Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 312 (2003) 873–876
BBRC
www.elsevier.com/locate/ybbrc

Cu/Zn superoxide dismutase can form pore-like structures

Jinhyuk Chung,[a] Hoichang Yang,[a] Mitchel D. de Beus,[a] Chang Y. Ryu,[a] Kilwon Cho,[b] and Wilfredo Colón[a,*]

[a] *Rensselaer Polytechnic Institute, Department of Chemistry, 110 8th street, Troy, NY 12180, USA*
[b] *Department of Chemical Engineering, Pohang University of Science and Technology, Pohang, Kyoungbuk 790-784, Republic of Korea*

Received 28 October 2003

Abstract

Mutations in Cu/Zn superoxide dismutase (SOD) are associated with familial amyotrophic lateral sclerosis (FALS), a neurodegenerative disease that is characterized by the selective death of motor neurons. Despite the genetic association made between the protein and the disease, the mechanism by which the mutant SOD proteins become toxic is still a mystery. Using wild-type SOD and three pathogenic mutants (A4V, G37R, and G85R), we show that the copper-induced oxidation of metal-depleted SOD causes its in vitro aggregation into pore-like structures, as determined by atomic force microscopy. Because toxic pores have been recently implicated in the pathogenic mechanism of other neurodegenerative diseases, these results raise the possibility that the aberrant self-assembly of oxidatively damaged SOD mutants into toxic oligomers or pores may have a pathological role in FALS.

Keywords: Aggregation; Amyloid; Amyotrophic lateral sclerosis; Neurodegeneration; Channel; Pore; SOD; FALS; Oxidative damage

Mutations in copper/zinc superoxide dismutase (SOD) are associated with 20% of all cases of familial amyotrophic lateral sclerosis (FALS) [1] and over 100 missense mutations have been identified to date [2]. It has been well established that pathogenic SOD mutations do not cause FALS by interfering with its normal function as a scavenger of the superoxide radical, but rather by acquiring one or more toxic properties [3]. There have been several hypotheses proposed to explain the toxicity of mutant SOD, including that SOD mutations cause FALS via oxidative damage mediated by the SOD-bound copper molecule, or alternatively, by inducing the formation of toxic SOD aggregates (reviewed in [3]). However, the mechanism by which SOD mutations result in motor neuron death has not been elucidated.

Most neurodegenerative diseases have been pathologically linked to the abnormal deposition of a specific protein into fibrillar inclusions known as amyloid [4]. It was generally believed that amyloid fibrils were toxic, and therefore, the cause of cell death in amyloid diseases. However, evidence has been accumulating in recent years pointing towards soluble oligomers as the toxic species [5,6]. One particular type of oligomer consisting of an annular pore-like structure has recently been proposed to be the toxic intermediate in Alzheimer's and Parkinson's diseases [7]. This is consistent with the observation that many proteins linked to various amyloid diseases possess the ability to permeabilize membranes via the formation of ion channels [8].

In this study, we sought to investigate whether SOD was able to self-assemble into pore-like structures. Here we show that upon oxidative damage, wild-type (WT) and several pathogenic mutants of SOD can form insoluble aggregates, as well as, pore-like oligomeric structures. As may be expected, the highly pathogenic SOD A4V mutant has the highest intrinsic tendency towards aggregation.

Materials and methods

SOD expression and purification. The SOD expression vectors were a gift from Joseph Beckman (Oregon State University). The SOD cDNA was cloned into a PET21 vector between the *Bam*HI and *Nco*I sites and transformed into *Escherichia coli* strain BL21 pLysS-competent cells

* Corresponding author: Fax: 1-518-276-4887.
E-mail address: colonw@rpi.edu (W. Colon).

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.11.008

as previously described [9]. Cells were grown in Luria–Bertani (LB) media to an OD_{600} of 0.8, at which time isopropyl-β-D-thiogalactoside was added to a final concentration of 0.3 mM. After 1 h of induction, cells were collected by centrifugation (8000*g*) at 4 °C and frozen at −80 °C. The frozen cells were resuspended in 50 mM phosphate buffer (pH 7) and 150 M NaCl, and then lysed by four cycles of sonication with 30 s bursts with a 1 min interval of cooling in ice. The purification of SOD was carried out as previously described [10].

