between the spectra of these mutants and the native Mn-TrSOD and mutant Q172D Mn-TrSOD is distinctly different from all the other mutants as well as the native. The Q172D mutant was dominated principally by the Fe(III) present in the sample (AAS results). The Mn(II) hyperfine structure was not resolved in the EPR spectrum of these SOD mutants probably because most of the bound metal was in the Mn(III) state.

The best characterized spectrum was obtained from H75I-MnTrSOD (Figure 27 B) which presented six lines, spaced 90 to 100 G apart, with nearly the same amplitudes. This is the hyperfine structure and arises from the magnetic interaction of the electron spin ($S = 5/2$) on Mn(II) and its nuclear spin ($I = 5/2$).¹²⁵

Figure 27 - EPR spectra of Mn-binding mutants (A) G73ATrSOD, (B) H75ITrSOD, (C) Q172DTrSOD, (D) M27VTrSOD, (E) double mutant (G74Q+Q149G)-TrSOD and (F) D150GTrSOD without background subtraction. Samples were buffered at pH 7.4 with 50 mM PBS.

Source: By the author.

3.7 Analysis of EPR, AAS and enzymatic activity in SOD enzymes

3.7.1 Native TrSOD

T. reesei SOD has previously been biochemically characterized.⁸⁸ Comparison of its sequence with those of other SODs (Figure 28) initially suggested that it was a Mn-SOD. However, the combined results from EPR, enzymatic activity, and AAS presented herein suggest that this enzyme is in fact a cambialistic SOD. One of the outstanding features of the TrSOD enzyme is its ability to uptake environmental Fe even when its activity with Fe is comparatively low (10 times) relative to Mn. This uptake of Fe was observed after expressing this protein with 100 mM of both FeSO₄ and MnCl₂. In order to understand this Fe-binding preference, we compared the amino acid sequence from TrSOD with other SOD sequences (see Table 28).

Table 28 shows that TrSOD presents all of the residues described as being highly conserved in Mn-SODs (A45, Q149, D150, G74, M27, V187, H73 and H71), three of the residues which are specific for dimers (N71, P151 and T145), three which are specific for tetramers (Q172, I26 and F70) and one residue (F70) specific for Fe. This Fe-specific F70 residue is also present in both cambialistic SODs from *propionibacterium shermanii*¹²⁶ and *Aeropyrum pernix*.¹²⁷ In effect, the F70 residue may confer on TrSOD the ability to bind and retain catalytic activity with Fe ion. The EPR spectrum of the native TrSOD shows Fe-based signals similar to those observed for *E. coli* and *P. shermanii* Fe-SOD.³⁹ The EPR spectrum of Mn-TrSOD was also observed and the AAS confirmed the presence of Mn in the active site of the protein. Clearly, this SOD has the ability to use either metal and acquires the two metals depending on their availability in the culture medium and is catalytically active with either metal. All these findings suggest that TrSOD behaves as a cambialistic enzyme.

Table 27 - A comparison of the residue position specific for dimers, tetramers, Mn, Fe, Fe-dimers, Mn-dimers, Fe-tetramers and Mn-tetramers described by Wintjens, Bleicher and Bachega and compared to TrSOD and the cambialistic SODs of *Propionibacterium shermanii*¹²⁶ (PDB: 1AR4) and *Aeropyrum pernix*¹²⁷ (PDB: 3AK3).

	Wintjens: Residue positions 1ISA ^a	Bleicher: Residue positions 3ESF ^b	Bachega: Residue positions 3ESF ^b	Tetramer cambialistic 1AR4 ^c	proteins SODs 3AK3 ^d	TrSOD
Dimers	T22, F118, N65 and P144	F121, N144, T24, P148, N68 and T142	F121, N144, T24, E156, P148, N68, and T142	N65	N68	N71, P151 and T145
Tetramers	Q165, I22 and F65	----	----	Q170, I23 and F67	Q174, I27 and F71	Q172, I26 and F70
Fe	D165	F67, A71, Q72, D170 and F78	F67, A71, Q72, D170, F78, A145, A70 and W74	F67 and A70	F69 and A74	F70
Mn	Q141, D142, G68, M23 and V180	G72, M25, D146, Q145 H73 and H71	G159, A43, G72, M25, D146, Q145, H73 and H71	V185, M25 and A41	V189, M27 and A46	A45, G161 V187 Q149, D150, G74, M27, V187, H73 and H71
Fe-dimers	Q69, A141, F64, A68 and F74	----	----	F67	F71	F70
Mn-dimers	D19, S137 and R64	----	----	----	----	----
Fe-tetramers	L72 and H141	----	----	----	----	----
Mn-tetramers	P143 and V169	----	----	V174	----	----

^aThe 1ISA numbering is used corresponding to the Mn-SOD of *E. coli*

^bThe 3ESF numbering is used corresponding to the TbSODB2 of *Trypanosoma brucei*

^cThe 1AR4 numbering is used corresponding to the cambialistic SOD of *P. shermanii*

^dThe 3AK3 numbering is used corresponding to the cambialistic SOD of *A. pernix*

Source: By the author.

3ESF	1	...	M	A	F	S	P	L	P	N	C	Y	D	G	L	A	K	G	I	S	K	E	Q	V	T	F	H	Y	D	K	H	H	M	G	Y	V	T	K	L	N	A	A	N	..	S	N	P	A	L	A	K	S					
1ISA	1	...	S	F	E	L	P	A	L	P	Y	A	K	D	A	L	A	P	H	I	S	A	E	T	I	E	Y	H	Y	G	K	H	H	Q	T	Y	V	T	N	L	N	L	I	K	..	G	T	A	F	E	G	K	S				
1AR4	1	...	A	V	T	L	P	E	L	P	D	Y	S	A	L	E	P	Y	I	S	G	E	I	M	E	L	H	H	D	K	H	H	K	A	Y	V	D	G	A	N	T	A	L	D	K	L	A	E	A	R	D	K	A	D			
3AK3	1	M	V	S	F	K	R	Y	E	L	P	P	L	P	Y	N	N	A	L	E	P	Y	I	E	E	I	M	K	L	H	H	Q	K	H	H	N	T	Y	V	K	G	A	N	A	L	E	K	I	E	K	I	L	K	G	E	I	
TrSOD-I	1	M	S	V	G	T	F	S	L	P	A	L	P	Y	A	D	A	L	E	P	S	I	S	A	Q	I	M	E	L	H	H	S	K	H	H	Q	T	Y	V	T	N	L	N	N	A	L	K	T	Y	S	T	A	L	A	A	N	D

3ESF	55	V	E	E	I	I	R	T	E	K	G	P	I	F	N	L	A	A	Q	I	F	N	E	N	F	Y	W	E	S	M	S	P	N	G	..	G	G	E	P	S	G	..	K	L	A	E	A	I	R	A	S	F	G	S	F	A	K	F	K	
1ISA	52	L	E	E	I	I	R	S	S	E	G	G	V	F	N	N	A	A	Q	V	W	N	E	T	F	Y	W	N	C	L	A	P	N	A	..	G	G	E	P	T	G	..	K	V	A	E	A	I	T	A	A	S	F	G	S	F	A	D	F	K
1AR4	55	F	G	A	I	N	K	L	E	K	D	L	A	F	N	L	A	G	H	V	N	E	S	V	F	W	K	N	M	A	P	K	G	S	A	P	E	R	P	T	D	..	E	L	G	A	A	T	D	E	F	F	G	S	F	D	N	M	K	
3AK3	59	Q	I	D	V	R	A	V	M	R	D	F	S	F	N	Y	A	G	H	I	M	E	T	I	F	W	P	N	M	A	P	P	G	K	G	G	G	T	P	G	G	..	R	V	A	D	L	I	E	K	Q	F	G	G	E	K	F	K		
TrSOD-I	58	V	P	S	Q	I	A	L	Q	A	A	I	K	F	N	G	G	H	L	N	E	S	L	F	W	E	N	L	C	P	A	S	S	P	D	A	D	P	A	S	A	F	E	L	T	A	E	I	A	K	T	M	G	S	L	D	K	F	K	

3ESF	111	E	E	F	T	N	A	A	V	G	H	F	G	S	G	W	A	L	V	Q	D	T	T	T	K	K	L	K	V	F	Q	T	H	D	A	G	C	P	L	T	E	A	D	L	K	P	I	L	T	C	D	V	W	E	H	A	Y	Y	I	D
1ISA	108	A	Q	F	T	D	A	A	I	K	N	F	G	S	G	W	T	L	V	K	N	S	D	G	K	..	L	A	I	V	S	T	S	N	A	G	T	P	L	T	..	T	D	A	T	P	L	L	T	V	D	V	W	E	H	A	Y	Y	I	D
1AR4	112	A	Q	F	T	A	A	T	G	I	Q	G	S	G	W	A	S	L	V	W	D	P	L	G	K	R	..	N	T	L	Q	F	Y	D	H	Q	N	N	L	P	..	A	G	S	I	P	L	L	Q	L	D	M	W	E	H	A	Y	Y	L	Q
3AK3	116	A	L	F	S	A	A	K	T	V	E	G	V	G	W	G	V	L	A	F	D	P	L	T	E	E	L	R	I	L	Q	V	E	K	H	N	V	L	M	T	..	A	G	L	V	P	I	L	V	I	D	V	W	E	H	A	Y	Y	L	Q
TrSOD-I	117	E	A	M	G	K	A	Y	V	D	N	I	W	K	V	I	N	W	K	T	A	E	Q	R	F	K	G	D	R	E	D	A	F	K	I	..	L	K	A	S																				

3ESF	171	Y	K	N	D	R	P	A	Y	V	Q	T	F	N	W	V	V	N	W	D	H	A	E	N	Q	F	T	R												
1ISA	166	Y	R	N	A	R	P	C	Y	L	E	H	F	W	A	L	V	N	W	E	F	V	A	K	N	L	A													
1AR4	171	Y	K	N	V	K	G	D	Y	V	K	S	W	N	V	V	N	W	D	D	V	A	L	R	F	S	E	A	R	V	A									
3AK3	175	Y	K	N	D	R	G	S	Y	V	E	N	W	N	V	V	N	W	D	D	V	E	K	R	L	E	Q	A	L	N	N	A	K	P	L	Y	L	L	P	Q
TrSOD-I	173	Y	L	N	G	K	A	Y	V	D	N	I	W	K	V	I	N	W	K	T	A	E	Q	R	F	K	G	D	R	E	D	A	F	K	I	..	L	K	A	S

Figure 28 - Protein-sequence alignment of five superoxide dismutases. (*Trichoderma reesei* SOD (TrSOD-I) numbering). Note that the *E. coli* MnSOD (1ISA) and the *T. brucei*⁸⁵ FeSOD (3ESF) were used as a typical proteins representative of Mn-dimers and Fe-tetramers and *P. shermanii*¹²⁶ (PDB: 1AR4) together with *Aeropyrum pernix*¹²⁷ (PDB: 3AK3) are representative of tetrameric cambialistic enzymes. Strictly conserved residues in all four SODs are colored in red and those that are highly conserved are colored in yellow. Alignment was carried out using ESPrict 3.0.

