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Disruption of mitochondrial membrane integrity induced by amyloid aggregates arising from variants of SOD1

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal progressive neurodegenerative disorder selectively affecting motor neurons; 90% of the total cases are sporadic, but 2% are associated with mutations in the gene coding for copper–zinc superoxide dismutase (SOD1). The causes of motor neuron death in ALS are poorly understood in general, but for SOD1-linked familial ALS (fALS), aberrant oligomerization of SOD1 mutant proteins has been strongly implicated. A growing body of evidence suggests that fALS-causing mutations destabilize the native structure of SOD1, leading to aberrant protein interactions for aggregation. In this work, we show that wild-type human SOD1 and two of its mutants (D101N, G72S) form amyloid like fibrils under destabilizing condition (in the presence of KSCN 0.2 M and DTT 50 mM) at 37 °C, pH 7.4. The formation of the aggregates was monitored by their ability to enhance the fluorescence of Thioflavin T (ThT) and their morphology was assessed by transmission electron microscopy (TEM). Furthermore, interaction of SOD1 aggregates with mitochondrial membrane of rat brain, as an *in vitro* biological model, with the aim of gaining an insight into possible mechanism of cytotoxicity at the membrane level was verified. Release of mitochondrial enzyme, malate dehydrogenase (MDH), upon exposure to SOD1 aggregates demonstrates that these aggregates could affect membrane permeability.

1. Introduction

An increasing body of evidence suggests that protein misfolding leading to aggregation is a key pathologic feature of various amyloid-related disorders, including Alzheimer's, Parkinson's, Huntington's, type II diabetes and Creutzfeldt- Jakob (prion) diseases [1,2]. Ordered protein aggregates, often referred to as amyloid fibrils, are commonly found in these diseases. Amyloid deposits may be involved in amyotrophic lateral sclerosis (ALS), a devastating rapidly progressive and inevitably fatal neurodegenerative disease, characterized by motor neuron degeneration and paralysis [3]. Approximately 10% of ALS cases are familial (fALS), the remaining cases being sporadic (sALS). The familial and sporadic diseases are clinically indistinguishable and so have been proposed to share common disease mechanisms [1].

Twenty percent of fALS cases are caused by point mutations in copper/zinc superoxide dismutase (SOD1) gene [4]. A growing body of findings supports the hypothesis that gain of toxic function in SOD1 aggregates is involved in pathogenesis rather than loss of its function [5–8]. SOD1 is present in aggregates in motor neurons of SOD1-linked fALS patients [7,9], mice models [10–12] and in some sALS patients [12–14].

SOD1 is a homodimeric protein, each subunit folds into an eight-stranded beta barrel [3], containing one catalytic copper ion, one structural zinc ion, one intra-subunit disulfide bond and two free cysteines [15]. More than 147 mainly missense mutations throughout the SOD1 structure have been associated with fALS [1]. Increased propensity toward aggregation has been reported for mutations that lead to metal and/or disulfide deficient SOD1, often under highly destabilizing conditions, which favor aggregation in general [3,11,16–20]. In addition, several studies have reported observation of aggregation for metallated ALS- associated mutant SOD1s, which may be caused by the loss of metals and/or dimer dissociation under destabilizing conditions [3,21–24]. Despite of wide studies, the exact mechanism of SOD1 aggregates pathogenesis in ALS and the relevance of such aggregations to human disease are not known. The fact that different amyloids arise from either the cytosolic or extra cellular proteins points to the plasma membrane as a potential primary target which is accessible to both

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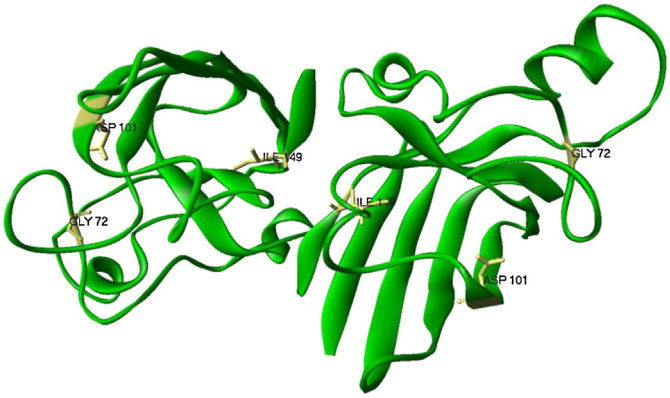

Fig. 1. Location of the investigated mutations (G72S, D101N, I149T) mapped on the Cu/Zn WT SOD1 structure (PDB ID: 2C9V).

compartments [25–28]. There appear to be a consensus on membrane permeabilization by amyloid oligomers being responsible for the deleterious effects on the neural cell membrane. In addition to plasma membrane as a primary target, internal organelles such as mitochondria also may be affected [29]. Recent reports indicate that mitochondrial dysfunction may play a critical role in the development of neurodegeneration in disorders like ALS, Parkinson and Alzheimer [30]. Moreover, there is clear evidence for binding of ALS-linked mutant SOD1 proteins to mitochondria, which may cause mitochondrial damage and cell death [31–34].

