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Original Contribution

Novel mechanisms for superoxide-scavenging activity of human manganese superoxide dismutase determined by the K68 key acetylation site

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ABSTRACT

Superoxide is the primary reactive oxygen species generated in the mitochondria. Manganese superoxide dismutase (SOD2) is the major enzymatic superoxide scavenger present in the mitochondrial matrix and one of the most crucial reactive oxygen species-scavenging enzymes in the cell. SOD2 is activated by sirtuin 3 (SIRT3) through NAD^+-dependent deacetylation. However, the exact acetylation sites of SOD2 are ambiguous and the mechanisms underlying the deacetylation-mediated SOD2 activation largely remain unknown. We are the first to characterize SOD2 mutants of the acetylation sites by investigating the relative enzymatic activity, structures, and electrostatic potential of SOD2 in this study. These SOD2 mutations affected the superoxide-scavenging activity in vitro and in HEK293T cells. The lysine 68 (K68) site is the most important acetylation site contributing to SOD2 activation and plays a role in cell survival after paraquat treatment. The molecular basis underlying the regulation of SOD2 activity by K68 was investigated in detail. Molecular dynamics simulations revealed that K68 mutations induced a conformational shift of residues located in the active center of SOD2 and altered the charge distribution on the SOD2 surface. Thus, the entry of the superoxide anion into the coordinated core of SOD2 was inhibited. Our results provide a novel mechanistic insight, whereby SOD2 acetylation affects the structure and charge distribution of SOD2, its tetramerization, and p53–SOD2 interactions of SOD2 in the mitochondria, which may play a role in nuclear–mitochondrial communication during aging.

1. Introduction

In mammalian cells, the mitochondrion is the major site of adenosine triphosphate production and a vital site for other diverse cellular functions, including metabolism, generation of reactive oxygen species (ROS)[2], stress response, and apoptosis [1–3]. Despite ongoing controversies, theoretically, the mitochondrion has been regarded as the biological clock for aging [4]. The imbalance between mitochondrial ROS production and antioxidant capacities underlies many oxidative stress- and age-related diseases, including neurodegenerative diseases, atherosclerosis, carcinogenesis, and diabetes [2,5–7]. The superoxide anion $O_2^{\bullet -}$ is the primary ROS produced by complexes I and III of the electron transport chain in the mitochondrion. $O_2^{\bullet -}$ is one of the toxic oxygen radicals that could further lead to formation of other oxidative species, such as hydrogen peroxide (H_2O_2), hydroxyl radical, and peroxynitrite ($OONO^-$), which inflict damage on both the mitochondria and the cell. In the mitochondrial matrix, the steady-state concentration of $O_2^{\bullet -}$ may be estimated to be as low as 10^{-10} M [8], which is believed to be maintained by the scavenging enzymes within the mitochondria, such as superoxide dismutase (SOD) and/or superoxide reductase [9]. However, Wang et al. [10] indicated that the mitochondria undergo bursts of superoxide production at a single mitochondrial level, which is called "superoxide flash," signifying the fluctuating concentrations of the superoxide molecules in the mitochondria. Among all the ROS-scavenging enzymes in the mitochondria, manganese superoxide dismutase (SOD2 or MnSOD) is the pivotal antioxidant enzyme that

Abbreviations: SOD2, manganese superoxide dismutase; ROS, reactive oxygen species; MD, molecular dynamics; PTM, posttranslational modification; Anh, acetic anhydride; MnTM-4-PyP, manganese(III) *meso*-tetrakis (*N*-methylpyridinium-4-yl) porphyrin.

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catalyzes the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen, protecting cells from oxidative stress. The intramitochondrial level of SOD2 is believed to be 10 to 40 μM. This dismutation reaction is considered to be fast, similar to diffusion ($k = 2.3 \times 10^9\ M^{-1}\ s^{-1}$), indicating that this reaction is catalyzed by a highly effective superoxide dismutase [8]. In various studies, many investigators have characterized different SOD2 mutants to illustrate the biological function and structure–activity relationship of SOD2; however, nobody has acquired a mutant with activity higher than that of wild-type SOD2 [11–15]. In recent years, several studies have reported that the antioxidative activity of the SOD2 is regulated by many posttranslational modifications (PTMs), including acetylation [16,17], methylation [18], phosphorylation [19], nitration [14,20], and glutathionylation [20]. These explorations have provided new methods to possibly obtain a higher-activity mutant form of SOD2 by changing the PTMs in SOD2.

