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Human Mn-superoxide dismutase inactivation by peroxynitrite: a paradigm of metal-catalyzed tyrosine nitration *in vitro* and *in vivo*

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Human MnSOD is a homotetramer and represents an essential mitochondrial antioxidant enzyme, which catalyzes the dismutation of superoxide radicals ($O_2^{\bullet -}$) at near diffusion-controlled rates. Under a variety of disease conditions and in the process of aging, nitric oxide ($^{\bullet}NO$) can outcompete MnSOD and react with $O_2^{\bullet -}$ to yield the potent oxidant peroxynitrite ($ONOO^-$). Then, peroxynitrite can promote the regio-specific nitration of MnSOD at active site tyrosine 34, which turns the enzyme inactive. In this review we assess the kinetic aspects of the formation of peroxynitrite in the presence of MnSOD and the biochemical mechanisms of peroxynitrite-mediated MnSOD nitration. In particular, the central role of the Mn atom in the reaction of the enzyme with peroxynitrite ($k = 1.0 \times 10^5$ M^{-1} s^{-1} per tetramer at pH = 7.4 and $T = 37$ °C) and the catalysis of nitration at the active site are disclosed. Then, we analyze at the atomic level of detail how a single oxidative post-translational modification in the enzyme, namely the nitration of tyrosine 34, results in enzyme inactivation. Herein, kinetic, molecular, structural biology and computational studies are integrated to rationalize the specificity and impact of peroxynitrite-dependent MnSOD tyrosine nitration *in vitro* and *in vivo* from both functional and structural perspectives.

Significance to metallomics

Human Mn-superoxide dismutase (hMnSOD) is a key mitochondrial antioxidant enzyme that catalyzes the dismutation of superoxide radicals. The Mn center plays a central role in catalysis together with an array of inner and second sphere amino acids that participate in the fine-tuning of the redox potential and the hydrogen bond network needed for optimal dismutation rates. Importantly, Mn also catalyzes a reaction that leads to enzyme inactivation *in vitro* and *in vivo*, namely, the peroxynitrite-dependent nitration of critical Tyr34. These observations are relevant in the context of the disruption of mitochondrial redox homeostasis observed in various disease states.

Introduction

History of SOD

In 1939, Mann and Keilin identified a blue-green protein of ~30 kDa containing copper which was denominated "hemecuprein".[1] Several similarly-structured proteins were found later, but no activity was assigned to them until 1969 when Fridovich's lab proposed an unsuspected enzymatic activity: the catalysis of superoxide radical ($O_2^{\bullet -}$) dismutation (eqn (1)).[2]

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

This discovery was of fundamental importance since it strongly suggested the formation of superoxide *in vivo* and the enzyme was named "superoxide dismutase" (SOD).[2] Soon after, the importance of such enzymes for bacteria to survive in the presence of oxygen was shown: strict anaerobic organisms lacked SODs.[3]

Following the discovery of the copper (and zinc)-containing SOD (Cu,Zn SOD), several other enzymes displaying the same activity were discovered. All proteins shared the presence of a redox active metal in its active site, such as iron, manganese or nickel.

In particular, a manganese-containing protein was found in bacteria and mitochondria, and was homologous to the family of iron-containing SODs (FeSODs).[4] After that, several manganese-containing SODs (MnSODs) were found in diverse sets of organisms from *Archaea* to humans (reviewed in ref. 5).

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Superoxide dismutation mechanism

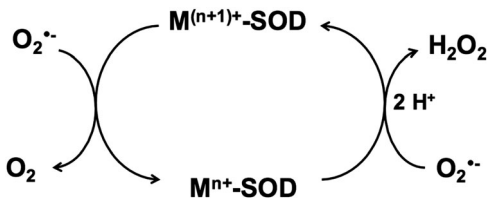
The mechanism by which SODs carry out the dismutation of superoxide involves a ping–pong oxidation/reduction cycle, which leads to the oxidation of superoxide to form molecular oxygen *via* a metal reduction and then the reduction of a second molecule of superoxide to form hydrogen peroxide, when the metal is re-oxidized.

The simplified mechanism of enzymatic superoxide dismutation is shown in Scheme 1.

Although MnSOD follows this general mechanism, the steps that lead to the actual release of oxygen and hydrogen peroxide in the two steps mentioned above have proven to be more complicated.[6] Overall, two different mechanisms have been proposed for the dismutation of superoxide by MnSOD as part of the phenomenon denominated "gating".[6,7] The gating in MnSOD was discovered by exposing the enzyme to different concentrations of superoxide, and distinctly different activities were observed depending on the initial concentration of the substrate. Schematically, pulse radiolysis studies showed that, when the concentration of MnSOD was nearly five times higher than the concentration of superoxide, the kinetics of the disappearance of the substrate were similar to the ones obtained in other SODs such as Cu,ZnSOD. But when the concentration of superoxide was higher than the concentration of the enzyme, a two-step decay was observed with a first order initial "burst" and a subsequent "zero order" phase.[6,8,9] While under normal cellular conditions, superoxide levels are kept in the nanomolar range,[10,11] basal formation rates are in the order of micromoles per second,[12–15] due to the presence of micromolar levels of MnSOD and a high dismutation rate constant (*vide infra*). Still, under cellular conditions that include mitochondrial dysfunction, mitochondrial superoxide formation rates can be largely increased: it is under these conditions (constant MnSOD levels under high superoxide fluxes) that the alternative catalytic mechanism may operate to attenuate the production of hydrogen peroxide bursts.[5,16,17]

MnSOD is a highly effective enzyme, performing a rapid catalysis with a k_{cat} near 4×10^4 s^{-1} and a k_{cat}/K_m near diffusion control of $\sim 1 \times 10^9$ M^{-1} s^{-1}.[18]

The rate constant of superoxide dismutation by SOD is not highly dependent on pH in the physiological range, whereas superoxide spontaneous dismutation varies highly with pH with rate constants ranging from 0 at high pH ($\sim$10), $\sim 1 \times 10^7$ M^{-1} s^{-1} at pH = pK_a = 4.8 to $\sim 2 \times 10^4$ M^{-1} s^{-1} at pH = 8 (*e.g.* in the mitochondrial matrix).[5]



Scheme 1 Redox cycle for superoxide dismutation. In MnSOD, M = Mn, n = 2 and the resting enzyme is in the Mn^{III} state.

Evolution of MnSOD

FeSOD is considered to be the most ancient SOD,[19,20] while MnSOD appeared later, exhibiting a degree of sequence and structural similarity, which suggest the existence of a common ancestor.[21,22] The differences in the sequences of both families of SODs account for the metal specificity and oligomeric structure observed.[22]

It is proposed that MnSOD would have thriven when the increase of oxygen in the atmosphere would make the use of Mn a less toxic option than Fe, leading to a new family of SODs that would have evolved from a common ancestor that incorporated Mn in bacteria and other ancient organisms.[23–25]

Eukaryotic MnSOD, which is found mainly in mitochondria, likely evolved from a common archaeal ancestor,[26,27] instead of a bacterial ancestor although the structures between different MnSODs share high homology (Fig. 1). This goes in agreement with the tetramerical structure mostly found in eukaryotes compared with the mostly dimerical structure of bacterial MnSOD.[5]

Cambialistic SOD

In microorganisms adapted to different growth conditions, such as anaerobes, microaerophiles or hyperthermophiles, an SOD capable of functioning with either Fe or Mn, known as "cambialistic", is found.[19] Such variants have the capacity to change the geometry of the coordination to the metal by the residues in the active site; in essence, trigonal bipyramidal coordination with five ligands for Mn and octahedral coordination with six ligands for Fe.[28,29] Evolutionarily, cambialistic SODs are supposed to be a common ancestor for both modern Fe-SODs and Mn-SODs.[30]

