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*The authors dedicate this review to the honor of Dr. Alexander Wlodawer on his 70th birthday, with admiration for his applications of structural biology to the understanding and treatment of disease.

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the progressive degeneration of spinal motor neurons. Although mutations in dozens of proteins have been associated with ALS, the enzyme, superoxide dismutase 1 (SOD1) was the first protein identified with the development of ALS and accounts for ~20% of familial cases. In experimental animals and patient samples, mutant SOD1 is found in cytoplasmic deposits implicating SOD1 aggregates as the toxic entities. Here we discuss the various biochemical and structure-based hypotheses proposed for mutant SOD1-associated ALS. Although much remains to be discovered about the molecular mechanism of SOD1 mediated toxicity, these hypotheses offer new avenues for therapeutic development.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a late-adult onset disease characterized by the loss of voluntary motor functions. Patients live an average of 3-5 years after the appearance of symptoms and very few are known to have survived for more than two decades [1]. Clinically the disease is identified by loss of motor function, which progresses in an unusually fast and unpredictable manner. Proteinaceous deposits are found in degenerating motor neurons of the cerebellum, cortex and spinal cord suggesting that protein aggregation is the underlying cause of the disease. Although 90% of all ALS cases are sporadic (sALS), 5–10% of cases are inherited and called familial ALS (fALS); these have a clear genetic linkage to a specific gene. Genome-wide association studies (GWAS) in the last decade have implicated nearly 30 genes in ALS pathogenesis. Of these, the majority code for the proteins TARDBP, SOD1, FUS, VCP and OPTN (Fig. 1). The gene encoding the cytosolic enzyme, superoxide dismutase 1 (SOD1) was first discovered to have mutations in ALS patients in 1993 [2]. Mutations in the SOD1 gene are found in the exons suggesting that their toxic effects are the result of malfunctions of the protein. Furthermore, large aggregates that stain as SOD1 are found in autopsy samples suggesting a pathological link between SOD1 aggregates and motor neuron death. The role of SOD1 in sporadic and non-SOD1 linked fALS is less clear. Biochemical studies and antibody reactivity suggest an altered SOD1 conformation is present in both sALS and non-SOD1-linked fALS patients [3–5]. SOD1 aggregates are also observed in the presence of mutant TDP-43 and FUS in patients and in cell culture suggesting cross talk between various proteins associated with ALS [6]. An oxidized form of wild-type SOD1 (wtSOD1) has also been detected in sporadic patient tissues [7]. Recently,

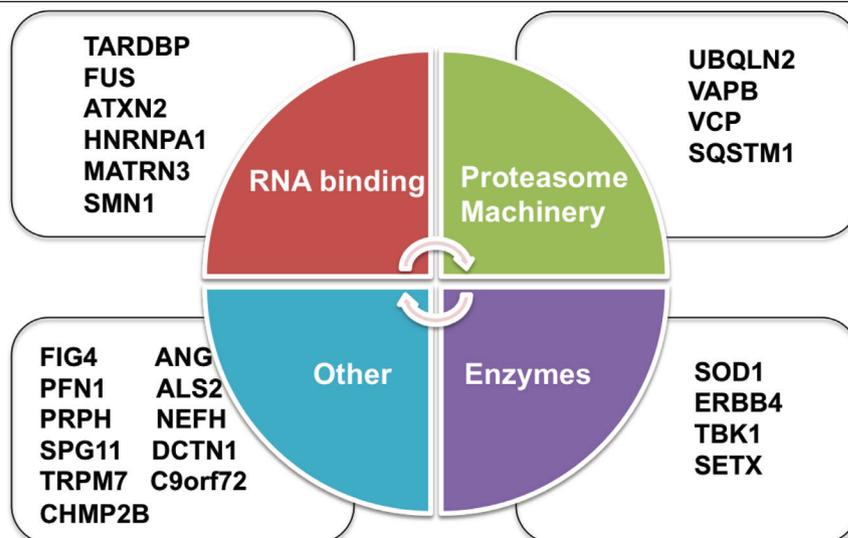


Figure 1. Different ALS-linked genes classified by function.