Source: By the author.

Cambialistic enzymes share conserved amino acids with highly specific Mn-SODs or Fe-SODs. TrSOD is 63% identical with a Mn specific SOD from *Aspergillus fumigatus*¹²⁸ (PDB code: 1KKC), and 42% and 39% identical with the cambialistic SODs from *P. shermanii* and *A. pernix*, respectively. Since the tetrameric enzyme TrSOD retains activity with both metals but with the Mn form being more active we classify it as belonging to the Mn-like sub-group.

These cambialistic SODs were found in microorganisms adapted to different growth conditions, including microaerophiles, aerobes, obligate anaerobes and hyperthermophiles. Modern Fe-SODs and Mn-SODs are believed to have evolved from a common ancestor via cambialistic SODs.¹²⁰ However, our knowledge about the determinants of the specific properties of this group of enzymes is still poor. In fact, crystal structures for only a few cambialistic SODs have been determined, those from the bacteria *Propionibacterium shermanii* (PDB:1AR4),³⁸ *Porphyromonas gingivalis* (PDB:1QNN),³⁹ *Clostridium difficile* (PDB: 4JYY),^{39,129} *Aeropyrum pernix* K1 (PDB: 3AK3)¹²⁷ and the recently resolved structures of *Streptococcus mutans* (PDB: 4YIP)¹⁰⁸ and *Streptococcus thermophilus* (4YIO).¹⁰⁸ Most of these cambialistic SODs possess almost exclusively the typical determinants of Mn-SODs similar to TrSOD. Since the

results showed here classify the TrSOD as a cambialistic enzyme, it will be referred to as “cambialistic TrSOD” throughout the remainder of this thesis.

Some cambialistic enzymes have been found as dimers and others have been found as tetramers (such is the case of TrSOD). Formation of dimers and tetramers in some cases, may simply be a way to provide thermal stability to the mature fold and/or prevent degradation from proteases.

MnSOD from *Saccharomyces cerevisiae* mitochondria (ScMnSOD) and another from *Candida albicans* cytosol (CaMnSODc), have many biochemical and biophysical properties in common, yet ScMnSOD is a tetramer and CaMnSODc is a dimer (alternatively it has been called a “loose tetramer”) in solution. Sheng, et al¹¹⁷ has reported that although CaMnSODc was found to crystallize as a tetramer, there is no indication from the solution properties that the functionality of CaMnSODc *in vivo* depends upon the formation of the tetrameric structure. In other words, the oligomeric state may not influence the catalytic activity of these SODs.

Molecular exclusion results showed that the cambialistic TrSOD at low concentrations (8.6 μ M) behaves as a dimer. This finding suggests that TrSOD in the cell could be active as a dimer and, according to the protein concentration, the oligomeric state could change (from dimeric to tetrameric) possibly co-existing but without affecting the activity of the enzyme.

3.7.2 Characterization of point mutants

EPR, AAS and enzymatic activity were measured for six point mutations of TrSOD (M27V, G73A, H75I and D150G designed to decrease Mn specificity; and L80F and Q172D designed to increase Fe specificity).

M27V, H75I, L80F and D150G-TrSOD mutants. These mutations did not cause a big impact on their catalytic activity compared to the native TrSOD. The higher activity detected in most of these mutants is explained by the higher metal content presented in the active site (AAS). The ability of the native enzyme to retain Fe in the active site over Mn was retained in all these mutant proteins that preferentially bind Fe over Mn. The EPR spectra variations from one mutant to another correspond to very small changes in the metal environment that are commonly reported in SODs.

Q172D-TrSOD mutant. This mutant under the three different conditions showed higher relative content of Mn and this may be a reason for its higher activity compared to the other mutants. Interestingly, this mutation is a clear example that the substitution of one amino acid by an Fe- specific residue is not enough to explain specificity in SOD enzymes.

G73A-TrSOD mutant. This mutant has a higher metal content but is not enzymatically active. This result is in good agreement with the EPR spectrum which has a more axial EPR signal in comparison with the more rhombic EPR signal presented by the native TrSOD. This type of signal is not unusual as it has been observed in SODs from other organisms when binding superoxide inhibitors such as azide. The azide ion is known to be a competitive inhibitor of the enzymatic activity of Fe-SOD and Mn-SODS.⁸⁰⁻⁸¹

The spectra of G73ATrSOD appears to be identical to crystals of the *E. coli* enzyme, of *Ps. Ovalis* and of the structure of the *Caenorhabditis elegans* MnSOD-3-azide complex.¹³⁰ These enzymes have been shown to form a 6-coordinate species with azide suggesting that the G73ATrSOD mutant also presents six- coordinate geometry in the active site which is an explanation of its lack of activity.

3.7.3 Characterization of (G74Q+Q149G) double mutant

To analyze the importance of the position and interactions of the glutamine, the double mutant (G74Q+Q149G) was tested. One of the most relevant difference in the Fe/MnSODs family is that the role of Gln149 in Mn-TrSOD is performed by Gln74 in Fe-specific SODs. The Gln74 in Fe-SODs is clearly analogous to the Gln at position 149 of Mn-TrSOD as their side chains occupy similar positions in the active site and its hydrogen bonding network. Cambialistic TrSOD has glycine at position 74 and it is believed that this glycine (alanine in some FeSODs) accommodates the Gln149 side chain allowing it to perform its function. By changing the position of this Gln149 to Gln74 (and placing a Gly at position 149) in TrSOD, we were interested to observe if this increased the specificity for Fe. AAS results for this mutant show that Fe-binding is maintained for the active site of this mutant protein. The EPR spectra of the double mutant has two additional g values of 3.7 and 3.5 that could indicate the variations caused by the mutation in the active site of the enzyme.

A series of similar mutants targeting the equivalent glutamine residue for the human mitochondrial form of MnSOD have been performed.^{62,131} The mutant Q143H-mito-MnSOD had a 150-fold decrease in activity. The mutant Q143A-mito-MnSOD had a 250-fold decrease in activity. This mutation (Q143H) resulted in a longer Mn-solvent distance with respect to Gln, giving an increased E_m . In this report, another water molecule seems to mediate interactions between the coordinated molecule and Gln side chain, but this new indirect hydrogen bond is likely weaker than the direct hydrogen bond associated with a higher E_m relative to MnSOD. It was demonstrated that this conserved glutamine may participate in roles other than proton transfer, such as stabilizing the active site.¹³¹

Aaron⁷² had analyzed the double mutant (G77Q+Q146A) and converted a Mn-specific SOD into a cambialistic SOD proving that the Gln, though important, is not the sole determinant of metal-ion specificity. This report explained that the interactions between the glutamine and the metal ion are different between this double mutant and specific FeSODs. The low activities reported for this previous mutant and for our double mutant seem to be related to the loss of hydrogen bonding interactions among amino acids of the second sphere of coordination (as those which were suggested for Bachega and analyzed here) that could be essential for the specificity for the metal. Overall, glutamine plays an important role in metal ion selectivity at the active site, but it is not the only factor that can influence metal specificity. In fact, we were not expecting a huge change in catalytic activity or selectivity of the metal with single point mutations, rather it could be necessary to mutate two, three or maybe all the amino acids specific for Mn to change the selectivity for the metal.

3.8 Crystallization tests, X-ray diffraction and refinement

3.8.1 Crystallization

Initially, for the native protein, needles were obtained after 72 h using the screen Crystal Index (the condition is based on 25% polyethylene glycol 3350, 0.1M Bis-Tris at pH 6.5 (Figure 29)). Improvement of the crystal quality and size was achieved by refining the initial crystallization condition (varying the pH).

Figure 29 – Crystals of TrSOD obtained from the screen Crystal Index in the condition: 25% (w/v) PEG 3350, 0.1M Bis-Tris at pH 6.5.

Source: By the author.

Small hexagonal and monoclinic crystals appeared within 7 to 15 days and grew to about 500 μm along the largest dimension. The best crystals for different mutant proteins appeared at different pH values as shown in Table 29. Figure 30 shows the best crystals obtained for each of the mutants for which crystallization was successful.

Table 28 - Crystallization conditions for native TrSOD and mutant proteins.

Samples	Precipitant	pH	protein concentration
G73A-TrSOD	PEG 3350 25% 0.1M Bis-Tris	5.7	8.7 mg/mL
L80F-TrSOD		6.5	6.3 mg/mL
H75I-TrSOD		6.9	6.3 mg/mL
D150G-TrSOD		6.7	8.7 mg/mL
M27V-TrSOD		6.1	8.1 mg/mL
(G74Q + Q149G)-TrSOD		6.0	10.3 mg/mL
Q172D-TrSOD		6.5	8.0 mg/mL
Native Mn-TrSOD		5.6	4.3 mg/mL

Source: By the author.

Figure 30 - Crystals obtained in the condition: 25% (w/v) PEG 3350, 0.1M Bis-Tris varying pHs. A: Crystals of mutant Q172D Mn-TrSOD at pH 6.5; B: Crystals of mutant M27V-Mn-TrSOD at pH 6.1; C: Crystals of mutant H75I-Mn-TrSOD at pH 6.9; D: Crystals of mutant L80F-Mn-TrSOD at pH 6.5; E: Crystals of mutant D150G Mn-TrSOD at pH 6.7 and F: at pH 6.9; G: Crystals of Native Mn-TrSOD at pH 6.3 and H: at pH 5.6.

Source: By the author.

Interestingly, changing the percentage of PEG 3350 did not improve the quality of the crystals. Although small crystals were easily obtained, several steps of refinement were necessary (changing the pH) in order to obtain reasonably sized

crystals for data collection. Reproducibility of crystals depended on the purification and some purification batches did not yield crystals at all. Several crystals' habit were obtained for each mutant and those that diffracted best after freezing were used for data collection.

Overall, all the crystals yielded good diffraction data except for those of the double mutant (G74Q+Q149G), which did not diffract well. Furthermore, the data collected for Q172D presented low resolution and the data was impossible to process.

3.8.2 Data collection and refinement

General descriptions for the crystal structures were separated into two different groups according to the metal used to grow the crystals: Mn-TrSOD and Fe-TrSOD structures.