Importance of MnSOD in eukaryotes

In 1995 Li and collaborators concluded that the deletion of the MnSOD gene in mice resulted in neonatal lethality within 1–2 weeks of birth, with the mice showing several pathological features such as myocardial injury, neurodegeneration and mitochondrial damage.[31] The deleterious effects of the knocking out of MnSOD extended to the MnSOD (–/+) heterozygous knockout mice, expressing 50% of the normal complement of MnSOD, which show oxidative damage and alterations in mitochondria, even though those effects are not fatal as in the complete knockout.[32]

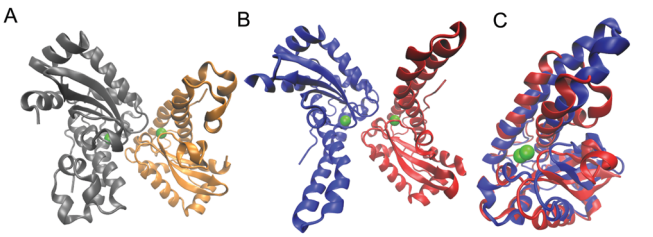


Fig. 1 Structural comparison between *E. coli* and human MnSOD. (A) Dimeric structure of *E. coli* Mn SOD (PDB code 1VEW). (B) Dimeric structure of human MnSOD (PDB code 1N0J). (C) Structural comparison between the monomers of human (blue) and *E. coli* (red) MnSOD. Mn atoms in all three structures are represented in green.

While the cytoprotective and antioxidant activities of MnSOD are evident in cells and tissues, the ultimate reasons for superoxide-mediated toxicity in biological systems have remained elusive for a long time. In particular, superoxide is not a strong oxidant[33] and does not have too many critical biological targets. Two of the best characterized processes accounting for superoxide toxicity are (a) the oxidative inactivation of Fe–S cluster-containing dehydratases[34–36] and (b) the diffusion-controlled reaction with nitric oxide ($^{\bullet}$NO) to yield peroxynitrite, a strong oxidant and nucleophile.[37]

In 1996, in a landmark discovery directly related to the theme of this review, it was observed that chronic allograft rejection in human renal transplants was associated with the nitration and inactivation of MnSOD[38] and it was later observed that nitration preceded the nephropathy described,[39] relating for the first time an oxidative posttranslational modification and loss-of-activity of the enzyme to a pathological state. The role of MnSOD nitration in other pathologies will be discussed in detail in the 'The relevance and mechanism of Tyr34 MnSOD nitration' section.

Alterations in active MnSOD levels are observed in other pathologies such as cancer, in which a decrease in MnSOD activity was observed[40–42] although the levels of protein can remain unaltered or be even increased.[43,44] Some studies showed that restoration of the levels of active MnSOD leads to a slower growth in tumors and alteration of the phenotype of cancer cell lines,[45–47] which points towards the importance of an active MnSOD in the development of this pathology. The decrease in enzyme activity in cancer could be due to mutations in the enzyme that could either decrease its activity or make the enzyme prone to inactivation. Examples of such mutations are I58T or L60F observed in cancer-derived cell lines (such as the Jurkat T cell leukemia-derived cell line),[48–50] which are more susceptible to thiol reagents or the widely described V9A polymorphism in the mitochondrial target sequence, which has been associated with the prevalence of some types of cancer such as breast or gastric cancers.[51,52] Also mutations in the promoter of the gene of MnSOD that could lead to a decrease in its expression have been described in tumor cells.[53,54]

The decrease in the levels of active MnSOD could also be related to its inactivation, as was observed in some cases such as pancreatic cancer, which was correlated with an increase of nitrated MnSOD.[55]

However, the biological role of MnSOD in cancer is not straightforward if considering chemotherapy. For instance, some reports show that, in some cases, MnSOD could play a protective role in cancer cells, as it was shown that in diclofenac-treated neuroblastoma and melanoma cells, a decrease in MnSOD levels and activity was observed with a concomitant increase in apoptosis.[56,57] Similar results were obtained by the use of tamoxifen, which also leads to the increase of reactive oxygen species in cancer breast cells by down-regulation of MnSOD.[58]

MnSOD induction by cytokines[59–62] or its overexpression[63,64] can have tissue protective effects under conditions such as ischemia/reperfusion injury,[63] neurotoxicity[65,66] or radiation damage,[61,67] further supporting the role of mitochondrial superoxide-mediated toxicity in pathology.

Manganese center

The Mn atom has a midpoint potential (E_m) for the Mn^{III}/Mn^{II} couple of nearly 1.5 V (in the high spin form in aqueous solution) *vs.* the normal hydrogen electrode (NHE).[68,69] Fe, which is also present in SOD, presents an E_m of 0.77 V in the high spin form in aqueous solution.[70] The lower E_m of the Fe couple explains its higher reactivity towards hydrogen peroxide (Fenton reaction, eqn (2)).

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + {}^{\bullet}OH + OH^- \quad (2)$$

The Fenton reaction generates strong oxidants including hydroxyl radicals (or high oxidation states of the Fe[71]). The E_m of the Mn couple results in a less reactive free metal ion for secondary oxidant formation under conditions of oxidative stress where hydrogen peroxide is present.

However, a high E_m also makes it inefficient in SOD activity. In order to catalyze both half reactions of superoxide dismutation, the E_m of the Mn^{III}/Mn^{II} couple should be nearly 0.36 V, half-way between the E_m values of SOD's two half-reactions.[72] Therefore, the active site configuration in MnSOD must allow depression of the potential in order to obtain the necessary E_m (nearly 1.1 V drop).[5,30,73]

In conclusion, the efficiency of this enzyme can be explained by both the arrangement of residues near the metal in the active site and the metal center itself.

MnSOD structure

All known manganese-containing SODs exist as either homodimers or homotetramers. Most MnSODs found in bacteria or other prokaryotes are dimers, and the ones found in most eukaryotes, including human cells, are tetramers. A homotetrameric MnSOD is described as a ''dimer of dimers''.[5]

Monomer structure of MnSOD

The monomeric structure of human SOD2 is composed of a mixed α-helix and β-sheet, which resembles the structure of Mn-SOD from *Thermus thermophilus*.[74,75] It is basically divided into 2 domains, a N-terminal helical hairpin domain and a C-terminal domain, containing a three-stranded antiparallel β sheet and five α helices. The N-terminal domain is composed of residues 1–84 and is formed primarily by two long antiparallel α helices separated by a tight turn, forming a helical hairpin structure. The C-terminal domain is a mixed α/β structure, with the central layer formed by a three-stranded antiparallel 8 sheet (residues 85–198).[76]

Nevertheless, the overall structure of the monomers and the spectral characteristics of human SOD2 are similar to those of the Mn-SODs from prokaryotes such as *E. coli* and *T. thermophilus* and eukaryotic cells such as *S. cerevisiae*, due to its highly conserved sequence among species.[77]

Active site of human MnSOD

The active site of human MnSOD contains a ring of positive charges surrounding the active site region that are suggested to

