ABSTRACT

Tracking SOD1 heterodimerization processes using fluorescence aggregation assays

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Copper-zinc superoxide dismutase (CuZnSOD) is an enzyme which is found throughout eukaryotic cells and in some areas of bacterial cells and is responsible for catalyzing the transformation of superoxides into hydrogen peroxide and dioxygen. Studies have found that inherited mutations in the gene encoding CuZnSOD, SOD1, are present in a portion of cases of amyotrophic lateral sclerosis (ALS), a neurodegenerative disease marked by motor neuron degeneration and cell death. Because of this, CuZnSOD is a well-studied protein, though much is still to be learned about the pathology of the disease and how SOD1 causes it. One such aspect of disease pathology that is yet to be explored fully is the role subunit exchange plays in protein aggregation. Many of familial ALS-causing mutations are autosomal dominant, meaning interactions between wild-type (WT) and mutant forms of SOD1 protein exist. Heterodimers, containing one WT subunit and one mutant subunit, have been observed in tissue samples of individuals affected by ALS, necessitating study of the process by which these heterodimers are formed. In order to study these properties of subunit exchange and heterodimerization, recent studies have used fluorescence aggregation assays in order to monitor formation of protein aggregates over time and study the types of conditions that result in heterodimerization of SOD1 protein. By tracking the kinetics of heterodimerization over time, much can be learned from the rate and products formed from this reaction. These fluorescence assays are a unique way of studying protein aggregation kinetics, and the findings indicate varying rates of aggregation according to certain mutations in SOD1. The findings from this set of research using aggregation assays serves as a launching point for understanding how the presence of both WT and mutant protein may affect or accelerate disease progression as a result of heterodimerization.

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TRACKING SOD1 PROTEIN HETERODIMERIZATION PROCESSES USING FLUORESCENCE AGGREGATION ASSAYS

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CHAPTER ONE

SOD1, ALS, and Protein Aggregation

Copper-zinc superoxide dismutase (SOD1) is an enzyme that was first discovered and characterized in the mid to late 20th century.[1] Within eukaryotic cells, SOD1 is primarily found in the cytoplasm, nucleus, and the intermembrane space of the mitochondria.[2] SOD1 is naturally found as a homodimer, with each subunit containing both a copper-binding site and a zinc-binding site, as well as an intrasubunit disulfide bond between Cys57 and Cys146. SOD1 subunits adopt a Greek-key β-barrel form containing seven connecting loops, of which two, termed the zinc loop and the electrostatic loop, are especially notable. The zinc loop contains all of the residues which coordinate the zinc ion, as well as one of the residues involved in the disulfide bond, while the electrostatic loop makes up a large part of the active site and controls the diffusion of reactants into the active site.[3]

SOD1 is primarily involved in the reduction of superoxide ions (O_2^-) that are produced in large part through cellular respiration (Schematic 1).[4] Leakage within the mitochondria during oxidative phosphorylation in the electron transport chain produces a large portion of superoxides

$$2O_2^- + 2H^+ \xrightarrow{\text{CuZnSOD}} O_2 + H_2O_2$$

Schematic 1. The reduction of superoxide ions involves the use of CuZnSOD and leads to the release of dioxygen and hydrogen peroxide.

found within the human body, which are reduced through the reduction and oxidation of the copper ion bound to SOD1. In the oxidation step, the first superoxide ion reduces the copper (II) ion, resulting in dioxygen which is released.[1,3] Shortly after, the second superoxide ion oxides the

copper (I) ion, regenerating the copper (II) ion. Protons which were bound within to active site residues are removed and rearranged in order to form the hydrogen peroxide from the recently oxidized O_2.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease which is marked by a degeneration of motor neurons.[2] A large majority of ALS cases are sporadic, however there is a set of cases that occur via inherited genetic factors, known as familial ALS (fALS), and most of the causes for fALS have been determined to be mutations in the gene encoding SOD1.[3,5,6] Most of the mutations in the *sod1* gene have been observed to be autosomal dominant in their nature. Disease onset typically occurs in later life, with the median age of onset being 55 years, and usually spreads throughout the body from a single focal point. Severity of fALS can be dependent on the mutation, and survival time can vary from as short as 2 years to as long as 20 years.[7,8] Because of its diverse set of mutations and wide variance in severity, there is still much to be discovered about SOD1 and how it affects the lives of those who live with fALS.

SOD1 Aggregation and Heterodimerization

Many of the mutations in SOD1 which contribute to neuron death and disease progression were also implicated in observations that SOD1 was present in cytoplasmic inclusions and Lewy bodies in spinal cord samples of patients with fALS.[9] These aggregates quickly became the center of attention for study on SOD1, and further study showed that samples from patients with heterozygous SOD1 mutations contained heterodimeric SOD1 protein, containing both a wild-type (WT) subunit as well as an ALS-variant subunit.[10–13] This discovery of heterodimerization,

combined with the observation of autosomal dominance, indicates an important area of study regarding interactions between WT and mutant SOD1 protein.