mutations in SOD1 were also found in sporadic forms of ALS such as the aggressive juvenile-onset ALS [8,9]. From these reports it appears that SOD1 plays a role that extends to ALS cases associated with mutations in other proteins, as well as to at least some sporadic cases. To date more than 160 different disease-associated mutations have been found in SOD1; these are spread over the entire 153 amino acid sequence (<http://alsod.iop.kcl.ac.uk>). Studies over the last two decades have elucidated key features of mutant SOD1 (mSOD1) aggregation. It is generally accepted that mSOD1 acquires toxic properties: mSOD1 does not lose its enzymatic activity [10] and knockdown of wild-type SOD1 does not cause ALS symptoms in mice [11]. Together these observations strengthen the hypothesis that disease mutations confer a toxic function that ultimately causes cellular dysfunction.

In this review, we summarize the various hypotheses proposed for mSOD1 toxicity. We focus on the biophysical studies of SOD1 mutants, and in particular the biochemical and structural data that suggest varying roles for different regions of the SOD1 protein in aggregation and ALS pathogenesis.

STRUCTURAL MODELS FOR CHANGES IN MUTANT SOD1

Several proteins associated with neurodegenerative diseases such as β -amyloid, α -synuclein and islet amyloid polypeptide do not have a native tertiary structure. However, SOD1 does have a tertiary structure and the effect of mutations on the native structure has been extensively studied. The native metal-bound SOD1 exists as a homo-dimer wherein each monomer has a Greek key architecture and binds one copper and one zinc ion (Fig. 2A). The monomer is composed of an eight-stranded β barrel and two loops – the metal binding loop (spanning residues 49-84) and the electrostatic loop (spanning residues 122-143) [12,13]. The simplest hypothesis for mutant SOD1-mediated neuronal damage is that the presence of each disease mutation increases the aggregation propensity of the protein. These aggregates accumulate over time and are toxic to neuronal cells. In support of this hypothesis, severity of the disease correlates with the aggregation propensity of the mutants. For example, patients with the A4V mutation survive only 6 months post symptom-onset and this mutation increases the aggregation propensity many fold. However, the increase in aggregation propensity does not correlate with an earlier age of disease onset [14,15]. Furthermore, not all disease mutations increase the aggregation propensity of the protein [14] (Table 1). Structural studies of more than a dozen different SOD1 constructs harboring disease-related mutations have been carried out and provide conflicting results. Overall, most mutations do not cause major structural alterations to the native SOD1 structure [16]. Crystal structures of A4V and I113T mutants show some structural deviations in the dimer interface that might contribute to increased aggregation propensity [17] and the structure of the G37R mutant shows altered subunit arrangement [18] while structures of D124V and H80R mutants show disrupted metal binding loops [19]. Mutations such as A4V, G93A, I113T and G37R also do not affect the enzymatic activity