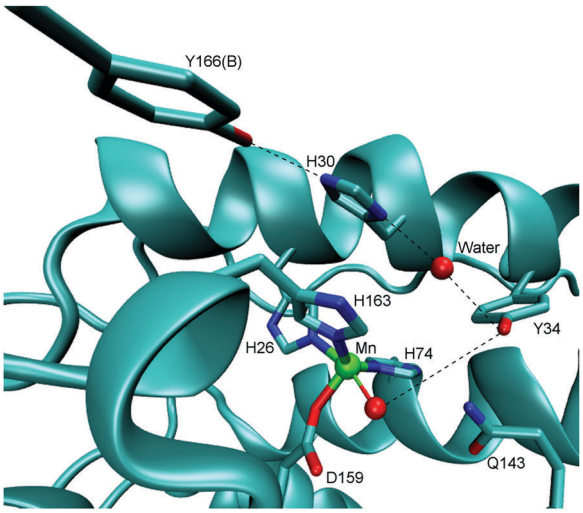


Fig. 2 Active site of human MnSOD showing the Mn center, the coordinating amino acids in the first sphere, and the O of the $^{-}OH/H_2O$, along with a second shell of amino acids that participate in a hydrogen bond network; these amino acids include Tyr34 at ~5.6 Å from the metal center and Tyr166 from chain B of the dimer. PDB code 1N0J.

attract and facilitate the entrance of the negatively-charged superoxide into the 10 Å deep access cavity leading to the Mn atom.[76,78]

The Mn^{III} in the resting enzyme state is penta-coordinated to three histidines (His26, His74 and His163), one aspartate (Asp159) and one hydroxyl ion. There is also a second shell of residues that participate in the structure of the active site and create a hydrogen-bond network composed of solvent molecules and amino acids, including Gln143, Tyr34, His30 and Tyr166, which are needed for proper activity[76,79,80] (Fig. 2).

Structural significance of Tyr34

Tyrosine 34 (Tyr34) has proven to be a key residue in the catalytic process since its mutation to phenylalanine leads to a decrease in k_{cat} by ~10-fold.[7,81–83] Indeed, kinetic and crystallographic studies performed with different mutants in position 34 showed that Tyr34 plays a role in the second half-reaction of the catalytic cycle, the oxidation of Mn^{II} to Mn^{III}, which involves a fast protonation of the product.[84] All five mutations studied (Y34A, Y34N, Y34H, Y34V and Y34F) showed a nearly 75-fold decrease in the constant of this hemi-reaction. In particular, it is proposed that Tyr34 plays a role in avoiding product inhibition, which is increased ~80-fold in mutants on this residue,[83] which can be explained by the decreased rate constant for the catalytic reduction of superoxide to hydrogen peroxide. At the structural level, modifications of this residue did not alter the overall active site arrangement, reinforcing the assumption that the role of this residue is functional rather than structural.[84]

The importance of Tyr34 was clearly inferred when it was substantiated that its oxidative posttranslational modification to 3-nitrotyrosine resulted in MnSOD inactivation,[85] and will be discussed in detail in the next section.

Quaternary structure

The quaternary structure of MnSOD varies from a dimer to tetramer, the latter being a dimer of dimers. The dimer is therefore the basic active conformation, whereas the tetrameric structure gives the enzyme enhanced stability (*i.e.* thermal and pH stability) since it would enhance the dimer interphase.[16,86]

The interface between monomeric subunits to form dimers is well characterized and highly conserved in all species examined, from *E. coli* to humans.[87] On the other hand, the interaction between dimers to form a tetramer is highly variable among the structures known, varying from no interaction in *E. coli* and *Bacillus stearothermophilus*[88] to a dimer–dimer interface in *Thermus thermophilus*[89] that bears no resemblance to the unusual dimer–dimer interface seen in the human enzyme.[76]

In humans the interphase is conformed by Tyr45, Leu49, Gln57, Ile58, Leu60, Gln61 and Leu64 (Fig. 3). The importance of these interphase residues has been thoroughly studied and it was determined that mutations on several of these residues lead to a decreased thermal resistance, tetramer disruption and overall more susceptibility to inactivation or decreased activity.[48,50,76,90]

MnSOD nitration

Mitochondria are organelles where the electron respiratory chain occurs and constitute the major sites of bioenergetic control in most mammalian cells. While most of the oxygen evolves into water in mitochondria, a fraction of it is reduced monovalently; therefore, mitochondria represent key intracellular sources of superoxide radicals under physiological and pathological conditions.[91,92] Since in mitochondria superoxide is formed at significant rates and nitric oxide can freely diffuse into it, these organelles are also major known sites of peroxynitrite formation and therefore mitochondrial proteins are major targets of this nitric oxide-derived oxidizing and nitrating species.[93]

Peroxynitrite ($ONOO^-$) is the product of the diffusion-controlled reaction between nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet -}$) ($k \sim 10^{10}$ M^{-1} s^{-1}).[94,95] This molecule is a potent

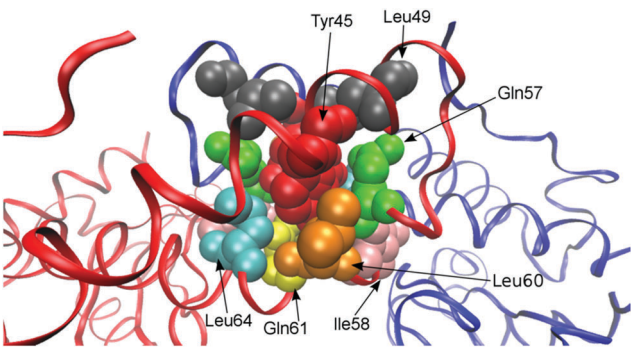


Fig. 3 Dimer–dimer interaction in human MnSOD. Residues responsible for the dimer–dimer interaction indicated for one dimer and the same color code is used on the same amino acids on the other dimer (PDB Code 5T30).

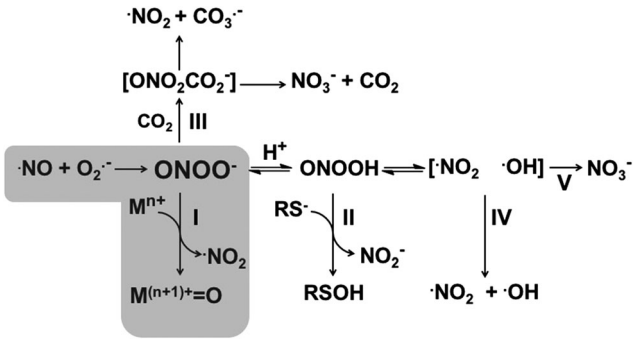


Fig. 4 Reactions of peroxynitrite with different biological targets. (I) One-electron oxidation of transition metal-containing centers (in the case of MnSOD, Mn). (II) Two-electron oxidations with a target (RS^-, *e.g.* deprotonated thiols). (III) $ONO_2CO_2^-$ formation by reaction with CO_2^-. (IV) Homolysis of ONOOH to radicals. (V) Recombination to NO_3^-. The relevant path for MnSOD nitration by peroxynitrite is highlighted in grey. Adapted from ref. 164.

one- and two-electron oxidant and nitrating species, with a redox potential at pH 7 (E_0') for the $ONOO^-/{}^\bullet NO_2$ and $ONOO^-/NO_2^-$ pairs of 1.4 and 1.2 V, respectively.[96,97] Peroxynitrite can react directly with several biological targets such as thiols,[98] carbon dioxide[99–103] and transition metal centers from either metalloproteins or other metal-compounds such as Mn-porphyrins[104,105] (Fig. 4). The reaction of peroxynitrite with its targets promotes, in all cases, to oxidation processes.[100] In the case of the reaction with CO_2, the peroxynitrite anion acts as a nucleophile[37] and ultimately promotes the formation of carbonate ($CO_3^{\bullet -}$) and nitrogen dioxide radicals (${}^\bullet NO_2$).