In order to learn more about the molecular underpinnings of pathology in ALS we have characterized the native SOD1 and three fALS- related mutants with respect to their ability to form amyloid like fibrils under destabilizing conditions. The mitochondria isolated from rat brain were then used as an *in vitro* model to examine the possible destructive effects of SOD1 aggregates. It is proposed that the organelle with its well-characterized membranes consisting of various biologically active components, combined with its exceptional biochemical composition and compartmental diversity, may provide an extremely useful model system for biophysical studies related to mechanism of cytotoxicity at the membrane level, leading to cell death.

2. Materials and methods

2.1. Preparation of wild type and mutants SOD1

The mutations (G72S, D101N, and I149T) reported to be linked to fALS disease are located in metal binding loop, beta barrel and dimer interface respectively (Fig. 1).

Mutations were performed by quick change mutagenesis. The WT and mutant enzymes were expressed in *Ecoli-BL21 (DE3) pLYsS* at 22° C for 16 h and purified by Ni-NTA Sepharose (Novagen). The metallization of enzymes was done by serial dialysis, consists of 3 main steps: metal removal, metal charging and remove of unbound metals as described [35].

2.2. Specific activity measurements

SOD1 activity was determined by measuring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich [36]. The reaction mixture (final volume of 1 ml) was composed of 12 mM methionine, 0.075 mM NBT, 0.001 mM riboflavin, 50 mM Na_2CO_3 and 100 μg protein in 20 mM phosphate buffer (pH 7.4). The mixture was placed in a light box for 10 min. The increase in absorbance due to formazan formation was recorded at 560 nm. One unit of SOD1 activity was defined as the amount of enzyme that inhibits the NBT photo-reduction by 50%.

2.3. Spectroscopic characterization

All fluorescence measurements were carried out using a Cary Eclipse VARIAN fluorescence spectrophotometer with excitation fixed at 280 nm and emission spectra were recorded between 295 and 400 nm. The excitation and emission slit widths were set at 5 and 10 nm, respectively. The concentrations of 0.05 mg/ml of proteins in phosphate buffer (20 mM, pH 7.4) were used.

CD spectra were recorded using an AVIV 215 spectropolarimeter (Aviv Associates, Lakewood, NJ, USA) and a 0.05 mm path cell. The samples were prepared at final concentration of 0.4 mg/ml in phosphate buffer (20 mM, pH 7.4) and spectra were recorded in the range of 195–260 nm.

2.4. Characterization of SOD1 aggregation

Samples for aggregation experiments contained 1 mg/ml of proteins in different buffer compositions including EDTA 5.0 mM, DTT 50 mM, NaCl 100 mM, GuHCl 1 M, KSCN 0.2 M, SDS 5 mM and TFE 30% (all as single or in combination) at 37° C (pH 7.4) and with agitation at 750 rpm. Formation of the amyloid fibrils was monitored by following the increase in the ThT fluorescence (15 μM protein solutions and 20 μM ThT). The fluorescence recordings were performed with excitation at 444 nm and emission at 485 nm. The excitation and emission slit widths were set at 5 and 10 nm, respectively.

2.5. Electron microscopy

5 μl of aliquots were taken from aggregated samples and applied to carbon 400-coated grids. After 45 s, the grids were washed with distilled water and then stained with 2% uranyl acetate and the samples were then observed by a CEM 902 AZEISS microscope.

2.6. Preparation of rat brain mitochondria

Mitochondria from the brains of male rats (250–300 g) were removed, washed and homogenized in isolation buffer (10 mM Tris–HCl, pH 7.4; 1 mM EDTA; 0.32 M sucrose). Homogenates were centrifuged at 3000 *g* for 10 min and mitochondrial fraction from the resulting supernatant was isolated according to [37], and stored at 15 mg/ml in isolation buffer in liquid nitrogen. Mitochondrial membrane integrity was confirmed by determination of specific activity of markers enzymes: MDH, glutamate dehydrogenase, rotenone-insensitive NADH cytochrome c reductase, cytochrome c oxidase, citrate synthase and adenylate kinase, as described previously [38].

2.7. Incubation of isolated mitochondria with WT and mutants SOD1 fibrillation products

In terms of affecting the oligomers and fibrillar aggregates of WT and mutant proteins (G72S, D101N) on mitochondrial membrane of rat brain, aliquots of the aggregation reaction, prepared by incubation of 1 mg/ml of the proteins at 37 °C and agitation at 750 rpm in the presence of Tris–HCl 50 mM, KSCN 0.2 M and DTT 50 mM, were taken. Sampling was performed after 6 and 48 h to obtain oligomer and fibrillar aggregates of SOD1, respectively as confirmed by ThT fluorescence analysis and TEM micrographs. Briefly, aliquots of solutions containing WT and mutants aggregation products, and buffer as control, were added to 200 μl of mitochondrial homogenate (1 mg/ml), followed by incubation for 30 min at 30 °C. The incubation time of 30 min was used to allow