Mn-TrSOD structures: The native superoxide dismutase from *Trichoderma reesei* (Mn-TrSOD) was solved and refined against data extending to 2.45 Å spacing. Native Mn-TrSOD was found in the space group of P1 with unit cell parameters $a = 73.72$ Å, $b = 112.96$ Å, $c = 162.94$ Å and angles of $\alpha = 88.49^\circ$, $\beta = 86.24^\circ$ and $\gamma = 89.94^\circ$. The Matthews coefficient of 2.36 Å³Da⁻¹ implies an estimated solvent content of 48% with twenty-four subunits related by non-crystallographic symmetry. These chains are labelled from A to X and the electron density maps are contiguous from residue 2 to about residue 210 for all modelled chains. However, some residues (the first residue at the N-terminus, the final 4 residues at the C-terminus, and the last 12 residues for the X and T chains) could not be observed.

The final structure of native Mn-TrSOD gave a crystallographic R- factor of 18.4% and a R_{free} of 21.2%. The Ramachandran plot indicates that backbone torsion angles for most non-glycine residues fall within energetically favorable and acceptable regions. No serious geometrical defects of either main chain or side chain properties were observed from the analysis with MolProbity. The refinement statistics are given in Table 30.

Table 29 - Data collection and refinement statistics for Native Mn-TrSOD.

Data collection and refinement	Native Mn-TrSOD
Beamline	APS (21-ID-G)
detector	MAR 300
Wavelength (Å)	0.9786
Temperature (K)	100
Space group	P1
Resolution range	93.90 - 2.45 (2.58 - 2.45) ^a
Unit cell axes (Å)	
a	73.72
b	112.96
c	162.94
Angles (α, β and γ)	88.49° 86.24° 89.94°
Total reflections	633293 (89341)
Unique reflections	187783 (18568)
Multiplicity	3.4 (3.3)
Completeness (%)	97.5 (96.7)
I/sig(I)	11.6 (5.6)
Wilson B-factor	20.91
R-factor	0.1849 (0.2095)
R-free ^b	0.2129 (0.2677)
Number of protein atoms	38604
Number of Mn ²⁺ ions	24
Number of solvent molecules	2038
Number of protein residues	4980
RMS(bonds(Å)) ^c	0.008
RMS(angles(°))	0.89
Ramachandran favored (%)	97
Clashscore	2.82
Average B-factor	28.86

^aIn parentheses, data for the outermost resolution shell are given.

^bFree *R*-factor calculated with 10% of the total reflections held aside through refinement for cross-validation.

^crmsd, rms deviation.

Source: By the author.

Mn-G73ATrSOD and Mn-M27VTrSOD belonged to the orthorhombic space group $P2_12_12_1$ with unit cell constants: $a = 75.1 \text{ \AA}$, $b = 110.6 \text{ \AA}$, $c = 180.9 \text{ \AA}$ and $a = 69.1 \text{ \AA}$, $b = 77.6 \text{ \AA}$, $c = 159.0 \text{ \AA}$, respectively. The Mn-L80FTrSOD mutant belonged to the orthorhombic space group $P22_12_1$ with the following unit cell constants: $a = 73.1 \text{ \AA}$, $b = 75.0 \text{ \AA}$, $c = 239.2 \text{ \AA}$. These three mutants had eight, four and six subunits in the asymmetric unit with respective Matthews coefficient of $2.50 \text{ \AA}^3\text{Da}^{-1}$, $2.20 \text{ \AA}^3\text{Da}^{-1}$ and $2.29 \text{ \AA}^3\text{Da}^{-1}$ which yielded estimated solvent contents of 51%, 44% and 46%, respectively. Each chain of these mutants binds one Mn ion and the final models of each contain 832, 976, and 329 solvent water molecules, respectively. The final structure of Mn-G73ATrSOD gave a crystallographic R-factor of 21.6% and a R-free of 24.7%. The RMSD's from the ideal values are 0.003 for bond lengths and 0.62° for bond angles. For Mn-M27VTrSOD the final values of R-factor and R-free are 16.2% and 18.5% respectively; and for Mn-L80FTrSOD, 24.7% and 29.7%, respectively. The Ramachandran plots for these three mutants indicate that the backbone torsion angles for most non-glycine residues fall within favourable and acceptable regions. A full list of data collection and refinement statistics for these mutants are reported in Table 31.

Table 30 - Data collection and refinement statistics for Mn-L80FTrSOD, Mn-73ATrSOD and Mn-M27VTrSOD.

	Mn-L80FTrSOD	Mn-M27VTrSOD	Mn-G73ATrSOD
Data collection and refinement			
Beamline	APS (21-ID-G)	I04	APS (21-ID-G)
detector	MAR 300	PILATUS 6M-F	MAR 300
Wavelength (Å)	0.9786	0.9795	0.9786
Temperature (K)	100	100	100
Space group	P 2 ₁ 2 ₁	P 2 ₁ 2 ₁ 2 ₁	P 2 ₁ 2 ₁ 2 ₁
Resolution range	47.9 - 2.0 (2.07 - 2.00) ^a	48.7 - 1.6 (1.67 - 1.62)	72.1 - 2.0 (2.1 - 2.0) ^a
Unit cell axes (Å)			
a	73.1	68.1	95.1
b	75.0	77.6	110.6
c	239.2	159.0	180.9
Total reflections	361289(12155)	711529 (68313)	1494155 (119526)
Unique reflections	85235 (8180)	107725 (10599)	120699 (11952)
Multiplicity	4.2 (2.9)	6.6 (6.4)	12.4 (10.0)
Completeness (%)	95	99.98 (99.95)	98 (100)
I/sig(I)	4.3 (0.4)	15.28 (1.55)	12.35 (0.62)
Wilson B-factor	38.40	18.71	39.37
R-factor	24.7 (41.1)	16.2 (26.6)	21.6 (37.4)
R-free ^b	29.7 (40.7)	18.5 (27.6)	24.7 (37.6)
Protein atoms	9822	6429	12198
Number of Mn ²⁺ ions	6	4	8
Solvent molecules	329	976	832
Protein residues	1266	829	1575
RMS(bonds(Å)) ^c	0.002	0.007	0.003
RMS(angles (°))	0.52	1.08	0.62
Ramachandran favored (%)	96	97	98
Clashscore	2.84	2.36	2.70
Average B-factor	53.84	26.20	52.61

^aIn parentheses, data for the outermost resolution shell are given.^bFree *R*-factor calculated with 10% of the total reflections held aside through refinement for cross-validation.^crmsd, rms deviation.

Source: By the author.

Fe-TrSOD structures: Under the best conditions, crystals diffracted at 3.1 Å, 2.03 Å, 1.85 Å and 1.4 Å resolution, for Fe-Q172DTrSOD, Fe-H75ITrSOD, Fe-D150GTrSOD and Fe-M27VTrSOD respectively. The data collected for Fe-Q172DTrSOD was not good enough to be processed.

The crystal for Fe-H75ITrSOD belonged to the space group P2₁2₁ with cell parameters of *a* = 73.43 Å, *b* = 75.17 Å, *c* = 241.8 Å and presented six molecules in the asymmetric unit with a Matthews coefficient of 2.33 Å³Da⁻¹ and an estimated solvent content of 47%. Mutants, Fe-D150GTrSOD and Fe-M27VTrSOD belonged to the orthorhombic space group P2₁2₁2₁ and presented four molecules in the asymmetric unit with respective Matthews coefficient of 2.27 Å³Da⁻¹ and 2.04 Å³Da⁻¹ and estimated solvent contents of 46% and 40% respectively. The unit cell parameters are *a* = 79.06

Å, $b = 82.03$ Å, $c = 133.72$ Å and $a = 62.74$ Å, $b = 78.17$ Å, $c = 158.38$ Å, respectively. Each chain of these mutants contains one Fe ion with each one containing 1308, 1230, and 1106 solvent water molecules, respectively. The electron density maps are contiguous for all modelled chains.

For each mutant, the introduced amino acid is well defined by the electron density. The final values of the R-factor and R-free for H75I are 15.8% and 18.7%, respectively; for D150G these values are 16.2% and 18.8%, respectively and for Fe-M27V 15.6% and 18.0%, respectively. The Ramachandran plot of each mutant indicates that the backbone torsion angles for most of the non-glycine residues fall within favourable and acceptable regions. A full list of data collection and refinement statistics for these mutants are reported in Table 32.

From the data presented in Tables 31 and 32 it is clear that the two crystal forms of the M27V mutant (in the presence of the different metals) are isomorphous. Furthermore, the L80F and H75I mutants, which both crystallize in space group $P2_12_1$ with six subunits in the asymmetric unit, are also isomorphous. On the other hand, the G73A and D150G mutants both present unique crystal forms, consistent with the significant structural changes that the mutations induce and which will be described below.

The atomic coordinates and structure factors of all the structures will be deposited in the Protein Data Bank (PDB).

Table 31 - Data collection and refinement statistics for Fe-D150GTrSOD, Fe-H75ITrSOD and Fe-M27VTrSOD mutants.

	Fe-D150GTrSOD	Fe-H75ITrSOD	Fe-M27VTrSOD
Data collection and refinement			
Beamline detector	APS (21-ID-F) MAR 225	APS (21-ID-G) MAR 300	I04 PILATUS 6M-F
Wavelength (Å)	0.9787	0.9786	0.9795
Temperature (K)	100	100	100
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₂ 12 ₁	P2 ₁ 2 ₁ 2 ₁
Resolution range (Å)	51.05 - 1.85 (1.916 - 1.85) ^a	40.67 - 2.03 (2.103 - 2.03) ^a	49.18 - 1.4 (1.45 - 1.4) ^a
Unit cell axes (Å)			
a	79.0	73.4	62.7
b	82.0	75.1	78.1
c	133.7	241.8	158.3
Total reflections	145435 (14714)	172417 (16501)	1381567 (114927)
Unique reflections	74311 (7420)	87292 (8584)	152494 (14895)
Multiplicity	2.0 (2.0)	2.0 (1.9)	9.1 (7.7)
Completeness (%)	99.21 (100.00)	99.82 (99.36)	99.27 (98.08)
I/sig(I)	11.27 (4.56)	17.82 (6.56)	13.54 (1.46)
Wilson B-factor	16.33	22.25	14.90
R-factor	16.2 (20.6)	15.8 (18.1)	15.6 (25.9)
R-free ^b	18.8 (25.1)	18.7 (21.7)	18.0 (32.3)
Number of protein atoms	6441	9792	6077
Number of Fe ³⁺ ions	4	6	4
Number of solvent molecules	1230	1308	1106
Number of protein residues	833	1266	786
RMS(bonds(Å)) ^c	0.003	0.004	0.013
RMS(angles(°))	0.81	0.81	1.41
Ramachandran favored (%)	97	97	98
Clashscore	2.90	1.13	3.34
Average B-factor	21.50	28.30	22.80

^aIn parentheses, data for the outermost resolution shell are given.