The reaction of peroxynitrite with proteins containing transition-metal centers occurs preferentially at the metal, in a process that usually kinetically prevails over that of amino acids; Cys, Trp and Met are the only reported amino acids that react directly with peroxynitrite,[100] reviewed in ref. 37 and 106. Of note, tyrosine does not react directly with peroxynitrite, but rather with peroxynitrite-derived radicals.[106]

The formation of peroxynitrite can potentially outcompete the MnSOD-catalyzed superoxide dismutation reaction ($k \sim 1 \times 10^9$ M^{-1} s^{-1} [9,107,108]) and peroxynitrite can, in turn, react with MnSOD to cause its nitration and inactivation.[109] The reactivity of peroxynitrite with different SOD types and SOD mimics (such as Mn-containing porphyrins) has been thoroughly studied in order to better understand the superoxide, nitric oxide and peroxynitrite interplay in the biological context. In particular, Mn porphyrins readily react with peroxynitrite since both Mn^{II} and Mn^{III} ions are Lewis acids that show preference for interaction with hard Lewis bases, highly charged and generally small nucleophiles such as carboxylates and other oxyanions.[105] In particular, Mn^{II} reacts with peroxynitrite, yielding nitrite and oxidizing Mn^{II} to $Mn^{IV}{=}O$, which can proceed to Mn^{III} by a one-electron reaction, yielding a molecule of water.[105,110] In the case of Mn^{III} upon reaction with peroxynitrite, the oxo-manganese compound is also formed and a molecule of ${}^\bullet NO_2$ is generated.[105] Several redox proteins such as flavoenzymes and other cellular reductants can react with Mn^{III} to generate Mn^{II} and therefore this state of the metal is considered to be the one responsible for the neutralizing effects on peroxynitrite in biological systems.[110] Regarding SODs, several studies have reported both the inactivation and oxidation of Cu/Zn-, Mn- and Fe-SOD variants *via* the reaction of peroxynitrite with the metal centers.[104,109,111–114]

The nitration and inactivation of MnSOD have been reported *in vivo* under a variety of inflammatory and other disease conditions or pharmacological interventions that are associated with mitochondrial dysfunction, and excess superoxide and nitric oxide (${}^\bullet NO$) formation such as in aging.[115,116] Table 1 shows some of the conditions where MnSOD has been found nitrated *in vivo* in both human conditions and animal models (mammals). Several techniques have been developed to detect MnSOD Tyr34 nitration *in vivo* including bioanalytical and immunochemical methods (reviewed in ref. 93) ranging from the development of MS-based techniques[117] to antibodies which specifically recognize Tyr34 nitrated MnSOD.[115]

The relevance and mechanism of Tyr34 MnSOD nitration

MnSOD has 198 amino acids, of which 9 are tyrosines (Fig. 5). Although several tyrosines are capable of being nitrated (Tyr2, Tyr9, Tyr11,Tyr34, Tyr45 and Tyr193),[118,119] the nitration of Tyr 34 is the one responsible for the inactivation of the enzyme representing a paradigmatic example of peroxynitrite-dependent ''loss-of-protein function'' and regio-selectivity of nitration *in vitro* and *in vivo*.[38,39,85,119–122]

The mechanisms by which both nitration and inactivation occur have been thoroughly studied in our laboratory and by others, both *in vitro* and using computational simulations.

In 2001, our group[114] studied the reaction of peroxynitrite with MnSOD, focusing on both the kinetic aspects of the reaction and the role of the metal center in the decomposition kinetics of peroxynitrite.

Studying the reaction between human MnSOD and peroxynitrite, a second order rate constant was determined by stopped flow spectrophotometry with a value of $(2.5 \pm 0.04) \times 10^4$ M^{-1} s^{-1} at 37 °C, pH 7.4, per monomer. The reported values were later challenged by another group studying *E. coli* MnSOD[118] reporting a much lower value (9.3×10^2 M^{-1} s^{-1} at 25 °C, pH 7.4, per monomer), at first glance incompatible with the reaction of peroxynitrite with a transition metal center-containing protein and the observed nitration events *in vivo*. Thus, we reevaluated the rate constant values for the peroxynitrite reaction with human MnSOD by competition kinetics using coumarin boronate,[123] a probe that reacts directly with peroxynitrite with a $k = 1 \times 10^6$ M^{-1} s^{-1} at 37 °C, pH 7.4.[124] The rate constants obtained per monomer were $(4.7 \pm 0.3) \times 10^4$ M^{-1} s^{-1} at 37 °C, pH 7.4, and $(1.85 \pm 0.21) \times 10^4$ at 25 °C, pH 7.4, confirming the original report. In Table 2 the established rate constant values of MnSOD with peroxynitrite are shown.

In ref. 114 the role of the Mn center in both the reaction with peroxynitrite and the nitration/inactivation of the enzyme was determined by the removal, replacement or restoration of the metal from the active site. In this sense, the reactivity of the enzyme with peroxynitrite was largely decreased in the

Table 1 Examples of conditions in which nitrated MnSOD was detected *in vivo*

Condition	Tissue and model	Method of detection	Ref.
Hyperglycemia	Hyperglycemic rat pancreatic beta cells and Langerhans islets	Immunodetection,[a] 2D-proteomics/MS detection[b]	140
ALS, Alzheimer's and Parkinson's disease	Cerebrospinal fluids of human patients with ALS, Alzheimer's and Parkinson's disease	Immunodetection	141
Aging	Aging rat skeletal muscle, cardiac sarcoplasmic reticulum membrane and aorta	Immunodetection, HPLC/UV Vis detection	115 and 116
LPS treatment/exposure	Mouse heart and rat eye tissue	Immunodetection	142 and 143
Chronic allograft rejection and nephropathy	Human and rat tubular epithelium	Immunodetection	38 and 39
Ischemia/reperfusion	Rat renal tissue	Immunodetection	144
Angiotensin II treatment	Angiotensin-infused rat renal tissue	Immunodetection	145
Lipoprotein unbalance	Cardiovascular tissue of Apo $E^{-/-}$ mice	Immunodetection	146
Cigarette smoke exposure	Mouse cardiovascular tissue	Immunodetection	146
Acetaminophen-induced hepatotoxicity	WT and nitric oxide synthase knock-out mouse hepatic tissue	Immunodetection	147 and 148
Tamoxifen treatment	Tamoxifen-treated breast cancer mouse cells	Immunodetection	58
Persistent pulmonary hypertension	Endothelial pulmonary cells of a hypertensive newborn lamb	Immunodetection	149
Asthma (allergies)	Human airway epithelial cells from asthmatic individuals/OVA sensitized lung tissue of mice	LC/MS detection, immunodetection, 2D-proteomics/MS detection	150 and 151
Hyperalgesia	Spinal tissue of NMDA-injected rats	Immunodetection	152

[a] Immunodetection encompasses methods that utilize antibodies that recognize protein-3-nitrotyrosine,[153] such as western blotting, immunohistochemistry and immunocytochemistry. [b] Proteomic and bioanalytical methods for protein-3-nitrotyrosine detection have been reviewed recently.[154]

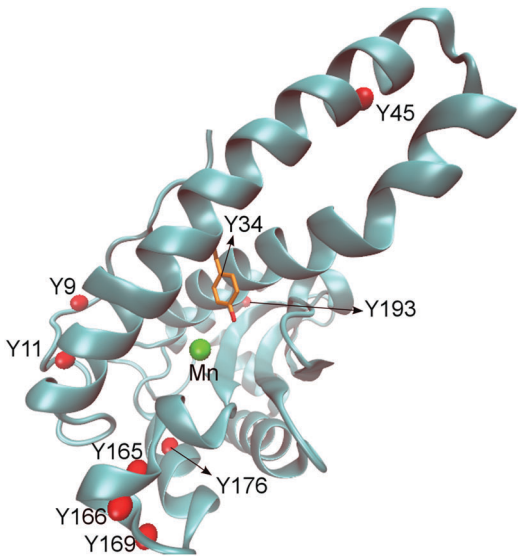


Fig. 5 Localization of tyrosine residues in human MnSOD (monomer). Tyr34 is represented in orange; Tyr9, 11, 45, 165, 166, 169, 176 and 193 are represented in red (showing the oxygen in the phenol group). Note that Tyr34 is adjacent to the Mn atom represented in green. Code PDB: 1LUV.