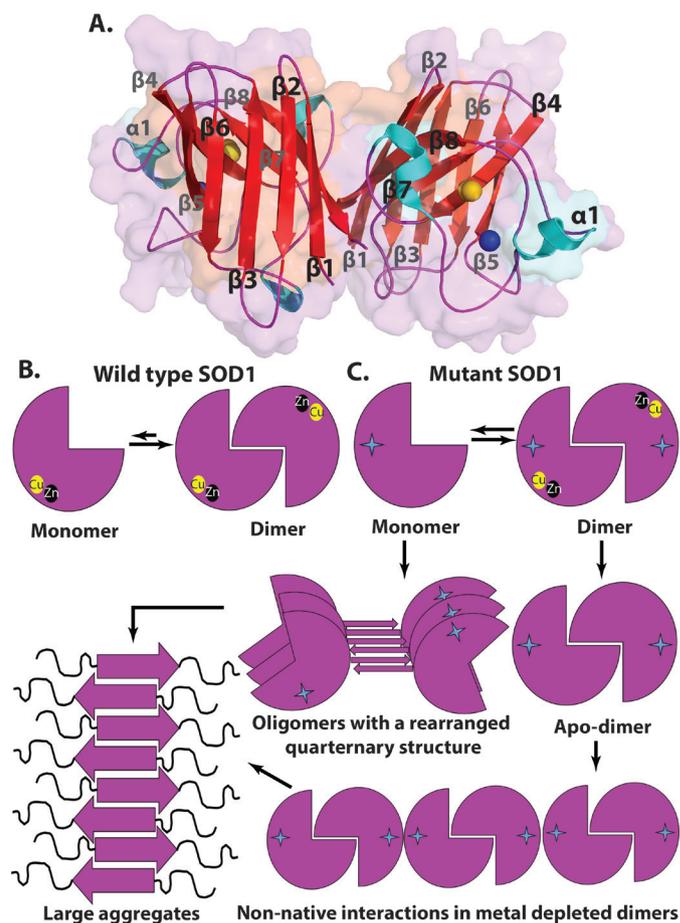


Figure 2. A) Crystal structure of metal-bound SOD1 (PDB 2C9S). Native SOD1 exists as a stable dimer. Each monomer is composed of an eight-stranded β barrel (red) and a metal binding loop (magenta) and an electrostatic loop. Cu and Zn are shown respectively as yellow and blue spheres. Strands facing towards the viewer are named in black and away from the viewer are named in gray. B) Wild type SOD1 is an exceptionally stable dimer and can be monomerized only by loss of metal ions. C) A disease mutation (blue cross) can gain a toxic function by different modes. It can increase the propensity to monomerize and these monomers in turn can form oligomers with non-native quaternary structure that may be toxic. Another mode of oligomerization involves apo dimers that harbor non-native contacts forming fibrous assemblies.

of the protein whereas G85R and H48Q mutations that are present in the metal binding loop lead to reduced activity [16]. In light of these conflicting reports, it is difficult to determine if increased aggregation propensity is the only effect of mutations that cause the disease; whereas protein aggregation may contribute to disease progression, it may not be the cause of the disease itself.

Another hypothesis is that the mutations introduce structural instability either by destabilizing the native structure or by reducing its metal binding affinity [20]. One line of evidence supporting this hypothesis is that large molecular weight aggregates are seen prior to disease onset in transgenic mice [21,22]. Additionally, large inclusion bodies containing insoluble SOD1 are found only in end-stage mice [23,24]. Biochemical and cell culture studies also show that SOD1 forms soluble oligomers that are sufficient to cause toxicity [25–29]. In-cell NMR and mutagenesis studies have shown that several mutants lack metal binding and are prone to oligomerization [30]. Structural studies of metal

Table 1. Comparison of several SOD1 mutations

Location of mutation	Mutations	Aggregation propensity vs. wt	Melting temperature vs. wt
β barrel	G37R	Similar	Similar
Electrostatic loop	N139K	High	Similar
Metal binding loop	H46R,D134N, H80R, D124V	High	Low
Dimer Interface	A4V, I113T	High	Low

depleted mutants also support this hypothesis. For example, in the crystal structures of the metal deficient mutants - H46R, S134N, D124V and H80R significant new inter-molecule interfaces are observed [19,31-33]. The buried surface area in these new interactions is identical to the native dimeric interface and thus proposed to be significant. Furthermore, these new interfaces can only occur by rearranging the metal binding loop. Therefore, reduced metal binding would increase flexibility and in principle stabilize these interactions. However, it is important to note that these interactions are observed only in the crystal structures and their physiological relevance has been unclear.