^bFree *R*-factor calculated with 10% of the total reflections held aside through refinement for cross-validation.

^crmsd, rms deviation.

Source: By the author.

3.9 The crystal structure of MnTrSOD

The final model of the native Mn-TrSOD consists of twenty-four polypeptide chains related by non-crystallographic symmetry together with twenty-four manganese ions and 3207 water molecules in the asymmetric unit. The 24 polypeptide chains are organized into six tetramers which have 222 (D₂) symmetry. The first tetramer will be used to describe most aspects of the structure. The tetramer is composed of subunits A, B, C and D and each subunit binds one Mn ion. Below we describe a subunit.

3.9.1 Subunit fold

Each subunit of *T. reesei* SOD consists of nine α -helices (H1-H9), three 3_{10} helices, one three-stranded (S1-S3) antiparallel β -sheet and loops connecting those secondary elements shown in Figure 31.

Figure 31 - Cartoon representation of the TrSOD monomer showing Mn ion (purple sphere) in the active site. The colors indicate each element corresponding to the secondary structure of the monomer. The N and C terminals of the monomer are labeled as N and C, respectively. This figure was prepared using chimera.

Source: By the author.

All these secondary structure elements form two domains in the subunit: an N-terminal domain, mainly made up of H1 (Ala24-Ala54) and H3 (Gln65-Glu83) which are long antiparallel α -helices connected by the short H2 helix (Val58-Ala63).

The C-terminal domain shows an α/β fold with six helices (H4 (Pro99-Trp109), H5 (Leu112-Gly125), H6 (Tyr170-Tyr173) H7 (Lys177-Val187), H8 (Trp190-Lys198) and H9 (Arg101-Ala210)), three 3_{10} helices (Pro91 to Ala93, Pro95 to Ser97 and Glu166 to Ala168) and three β -strands S1 (Gly130-Glu137), S2 (Gly140-Lys147) and S3 (Val157-Asp163).¹³¹ The Mn ion in the active site is bound between these two domains (shown in Figures 31 and 32).

Figure 32 - The secondary structure of one monomer of TrSOD superimposed on its amino acid sequence. The secondary structure is indicated above the sequence. The figure was generated with the PDBsum server.

Source: By the author.

All single-metal binding Fe/Mn-SODs fall into the same subfamily with respect to their fold topology; however, a remarkable structural difference is seen at a junction between the N-terminal helices, classifying these SODs into three different groups: Group 1; SODs that lack an intervening helix H2, and helices H1 and H3 are directly connected with a short loop. (e.g., Mn-SODs from human mitochondria,^{34,132} Fe-SOD from *Mycobacterium tuberculosis*¹³³ and cambialistic SOD from *Propionibacterium shermanii*³⁸). Group 2; SODs that have an intervening fragment containing a short helix H2 between the two helices H1 and H3. (e.g., Fe-SODs from *Escherichia coli*⁴⁴, cambialistic SOD from *Porphyromonas gingivalis*³⁹). Group 3; SODs with longer loop between H1 and H2 (e.g., Fe-SOD from *Aquifex pyrophilus*¹³⁴). An example of each group is shown in the Figure 33.

Figure 33 - Ribbon diagram of different SOD monomers showing differences in the N terminal domain (red). Example of the group 1 correspond to the Fe-SOD from *M. tuberculosis*, example of the group 2 correspond to the Fe-SOD from *E. coli* and example of the group 3 correspond to the Fe-SOD from *A. pyrophilus*. The N and C terminals of each monomer are labeled as N and C, respectively. This figure was prepared using chimera.

Source: By the author.

The crystal structure of the native SOD from *T. reesei* clearly shows that its chain folding belongs to the first group. It is important to mention that helix 2 in TrSOD protein is an extension of helix 3. These helices project out into the solvent region making the shape of the monomer look like a letter 'L'.

Comparing the overall conformation of Mn-TrSOD with other SODs, we have found two noticeable differences:

Conformational differences exist for the L2 loop (that connect the N-terminal domain with the C-terminal domain, between H3 and H4) which shows the most variation in the primary sequence across species. L2 loop in Mn-TrSOD has an extended form (including two short 3_{10} helices) and plays a key role in forming a compact tetramer. Within the monomer, it is not involved in interactions with any other regions.

Another noticeable difference involves the last twelve residues of Mn-TrSOD which are not normally present in other Fe/Mn-SOD sequences and form a unique α -helix, which is rather irregular in structure, (H9). Although the MnSOD from *A. fumigatus*¹²⁸ is even longer than TrSOD, having an additional nine residues at its C-terminus, these were not observed to be structurally ordered. A comparison of the last helices of SODs from *T. reesei* and *A. fumigatus* is shown in Figure 34.

Source: By the author.

3.9.2 The active site geometry

The active site geometry is well defined by the electron density map (Figure 35) and is effectively identical to that reported for homologous SODs.^{38, 127, 129} The Mn is penta-coordinated by four residues (His30, His78, Asp167, and His163) and a solvent

molecule that has been proposed to be a water molecule. The four ligands which coordinate the Mn ion are invariant amongst all Fe and Mn-SOD whose structures have been determined.

Two ligands are provided by each domain: The N-terminal domain provides His30 (H1) and His78 (H3), both coordinated via their N ϵ^2 nitrogen. The C-terminal domain provides Asp167 (coordinated via its O δ^2 oxygen) and His163 (in the 3₁₀-helix, after S3, coordinated via its N ϵ^2 nitrogen). The coordination is trigonal bipyramidal which is in good agreement with all Fe/Mn-SODs reported to date. Three of the four atoms (His78 (N ϵ^2), Asp163 (O δ^2) and His167 (N ϵ^2)) form the trigonal planar base and His30 (N ϵ^2), is coordinated in an axial position. Interatomic distances between the metal ion and each ligand scarcely diverge among SODs.

The area around the active site is fairly hydrophobic with two tryptophan residues (131 and 165) 5.1 Å and 5.3 Å from the metal, respectively. Trp131 forms a hydrogen bond to Gln149, which in turn forms a hydrogen bond with the hydroxyl group of Tyr38. The Tyr38 is a highly conserved amino acid that forms a hydrogen bond with His34 via a water molecule. His34 from one subunit makes a hydrogen bond with Tyr170 of the other subunit. The classical hydrogen bonded network extends further from the metal bound solvent of one subunit to the other subunit allowing the communication between the two metal sites.^{33, 37} The position of the Mn defines a cavity inside the protein which is hidden and protected by three additional residues, His35, Tyr38, and Trp165. These three residues are involved in the control of substrate and product transportation.

Figure 35 - $2F_{\text{obs}} - F_{\text{calc}}$ electron density map around the Mn binding site of one subunit of Mn-TrSOD contoured at 1.5σ . The amino acid residues surrounding the active site are drawn in a stick representation. The manganese ion (purple sphere) is bound by His30, His78, Asp163, and His167. The fifth coordination site is occupied by a water molecule (red sphere) which binds Gln149.

Source: By the author.

3.9.3 Monomer-Monomer interactions

The subunits A, B, C, and D of Mn-TrSOD associate to form dimers, which further oligomerize to form tetramers. There are three different interfaces for each monomer: The first interface is formed by monomers A with B (A/B which is the same as that between monomers C and D (C/D)), the second interface is formed by monomer A with C (A/C=B/D) and the third interface is formed by monomer A with D (A/D=B/C).

Interface A/B (C/D):

The chains labeled A and B (C/D) associate about a non-crystallographic local 2-fold axis. The interface includes interactions involving residues from helix 1 (H1) and residues 166 to 176. The latter region is a loop which contains two single-turn helical segments, 166 to 168 (3_{10} helix) and 170 to 173 (H6), which are close to the active site (Figure 37(A)) and contain many highly conserved residues and which provide one of the Mn ligands (His167).

This dimer interface is highly conserved in all Fe/Mn-SOD structures.^{33, 117} The conserved dimer A/B (C/D) in TrSOD presented an interface area of 1046 Å² with a total binding energy of -20.26 Kcal/mol and with three highly conserved hydrogen bonds (calculated using the PISA¹³⁵ server and shown in Figure 36 (B)). In addition to the hydrogen bonds involving protein groups, there are many more involving well defined water molecules which provide intricate bridging interactions between the subunits.

Figure 36 - Native Mn-TrSOD (A) dimer interface A/B (C/D). Region 166 to 176 contains a 3₁₀ and the H6 helices (highlighted in blue). The N and C terminals of each monomer are labeled as N and C, respectively. The Mn ion is highlighted in red. (B) Information about the interaction radar generated by PISA.

Source: By the author.

Interestingly, this conserved dimer interface suggests a possible communication pathway between the two active sites in Fe/Mn-SODs. In order to understand the monomer-to-monomer communication and/or possible cooperative behavior between these two monomers, Edwards⁶⁹ investigated the role of the hydrogen bond between His30 and Tyr163 (His34 and Tyr170 in TrSOD numbering) in MnSOD from *E. coli*. They performed point mutations (H30A, H30N and Y163F) indicating that these amino acids are responsible for aspects of the catalytic mechanism including interaction or protonation of substrate in the active sites. Consequently, catalytic activity of superoxide dismutases is crucially dependent on their quaternary structure.

TrSOD presented a way of communication between the active site of each monomer. It leads to active-site coupling, via the bridging loop metal(A)-His167A, Glu166B-His167B-metal(B), linking subunit A with B (shown in Figure 37). For Fe/Mn

SODs in general the dissociation of dimers into monomers results in the loss of enzymatic activity and so the integrity of the interface seems to be crucial.

Figure 37 - Mechanism of communication between monomers. This mechanism is presented in all Fe/MnSODs, the amino acids (His167 and Glu166) responsible for this communication are highly conserved. The Mn ion is highlighted in purple. Interactions are colored in red.
Source: By the author.

Interface A/C (B/D)

The A/C (B/D) interface is formed mainly by the protruding helices from the amino terminal domain that cross each other at an angle of about 150°. This interface will be called the “tip of the helical hairpin” (Figure 38). Important interactions in this interface include hydrogen bonds between Asn56 of one subunit and Ser2 of the other and between symmetrically related Gln65. Several Van de Waals interactions also exist. This interface is tightly packed and the total binding energy is -21.05 Kcal/mol with an interface area of 1132 Å² similar to the A/B (C/D) interface.

Figure 38 - Native Mn-TrSOD (A) dimer interface A/C (B/D). The N and C terminals of each monomer are labeled as N and C, respectively. The Mn ion is highlighted in red. (B) Information about the interaction radar generated by PISA.

Source: By the author.

Interface A/D (B/C)

The A/D (B/C) interface is quite loose involving only two salt bridges between Arg142 and Glu137 of opposite subunits (Figure 39A). The total binding energy for this interaction is less than 1 Kcal/mol (according to PISA shown in Figure 39B). It is of very little importance for stabilizing the tetramer and may allow a degree of flexibility for subunit packing. This interface is probably largely the indirect result of the stabilities given by the A/B (C/D) and the A/C (B/D) interfaces.