apo-enzyme. Then, the replacement of Mn with Zn decreased the extents of enzyme nitration, leading to the conclusion that Mn not only participates in the reaction with peroxynitrite, but is also involved in the nitration of Tyr34, and subsequent inactivation of the enzyme. Restoration of the Mn levels to the apo-enzyme recapitulated the extents of peroxynitrite reactivity and nitration yields to those of the native enzyme. These results were also in line with the reported redox chemistry of Mn-porphyrins with peroxynitrite, involving the peroxynitrite-mediated oxidation of the Mn^{III} center to a Mn^{IV}=O species.[105,110]

Table 2 Second order rate constants of the MnSOD reaction with peroxynitrite

MnSOD	k (M^{-1} s^{-1}) (per monomer)	T (°C), pH	Ref.
Human	$(2.5 \pm 0.04) \times 10^4$	37, 7.4	114
	$(1.85 \pm 0.21) \times 10^4$	25, 7.4	123
	$(4.7 \pm 0.3) \times 10^4$	37, 7.4	123
E. coli	$(4.6 \pm 0.2) \times 10^4$	37, 7.4	114

The mechanisms of the MnSOD reaction with peroxynitrite and Tyr34 nitration were further assessed using QM-MM simulations at the atomic level of detail and thermodynamical calculations.[123] The process was divided into four elemental steps: peroxynitrite binding to the MnSOD active site (Step 0), formation of $^{\bullet}NO_2$ (Step 1), tyrosyl radical formation (Step 2) and tyrosine nitration (Step 3):

$$Mn^{III}\text{–SOD} + OONO^- \rightarrow Mn^{III}\text{–SOD–OONO (Step 0)} \quad (3)$$

$$Mn^{III}\text{–SOD–OONO} \rightarrow Mn^{IV}\text{=O–SOD} + {}^{\bullet}NO_2 \text{ (Step 1)} \quad (4)$$

$$TyrOH + Mn^{IV}\text{=O–SOD} \rightarrow TyrO^{\bullet} + Mn^{III}(OH)\text{–SOD (Step 2)} \quad (5)$$

$$TyrOH + {}^{\bullet}NO_2 \rightarrow TyrO^{\bullet} + HNO_2 \text{ (Step 2}') \quad (6)$$

$$TyrO^{\bullet} + {}^{\bullet}NO_2 + (H_2O) \rightarrow 3\text{-}NO_2\text{–TyrOH (Step 3)} \quad (7)$$

In the first step, peroxynitrite diffuses through the active site and binds to the Mn^{III}, yielding a Mn–peroxynitrite adduct. The second step involves a homolytic cleavage of the peroxynitrite O–O bond. This step has a calculated energy barrier of ~9 kcal mol^{-1}, which yields a Mn^{IV}=O intermediate and free $^{\bullet}NO_2$. The fate of the Mn^{IV}=O intermediate presents two alternatives. A first possibility is that it abstracts a hydrogen atom from Tyr34 to yield Mn^{III}–OH (which releases a hydroxyl to yield the

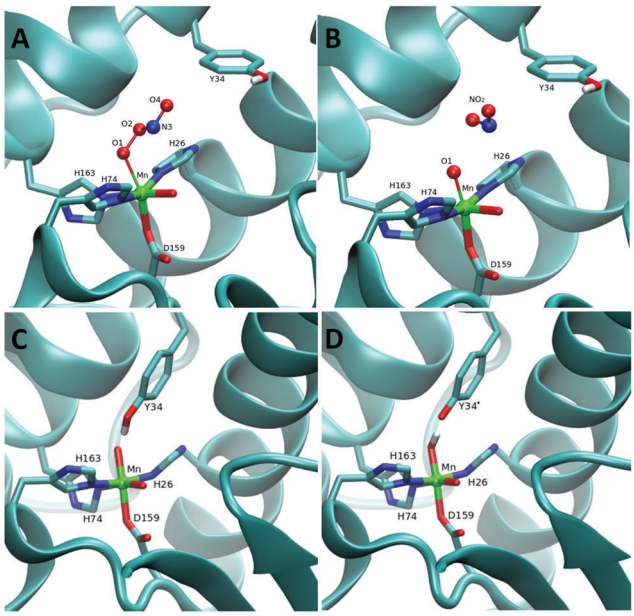


Fig. 6 Mechanism of the reaction of peroxynitrite with the active site of MnSOD. (A and B) Steps 0 and 1 showing the formation of the Mn–peroxynitrite adduct and its homolysis to yield Mn^{IV}=O and $^{\bullet}NO_2$, respectively. (C and D) Step 2 showing the interaction of Tyr34 with Mn^{IV}=O and subsequent formation of a tyrosyl radical and Mn^{III}(OH). Adapted from ref. 123.

protein resting state) and Tyr34 radicals. This process which involves a small 5 kcal mol^{-1} barrier could account for the observed catalyzed nitration, which leads to enzyme inactivation. The $^{\bullet}NO_2$ generated is free to diffuse out of the MnSOD active site, or it can also react with tyrosine to yield a tyrosyl radical (Step 2′). Another $^{\bullet}NO_2$ molecule can then react with the tyrosyl radical to form 3-nitrotyrosine, given that energy barriers for both these steps are also small, less than 10 kcal mol^{-1}. Altogether, these steps account for the production of both site-specific and diffusible nitrating species,[123] making the nitration of Tyr34 a specific but <100% yield process. Graphical representations of steps 1 and 2 are depicted in Fig. 6.

As an alternative mechanism,[118] it was proposed that peroxynitrite was capable of reducing the metal center of MnSOD as Mn^{II} was detected by low temperature EPR techniques after enzyme exposure to a large excess of peroxynitrite. This finding was unexpected according to the known oxidative chemistry of peroxynitrite towards Mn-containing centers,[105] summarized in the reaction scheme of Fig. 4. The detection of Mn^{II} in ref. 118 was probably due to the exposure of the enzyme to extremely high concentrations of pure peroxynitrite (*e.g.* 20 mM), which can yield enough superoxide[94] (secondary to the equilibrium of peroxynitrite with its precursor species) to reduce Mn^{III}. Indeed, Mn^{II} was not detected in MnSOD when exposed to biologically-relevant concentrations of peroxynitrite.[123] Finally, the hypothetical one-electron reduction of Mn^{III} by peroxynitrite[118] to yield a nitrosodioxyl radical (eqn (8)) was further ruled out on thermodynamic grounds.[123]

$$Mn^{III}\text{–SOD} + OONO^{-} \rightarrow Mn^{II}\text{–SOD} + OONO^{\bullet} \quad (8)$$

Calculations indicate that the reaction in eqn (8) is highly endergonic (ΔE = +45.7 kcal mol^{-1}), and therefore thermodynamically very unfavorable.

Altogether, the global reaction mechanism indicated in eqn (3)–(7) and Fig. 6 fully rationalizes the reaction of MnSOD with peroxynitrite and the subsequent nitration of active site Tyr34.