In addition to studies investigating the effect of disease-linked mutations on the global structure of SOD1, research groups have analyzed the SOD1 protein sequence to identify segments that affect the aggregation kinetics. Aggregates isolated from the spinal cords of transgenic mice have been shown to contain full-length SOD1 that is devoid of metals [34,35] but limited proteolysis and atomic force microscopy experiments suggest that the core of large aggregates formed by SOD1 is composed of residues 1-63 [36]. Molecular dynamic simulations, mass spectrometry and hydrogen/deuterium (H/D) exchange studies suggest local unfolding of the β -barrel and exceptional flexibility in the β strands 3 and 4 corresponding to residues 29-36 and 41-48 [37-39]. These strands compose the β barrel and thus their rearrangement likely induces changes in the structure to enable secondary interactions that are important for aggregation. In vivo models and cell culture studies also suggest that the N-terminal region of SOD1 plays an important role in toxicity, in particular residues such as Trp32 that may be important sites for post translational modifications [8,40,41]. Another study with shorter segments identified 4 different segments that are important for aggregation [42]. Mutations in two of these segments - 101-107 and 147-153 changed the aggregation propensity suggesting that these segments play an important role in the initiation of aggregation. The segment 147-153 lies at the dimer interface and this peptide segment can seed fibril formation of the full-length protein in vitro [42], suggesting that it plays an important role in aggregation.

SOD1 monomer also has 4 cysteine residues (Cys 6, Cys 57, Cys 111, and Cys 146) with Cys 57 and Cys 146 engaged in a disulfide bond. The role of these Cys residues in SOD1 aggregation has been explored (reviewed in [23,43]. SOD1 mutations increase the aggregation propensity of disulfide-reduced apo-SOD1 [44,45] and aberrant disulfide-linked oligomers are detected in spinal cord homogenates of transgenic mice [46]. However, mutating the cysteine residues does not change the aggregation propensity of

the protein [47]. Also, cysteine residues are not conserved across different species. Taken together, these reports suggest that disulfide reduction is an important albeit non-critical step in the toxic aggregation pathway.

Overall, these biochemical and structural studies suggest that SOD1 mediated toxicity is likely caused by a combination of different events such as aberrant disulfide reduction, the presence of a disease-associated mutation and reduced metal binding (Fig. 2). One or more of these events results in a shift in the equilibrium toward pathogenic aggregation.

TOXIC SOD1 SPECIES - LARGE AGGREGATES OR SOLUBLE OLIGOMERS

The molecular stoichiometry of the toxic species in neurodegenerative diseases has been intensely debated. While large aggregates are considered to be the pathological hallmark of Alzheimer's or Parkinson's disease and ALS, evidence for small oligomers as the toxic entities has also been presented.

In case of SOD1, evidence for both large aggregates and small oligomers as the toxic species has been given. Large inclusions are a defining histological feature of patients and detergent insoluble aggregates are formed in animal models as well. However, the cause and effect relationship between insoluble aggregates and motor neuron death is unclear. Several studies of SOD1 suggest that small oligomers may also be toxic. In these experiments, disulfide reduced forms of mutant SOD1 were detected that may be the precursor species for the large aggregates [25,26,28,29]. The minimum toxic species has been proposed to be trimeric with a non-native quaternary assembly [48]. Similar trimeric assemblies have been proposed for other aggregating proteins such as tau [49], amyloid- β [50] and α -synuclein [51]. The structural details of these toxic oligomers have been elusive till now and it is also not known if the native SOD1 structure is intact or not.

PRION-LIKE BEHAVIOR OF SOD1

It is increasingly appreciated for neurodegenerative diseases that protein aggregates can transfer from cell to cell much like the aggregated forms of the PrP prion protein. Protein aggregates of α -synuclein [52-54], tau [55], β -amyloid [56,57] have been shown to act as templates for seeding native protein into aggregates as do prion proteins, a behavior termed 'Prion-like'. However, the behavior of these proteins differs from the canonical prion proteins, as

there is no evidence of transmission of disease between individuals by transfer of aggregates.