Demetallation of SOD. Purified SOD was demetallated as previously described [11]. SOD samples were then dialyzed against 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris) buffer at pH 7.8. All SOD samples were prepared fresh just before the experiment.

SOD aggregation and kinetic observations. SOD aggregation was achieved by mixing 10 μM SOD with 0.8 mM copper sulfate ($CuSO_4$) and 10 mM hydrogen peroxide (H_2O_2) in 20 mM Tris at 37 °C (pH 7.4). To monitor the kinetics of aggregation, the SOD solution was equilibrated in a quartz cuvette at 37 °C for 5 min within the sample chamber of a Hitachi F-4500 fluorimeter, at which point a freshly made solution containing the $CuSO_4$, H_2O_2, and Tris was added. Using a small stir bar, the solution was mixed for 5 s, and the light scattered at a 90° angle was immediately monitored for 1 h at an excitation and emission wavelengths of 350 with 2.5 nm slit width. Afterwards, the aggregation mixture was centrifuged at 18,000*g* and the SOD aggregate was then resuspended in 50 μL fresh water for storage at room temperature.

Atomic force microscopy and the sample preparation. Aggregated pellet resuspended in 50 μL fresh water was sonicated for 10 min. The suspension was then spun at ~18,000*g* for 30 s. A 20 μL drop of the supernatant was placed onto a freshly cleaved mica and it was dried by slow evaporation over 3 h followed by 30 min vacuum-drying before imaging using tapping mode AFM in a Multimode Nanoscope IIIa instrument (Digital Instrument/Veeco Metrology Group, Santa Barbara, CA). Silicon tips with a radius of ~10 nm (nominal spring constant = 31–43 N/m, resonant frequency = 160–210 kHz) were used as probes.

Results and discussion

The presence of oxidatively damaged aggregated SOD in transgenic mice expressing FALS-related SOD mutants [12] and the demonstration that copper-mediated oxidative damage can induce SOD mutant aggregation in vitro [13,14] suggest that oxidative damage of SOD may unleash its toxic effect via the formation of an abnormally self-assembled SOD species. It has been well established that when SOD is incubated with copper and H_2O_2, the latter reduces Cu^{2+} to Cu^{+}, resulting in oxidative damage to SOD (reviewed in [15]). Therefore, to explore whether oxidatively damaged SOD can form pore-like structures in vitro, we added $CuSO_4$ and H_2O_2 to solutions of metal-depleted (apo) WT SOD and three pathogenic SOD mutants (A4V, G37R, and G85R). These experiments were carried out using apo SOD because recent studies suggest that the toxic effect of SOD mutants may involve the apo protein [16,17].

Upon exposing SOD to $CuSO_4$ and H_2O_2, SOD mutants began to aggregate within seconds. As expected, control experiments showed that aggregation did not occur when $CuSO_4$ and H_2O_2 were added independently, indicating that the presence of both reagents was required for aggregation to occur (data not shown). The aggregation of SOD was monitored by measuring the signal of the light scattered at a 90° angle after excitation at 350 nm (Fig. 1A). Under the oxidizing conditions used in our study even the WT SOD protein exhibited mild aggregation, as did the G37R SOD mutant, which is involved in very mild cases of FALS [18]. Interestingly, the extent of SOD aggregation revealed a clear trend consistent with the known disease severity of the mutants (Fig. 1B) [18]. Due to the limited number of mutants studied, it is not possible to assess here the physiological relevance of this trend.

The SOD aggregates were incubated in fresh water and imaged at various times by AFM. Remarkably, the morphology of the SOD aggregates, including those of WT SOD, revealed numerous pore-like structures with a diameter of about 19 nm and an inner diameter of 5 nm (Fig. 2). When the samples were analyzed by AFM after incubating the SOD aggregates for 10 days, the pores were largely absent and replaced by amyloid-like fibrils (J.C. et al., unpublished results). It is intriguing that these annular structures are very similar to those formed by β-amyloid and α-synuclein [7], which are associated with Alzheimer's and Parkinson's diseases, respectively.