Molecular basis of the inactivation

The pivotal role of Tyr34 in the catalysis[84] is consistent with the loss-of-activity observed upon its nitration. In 2006,[80] human NO_2Tyr34-MnSOD was crystallized and structural analysis showed that the enzyme active site was not conformationally altered and that the nitro group in Tyr34 pointed towards the Mn center (Fig. 7). The bulk of the nitro group occupying part of the narrow active site of MnSOD (~7–8 Å diameter) would not allow the entrance of superoxide near the Mn center. Fig. 8 graphically presents the blockage of the nitro group to the superoxide diffusion towards the metal center.[123]

In addition, in Tyr34 nitrated MnSOD the hydrogen bond network, which is crucial for the activity, seemed to be altered as well[80] (Fig. 7); this phenomenon could interfere with the proton transfer crucial during catalysis.[9,82,125]

An additional possibility for the observed enzyme inactivation is the fact the tyrosine nitration causes a decrease in pK_a of about 3 pH units in the phenolic –OH compared to that of unmodified tyrosine.[126] Thus, at physiological pH a high proportion of the –OH in nitro-Tyr34 becomes dissociated into the phenolate form[127] and generates an electrostatic repulsion that would prevent the access of negatively charged superoxide to the active site.

In 2011, Moreno *et al.* explored, using computational simulations, the molecular basis of the inactivation of MnSOD

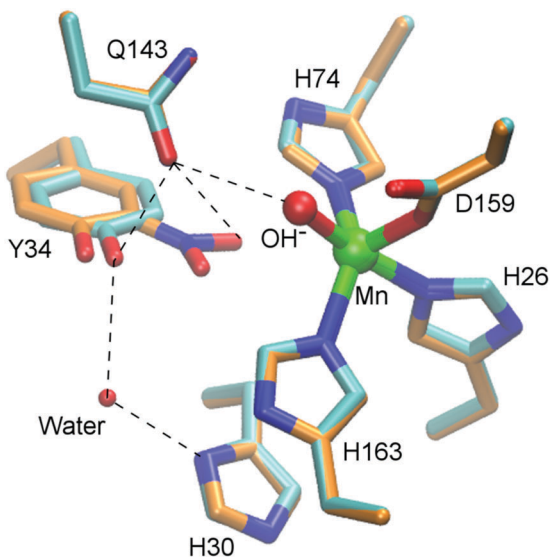


Fig. 7 Comparison of the active site of native and nitrated MnSOD. Molecular structures of the active site of native MnSOD (C atoms in cyan) and Tyr34 nitrated MnSOD (C atoms in orange). Dotted lines indicate a disturbed hydrogen bond network generated upon nitration. Adapted from ref. 80.

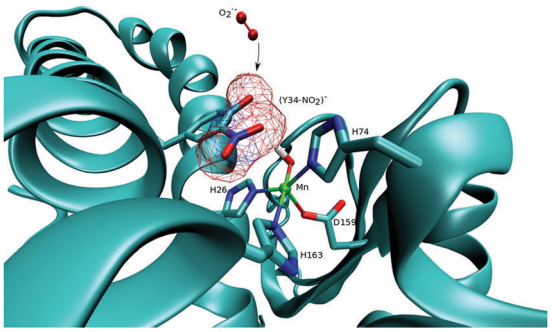


Fig. 8 Active site and entrance channel of MnSOD showing the nitro group in Tyr34 blocking the access to the active site to a molecule of superoxide. Reprinted with permission from V. Demicheli, *et al.*, *Biochemistry*, 2016, **55**(24), 3403–3417, DOI: 10.1021/acs.biochem.6b00045. Copyright 2016 American Chemical Society.

by nitration of Tyr 34.[128] Molecular dynamics simulations (MD) are extremely useful to obtain detailed atomistic level information.[129] These techniques allow understanding the temporal evolution of the protein structure and dynamics and the free energy profiles of the migration process. The ligand migration to the active site is determined by the presence of specific residues acting as attractors or blocking the entry process. This phenomenon is dynamic and is determined and controlled by the protein motions, complementing the crystallographic data from a structural biology viewpoint.

In this work, a detailed study of the superoxide entrance into the active site was performed, comparing the free energy profiles of this process in native or nitrated MnSOD. The results showed that the incorporation of the bulky negatively polarized substituent in Tyr34 produces steric and electrostatic repulsion effects which are reflected in the dramatic increase of the free energy profile for superoxide access to the metal center (Fig. 9). MD simulations showed that while Tyr34 assists superoxide for its entrance by pushing it into the active site, nitrated Tyr34 gets closer to Mn than what was earlier shown by the crystal structure causing the blockage of the channel[128] (Fig. 9).

From the biochemical, crystallographic and molecular dynamics studies, it can be concluded that a sum of the changes listed (namely, disruption of the hydrogen bond network, steric restriction and electrostatic repulsion) jointly leads to the loss of MnSOD activity by the nitration of a single tyrosine residue (Tyr34).

Peroxynitrite formation in the presence of MnSOD

In spite of the *in vitro* and *vivo* observations supporting the peroxynitrite-mediated nitration of Tyr34 in MnSOD, the actual formation of peroxynitrite in the presence of SOD is, at first glance, far from obvious. Peroxynitrite formation requires the reaction of superoxide with nitric oxide; even though both radicals can be simultaneously present in mitochondria, levels of MnSOD, which based on the specific activity in this organelle, mitochondrial volume and protein concentration are estimated to be between 10 and 30 μM,[15,114,130] could largely limit the amounts of superoxide available due the fast dismutation reaction. However, nitric oxide becomes one of the few biomolecules that could kinetically outcompete MnSOD, as the

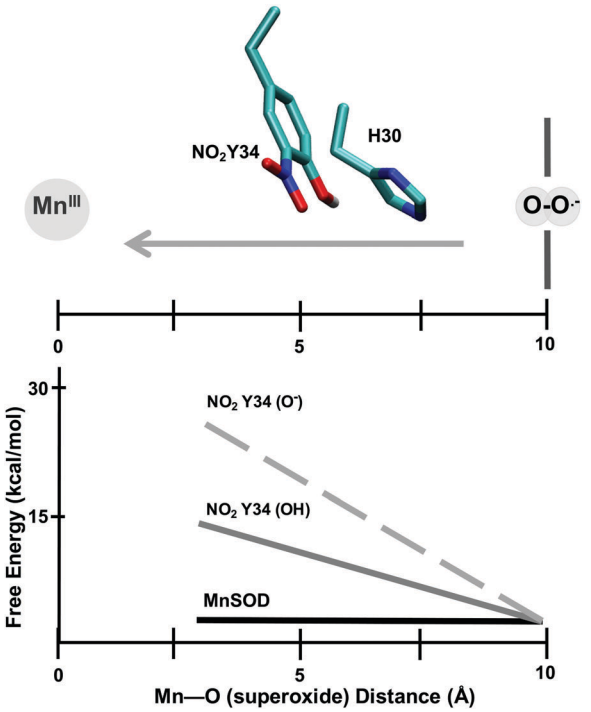


Fig. 9 Free energy profile for superoxide migration along the diffusion pathway in the MnSOD access channel. Upper panel: schematic representation of the active site of nitrated Tyr34 in MnSOD and the pathway of superoxide from the entrance to the Mn center. Lower panel: free energy estimation of superoxide as it approaches the Mn center comparing the unmodified (black line) and nitrated forms in the phenol (full grey line) or the phenolate forms (dashed grey line). The free energy scale values represent estimates obtained from ref. 128.