Lysine acetylation has emerged as a key regulatory mechanism of protein function in diverse biological processes, including histone degradation [21], chromatin remodeling, transcription, and metabolism pathways in the mitochondria [22]. Compared with cytosol, the mitochondria are a preferable environment for the acetylation reaction because of the higher concentration of acetyl-CoA (3- to 50-fold that of cytosol), which provides the acetyl group for acetylation, and the high pH (pH 8.0) [23], at which the nonenzymatic process of acetylation may occur. Recently, large-scale proteomic approaches have identified numerous mitochondrial acetylated proteins in both humans and mice [24–26], of which many are the enzymes involved in the intermediary metabolic processes, including fatty acid metabolism, urea cycle, tricarboxylic acid cycle, and gluconeogenesis. According to Xiong and Guan, these metabolic enzymes are affected by acetylation in a coordinated manner through various mechanisms, such as inhibition, activation, and protein destabilization [27]. Meanwhile, the cross talk between acetylation and phosphorylation/methylation regulates the key enzymes of the metabolism in response to different physiological stimulations [28]. SOD2 functions as the first responsive enzyme in the mitochondria to cope with the surge of superoxide molecules and is activated by deacetylation of specific lysine (K) residues, including K53, K68, K89, K122, and K130, which are identified in both humans and mice by mass spectrometry [17,29–31]. As shown in Fig. 1, these lysine residues (marked red) are conserved among various species, including yeast, *Drosophila*, mouse, rat, and bovine. One intriguing problem is that the acetylation sites identified in SOD2 in humans are different from those in mice; for instance, K122 is the acetylation site in mice, whereas K68 is the acetylation site in humans [30]. The deacetylation of these lysine residues in SOD2 is catalyzed by SIRT3 [30], a mitochondrial deacetylase [32] of the sirtuin family that functions in the regulation of antioxidative responses [33–35]. Although the deacetylation-mediated SOD2 activation seems to be clearly illustrated in the aforementioned studies, the mechanism underlying this activation by which the acetylation site exactly determines the SOD2 activity in humans and whether there may exist any unknown sites that are also involved in SIRT3-mediated deacetylation of SOD2 remain to be elucidated. Meanwhile, the mechanism underlying the acetylation-dependent SOD2 activity remains unclear. To answer these questions, one explanation that is consistent with the model proposed by Fridovich et al. in 1983 Q2 indicated that deacetylation of lysine residues could form a cationic region to attract the negatively charged superoxide molecules, which increases the SOD2 activity [36]. However, more experiments are needed to demonstrate the existence of such a mechanism. In this study, we aimed to further investigate the SOD2 acetylation mechanisms. By applying the novel method of nonenzymatic covalent conjugation to add acetyl groups to lysine residues, we confirmed that lysine acetylation decreased the SOD2 activity in vitro. We identified more potential acetylation sites, such as K154 and K194, in SOD2. In addition, we investigated the details of the contributions of each of these lysine residues to SOD2 acetylation and activity in HEK293T cells. In this work, we highlighted K68 as the most important among the investigated acetylation sites in modulation of SOD2 activity in human cells. We also studied the mechanism by which the acetylation of these lysine residues affects the SOD2 activity. Through molecular dynamic simulations, we revealed that K68 mutations could cause significant conformational changes in the coordinated active core and alter the entire charge distribution of SOD2. We found that these mutations also affected the tetramer stability and protein–protein interactions of SOD2. These findings imply a novel function of lysine acetylation in SOD2 that is important for the enzymatic activity of SOD2 and disturbs the overall SOD2 structure and tetramerization. Thus, the endogenous SOD2 activity in human cells is regulated in a combined manner.

2. Materials and methods

2.1. Protein expression, purification, and site-directed mutagenesis

For protein expression in *Escherichia coli*, full-length SOD2 cDNA was subcloned into the pET26b(+) vector and the plasmid was transformed into BL21(DE)3 competent cells. Bacterial cells were grown in Luria–Bertani broth containing 50 μg/ml kanamycin. Isopropyl β-D-1-thiogalactopyranoside (0.6 mM) was added when the OD_{600} reached 0.6. The expression of His-tagged SOD2 was induced at 16 °C overnight. The *E. coli* cells were then collected and lysed with

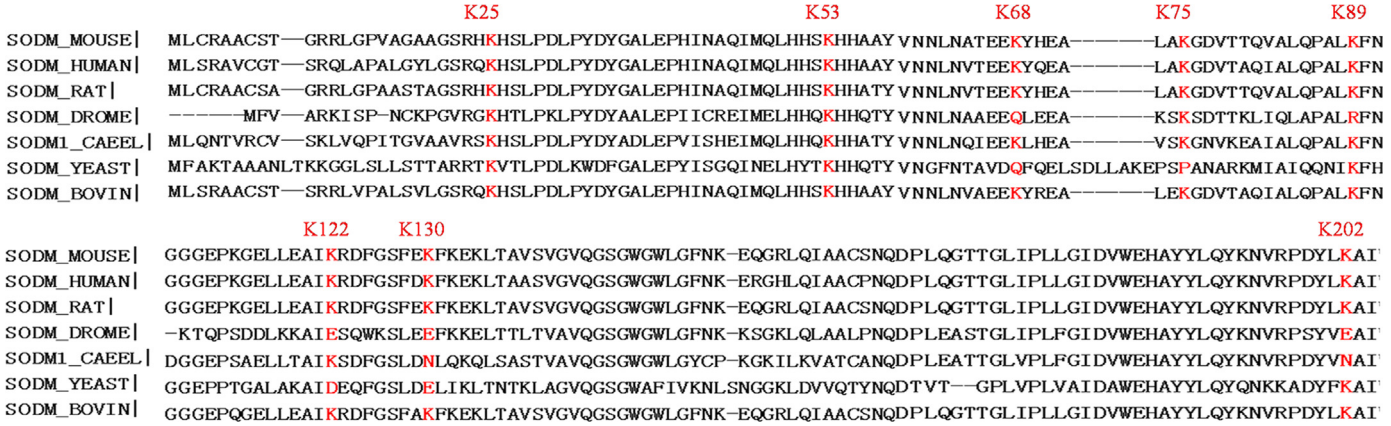

Fig. 1. The sequence alignment of SOD2 from various species. SOD2 sequences from various species were aligned and acetylated lysines that have been reported in mouse and human are marked red. DROME refers to *Drosophila melanogaster*, and CAEEL refers to *Caenorhabditis elegans*.

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