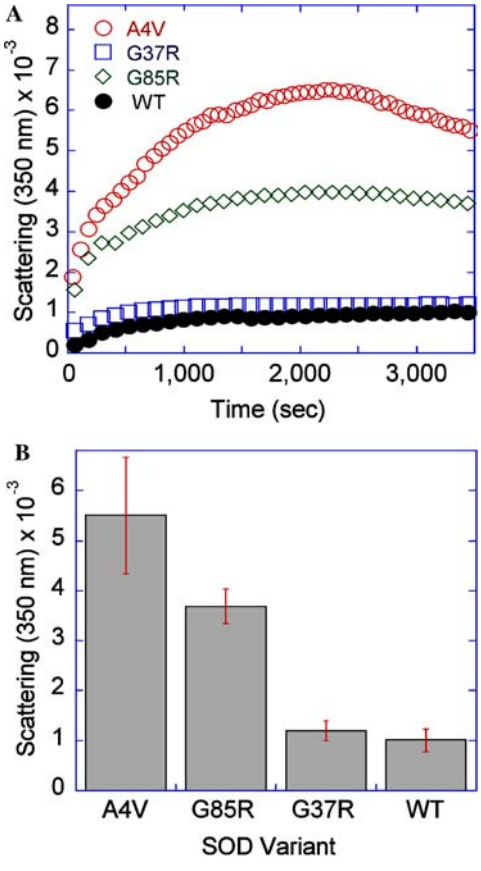

Fig. 1. (A) Time-course of apo SOD (10 μM, 20 mM Tris, pH 7.4, 37 °C) aggregation after the addition of $CuSO_4$ (0.8 mM) and H_2O_2 (10 mM). (B) Extent of SOD aggregation after 1 h. Error bars indicate standard deviation based on at least three replicates. Numbers inside the bars indicate the mean survival associated with each mutant [18].

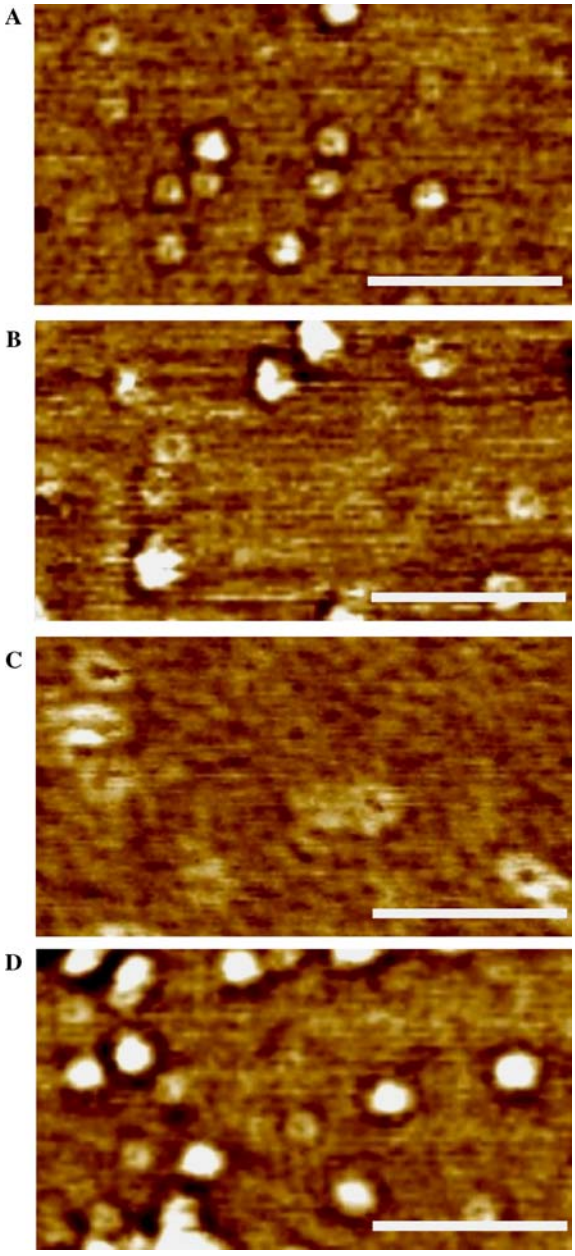

Fig. 2. AFM phase image of A4V (A), G37R (B), G85R (C), and WT (D) SOD after incubating the aggregates in water for three days at room temperature. The bar indicates 100 nm.