rate constant of peroxynitrite formation is about 10-fold higher than that of the MnSOD-catalyzed dismutation ($\sim 10^{10}$ *vs.* 10^9 M^{-1} s^{-1}, respectively). Indeed, the exposure of MnSOD to biologically-relevant fluxes of nitric oxide and superoxide led to a time-dependent tyrosine nitration and loss of enzyme activity[109] (Fig. 10). The mechanism of nitration involved the intermediacy of the tyrosyl radical, since a protein radical adduct was detected with the spin trap DMPO and revealed by immunospintrapping.[131] Interestingly, the intracellular reductant glutathione known to react with peroxynitrite ($k = 1.35 \sim 10^3$ M^{-1} s^{-1} [96]) and inhibit nitration reactions in the bulk phase mainly by the fast consumption of nitrogen dioxide ($k = 1.9 \times 10^7$ M^{-1} s^{-1})[132] was unable to completely inhibit nitration. The lack of a total protective effect of glutathione on enzyme nitration and inactivation underscored the regio-selectivity of Tyr34 nitration. Additionally, under exposure conditions to superoxide and nitric oxide fluxes, MnSOD Tyr34 could be nitrated following the reaction of excess nitric oxide with the tyrosyl radical to yield a transient 3-nitrosotyrosine that would be further oxidized to 3-nitrotyrosine by oxidation steps mediated by the Mn^{IV}=O species with the intermediacy of an iminoxyl radical.[133]

Based on kinetic simulations previously performed in ref. 109 for Cu,ZnSOD exposed to simultaneous fluxes of superoxide and nitric oxide, we adapted the calculation with the

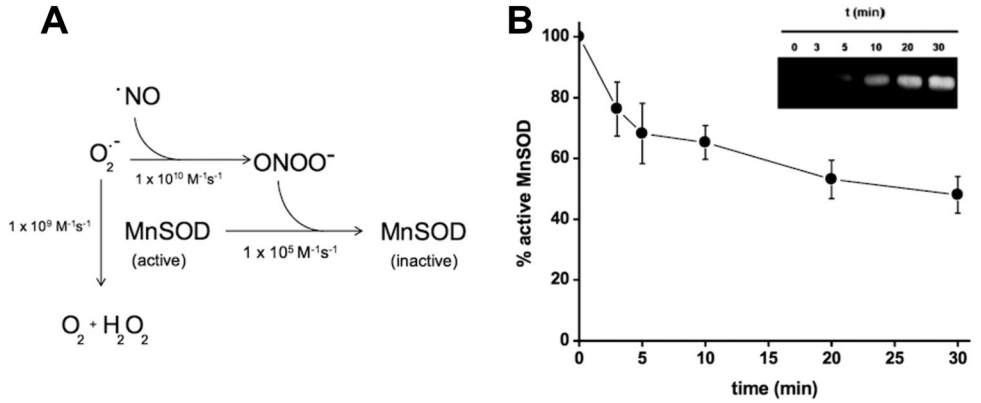


Fig. 10 Nitration and inactivation of MnSOD in the presence of fluxes of superoxide and nitric oxide. (A) Proposed reactions leading to MnSOD nitration and inactivation in the presence of simultaneous fluxes of superoxide and nitric oxide. (B) Time-dependent nitration and inactivation of human MnSOD (5 μM) exposed to simultaneous fluxes (10 μM min^{-1}) of superoxide and nitric oxide. Adapted from Demicheli *et al.*, **42**(9), 1359–1368. Copyright (2007), with permission from Elsevier.

appropriate kinetic information for MnSOD (Table 3) and evaluated the temporal course of MnSOD inactivation and transient levels of superoxide, nitric oxide and peroxynitrite (Fig. 11). The simulation clearly shows that MnSOD is inactivated (Fig. 11A) and peroxynitrite increased (Fig. 11B) from the beginning of the process and continuously as a function of time. Then, while superoxide can initially be readily dismutated by the existing levels of MnSOD (superoxide was kept in the pM range for the first 30 minutes, Fig. 11C), the transient levels of nitric oxide become 10^6 fold higher (μM range, Fig. 11D) and therefore, by mass action law, starts to outcompete MnSOD to yield peroxynitrite (nM range) ultimately responsible for MnSOD inactivation (see Fig. 10A and Table 3, eqn (7)). Upon enzyme full inactivation (~50 min), superoxide levels dramatically increase, whereas nitric oxide levels continue to diminish until both reach similar concentrations (nM range). The overall conclusion provided by this kinetic model is that peroxynitrite can be readily formed from superoxide and nitric oxide fluxes even in the presence of MnSOD and that the peroxynitrite-mediated MnSOD Tyr34 nitration triggers a "vicious cycle" by which enzyme inactivation facilitates further peroxynitrite formation and nitration reactions (Fig. 10 and 11).

Other possible tyrosine nitration mechanisms

Even though protein tyrosine nitration *via* peroxynitrite has been widely reported, other biochemical sources capable of generating nitrogen dioxygen can cause it.[134] In fact, there are reports on MnSOD nitration by peroxynitrite-independent, nitrogen dioxide generating systems.[118,123] Interestingly, in those systems, upon such exposure of MnSOD to hemeperoxidase–hydrogen

Table 3 Selected reactions during the exposure of MnSOD to simultaneous fluxes of nitric oxide and superoxide. (f) and (r) represent forward and reverse kinetic constants, respectively

	Reaction	k	Ref.
(1)	$O_2^{\bullet-} + {}^{\bullet}NO \rightarrow ONOO^-$	1×10^{10} M^{-1} s^{-1}	155
(2)	$Mn^{III}SOD + O_2^{\bullet-} \rightarrow Mn^{II}SOD + O_2$	1.5×10^9 M^{-1} s^{-1}	5, 7 and 18
(3)	$Mn^{II}SOD + O_2^{\bullet-} + 2H^+ \rightarrow Mn^{III}SOD + H_2O_2$	1.5×10^9 M^{-1} s^{-1}	5, 7 and 18
(4)	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$	2.5×10^5 M^{-1} s^{-1}	156
(5)	$ONOO^- \rightarrow NO_3^-$	0.63 s^{-1}	157
(6)	$ONOO^- \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}OH$	0.27 s^{-1}	157
(7)	$MnSOD + ONOO^- \rightarrow MnSOD_{inactive}$	1.0×10^5 M^{-1} s^{-1}	114 and 123
(8)	${}^{\bullet}OH + NO_2^- \rightarrow OH^- + {}^{\bullet}NO_2$	1.0×10^{10} M^{-1} s^{-1}	158
(9)	${}^{\bullet}OH + {}^{\bullet}NO \rightarrow NO_2^- + H^+$	2.0×10^{10} M^{-1} s^{-1}	158
(10)	${}^{\bullet}OH + {}^{\bullet}NO_2 \rightarrow ONOO^- + H^+$	4.5×10^9 M^{-1} s^{-1}	159
(11)	${}^{\bullet}OH + O_2^{\bullet-} \rightarrow O_2 + OH^-$	7×10^9 M^{-1} s^{-1}	158
(13)	$2{}^{\bullet}NO + O_2 \rightarrow 2{}^{\bullet}NO_2$	2.9×10^6 M^{-2} s^{-1}	160
(14)	${}^{\bullet}NO + {}^{\bullet}NO_2 \rightleftharpoons N_2O_3$	1.2×10^9 M^{-1} s^{-1} (f) 8.4×10^4 s^{-1} (r)	158
(15)	$2{}^{\bullet}NO_2 \rightleftharpoons N_2O_4$	4.5×10^8 M^{-1} s^{-1} (f) 6.9×10^3 s^{-1} (r)	158
(16)	$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+$	8.2×10^4 s^{-1}	160
(17)	$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$	1×10^3 s^{-1}	161
(18)	$NO_2^{\bullet} + O_2^{\bullet-} \rightleftharpoons O_2NOO^-$	4.5×10^9 M^{-1} s^{-1} (f) 1.1 s^{-1} (r)	162
(19)	$O_2NOO^- \rightarrow NO_2^- + O_2$	1.3 s^{-1}	162
(20)	$N_2O_3 + ONOO^- \rightarrow NO_2^- + 2{}^{\bullet}NO_2$	3.1×10^8 M^{-1} s^{-1}	163
(21)	$ONOO^- + {}^{\bullet}OH \rightarrow ONOO^{\bullet} + OH^-$	4.8×10^9 M^{-1} s^{-1}	159