A unique aspect of protein aggregation processes in general is the way aggregates form. Protein aggregates can take on two forms, amorphous or fibrillar, and which structure they tend towards is dependent on how the β-sheets of the protein stack. In amorphous aggregates, β-sheets between discrete proteins are more randomly mixed and stacked, however, the formation of fibrillar aggregates is much more highly ordered.[14] Disorder due to mutations in the zinc and electrostatic loops of SOD1 causes several of the β-strands on the protein to form hydrogen bonds between subunits, self-assembling into β-barrels that form a filamentous array.[1] These filaments are the primary form of aggregate found in patients with ALS and are the first aggregates that are formed, but enough time will allow fibrils to break down into more amorphous forms. The formation of either amorphous or fibrillar aggregates is stochastic in nature, which explains the variability seen in previous fluorescence aggregation assays and points to a model where SOD1 fibrillization is a matter of probability, at least in some part.[15]

Heterodimerization

The heterodimerization between WT SOD1 and ALS-variant SOD1 might inherently be a cause for disease pathogenesis.[10,16–18] The heterodimerization between WT hSOD1 and ALS-variant hSOD1 in HEK293 cells promotes toxicity, regardless of SOD1 aggregation.[18] The co-expression of WT human SOD1 (hSOD1) in G85R hSOD1 transgenic mice promotes ALS, unlike mice expressing just G85R hSOD1, and overexpression of WT hSOD1 along with mouse G86R SOD1 mice reported no evidence of heterodimerization (or aggregation) as the mouse and human protein likely do not heterodimerize.[16,17]

Heterodimerization might explain the resulting toxicity between WT SOD1 protein and mutant SOD1 protein interactions.[7,10,19] For example, the co-expression of WT SOD1 appears to be a requisite for pathogenesis in the A4V SOD1 transgenic mouse.[20,21] In the G93A SOD1 transgenic mouse, the co-expression of human WT SOD1 only slightly accelerated the onset of neurodegeneration (by 15 ± 32 days) but significantly increased the progression of symptoms, compared to mice expressing only G93A SOD1 (duration was 40.2 ± 6.5 compared to 24.0 ± 11.6 days for G93A mice and G93A/hWT mice respectively).[16,22] Therefore, it is possible that the WT SOD1 protein is not a mere spectator in SOD1-linked fALS, but rather activates mutant SOD1 and works synergistically with it.[10,23]

At the molecular level, little is known regarding SOD1 heterodimerization, including the mechanism(s) of heterodimerization.[7,19] Nearly all biophysical and biochemical studies of ALS-variant SOD1 have focused on homodimeric mutant SOD1 or WT SOD1, however the importance of studying heterodimerization is evident in studies which can relate certain observations in clinical phenotype to SOD1 heterodimerization.[24–28] For example, a previous study demonstrated that the rate and free energy of heterodimerization between WT SOD1 and ALS-variant SOD1 can be measured using capillary electrophoresis (CE).[7] The study reported a strong correlation between patient survival time after diagnosis and ΔG_{Het} of heterodimerization, justifying the importance of understanding the interactions between mutant and WT SOD1.

In theory, there are two basic types of heterodimerization: *de novo* heterodimerization and subunit swapping (Figure 1). In *de novo* heterodimerization, monomeric WT SOD1 and monomeric ALS-variant SOD1 heterodimerize before ever homodimerizing, assuming both the ALS-variant SOD1 and WT SOD1 are expressed at the same time and in the same space. In

contrast, heterodimerization may also occur by subunit swapping of homodimeric WT or mutant SOD1. Here, multiple mechanisms are possible (dissociative, associative, mixed) (Figure 1). Because of SOD1's long turnover rate in the central nervous system, this subunit form of heterodimerization undoubtedly occurs in the context of ALS.[29]

Another important aspect of heterodimerization in SOD1 systems is the rate of aggregation *in vivo* of different mutant forms. A previous study which used Kaplan-Meier estimators to quantify rates of protein aggregation found that some mutations formed fibrillar aggregates faster than WT while other mutations formed fibrillar aggregates slower, and when compared to clinical survival times, found that the results more closely correlated to observed phenotype than previous metrics.[8] A practical extension of this work might study if WT-mutant heterodimers aggregate at