It will be very important to determine whether these SOD pores also share their toxic ability to permeabilize membranes via the formation of ion channels, as has also been shown for many amyloidogenic proteins [8]. Perhaps this could explain various key phenotypic features in ALS, such as mitochondrial dysfunction and increased cytosolic calcium levels [19]. It has been proposed that mitochondrial dysfunction via the activation of mitochondrial permeation transition pores (MPTP) may be the key step in committing motor neurons to cell death in ALS [20]. Furthermore, the increased immunoreactivity of SOD around the outer membranes of mitochondria [20], and the involvement of SOD aggregates in expanding the intermembrane space of mitochondria [21], suggests that this may be the determining site of SOD damage in FALS.

The fact that FALS and sporadic ALS are virtually pathologically indistinguishable suggests that although different genetic and/or environmental factors may trigger the disease, a common toxic species may exist. Although SOD has only been linked to a small percentage of ALS cases, our results suggest a plausible mechanism by which SOD may be the common toxic agent in ALS. Our demonstration that metal-depleted WT SOD can be induced to self-assemble into pores upon oxidative damage suggests that SOD mutations may not always be required for SOD toxicity, as situations may exist where oxidative stress leading to metal loss, copper misloading, or other cellular factors may lead to the formation of toxic WT SOD pores. Even though the role of these pores in familial and sporadic ALS remains to be tested, the fact that SOD mutants form these structures much faster than WT SOD, and the growing evidence that mis-assembled oligomers and pores are linked to toxicity in other neurodegenerative disease, provides a compelling argument for the potential significance of these putative SOD pores. It will be interesting to see, as the toxicity of these pores is tested and other genetic factors associated with ALS are discovered in the future, whether SOD will turn out be the common culprit in ALS.

Acknowledgments

This work was supported by a NIH Grant (R01NS42915) to W.C. H.Y. and K.C. were supported by the Ministry of Science and Technology of Korea (National Research Laboratory Project) and the Ministry of Education of Korea (BK21 Program). C.R. thanks the NSF NSEC Program (NSF DMR-0117792) for the AFM support.

References

[1] D.R. Rosen, T. Siddique, D. Patterson, D.A. Figlewicz, P. Sapp, A. Hentati, D. Donaldson, J. Goto, M. More, Mutations in Cu, Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis, Nature 362 (1993) 59–62.
[2] P.M. Andersen, K.B. Sims, W.W. Xin, R. Kiely, G. O'Neill, J. Ravits, E. Pioro, Y. Harati, R.D. Brower, J.S. Levine, H.U. Heinicke, W. Seltzer, M. Boss, R.H. Brown Jr., Sixteen novel mutations in the Cu/Zn superoxide dismutase gene in amyotrophic lateral sclerosis: a decade of discoveries, defects and disputes, ALS and other motor neuron disorders 4 (2003) 62–67.
[3] D.W. Cleveland, J.D. Rothstein, From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS and other motor neuron disorders, Nat. Rev. Neurosci. 2 (2001) 806–819.
[4] M.D. Kaytor, S.T. Warren, Aberrant protein deposition and neurological disease, J. Biol. Chem. 274 (1999) 37507–37510.
[5] M. Bucciantini, E. Giannoni, F. Chiti, F. Baroni, L. Formigli, J. Zurdo, N. Taddei, G. Ramponi, C.M. Dobson, M. Stefani,

Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases, Nature 416 (2002) 507–511.
[6] B. Caughey, P.T.J. Lansbury, Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders, Annu. Rev. Neurosci. 26 (2003) 267–298.
[7] H.A. Lashuel, D. Hartley, B.M. Petre, T. Walz, P.T. Lansbury Jr., Neurodegenerative disease: amyloid pores from pathogenic mutations, Nature 418 (2002) 291.
[8] B.L. Kagan, Y. Hirakura, R. Azimov, R. Azimova, M.C. Lin, The channel hypothesis of Alzheimer's disease: current status, Peptides 23 (2002) 1311–1315.
[9] J.P. Crow, J.B. Sampson, Y. Zhuang, J.A. Thompson, J.S. Beckman, Decreased zinc affinity of amyotrophic lateral sclerosis-associated superoxide dismutase mutants leads to enhanced catalysis of tyrosine nitration by peroxynitrite, J. Neurochem. 69 (1997) 1936–1944.
[10] L.J. Hayward, J.A. Rodriguez, J.W. Kim, A. Tiwari, J.J. Goto, D.E. Cabelli, J.S. Valentine, R.H. Brown Jr., Decreased metallation and activity in subsets of mutant superoxide dismutases associated with familial amyotrophic lateral sclerosis, J. Biol. Chem. 277 (2002) 15923–15931.
[11] B. Sutter, P.L. Bounds, W.H. Koppenol, The preparation of apo-Cu,Zn superoxide dismutase by ion-exchange chromatography on iminodiacetic acid–Sepharose, Protein Expr. Purif. 19 (2000) 53–56.
[12] P.K. Andrus, T.J. Fleck, M.E. Gurney, E.D. Hall, Protein oxidative damage in a transgenic mouse model of familial amyotrophic lateral sclerosis, J. Neurochem. 71 (1998) 2041–2048.
[13] S.Y. Kim, O.J. Kwon, J.W. Park, Inactivation of catalase and superoxide dismutase by singlet oxygen derived from photoactivated dye, Biochimie 83 (2001) 437–444.
[14] R. Rakhit, P. Cunningham, A. Furtos-Matei, S. Dahan, X.F. Qi, J.P. Crow, N.R. Cashman, L.H. Kondejewski, A. Chakrabartty, Oxidation-induced misfolding and aggregation of superoxide dismutase and its implications for amyotrophic lateral sclerosis, J. Biol. Chem. 277 (2002) 47551–47556.
[15] S.L. Jewett, A.M. Rocklin, M. Ghanevati, J.M. Abel, J.A. Marach, A new look at a time-worn system: oxidation of CuZn-SOD by H_2O_2, Free Radicals Biol. Med. 26 (1999) 905–918.
[16] M.J. Lindberg, L. Tibell, M. Oliveberg, Common denominator of Cu/Zn superoxide dismutase mutants associated with amyotrophic lateral sclerosis: decreased stability of the apo state, Proc. Natl. Acad. Sci. USA 99 (2002) 16607–16612.
[17] A. Okado-Matsumoto, I. Fridovich, Amyotrophic lateral sclerosis: a proposed mechanism, Proc. Natl. Acad. Sci. USA 99 (2002) 9010–9014.
[18] T. Juneja, M.A. Pericak-Vance, N.G. Laing, S. Dave, T. Siddique, Prognosis in familial amyotrophic lateral sclerosis: progression and survival in patients with glu100gly and ala4val mutations in Cu, Zn superoxide dismutase, Neurology 48 (1997) 55–57.
[19] D. Jaarsma, E.D. Haasdijk, J.A. Grashorn, R. Hawkins, W. van Duijn, H.W. Verspaget, J. London, J.C. Holstege, Human Cu/Zn superoxide dismutase (SOD1) overexpression in mice causes mitochondrial vacuolization, axonal degeneration, and premature motoneuron death and accelerates motoneuron disease in mice expressing a familial amyotrophic lateral sclerosis mutant SOD1, Neurobiol. Dis. 7 (2000) 623–643.
[20] C. Bendotti, N. Calvaresi, L. Chiveri, A. Prelle, M. Moggio, M. Braga, V. Silani, S. De Biasi, Early vacuolization and mitochondrial damage in motor neurons of FALS mice are not associated with apoptosis or with changes in cytochrome oxidase histochemical reactivity, J. Neurol. Sci. 191 (2001) 25–33.
[21] C.M. Higgins, C. Jung, Z. Xu, ALS-associated mutant SOD1-G93A causes mitochondrial vacuolation by expansion of the intermembrane space and by involvement of SOD1 aggregation and peroxisomes, BMC Neurosci. 4 (2003) 16.