Figure 39 - Native Mn-TrSOD (A) dimer interface A/D (B/C). The N and C terminals of each monomer are labeled as N and C, respectively. The Mn ion is highlighted in light blue. (B) Information about the interaction radar generated by PISA.

Source: By the author.

3.9.4 Quaternary structure

SODs can be classified, according to their quaternary structure, into dimers (e.g. FeSODs from *E. coli*) and tetramers (Fe-SODs from *M. tuberculosis*, Mn-SOD from human mitochondria). As already mentioned, tetramers are mainly formed by interactions between two highly conserved dimers A/B (C/D) by employing interactions made by the N-terminal domains (the A/C interface). TrSOD presents the conserved amino acid sequence corresponding to the tetrameric structure (shown in section 3.7) which was confirmed by molecular exclusion and DLS. All the theoretical and experimental analyses clearly indicated that TrSOD is a tetrameric enzyme in solution. The tetrameric structure of TrSOD is more similar to the Mn-SOD from *A. fumigatus*¹²⁸ (with 63% of similarity) than to all other Fe/Mn-SOD structures (Figure 40).

Although the structures possess a very similar overall fold, differences between their amino acid sequences could vary from one to another. The most relevant difference in fungal SOD amino acids (that have been characterized) is the existence of a higher degree of identity to the tetrameric Mn-SODs than to the other types of SODs. This is also the case for TrSOD that even though it presents cambialistic behavior, its higher preference for Mn is relevant.

Figure 40 - Comparison of the quaternary structure of (A) TrSOD tetramer and (B) Mn-SOD from *A. fumigatus*. Chains A is colored in yellow, B is colored in purple, C is colored in orange and D is colored in blue. The N and C terminals of each monomer are labeled as N and C, respectively. The Mn ion is colored in red.

Source: By the author.

Figure 41 - Electrostatic surface potential for the native TrSOD protein.

Source: By the author.

Tetrameric TrSOD protein is also compact and closed (Figure 41) and these features may protect its active site from higher water concentrations or competitive substrates. All tetrameric SODs show 222-point symmetry in their subunit arrangement but their subunit orientations are different from one another.

TrSOD presented six tetramers in the asymmetric unit which are extremely similar (after superposition of the first TrSOD tetramer with the other five tetramers) with root mean square deviations (RMSD) of 0.39 Å (for first tetramer with the second tetramer), 0.34 Å (for the first tetramer with the third tetramer), 0.41 Å (for the first tetramer with the fourth tetramer), 0.37 Å (for the first tetramer with the fifth tetramer) and 0.37 Å (for the first tetramer with the sixth tetramer). The RMSD varies from one to

another due to the obvious differences in the A/D (B/C) interfaces that gives flexibility to the quaternary structure. Figure 42 shows the six tetramers that were found in the asymmetric unit.

Figure 42 - Schematic drawing of the 24 subunits (arranged into six tetramers) found in the asymmetric unit. The metal is colored in red.

Source: By the author .

The first TrSOD tetramer will be used to compare similarities and differences of the native TrSOD structure with the structure of all mutants that were resolved. Six structures of mutants were resolved and in order to facilitate the comparison, we classified the mutants, according to the metal added when expressing the enzymes, into two different sub-groups:

Mn-SODs (Mn-M27VTrSOD, Mn-L80FTrSOD and G73ATrSOD) and
Fe-SODs (Fe-M27VTrSOD, Fe-H75ITrSOD, Fe-D150GTrSOD)

3.10 Comparison between native Mn-TrSOD and the Mn-SOD mutants

3.10.1 Structural comparison between native Mn-TrSOD and Mn-M27VTrSOD mutant

The M27V mutant was made to favour Fe selectivity as methionine is observed in most Mn-specific SODs and valine in most Fe-specific SODs. In the Mn-M27VTrSOD crystal structure there are four protein molecules in the asymmetric unit that form the obligate tetramer which is very similar to that of native Mn-TrSOD (Figure 43); the RMSD between the C α 's of Mn-M27VTrSOD tetramer with the native TrSOD is 0.61Å. This indicates that there is no significant change in the quaternary structure as a result of the mutation. The main-chain conformation for most residues of Mn-M27V mutant fall within the favored regions of the Ramachandran plot. This was verified with MOLProbity¹³⁵ which indicated that 97.1% of the amino acids are in favored regions of the Ramachandran plot and 100% of the amino acids were in the allowed regions.

Figure 43 - The Fe-M27V mutant. (A) The asymmetric unit composed of one tetramer. (B) Superposition of all monomers found in the asymmetric unit. The N and C terminals of each monomer are labeled as N and C, respectively. The metal ion is colored in red.

Source: By the author.

The four residues that bind the Mn were well defined during the refinement process (Figure 44). The Met27 is located three residues away from His30, which is a ligand binding residue. The location of this mutation and the surrounding protein side chains show that the active site coordination distances are similar for both native Mn-TrSOD

and Mn-M27VTrSOD except for Asp163 with respect to the metal ion (1.94 Å in the native protein and 2.10 Å in the M27V mutant). This may be within experimental error.

Figure 44 - $2F_{\text{obs}} - F_{\text{calc}}$ electron density map of the M27 position (three residues away from the active site) contoured at 1.5σ in the subunit A of (A) native Mn-TrSOD and V27 position in the subunit A of (B) Mn-M27VTrSOD. The amino acid residues surrounding the active site and the mutant in the position 27 are drawn in a stick representation. The manganese ion is highlighted in purple. The fifth coordination site is occupied by a water molecule (red sphere) which binds a Gln149.

Source: By the author.

The residue substitution is accommodated in the three-dimensional structure by small local conformational changes without affecting the activity of the enzyme. Trp82 is slightly shifted in order to occupy the space left by the absence of the Met27 side chain (Figure 45).

Figure 45 - Superposition of the amino acids around the position 27 in native Mn-TrSOD (gray) and Mn-M27VTrSOD mutant (yellow) showing the local changes. Mn ion is shown in yellow.

Source: By the author.

3.10.2 Structural comparison between native Mn-TrSOD and Mn-L80FSOD mutant

Mn-L80FTrSOD carries a Leu80 to Phe mutation in helix 3 (H3) and was made in order to increase selectivity for Fe given that phenylalanine at this position is characteristic of such enzymes. The crystal structure presented one full tetramer and half of a second one in the asymmetric unit. The latter is completed by the crystallographic two-fold along *a*. Monomers that compose the tetramer were superimposed and gave an RMSD of about 0.3 Å while superposition of the whole tetramer against the native TrSOD gave an RMSD of 0.42 Å showing that both the tertiary and quaternary structures are very similar (Figure 46 (A and B)).

The replacement of L80 with Phe (which is two residues away from His78, one of the metal binding residues) caused little impact on the active site (shown in Figure 46(C and D)).

The side chain at position 80 adopted a very similar conformation to the native valine but because of its larger size, Phe205 and W133 move slightly to accommodate Phe80 (shown in the Figure 46(E)). The variation in the side chain of Phe205 causes a rearrangement to Arg203 which normally forms a hydrogen bond with Val153. As a consequence, Val153 changes its normal position and in order to form a hydrophobic contact Phe205. Enzymes that contain Fe (Fe-SODs) have a helix in this region. In summary, a single amino acid substitution leads to a complex unpredictable local rearrangement. However, this appears not to significantly perturb the active site metal and its catalytic capability.

Figure 46 - The Mn-L80FTrSOD mutant. (A) The asymmetric unit composed of one tetramer and half of the second one. Subunit A is colored in gray, Subunit B is colored in purple, subunit C is colored in pink, subunit D is colored in blue, subunit E is colored in yellow and subunit F is colored in green. The metal ion is colored in red. (B) Superposition of all monomers found in the asymmetric unit. The N and C terminals of each monomer are labeled as N and C, respectively. The metal ion is colored in pink (C) $2F_{\text{obs}} - F_{\text{calc}}$ electron density map of the L80 position contoured at 1.5σ (two residues away from the active site) in the subunit A of native Mn-TrSOD and (D) F80 position in the subunit A of Mn-L80FTrSOD contoured at 1.5σ . The metal ion is colored in purple. (E) Superposition of the two subunits A of native Mn-TrSOD and L80FTrSOD mutant showing the local changes around the 80 position.

Source: By the author.

3.10.3 Structural comparison between native Mn-TrSOD and Mn-G73ASOD mutant

3.10.3.1 Overall structure of Mn-G73ATrSOD

The mutation G73A is located in helix 3 (H3) and was made in order to eliminate a residue which is characteristic of Mn containing enzymes and replace it with one which is common in Fe containing enzymes. The crystal structure has 8 monomers in the asymmetric unit forming a single octamer. The electron density maps are contiguous for all modeled monomers (except for the last 12 amino acids corresponding to helix 9 (H9)). The RMSD between the Ca's of the eight subunits range between 0.62 Å and 1.21 Å while the RMS deviation between one monomer of the native TrSOD and the eight subunits range between 1.34 Å and 2.07 Å. The regions of greatest difference between monomers (shown in Figure 47 (A and B)) are concentrated in the N-terminal domain in the “tip of the helical hairpin” between the first and second helices, residues 47-72 (involved in dimer-dimer contacts). Ala73 and the four residues that coordinate bind the Mn are well defined in the electron density map and were well behaved during the refinement process (Figure 47 (D)).

Figure 47 - (A) Superposition of the eight subunits present in the asymmetric unit. The N and C terminals of each monomer are labeled as N and C, respectively. (B) Superposition between the monomers A of native Mn-TrSOD and G73A mutant showing the local changes around the “tip of the helical hairpin”. The N and C terminals of each monomer are labeled as N and C, respectively. (C) $2F_{\text{obs}} - F_{\text{calc}}$ electron density map of the G73 position contoured at 1.5 σ (five residues away from the active site) in the subunit A of native Mn-TrSOD and (D) A73 position in the subunit A of Mn-G73ATrSOD. The Mn ion is highlighted in red (A and B) and purple (C and D).

Source: By the author.

There are noticeable differences in the active site of the monomers of the G73A mutant. In the active site of monomer F, the metal is coordinated by six ligands: the five normal ligands (present in all SODs) plus an additional water molecule, which was found in the equatorial plane (Figure 48 (A)). Also, the active site of monomer G (which is coordinated by the common five ligands) is surrounded by several additional water molecules that are not bound directly to the Mn ion (Figure 48 (B)) but are close proximity and not normally observed in the native structure. These findings suggest that the active site of the Mn-G73ATrSOD mutant may be more exposed to solvent than normal.

