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Exploring the molecular basis of human manganese superoxide dismutase inactivation mediated by tyrosine 34 nitration

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ABSTRACT

Manganese Superoxide Dismutase (MnSOD) is an essential mitochondrial antioxidant enzyme that protects organisms against oxidative damage, dismutating superoxide radical ($O_2^{\cdot-}$) into H_2O_2 and O_2. The active site of the protein presents a Mn ion in a distorted trigonal–bipyramidal environment, coordinated by H26, H74, H163, D159 and one $^-$OH ion or H_2O molecule. The catalytic cycle of the enzyme is a "ping-pong" mechanism involving Mn^{3+}/Mn^{2+}. It is known that nitration of Y34 is responsible for enzyme inactivation, and that this protein oxidative modification is found in tissues undergoing inflammatory and degenerative processes. However, the molecular basis about MnSOD tyrosine nitration affects the protein catalytic function is mostly unknown.

In this work we strongly suggest, using computer simulation tools, that Y34 nitration affects protein function by restricting ligand access to the active site. In particular, deprotonation of 3-nitrotyrosine increases drastically the energetic barrier for ligand entry due to the absence of the proton.

Our results for the WT and selected mutant proteins confirm that the phenolic moiety of Y34 plays a key role in assisting superoxide migration.

Introduction

Superoxide dismutases (SODs) are enzymes that protect biological systems against oxidative damage caused by superoxide radical ($O_2^{\cdot-}$) which is generated during aerobic metabolism by one electronic reduction process of molecular oxygen through different mechanisms like the catalytic production by flavoproteins like xanthine oxidase [1,2], the mitochondrial respiratory chain [3] or activation of NADPH oxidase [4,5].

There are at least three unrelated families of SODs found in nature: the structurally homologous mononuclear active site iron SODs or manganese SODs (MnSOD)[1], the binuclear copper/zinc SODs and the mononuclear nickel SOD [6–9].

MnSOD is found in mitochondrial matrix and chloroplast of eukaryotes and in the cytoplasm of bacteria. This enzyme was found to be essential in mammals as shown by knockout mice experiments where individuals that did not express MnSOD died within 10 days after birth [10]. Several crystal structures have been published of the wild type (WT) enzyme [11–13] and the active site consists of one Mn ion per unit in a distorted trigonal–bipyramidal environment, coordinated by three histidine residues, one aspartate and one $^-$OH ion or H_2O molecule (Fig. 1) [12,14].

MnSOD is a homotetramer (96 kDa) that has a ring of positive electrostatic charge surrounding the active site, which is suggested to enhance attraction for negatively charged superoxide. Proximal to the catalytic Mn site is the substrate access cavity, which is characterized by a hydrogen-bonded network that is comprised of solvent molecules and several key residues such as Q143, Y34, H30 and Y166, which are needed for proper activity [13,15,16]. Fig. 2 shows the ligand migration pathway to the active site in one monomer in the dimeric structure. Since Y34 is located in the access tunnel, this residue is known to play a central role in the ligand migration process [17].

The proposed MnSOD catalytic cycle consists of a "ping-pong" mechanism which oscillates between metal Mn^{3+}/Mn^{2+} redox states as is schematically depicted in Eqs. (1 and 2) [18,19].

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[1] *Abbreviations used:* MnSOD, manganese superoxide dismutases; WT, wild type; NY, 3-nitrotyrosine; MSMD, multiple steered molecular dynamics.

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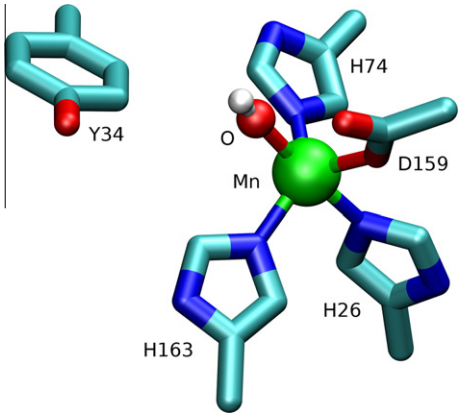

Fig. 1. Active site of MnSOD, showing the Mn center, the three histidines and aspartate residues, the O of the $^{-}OH/H_2O$ and the second sphere residue tyrosine at around 5.6 Å from the metal center.

$$Mn^{3+} + O_2^{\cdot-} \rightarrow Mn^{2+} + O_2 \quad (1)$$

$$Mn^{2+} + O_2^{\cdot-} + 2H^{+} \rightarrow Mn^{3+} + H_2O_2 \quad (2)$$

In the WT enzyme, the high values of the kinetic constants for both half-reactions (1.5 and $1.1 \times 10^9\ M^{-1}s^{-1}$, respectively) [17] might be explained by the presence of a big entry channel which freely leads the superoxide to the active site. Decreasing of kinetic constants could be promoted by the introduction of residues which alter the original tunnel features.

Recent computational studies revealed that the first step, the oxidation of $O_2^{\cdot-}$ to O_2, most likely occurs by an associative mechanism, while the second step seems to take place by a second sphere electron transfer mechanism, i.e., without direct coordination of the second $O_2^{\cdot-}$ substrate to the metal center. These results also interestingly suggest that Y34 plays a key role in the second step catalysis [20,21].

