Biochimie 97 (2014) 181–193

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Mechanistic study of CuZn-SOD from *Ipomoea carnea* mutated at dimer interface: Enhancement of peroxidase activity upon monomerization

Panchanand Mishra [a], Anshuman Dixit [b], Mamata Ray [a], Surendra Chandra Sabat [a,*]

[a] Department of Gene Function and Regulation, Stress Biology Laboratory, Institute of Life Sciences, Bhubaneswar 751023, Odisha, India
[b] Department of Translational Research and Technology Development, Drug Design and Discovery, Bioinformatics, Institute of Life Sciences, Bhubaneswar 751023, Odisha, India

ARTICLE INFO

Article history:
Received 6 February 2013
Accepted 14 October 2013
Available online 26 October 2013

Keywords:
Dimeric interface mutation
MD simulation
Peroxidase activity
Superoxide dismutase activity

ABSTRACT

The enzymatically active monomeric form of CuZn-superoxide dismutase has always been of interest to decipher the structure–function relationship in this class of enzymes. In the present study, spectroscopic and enzymatic characteristics of the dimeric and monomeric forms of recombinant *Ipomoea carnea* CuZn-superoxide dismutase were made to decipher their stability and altered catalytic properties. The monomeric form of protein was produced through site directed mutagenesis by replacing a conserved hydrophobic leucine with a polar lysine residue at the dimer-interface. Spectral characteristics of both the forms (monomer and dimer) showed the presence of novel electronic transitions. Superoxide scavenging activity of the mutated form was reduced to nearly half of the activity found in the native enzyme. Concomitantly, compared to native form the mutated enzyme showed an increase in peroxidase activity. High temperature dependent circular dichroism spectral analysis, differential scanning calorimetric profile, and the measurement of temperature dependent superoxide scavenging activity indicated an increased susceptibility of the mutated form to higher temperature as compared to the native form. The inhibitor studies like hydrogen peroxide, diethyldithiocarbamate and phenylglyoxal also indicate higher susceptibility, which might be due to, altered arrangement of active site residues as a consequence of the mutation. Molecular modeling and MD simulation studies further indicated that this specific mutation induces loss of hydrophobic interaction at dimer interface, resulting in the observed instability of the dimeric form. Increased peroxidative activity of the enzyme, upon monomerization may have physiological implication essentially in presence of high concentration of H_2O_2, as in case of plant cells specifically under stress conditions.

1. Introduction

Superoxide dismutases (EC 1.15.1.1), belong to a family of metallo-enzymes, which catalyze the disproportionation of superoxide anion radical ($O_2^{\cdot-}$) in a two-step reaction kinetics resulting in formation of molecular oxygen (O_2) and H_2O_2 [1]. Therefore, SODs play an important protective role in cells by preventing the reactive oxygen species (ROS) mediated oxidation of cellular components. Based on the presence of metal ion cofactor(s) in their active site, SODs are classified as CuZn, Mn, Fe, Ni, cambialistic (Fe/Mn or Fe/Zn) SODs, and heme co-ordinated SOD [2]. The latter three forms are, however, reported in prokaryotes only [3]. Iso-forms of CuZn-SOD, the most abundant SOD in higher plant cells are found in chloroplast, cytosol, peroxisome, and in extra cellular space [3,4].

Eukaryotic CuZn-SODs are in general homodimer and non-covalently bonded by identical sub-units containing a copper (Cu^{2+}) and a zinc (Zn^{2+}) ion [5]. The oxidized copper ion (Cu^{2+}) serves as the redox partner of $O_2^{\cdot-}$, whereas Zn^{2+} appears to stabilize the dimer assembly and plays a role in electrostatic

Abbreviations: BSA, bovine serum albumin; DSC, differential scanning calorimetry; DCFH-DA, 2′,7′-dichlorfluorescein-diacetate; DDC, diethyldithiocarbamate; DTPA, diethylenetriamine pentaacetic acid; fALS, familial Amyotropic Lateral Sclerosis; H_2O_2, hydrogen peroxide; ICP-MS, Inductively Coupled Plasma Mass Spectrometry; IcSOD, *Ipomoea carnea* CuZn SOD; IPTG, isopropyl β-D-1-thiogalactopyranoside; MALDI-TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrum; (T_m), melting temperature; Pgx, phenylglyoxal; KCN, potassium cyanide; RMSD, root mean square distance; NaN_3, sodium azide; SOD, superoxide dismutase; TFA, trifluoroacetic acid.

* Corresponding author. Institute of Life Sciences, Nalco Square, Chandrasekharpur, Bhubaneswar 751023, Odisha, India. Tel.: +91 674 2302059; fax: +91 674 2300728.
E-mail address: surendrachandra@gmail.com (S.C. Sabat).

0300-9084/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.biochi.2013.10.014

stabilization of the enzyme [6]. Based on the observation that the native sub-units exhibit identical activity, it has been suggested that the active sites in each of the sub-units can function independently [7]. In addition to the presence of dimeric forms, natural occurrence of monomeric form of CuZn-SOD, like those found in bacterial system is also reported in plant species [8,9], suggesting physiological relevance of the monomeric structure of the enzyme. Prokaryotic CuZn-SOD shows larger structural variability than eukaryotic enzymes in terms of their active site channel organization and arrangement of amino acid residues at dimer interface [10].

In humans, mutations in the dimeric interface of CuZn-SOD (SOD 1) that cause destabilization of the quaternary structure of dimeric CuZn-SOD and eventually lead to misfolding or formation of aggregates that may lead to fALS disease [11]. These mutant SODs generally retain their full activity; however there is a toxic gain of oxidative function. Another interesting feature that has attracted some of these aberrant CuZn-SODs for investigation is the stimulation of disproportionation of $O_2^{\cdot-}$ anion along with additional peroxidative activity [12,13]. Hence, these results provide rationale to study of peroxidase action of the CuZn-SODs and their mutant form, both under *in vitro* and *in vivo* conditions.