It is interesting that Mn-G73ATrSOD was the only mutant which clearly and definitively lost catalytic activity. The explanation for this phenomenon is based on the reaction mechanism for which two proposals exist: (1) the 5-6-5 mechanism⁴⁸ where the metal is six-coordinated when bound to the substrate and five-coordinated when not and (2) the associative displacement mechanism,¹³⁶ where the association of superoxide is concomitant with the displacement of one of the oxygen ligands.¹³⁷ In both mechanisms, the superoxide substrate coordinates to the metal center from the equatorial plane, and the coordination site is the same as that of the additional water molecule in the F monomer of G73A-TrSOD octahedral structure. Thus, it can be assumed that the formation of a six-coordinate water, which blocks substrate access to the metal center, inhibits superoxide binding. This result is in good agreement with the EPR spectra showed for this mutant which is very different from all others presented here and is consistent with a distorted octahedral environment. Thus easier access to the mutant protein active site by solvent may be one the principal reason why G73ATrSOD lost activity.

Figure 48 - $2F_{\text{obs}} - F_{\text{calc}}$ electron density map (A) of the active site of the subunit F of Mn-G73ATrSOD contoured at 1.5σ and (B) chain G in Mn-G73ATrSOD. The manganese ion is highlighted in purple. The fifth coordination site is occupied by a water molecule (red sphere) which binds a Gln149. In the F subunit an additional water molecule is found bound to the metal generating an octahedral coordination. The Mn ion is colored in purple.

Source: By the author.

The geometry of the hexa-coordinate active site (distorted octahedral) has been found also in other SODs: the cryo-trapped form of MnSOD from *E. coli*, the MnSOD of *Saccharomyces cerevisiae*, the Fe-bound form of the cambialistic SOD enzyme of *Aeropyrum pernix* K1, the MnSOD-3-azide complex of *Caenorhabditis elegans*, etc. In all cases this form of co-ordination leads the lowering or complete loss of catalytic activity.

In addition, a hydrogen bond between Tyr38-OH and the “extra” water molecule in subunit F suggests a subtle role for Tyr38 in orienting and stabilizing metal-superoxide interactions. This conserved Tyr residue, which is part of the outer sphere of the active site, has been shown by mutational studies not only to play a critical role in catalysis but also to reduce azide and hydroxide affinity in Fe/MnSODs.¹³⁸⁻¹³⁹ The OH of Tyr38 in monomer F was found to have shifted toward the original water molecule upon Mn binding (Figure 49). This shift is analogous to that of Tyr34 in *E. coli* Mn-SOD (Tyr 38 in TrSOD numbering) upon binding of hydrogen peroxide to the central metal.⁷⁴ This conserved tyrosine re-orientates itself to accommodate the azide molecule in *E. coli* SOD. Mutation of Tyr34 (which eliminates the monomer-monomer hydrogen bond) leads to activity 30-40% of that of the wild type.⁶⁹ These findings lead to the hypothesis that the substrate is competing with water molecules for access to the active site in Mn-G73ATrSOD.

Figure 49 - Superposition of the residues around the active site of monomer A of native MnTrSOD (yellow) and monomer F of G73ATrSOD mutant (blue). Note that Tyr38 of G73A mutant moved slightly towards the original water molecule.

Source: By the author.

The distances between the Mn ion with respect to the ligands are similar in both native TrSOD and G73ATrSOD mutant. Therefore, there may not be significant alterations to the active site beyond the increased likelihood of acquiring a sixth ligand.

3.10.3.2 The G73ATrSOD monomer

Ala73 is situated near the conserved dimer interface and five residues away from an active site ligand (H78). In native Mn-TrSOD Gly73 is flanked by Gly72 and Gly74 (also present in Fe tetramers). This GGG pattern occurs in the middle of helix H3 and has been reported⁴⁵ as an unusual feature that can be expected to locally weaken the structure of the α -helix and may be required for packing constraints. In the native TrSOD this region presents a tighter helical turn, slightly reducing its diameter when compared with the same helix from other organisms (Figure 50). Other SODs have alanine in this position (e.g. dimer Fe-SOD from *E. coli* (PDB:1ISA), dimer Mn-SOD from *Bacillus anthracis* (PDB: 1XUQ), cambialistic tetrameric enzyme from *Aeropyrum pernix K1* (PDB: 3AK3), etc.).

Figure 50 - (A) Residues 71-74 of monomer A in the Mn-G73ATrSOD mutant. Residues in this positions are making a helix turn which is of reduced diameter than the other turns from other organisms. (B) Residues analogues (NGG) of monomer A in the SOD from *Bacillus anthracis*. (C) Residues analogues (NAA) of monomer A in the Fe-SOD from *E. coli*. (D) Residues analogues (YAG) of monomer A in the Fe-bound SOD from from *Aeropyrum pernix* K1. The N terminal of each structure is labeled as N. The metal ion is colored in blue. (the three pattern residues are showed in red)

Source: By the author.

The residue substitution, Ala73, is accommodated in the three-dimensional structure by small local conformational changes that resulted in a big effect in the tertiary structure. Ala73 in the mutant protein has ϕ and ψ values of -66.7° and -41.6° respectively, which have changed only slightly from the values of -68.8° and -37.9° for glycine in the native protein. Figure 51 shows the results of this small change caused by the mutation G73A. Ala73 has a methyl group that would cause steric hindrance with the carbonyl of Lys69 if there were no structural alteration (Figure 51B). In order to adopt a sterically permissible conformation the whole of the α -helix prior to Lys69 swivels slightly, whilst retaining a good hydrogen bond between the Ala-73 backbone amide and the carbonyl group of Lys 69. The result is that close to Lys 69 the movement is very subtle but at the tip of the helical hairpin, the movement is dramatic.

The shift in position of the “tip of the helical hairpin” that connects the two long helices in the N terminal domain is between 5.3 to 6.8 Å (depending on the subunit chosen) when compared with the native structure (Figure 52). As can be seen in the figure, the movement of H3 takes H1 with it. This movement would be expected to influence the A/C (B/D) interface which is dominated by contacts involving this part of

the structure. An analysis of the consequences for the quaternary structure is given below.

Figure 51 - Cartoon representation of the differences between native TrSOD and G73A mutant caused by Ala73. (A) native TrSOD showing the distance between "CA" of Gly73 and "O" of Lys69 which is 3.3 Å. (B) Simulation of the mutation G73A in the native TrSOD. Note that the methyl group of the Ala73 causes a steric hindrance with the carbonyl of Lys69 giving a distance of 2.6. (C) Neighborhood around the 73 position in the mutant G73A -TrSOD. Note that the distance between the methyl group of Ala73 and carbonyl of Lys69 is 3.2. In this mutant, Lys69 moves slightly to avoid the steric hindrance.

Source: By the author

Figure 52 – Superposition of monomers from native MnTrSOD and G73ATrSOD. The separation distance between the “tip of the helical hairpin” of these monomers is 5.4 Å. In general, this distance varies from one monomer to another from 5.3 Å to 6.8 Å.

Source: By the author

3.10.3.3 Monomer-monomer interactions in Mn-G73ATrSOD

The highly conserved A/B interface is very similar in the G73A mutant when compared to the native TrSOD and all other Fe/Mn-SODs. On the other hand, the A/C and B/D interfaces are significantly altered. The former (A/C) involves novel contacts and the latter (B/D) is completely lost. The normal A/D contact is also no longer present. The lack of the B/D and A/D contacts are the consequence of an altered quaternary structure, itself the result of the distortion of the helical hairpin which forms the basis of the A/C contact.

The novel A/C interaction still involves the “tip of the helical hairpin” (Figure 53), but the hairpins now cross at an angle of 180° compared with 150° found in the native structure. This new interaction presents two new hydrogen bonds between Gln61 (monomer A) and His75 (monomer C) and between Ile68 (monomer A) and Gln65 (monomer C). The interface area is 1046 Å² with a total predicted binding energy of -18,21 Kcal/mol (according to PISA), similar to that estimated for the native interactions (Figure 53).

Figure 53 - (A) dimer interface A/C of the Mn-G73ATrSOD mutant (left), the Mn ion is highlighted in purple. The N and C terminals of each monomer are labeled as N and C, respectively. Information about the interaction radar generated by PISA (right).

Source: By the author.

The new interactions between A and C together with the lack of contacts between A and D, B with C and B with D result in an open tetramer. This new open tetramer has point group symmetry 2, different from the native protein, which has 222 (D2) symmetry 222. In the open tetramer the normal A/B and C/D interfaces are conserved. A comparison of the open tetramer of G73ATrSOD with the native structure is shown in Figure 54.

Figure 54 - Comparison of the quaternary structure of (A) one tetramer of the native TrSOD and (B) the open tetramer of Mn- G73ATrSOD. The N and C terminals of each monomer are labeled as N and C, respectively. The Mn ion is shown in red.

Source: By the author.

The asymmetric unit presented two open tetramers. Each open tetramer has two free subunits (B and D) whose helical hairpins do not make contact. (Shown in

Figure 54 (right)). However, as can be seen in Figure 55, these are available to make contact with equivalent subunits from the other open tetramer in the following manner: Subunit B (tetramer 1)- Subunit B' (tetramer 2) and Subunit D (tetramer 1)-subunit D' (tetramer 2). This results in the formation of an octamer (Figure 55). DLS measurements in solution had already shown (section 3.3) that at higher concentrations the G73A mutant forms higher molecular weight oligomers which are compatible with the octamers observed here in the crystal structure. Furthermore, this mutant is unique in showing such behaviour, consistent with the fact that it is the only one to present an octameric oligomer in the crystal.

The asymmetric unit presented two open tetramers. Each open tetramer has two free subunits (B and D) that are not making any interaction in the structure (Shown in Figure 54 (right)) but in high concentrations (DLS results) tetramers can make a new interaction: Subunit B (tetramer 1)- Subunit B' (tetramer 2) and Subunit D (tetramer 1)-subunit D' (tetramer 2) allowing the formation of an octamer (Figure 55).

Figure 55 - Octameric structure of Mn-G73ATrSOD formed by two open tetramers. Each subunit has different colors.

Source: By the author.

The point group symmetry of the octamer is 222, with a dimer (presenting a local two-fold) in its asymmetric unit. The A/B dimer which forms the point group in the asymmetric unit is the classical dimer found in all Fe/Mn-SODs, a consequence of the preservation of the classical A/B interface.

3.10.3.4 B/B' (D/D') interactions

Subunits B and B' form a new interface not present in the classical tetramer. Five hydrogen bonds are observed at this interface as shown in Figure 56. The total binding energy for the interaction between the two monomers (B of one tetramer and B' from the other) is estimated to be -13.64 Kcal/mol. These new interactions therefore seem to be strong enough to maintain the octameric particle intact, which is consistent with the DLS results. For comparison the binding energy for the classical dimeric interface (A/B) in this case is calculated to be -17.69 Kcal/mol.