Protein tyrosine nitration is a covalent post-translational protein modification derived from the reaction of proteins with nitrating agents. One of the most relevant nitrating agents *in vivo* is peroxynitrite ($ONOO^-$), a potent oxidant formed by the diffusion-controlled reaction between nitric oxide ($^{\bullet}NO$) and superoxide radical ($O_2^{\cdot-}$) which is involved in a variety of disease states [22–24]. The nitration of protein tyrosines may alter the structure and/or the function of proteins [25–29], although the mechanistic details of the changes for specific proteins at the molecular level are just being unraveled [30].

Human MnSOD is a prime example of a tyrosine nitrated protein with functional consequences *in vivo*. MnSOD contains nine tyrosine residues, one of which (Y34) is highly conserved phylogenetically and is located only a few Å from Mn [13]. The site-specific nitration at this residue that is observed in many inflammatory and degenerative diseases including chronic organ rejection, ischemia/reperfusion, arthritis, atherosclerosis and tumorigenesis [31,32], results in enzyme inactivation [33]. Indeed, *in vitro* studies from several groups indicate that Y34 post- and even co-translationally nitrated human MnSOD is inactive [34–38] and that the Mn atom efficiently directs peroxynitrite and nitric oxide-mediated nitration in the presence of $O_2^{\cdot-}$ to the adjacent Y34 [22,36,37] which is at only 5 Å from the metal center. The detoxification function of MnSOD is essential to normal cell and mitochondrial homeostasis and therefore significant decreases in its activity as observed during tyrosine nitration results in mitochondrial oxidative stress with potential consequences in bioenergetics and signaling of cell death [32,39].

Understanding how MnSOD Y34 nitration (or replacement) alters MnSOD function at the molecular level is not an easy task. The replacement of Y34 by other amino acids results in a large reduction in catalysis efficiency [17,34,40,41], confirming a key role of this conserved amino acid in the dismutation reaction mechanism. For example, *in vitro* enzyme kinetics studies obtained Y34F mutant, yielded values of k_{cat}/K_M that are similar to those of the WT ($\approx 800\ \mu M^{-1}s^{-1}$), but there is a decrease of ten fold in the k_{cat} value, [42] suggesting the crucial role of Y34 hydroxyl group in both substrate binding and catalysis. Also, replacement of Y34 ortho hydrogen by a fluorine atom (3-fluorotyrosine) [41] which represents a minor steric change reduces the catalytic activity k_{cat}/K_M to $30\ \mu M^{-1}s^{-1}$. Finally, it should be noted that the actual observed k_{cat}/K_M value of $\approx 800\ \mu M^{-1}s^{-1}$ for the WT enzyme, is close to that expected for a diffusion-controlled reaction, strongly suggesting that superoxide access to the Mn active site is the rate limiting step [43]. This point confirms the idea that an obstruction or a charge density modification in the channel access could be the reason for the lack of activity in modified MnSOD enzymes.

Based on these results, several hypotheses have been put forward to explain the effect of Y34 nitration. The crystal structure of nitrated human MnSOD has been published [16] and shows that replacement of Y34 by 3-nitrotyrosine (NY) does not cause significant conformational changes of active-site residues or solvent displacement. This modification, however, has other effects; for example in a change in the pKa of the tyrosine phenol group, from around 10 to 7.5 [22,34,44], which may interrupt the hydrogen bonding network of the Y34-Q143 pair, affecting the catalytic

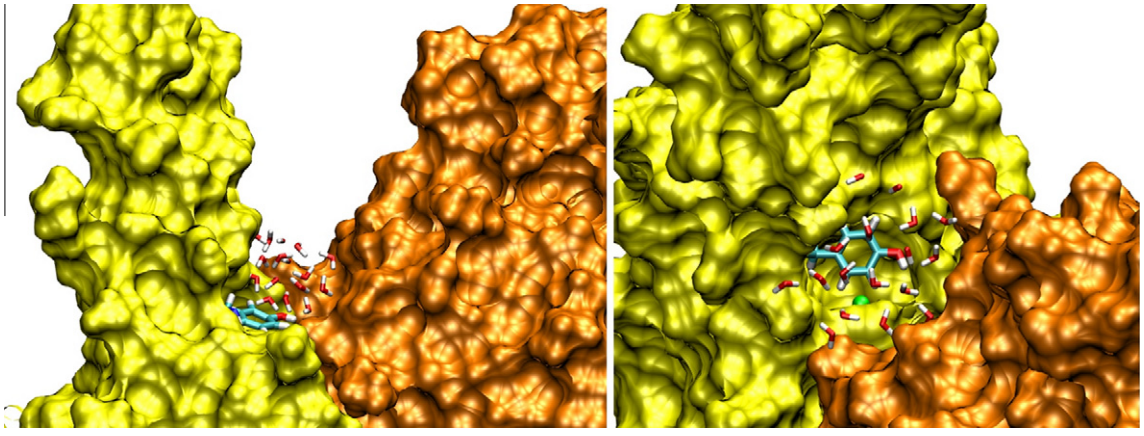

Fig. 2. Two different view (left and right panel) of surface contour analysis of the dimeric WT-MnSOD (each subunit depicted in different colors, Mn coordinated residues were excluded of the analysis). Manganese atom (green), Y34 (cyan), and several water molecules (red/white) are depicted in order to shed light on the accessibility to the active site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)