Ipomoea carnea (*I. carnea*) subsp. *fistulosa* of the Convolvulaceae family (morning glory) is a toxic plant. The toxic principles of the plant have been identified as two nortropane alkaloids, calystegines B2 and C1 and an indolizidine alkaloid swainsonine [14]. The plant serves as one of the most suitable species for phyto-extraction of cadmium from soil [15]. However, the plant has not been thoroughly examined to understand its antioxidative potential. Although, different species of *Ipomoea* i.e., *Ipomoea batata* shows few interesting facts about the structure and functional aspects of the enzyme [16], there is dearth of information on antioxidative enzymes of *I. carnea*, that grows abundantly in all kinds of agronomic conditions.

In the present study, the structure–function relationship of dimeric recombinant *I. carnea* CuZn-SOD and its monomeric variant have been explored. The monomeric form of the protein was obtained by site directed mutagenesis of one leucine (Leu) with a charged lysine (Lys) residue. The resulting dimer showed low stability with decrease in superoxide scavenging activity. However, there was an increase in peroxidase activity in mutated form as compared to wild type. These observations led us to perform detailed molecular modeling studies that connect different experimental observations of the mutant protein and revealed the characteristic structural changes of the protein, leading to disruption of residue–residue contacts and monomerization of the native protein.

To the best of our knowledge this is the first report, where peroxidase activity of a plant CuZn-SOD has been studied *in vitro* and further extended to its monomeric variant. The present study has re-examined the role of subunit–subunit interactions to decipher the stability and catalytic properties of the enzymes. In this report, the wild type *I. carnea* CuZn-SOD (IcSOD) has been described as Wild Type or WT, while the mutant one as mutant or MT or variant.

2. Materials and methods

2.1. Plant material and chemicals

The leaves of *I. carnea* that grows widely in the Institute of Life Sciences campus, Bhubaneswar, India were collected, washed thoroughly under tap water followed by distilled water before use in the experiments.

Unless otherwise indicated, all analytical grade reagents were procured from Sigma. The expression vector pQE30-UA, *Escherichia coli* M15 cells, Ni-NTA columns and matrix were procured from Quiagen. DNA modifying enzymes, Taq DNA polymerase, AMV reverse transcriptase, pGEM T-Easy vector and *E. coli* JM109 competent cells were obtained from Promega. Chromatography columns were procured either from Amersham Pharmacia or Pierce.

2.2. PCR amplification and construction of WT (pQE30-UA-WT) and the variant (pQE30-UA-mutant) vectors

Total RNA was isolated from *I. carnea* leaves using TRI reagent (Sigma), according to manufacturer's instructions. The single stranded cDNA was synthesized from 2 μg of RNA using oligo (dt) primer and AMV reverse transcriptase [17]. Second cDNA fragment corresponding to the ORF of the cytosolic IcSOD was PCR amplified using the first strand cDNA as template, with degenerate primer pair (Supplementary Table S1). The PCR product was first cloned into the pGEM T-Easy vector, and nucleotide sequence of the insert was verified by auto-sequencing with SP6 and T7 primer pairs (TCGA, New Delhi, India). The recombinant plasmid (pGEMT-Easy-*IcSOD*) was used as the template to amplify IcSOD gene (*IcSOD*), using same set of forward and reverse primers. The gene was subcloned into expression vector pQE30-UA (*pQE30-UA-IcSOD*), and transformed into competent *E. coli* JM109 cells. The correct orientation of the gene in the positive colonies, were screened by colony PCR using vector specific forward and gene specific reverse primer pairs (Supplementary Table S1). The presence of insert was additionally confirmed by restriction digestion using *BamHI* and *HindIII*.

Based on deduced protein sequence alignment (Supplementary Fig. S1), a variant plasmid replacing Leu to Lys at 49th position (Leu-49Lys) was constructed following *in vitro* site-specific recombinant PCR [18], using sense and antisense sequence of the required mutated region and −5′ and −3′ flanking region primers (Supplementary Table S1). Positive clones were selected and sequenced to confirm the mutation. The native and the variant plasmids were transformed into competent *E. coli* M15 cells for recombinant expression of the proteins.

2.3. Overexpression and purification of WT and variant recombinant IcSOD protein

The recombinant plasmids (*pQE30-UA-WT* and *pQE30-UA-mutant*) were expressed in *E. coli* host (M15) under IPTG induction. Presence of the recombinant protein in the soluble fraction was checked and purified to homogeneity following published protocol [19]. Inclusion of Cu (copper sulfate) and Zn (zinc sulfate) in the growth media and reconstitution of these metal cofactors in purified protein were performed following established protocol [20–22]. Purity of the protein was analyzed on 15% SDS-PAGE taking 1 μg of protein [23]. Presence of poly-histidine tag was confirmed with Western blotting using Penta-His antibody as described earlier [19]. Protein concentration was determined following Bradford [24].

2.4. Determination of native and sub-unit mass

The native molecular weight of the purified WT and the variant IcSOD was determined by gel filtration chromatography on a Bio-Sil 250 column (Biorad), equilibrated with 5 mM K-PO_4 (pH-7.2) buffer containing 150 mM NaCl and pre-calibrated with standard proteins. The subunit size (monomer) was determined in 15% SDS-PAGE and also by MALDI-TOF-MS. Protein samples (100 pmol) were dissolved