Figure 56 – (A) The new interactions observed between B and B' (D and D') present in the octameric structure of Mn-G73ATrSOD. The N and C terminals of each monomer are labeled as N and C, respectively. (B) Information about the interaction radar generated by PISA (B).

Source: By the author

The G73ATrSOD mutant shows the unexpected importance of the glycine residue in maintaining the tertiary and quaternary structures of the protein. As mentioned above, the octameric G73ATrSOD protein is inactive which is explained by small changes caused by the mutation that appear to increase solvent accessibility to the active site allowing an extra solvent molecule to act as a competitive inhibitor. Figure 57 shows a surface representation showing that the rearrangement which leads to an altered quaternary structure also increases access to the active site metal.

Figure 57 – (A) Comparison of the mutant G73A and native TrSOD domains. Ribbon representations and electrostatic surface potentials for the (A) G73A mutant and (B) native TrSOD protein. (C) Electrostatic surface of both, native and G73A mutant after moving 90°. Note that the mutant clearly has a higher solvent accessibility compared to the native which is a closed tetramer.

Source: By the author

Experiments with an I58T mutation in human mitochondrial Mn-SODs,¹⁴⁰ shows the destabilization of the tetramer and a concomitant reduction. This is a clear example that amino acids such as G73 and I50, which are near the tip of the helical hairpin are important in determining the oligomerization state and structural stability of Fe/Mn-SODs, with consequences for their catalytic activity.

In many systems, the biological role of oligomerization is not clear and sometimes may be an artifact of the solution conditions, principally the high protein concentration used in crystallography. In the case of G73ATrSOD mutant, our DLS results show that, at low protein concentrations, the enzyme is tetrameric but at high concentrations the protein becomes octameric suggesting that open tetramers at high concentrations can bind each other to stabilize a new oligomeric state.

It is perhaps surprising that the substitution of a glycine by an alanine can lead to such dramatic consequences. However, this is not unprecedented.

An important natural point mutation that has been shown to have an impact on oligomerization is G93A in human Cu/Zn-SOD. This point mutant facilitates amyloid fiber formation and is known to correlate with the development amyotrophic lateral sclerosis (ALS).¹⁴¹ Another example is the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS).¹⁴² The substitution of G96 by alanine leads to an enzyme which is not inhibited by glyphosate due to a steric clash between the methyl group of the alanine and the phosphonate group of the glyphosate. This difference between the natural plant EPSPS and the gene introduced by transgenesis is the basis for the massive production of transgenic soybeans in the world.

The connection between the amino acid sequence of a protein and its overall structure and function is complex, and not completely understood. On the one hand it is not surprising that just one mutation can alter the oligomeric state and activity of a protein but on the other it can be challenging to predict this *a priori*. Computational analyses may help but it is not possible to predict with total confidence whether an amino acid substitution will be harmless or not, as it was in this case.

3.11 Comparison between structure of the native Mn-TrSOD and the Fe-SOD mutants.

3.11.1 Structural comparison between native Mn-TrSOD and Fe-M27VTrSOD mutant

The Methionine to Valine mutation at position 27 is in the first helix (H1) of the monomer. This mutant includes four protein molecules in the asymmetric unit that form the obligate tetramer characteristic of TrSOD. The overall structure of Fe- M27VTrSOD is not significantly different from the wild-type enzyme with a RMSD for the Ca's of 0.52

Å. Also, the main-chain conformation for most residues of M27V fall within the allowed regions of the Ramachandran plot; the only exception is Lys139.

The four residues that bind the Mn were well defined in the electron density map, however there is no electron density for the last 12 amino acids in the C-terminal domain (different from Mn-M27V mutant) which could indicate disorder in the crystal. Otherwise, all important aspects of this structure are the same as those reported above for the Mn-M27VTrSOD mutant.

3.11.2 Structural comparison between native Mn-TrSOD and Fe-H75IVSOD mutant

The mutation of histidine to leucine at position 75 (H75I) is positioned in helix 3 (H3) and was designed to eliminate a residue which is characteristic of Mn-specific enzymes. Fe-H75ITrSOD has, in the asymmetric unit, one full tetramer and half of a second one, which is completed by crystallographic two fold symmetry along the a axis. Superposition of the structures of the Fe- H75ITrSOD and native Mn-TrSOD showed that global structural alignment yields an RMSD of only 0.37 Å° for 210 Cα's atoms and the hydrogen-bonding network around the metal atom also aligns very well. Superposition of the six monomers found in the asymmetric unit showed no significant variation (Figure 58).

Figure 58 – The Fe-H75ITrSOD mutant. (A) The asymmetric unit composed of one tetramer and half of another one. (B) Superposition of all monomers found in the asymmetric unit.

Source: By the author.

The electron density for the Isoleucine side chain together with the amino acids around the active site are well defined and shown in Figure 59. There is no significant conformational alteration in the active site of the mutant. Bond distances between the metal ion and the five ligands are similar compared with the native TrSOD except for the distance between the metal ion and the water molecule (2.25 Å and 2.03 Å for the native TrSOD and H75 mutant, respectively). However, the water position is, in general, less well determined than other atoms and so this difference may well not be significant. The point mutation is accommodated in the three-dimensional structure by small local conformational changes without affecting the activity of the enzyme (Figure 60). The small adjustments affect, primarily residues 4 to 9, which form an extended region which interacts with the first and third α -helices (H1 and H3) by van der Waals contacts and hydrogen bonds.

Figure 59 - (A) $2F_{\text{obs}} - F_{\text{calc}}$ electron density map of the H75 position (three residues away from the ligand His78) in the A subunit of native Mn-TrSOD and (B) I75 position in the A subunit of Fe-H75I-TrSOD.

Source: By the author.

Figure 60 - Superposition of the A subunits of native Mn-TrSOD and the Fe-H75I-TrSOD mutant showing the local changes around H75. The native protein is colored in yellow and mutant H75I is colored in green. The Fe ion is colored in purple.

Source: By the author.

3.11.3 Structural comparison between native Mn-TrSOD and Fe-D150GSOD mutant

Mutation of Asp150 to Gly was undertaken in order to eliminate a residue which is typical of Mn- containing enzymes and replace it with a residue commonly found in Fe-containing enzymes, in the hope of increasing the selectivity for the latter metal. Residue 150 is positioned in the loop that connects β -strands S2 and S3. D150GTrSOD has four protein subunits in the asymmetric unit. The crystallographically independent subunits making up the tetramer of D150G, superimpose on one another with an RMSD for their C α 's atoms of ~ 0.7 Å, indicating that they adopt effectively identical three-dimensional structures. However, the whole D150G tetramer superimposes on the native tetramer with a high RMSD for C α 's of 3.6 Å. This implies that although the tertiary structure of each subunit has been maintained, the relative orientation of the subunits has changed, indicating alteration of the quaternary structure. These changes to the structure of the D150G mutant are reflected in the symmetry of the tetramer. The mutant has lost two of the two-fold axes of the point group 222, being to point group 2 (Figure 61). This is not unprecedented, and to the knowledge of these authors there is at least one other case where such behavior has been noted in the peanut lectin structure.¹⁴³

Figure 61 - (A) Four subunits of the mutant D150G. These subunits are similar to each other. (B) Ribbon representation of the mutant D150G with 2 symmetry. (C) Cartoon representation of the native TrSOD. The native protein presents a 222 symmetry. It is noticeable the change in symmetry of the D150G mutant comparing it with the native protein TrSOD. The metal ion is colored in black.

Source: By the author.

The four residues that bind the Fe were well defined in the final electron density (Figure 62). The aspartic acid residue at position 150 is located one residue away from Glutamine 149 that binds a water molecule which is a direct ligand to the metal. The location of this mutation and the surrounding protein side chains show that the active site coordination distances are similar for both native Mn-TrSOD and Fe-D150GTrSOD.

Figure 62 - (A) $2F_{\text{obs}} - F_{\text{calc}}$ electron density map of the Asp150 position in the subunit A of native Mn-TrSOD and (B) Gly150 position in the subunit A of Fe-D150GTrSOD contoured at 1.5σ . Source: By the author.

Although the active site is highly conserved in this mutant, the conformation of several of the residues following the mutation itself (Pro151, Val152 and Val153) have changed. These residues are part of the loop (147 to 157) that connects β -strands S2 and S3. Changes in this loop cause Arg201 (at the beginning of helix 9 (H9)) to lose a hydrogen bond with the carbonyl group of Val153 leading to a re-orientation of the last helix (H9) of the C-terminal domain (Figure 63). The electron density of these changes are well defined (Figure 64).

Figure 63 - Superposition of the native Mn-TrSOD and the Fe-D150GTrSOD monomers. Native protein is colored in blue while mutant D150GTrSOD is colored in red. Noticeable differences in the “tip of the helix hairpin” and the rearrangement of the helix 9 of the C terminal domain is showed. The metal ion (Fe or Mn) is colored in blue.

Source: By the author.

Figure 64 - (A) $2F_{\text{obs}} - F_{\text{calc}}$ electron density map of the active site and the neighborhood of Asp150 in subunit A of native Mn-TrSOD and (B) $2F_{\text{obs}} - F_{\text{calc}}$ electron density map of the active site and the neighborhood of Asp150 in subunit A of Fe-D150GTrSOD. (C) superposition of both native and the D150G mutant showing the differences between them. There is a hydrogen bond between the carbonyl group of Val153 and Arg201 in the native TrSOD which is lost in the D150GTrSOD mutant.

Source: By the author.

Changes in the orientation of the neighboring residues leading to the loss of an interaction between Val153 with Arg201 is not the only noticeable difference in this mutant enzyme. The lack of D150 resulted in loss of salt bridges with lysine residues K147 and K69. This resulted in K147 (of monomer A) undergoing a conformational change and forming a new salt bridge with Asp148 from monomer B, an interaction which is not seen in the native protein. Furthermore, the helix 9 (H9) suffers a large conformational change (Figure 64) to adopt a new position leading to a salt bridge between Lys69 and the C-terminal carboxylate (Figure 65). These rearrangements satisfy both of the lysine residues which had lost their charge compensating interactions as a result of the D150G substitution.

Figure 65 – (A) Interactions between Lys147 and Lys69 with Asp150 in the conserved interface A/B of the native TrSOD. (B) New interactions of Lys69 and Lys147 in D150G-TrSOD mutant. Lys147 of one subunit makes a new interaction with ASP148 of the other subunit. The changes caused by the Gly150 in the subsequent residues (Pro151, Val152, Val153-colored in blue) allowed the Helix 9 (colored in blue) to rearrange making a new interaction between Leu212 and Lys69.