Several lines of evidence for transmission of SOD1 aggregates have been found. SOD1 is abundant in the cytoplasm but has been reported also to be secreted extracellularly *in vitro* [58–60]. Additionally, co-culturing of non-neuronal cells such as astrocytes expressing mSOD1 induces toxicity in motor neurons expressing unmutated wtSOD1 [61,62]. However, it is not clear if the toxic effect on motor neurons is due to mSOD1 transmission or through a signaling cascade. Overexpression of human wtSOD1 in mice expressing the familial mutant G85R accelerates the disease onset suggesting recruitment of wtSOD1 in aggregation [63]. It has also been shown that spinal cord homogenates from mSOD1 transgenic mice can seed aggregation of the protein *in vitro* [64]. Finally, injection of spinal cord homogenates in mice induces pathology that spreads to distant regions of the spinal cord [65–68]. Although mutant SOD1 forms intracellular inclusions it is possible that a soluble, transferred form is sufficient for spread of pathology. Taken together, these studies suggest that SOD1 aggregation may initiate spontaneously in a section of motor neurons and could then be sequentially transferred from cell to cell. This hypothesis is also supported by the clinical disease progression, which starts with loss of a subset of motor neurons and then progresses through connected anatomical regions.

COMPARISON OF SOD1 WITH OTHER ALS-LINKED PROTEINS

Gene sequencing has associated nearly 30 different genes with ALS. A majority of these including TARDP, FUS, OPTN, VCP and hnRNP are RNA binding proteins that are also capable of forming aggregates in response to stress [69–71]. In these cases, the pathology appears to derive from both a loss of native function and gain of toxic function as disease causing mutations often render the protein functionally inactive [72–74]. Indeed the mechanism of toxicity of RNA binding proteins seems to differ from that of SOD1. For RNA binding proteins defects in nuclear pore assembly and defective RNA processing [73,75,76] have been found whereas for SOD1, mitochondrial defects [77–80], golgi fragmentation [59], defects in proteasome machinery and axonal transport [77,81] have been found. These differences highlight an important realization that even though all forms of ALS are associated with protein aggregation, the mechanisms that cause toxicity are likely to differ from case-to-case, depending on the proteins that are mutated.

Another aspect that differentiates SOD1 from other ALS-associated proteins such as TDP43, FUS and VCP is the exclusivity of mutant SOD1 to ALS. For example, aggregates of TDP43 are also found in Alzheimer's and frontotemporal dementia (FTD) cases [82,83], FUS is also associated with FTD [84] and VCP mutants are found in inclusion body myopathy with early-onset Paget disease and frontotemporal dementia (IBMPFD) [85]. In contrast, SOD1 mutants are associated only with ALS and there is no evidence of SOD1 aggregation in any other neurodegenerative disease. As all forms of ALS cases are clinically similar, researchers have studied the mechanism of disease

onset in models of familial cases in hopes that the information could be used to design drugs that would be effective against all forms of the disease. However, as more genes are implicated in ALS, it appears that even though protein aggregation is a central theme, the downstream effects of these aggregates may differ. Thus, it may be necessary to understand the toxic properties and disease pathway of each ALS-associated protein.

THERAPEUTIC DEVELOPMENT FOR SOD1-RELATED ALS

Various strategies have been explored to protect against SOD1 aggregation and to prevent neuronal loss. Among these siRNA-based gene silencing that reduces mutant SOD1 protein production was found to be well tolerated in phase 1 clinical trials [86,87]. A similar approach was also shown to be effective in a C9orf72 mouse model [88] and thus may be applicable to all forms of ALS.

In animal models, immunization approaches using antibodies that bind to a disease-specific conformation have been shown to be marginally effective [89]. The anti-SOD1 antibodies tested increased the life span of transgenic mice by 6–9 days and thus provide proof of concept for immunization therapy. Full-length SOD1 and its shorter segments such as peptide 143–151 that lies at the dimer interface have also been used to elicit immune response in animal models [90,91]. This vaccination approach was successful in delaying disease onset by 28 days and increased the overall lifespan of transgenic mice by 40 days.