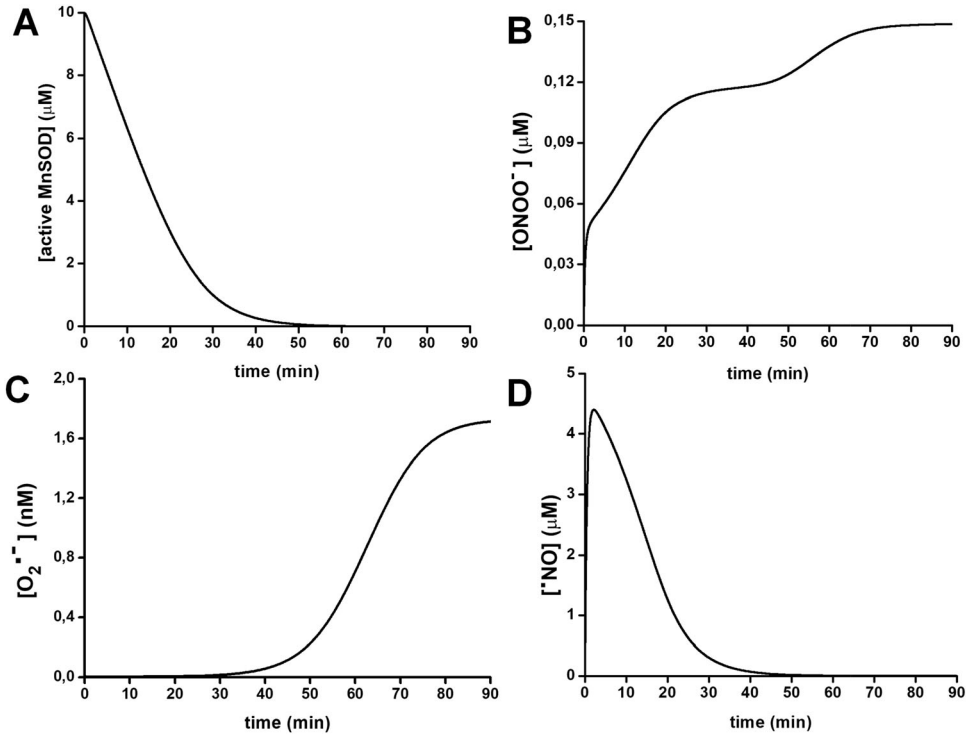


Fig. 11 Transient levels of active MnSOD, peroxynitrite, superoxide and nitric oxide generated using computer assisted simulations. Levels of (A) active MnSOD, (B) peroxynitrite, (C) superoxide and (D) nitric oxide during the simultaneous generation of superoxide and nitric oxide (10 μM min^{-1}) in the presence of MnSOD (10 μM). The kinetic simulation was based on the reactions presented in Table 3 and performed using the program GEPASI,[165–167] as reported previously.[109,123]

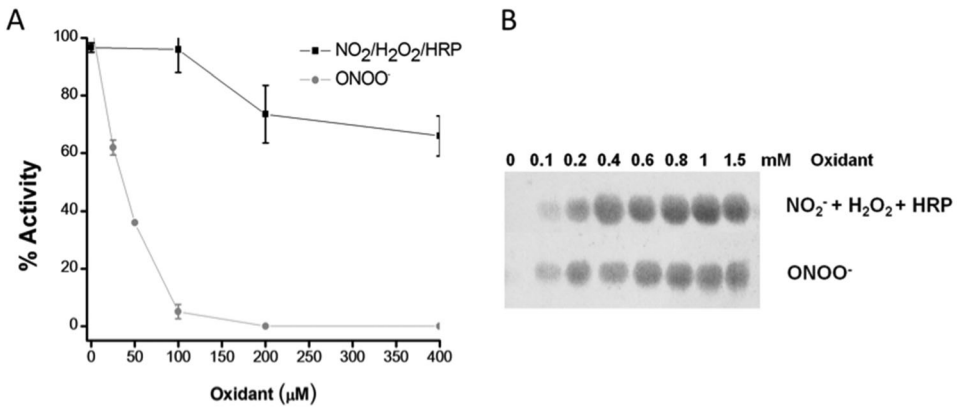


Fig. 12 Comparison of the nitration and inactivation of human MnSOD exposed to two different nitrating agents. (A) Activity of MnSOD exposed to peroxynitrite (grey circles) and nitrite, hydrogen peroxide and HRP (black squares). (B) Immunochemical detection of 3-nitrotyrosine of human MnSOD comparing both nitrating agents. Reprinted with permission from V. Demicheli, *et al.*, *Biochemistry*, 2016, **55**(24), 3403–3417, DOI: 10.1021/acs.biochem.6b00045. Copyright 2016 American Chemical Society.

peroxide–nitrite (Fig. 12), tyrosine nitration is observed but activity is minimally affected.[118] Peptide mapping–mass spectrometry analysis of the samples shows the nitration of solvent-exposed Tyr9 and 11, while critical Tyr 34 was unmodified (see Fig. 5).[118,123]

From a biological perspective, taking into consideration the lack of "professional" hemeperoxidases in mammalian mitochondria, this nitration mechanism may become relevant upon activation of the peroxidatic activity of cytochrome *c*,[135–137] but would not cause MnSOD inactivation. Thus, with the available data, Tyr34 nitration coupled to MnSOD inactivation is indicative of peroxynitrite formation and reactions in mitochondria and cells.

A final consideration in the context of MnSOD nitration involves the role of the carbonate radical formed in 35% yield from the reaction of the peroxynitrite anion with carbon dioxide ($k = 3 \times 10^4$ M^{-1} s^{-1}) (Fig. 4). Since carbon dioxide is present at high concentration in mitochondria (∼ mM),

it represents a major route of the fate of peroxynitrite *in vivo*. It has been proposed that peroxynitrite-derived carbonate radicals could diffuse through the MnSOD access channel and, being a good one-electron oxidant, attack Tyr34 and lead to tyrosyl radicals, which would react with nitrogen dioxide to yield nitro-Tyr 34.[118] The characterization and significance of the carbonate radical reaction with MnSOD and its potential relation to Tyr34 nitration await further studies.

Conclusions

Human MnSOD Tyr34 nitration and inactivation occur *via* a peroxynitrite-mediated process. This posttranslational modification in MnSOD is observed *in vivo* and is likely to participate in the disruption of mitochondrial redox homeostasis. The kinetics and mechanisms of enzyme nitration and inactivation have been disclosed at the atomic level of detail. Site-specificity in SOD tyrosine nitration involves the participation of transition metal centers that go beyond Mn, such as that recently observed in the case of *T. cruzi* FeSOD.[113] Further work should be extended to other FeSODs and cambialistic SODs that may be inactivated by nitration in a Tyr residue homologous to Tyr 34 in humans or, alternatively, by-pass the nitration process by subtle changes in the protein structure.[29,113,138,139]

The case of human MnSOD constitutes a paradigmatic example coupling a transition metal-assisted oxidative modification of a single tyrosine residue with enzyme inactivation *in vivo*.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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