Source: By the author.

On the other hand, there are also differences to the N-terminal domain in the mutant. The second helix, H2, forms a continuous helix together with H3 whereas in the native there is a small distortion allowing them to be described as separate helices. This alteration could be a consequence of the re-arrangement of the final helix (H9, at the C-terminus) which presents a new position in the structure as described above (Figure 64). This has consequences for the monomer-monomer contacts which were analyzed using the PISA program.

Interactions A/B (C/D): By comparing the interfaces of D150GTrSOD with respect to the native protein, it becomes clear that the former presents the two highly conserved dimer interfaces, (A/B and C/D) as expected. The total estimated binding energy for this interface was -15.5 Kcal/mol with an interface area of 858 Å² as shown in Figure 66. However, different from the native protein, when the conserved dimers A/B and C/D interact to form a tetramer this occurs in a way very different from that seen previously.

Figure 66 - (A) A/B (C/D) dimer interactions found in Fe-D150GTrSOD mutant. (B) Interface parameters generated by jsPISA.

Source: By the author.

Interactions A/C: Contrary to the conserved interfaces described above, the new A/C interface (shown in Figure 67) has a lower total binding energy of -8.7 Kcal/mol compared with the native TrSOD (-21.05 Kcal/mol). However, the buried surface area (BSA) of the A/C interface is 856.6 Å². Relative to the surface area of an

isolated subunit (10097.0 Å²), the buried fraction (8.7%) is still typical of a strong association. The A/C interface is created by a contact between two H1/H3 hairpins, one from each subunit, but using the opposite side to that which is usually employed (a switched interface). The residues normally observed as contributing to the interface are Ile68, Leu46, Ser50, Ala54 from both subunits while the residues found in A/C interface of the D150GTrSOD mutant are those from the back of the helices (Thr48, Thr51, Ser60, Ala63) as shown in Figure 67. This result was totally unexpected and reveals a degree of plasticity at the interface which would have been impossible to predict *a priori*.

Figure 67 - (A) A/C interactions found in native TrSOD. (B) A/C Interface found in the mutant D150GTrSOD showing that the contact areas between the subunits use the opposite sides of the helical hairpin. (C) Superposition of the A/C interface between the native (yellow) and D150GTrSOD mutant (purple). Only one subunit superposes well due to the inversion of the contacts by the helical hairpin (D) parameters generated by jsPISA.

Source: By the author.

Interactions B/D: This new interface (shown in Figure 68) has a lower total predicted binding energy of -5.4 Kcal/mol relative to the native TrSOD (-21.05 Kcal/mol). The new location of the H9 helix prevents direct interaction between the tips of the two helical hairpins. Both H9 helices occupy positions between the hairpins, driving them apart and thus generating completely new inter-subunit contacts. (Figure 68A). This interaction is weak, including just one hydrogen bond.

The A/C and B/D interfaces show less than 50% of radar area (Figure 68B), which as the PISA program recommends, may indicate potential artifacts of crystal packing. However, this mutant was shown to be a tetramer in solution, suggesting that these interactions are indeed relevant to the formation of the true oligomeric particle. The D150G mutation does not, however, seem to have a major impact on enzymatic activity since the specific activity of the mutant enzyme is only slightly lower than that of the native enzyme.

Figure 68 - (A) A/C (B/D) interface found in native TrSOD. (B) B/D Interface found in the mutant D150GTrSOD showing that the contact areas between the subunits is prevented by the helix 9 of opposite sides of the helical hairpin. (C) Interface parameters were analyzed by jsPISA.

Source: By the author.

Surprisingly, these two new interfaces A/C and B/D are different from one another and also different from those normally encountered in tetramers. The consequence of this alteration to subunit interactions is the loss of two 2-fold axes and a reduction in the symmetry of the oligomer. Presumably the alteration to the tertiary structure induced by the mutation and particularly the difference in the orientation of the C-terminal helix makes it impossible to form similar interactions via helical hairpins at both interfaces simultaneously. Hence the reduction in symmetry.

Other SOD structures (e.g., the tetramer Fe-SOD from *Aquifex pyrophilus*¹³⁴) present differences in their A/C interface (called A/D in *A. pyrophilus*) due to large conformational differences observed in loop L2 (that connect H3 and H4 and was widely discussed in section 3.9.1). However, what is particularly interesting in the case of the D150G mutation is that it generates a tertiary structural which is capable of generating two different interfaces involving the helical hairpin. In one case the opposite face of the hairpin is recruited to form a new interface and in the other the C-terminal helix H9 is inserted between the hairpins. This suggests plasticity at the A/C interface. As with the G73A mutation, the change to the quaternary structure was not anticipated when the point mutation was chosen and would have been very difficult to predict *a priori*.

Several studies have investigated (in terms of stability and catalysis) the role of the dimeric and tetrameric interfaces.¹⁴⁴⁻¹⁴⁵ Mutations at the A/C (B/D) interface (I58T) and A/B (C/D) interface (Y166F) of human MnSOD resulted mainly in tetramer dissociations (dimeric proteins) and loss of activity, respectively. Another mutation in human Mn-SOD (E162A)⁶⁵ in the tetrameric interface resulted in an intact MnSOD retaining its activity, oligomeric state, and metal ion specificity. Here we show that other mutations (G73A, D150G) near the active site have large effects on activity and oligomeric state. Furthermore, our study shows that an amino acid does not necessarily need to be part of the oligomeric interface to have an effect on activity and oligomeric state.

Oligomerization plays an important role in the function of many proteins, including SODs. There are several advantages (structural, mechanistic, and physicochemical in addition to new opportunities for functional control) that may be conferred by oligomerization and that may have been selected for through evolution. The family of Fe/Mn-SODs includes homodimers and homotetramers. The interface found in homodimers is highly conserved and is also present in tetramers. The active site and

the region surrounding it, as well as the monomer-monomer interface, are essentially indistinguishable from humans to bacteria. For tetramers the dimer-dimer interface can vary across species. Additionally, Fe/Mn dimers are associated with quite different amino acid signatures compared to tetrameric Fe/Mn-SODs suggesting more time for divergence within this last group. Tetrameric TrSOD associates into dimers at similar concentrations (20 μ M) found in cells but at higher concentrations they form a dimer of dimers (tetramer) and associate more tightly by virtue of extensive interactions between the α -turns (in the N-domains) of adjacent subunits.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

4.1 Conclusions

The structural basis for metal specificity, determined by differences in amino-acid sequence between Mn-SODs and Fe-SODs, is largely unknown. In recent years, considerable success has been achieved through mutations of amino acids corresponding to the second sphere of coordination, evaluating the influence on the reduction potential of Fe/Mn-SODs, thereby enabling detailed investigations into the origin of the strict metal specificities displayed by some enzymes. In the present dissertation the role of several structurally conserved residues around the active site, which appear not to participate directly in any of the proposed catalytic mechanisms, were explored.

We provide new features of the enzyme TrSOD which are important for understanding functional aspects of the Fe/Mn-SOD family as a whole. Our results have shown that the superoxide dismutase from *Trichoderma reesei* has the ability to bind either Fe or Mn in its active site and is catalytically active with either metal ion which is the principal reason to conclude that this enzyme belongs to the group of cambialistic SODs. Cambialistic SODs can be either dimers or tetramers. In the case of TrSOD, it presented a mixture of dimers and tetramers in low concentrations, the two forms remaining in equilibrium. However, its amino acid sequence and crystal structure suggest that it is most appropriately classified as a tetrameric protein.

Additionally, the complete structure of the cambialistic TrSOD has been solved for the first time and it presents an extra helix in the C-terminal domain of each subunit. The C-terminal extension (H9) of the structures (native TrSOD and mutants) showed it to be structurally variable but usually it occurs in the form of an α -helix (H9). Moreover, depending on the mutant, the location and orientation of this α -helix (H9) can change and in some cases it is found to be disordered. Therefore, based in our analysis, we conclude that the helix (H9) is a region of large structural variability which we describe for the first time in the family of Fe/Mn-SODs.

We also show examples of how the oligomeric state of the enzyme is dependent on particular structural features of the protein. In our results, a one residue substitution was shown to be sufficient to convert a patch on the protein surface into a protein

interface (Interface A/C of mutant D150G) changing the interactions between dimers and also compromising the symmetry of the protein. These changes in the interfaces are caused by the structural plasticity that the protein presents allowing for the accommodation of significant structural changes to take place which alter inter-subunit contacts without affecting the oligomeric state (D150G-TrSOD).

There is a wide range of mechanisms through which distant mutations can impact oligomerization via conformational changes, which can in turn influence interface coupling. Our results also show that the substitution of a single residue can be sufficient to drive the native oligomer towards larger multimeric complexes, e.g., the tetrameric TrSOD becomes an octamer as a consequence of the single G73A mutation. The octameric G73A mutant presents 222 symmetry and has no activity. The principal reason for this new octomeric state is one of stability: the protein loses one dimer interface making it possible for two tetramers to interact by new dimer interactions, thus resulting in a dimer of tetramers (or an octamer). Subsequently, there is a lack of activity triggered by the open structure formed by the new oligomer. It is clear that, as the structure opens, the enzyme exposes its active site to solvent molecules which then act as competitors of the substrate.

Although it is not surprising that only one residue substitution can influence oligomerization, predicting which amino acids will favor higher oligomeric states is almost impossible and some point mutations can cause very little impact on the structure e.g., M27VTrSOD, H75ITrSOD and L80FTrSOD which result in small local rearrangements to accommodate the mutation in the three-dimensional structure without affecting activity or oligomerization.

Collectively, this dissertation provides compelling evidence that changes in oligomerization of Fe/Mn-SODs and the ability to either stabilize or destabilize hydrogen-bonding interactions in TrSOD is accomplished by residues (G73A and D150G) that are far from the active site. This evidence is the first step to understanding the relationship between catalytic activity and oligomerization; and understanding such changes can directly aid the engineering of protein interactions as well as contribute to knowledge of the evolutionary pathways that generate them.

4.2 Future Perspectives

The analyzed point mutations open the door to understanding the function of Fe/Mn-SODs as a whole. Given that the original objective of this study was to identify which residues are most relevant for the determination of metal ion selectivity, based on the results obtained so far it would be of interest to continue the work in the direction of generating mutants which include two, three, four, etc. substitutions and evaluate their impact on catalytic activity, metal ion specificity, redox potential, inhibitor specificity and oligomerization.

Furthermore, computational simulations will be advantageous in exploring the geometric and electronic properties of the active sites together with theoretical methods (quantum mechanical and molecular mechanics (QM/MM)) which can estimate reduction potentials and serve as a guide for determining the amino acid mutations which may be responsible for selectivity of metal.

Ideally both the experimental and theoretical approaches should be combined and executed simultaneously.

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