With the advent of stem cell differentiation and induced pluripotent stem cells (iPSCs), patient derived motor neurons are being used to discover small molecule therapeutics. Patient-derived motor neurons show reduced survival and recapitulate the defects seen in transgenic mouse models such as reduced axon transport and mitochondrial defects [77,92]. Stem cell-derived motor neurons offer a fast method for high throughput screening of potential therapeutics. Indeed the first few studies have yielded several small molecules that inhibit aggregation *in vitro* and alleviate the toxic effects in cell culture [93,94].

Intriguingly, all ALS-linked mutant proteins are expressed ubiquitously in all tissues yet they aggregate only in the spinal cord. In this regard, some studies have focused on identifying a protective factor that may be present in non-neuronal tissues. In one study, a chaperone MIF2 (macrophage inhibitory factor) that expresses in non-neuronal tissues was discovered that inhibits SOD1 aggregation [95]. In a parallel approach, over expression of chaperones such as HSP1 [96] and Hsp110 [97] specifically in spinal motor neurons conferred protection. In a related study, a small molecule inhibitor, Sephin1 was developed that activates the proteasome system and increases chaperone production [98]. Sephin1 reduced the motor deficits and insoluble aggregate formation in transgenic mice expressing G93A mSOD1. Although these results are preliminary, they suggest that over-expression of individual chaperones or stimulating chaperone production have therapeutic potential.

A promising approach to therapy for amyloid diseases is stabilizing the native structure of proteins such that the equilibrium shifts away from aggregation. This approach has most successfully been applied for transthyretin [99] and light chain amyloidosis [100] but it remains unexplored for SOD1 till now. A few studies have shown that stabilizing the native structure of SOD1 by chemical crosslinking or by small molecules delays aggregation [101–103]. Intriguingly, when these compounds were co-crystallized with SOD1 they were found to bind not at the dimer interface but to the β -barrel, in particular β -strands 2, 3 and 6 suggesting that these strands play a role in aggregation.

CONCLUSIONS

Since its discovery as an ALS-linked protein, SOD1 has been extensively studied *in vitro*, in cell culture, and in animal models. Overall, various hypotheses such as increased aggregation propensity, dimer destabilization and oligomerization have been proposed for mSOD1 toxicity that may not be mutually exclusive. It is conceivable that under different conditions such as presence of a mutation, oxidative stress, and aberrant metal binding different mechanisms for toxicity are initiated.

Critical questions that remain unanswered include: the structure of the toxic species, the role of SOD1 in sporadic ALS, the mechanism of prion-like, intercellular spread of aggregates, and whether SOD1 and other ALS-linked proteins share a common pathogenic pathway. Answering these critical questions will ultimately help to defeat this devastating disease.

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Aspekt toksyczności SOD1 w stwardnieniu zanikowym bocznym

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Słowa kluczowe: dysmutaza ponadtlenkowa; stwardnienie zanikowe boczne (ALS)

STRESZCZENIE

Stwardnienie zanikowe boczne (ALS) to śmiertelna choroba neurodegeneracyjna charakteryzująca się postępującą degeneracją neuronów motorycznych rdzenia kręgowego. Z ALS powiązано obecność mutacji w wielu białkach, jednak to dysmutazę ponadtlenkową 1 (SOD1) jako pierwszą powiązано z rozwojem ALS; jest ona odpowiedzialna za około 20% rodzinnych wystąpień tej choroby. U zwierząt doświadczalnych i w próbkach pobranych od pacjentów zmutowana forma SOD1 została wykryta w złogach cytoplazmatycznych, co świadczy o toksyczności jej agregatów. W niniejszym artykule przedstawiono różne hipotezy oparte na danych biochemicznych i strukturalnych wyjaśniające rozwój ALS związany ze zmutowaną formą SOD1. Choć molekularne mechanizmy leżące u podstaw toksyczności SOD1 są w znacznym stopniu nieznanne, przedstawione modele mogą wskazywać na nowe możliwości opracowywania skutecznej terapii.