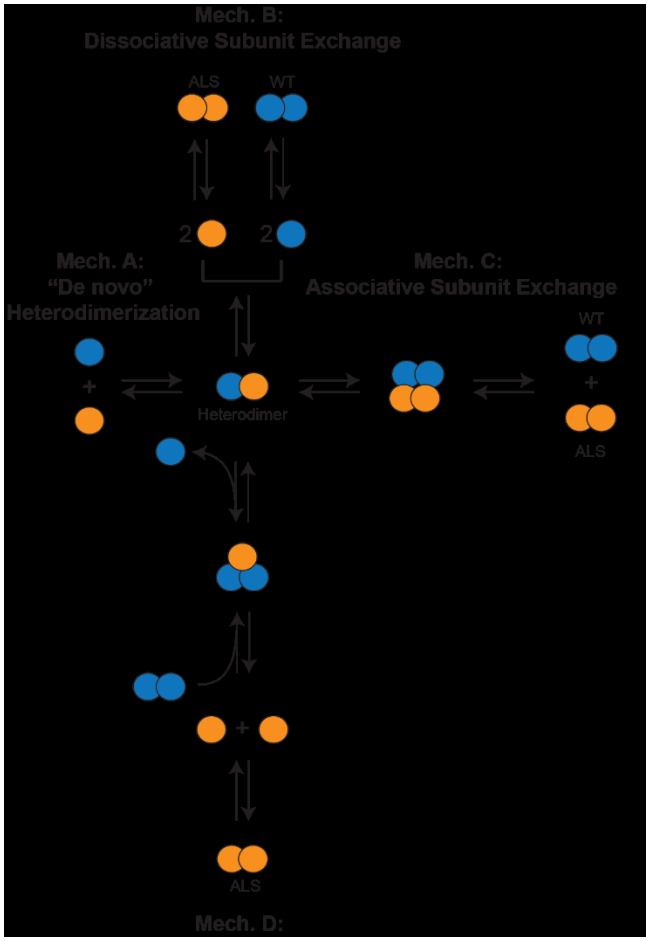

Figure 1. Possible mechanisms of heterodimerization between ALS-variant SOD1 and WT SOD1.

a rate in between the two homodimer forms or if something else entirely occurs as a result of heterodimerization.

Fluorescence Monitoring

Many of the techniques used to monitor protein aggregation have relied on the use of fluorescence. Certain organic fluorescent dyes, such as Thioflavin-T (ThT), become more emissive as a result of compound aggregation, as opposed to a general observation that most other dyes are quenched by aggregation.[30–32] Because most organic compounds have planar structures, they are more emissive when they are free in solution. However, for organic compounds with freely rotating groups, the aggregation of compounds, like ThT, restricts the rotational movement of the molecule and increases the amount of emission as a result. This makes ThT a powerful tool when studying protein aggregation, as it can be used to mark and monitor the formation of fibrils in solution without measuring emission prior to aggregation. Other fluorescent dyes which do not have this characteristic would not show an increase in emission as a result of protein aggregation making them of little use in aggregation studies. ThT works best for tracking fibrillar amyloid formation as a result of the shape of the aggregates but cannot bind to amorphous aggregates.[14,15]

CHAPTER TWO

Methods

This study investigates the *in vitro* aggregation of mutant SOD1 in the presence of WT SOD1. The H46R and D101N variants of SOD1 were chosen because (i) H46R and D101N SOD1 do not readily form fibrillar aggregates *in vitro*, and (ii) H46R and D101N variants of SOD1 exhibit drastically different clinical phenotypes (with survival times of ~17 and ~2.5 years, respectively), despite their apparently similar aggregation propensities.[8,33,34]

SOD1 Purification, Demetallation, and Biophysical Characterization

Wild type or fALS-variant SOD1 was recombinantly expressed in *S. cerevisiae*, purified, and demetalated, as previously described.[35] Purification was performed by successive ammonium sulfate precipitation, hydrophobic interaction chromatography, ion-exchange chromatography, and size-exclusion chromatography. Demetallation of purified SOD1 proteins was achieved through sequential dialysis in three buffers over the course 6 days: i) 0.1 M sodium acetate, 10 mM EDTA, pH 3.8; ii) 0.1 M sodium acetate, 10 mM NaCl, pH 3.8; and iii) 0.1 M sodium acetate, pH 5.5, as previously described.[36] Metal content of the protein was measured with inductively coupled plasma- mass spectrometry (7900 ICP-MS, Agilent Technologies, Santa Clara, CA).

Thioflavin-T (ThT) Fluorescence Aggregation Assays

High throughput ThT aggregation assays were performed in a 96-well microplate on a Fluoroskan Ascent™ FL microplate fluorometer (Thermo Scientific™, Waltham, MA). SOD1

(mutant or WT) was first transferred to aggregation buffer (10 mM potassium phosphate, 5 mM EDTA, pH 7.4) via centrifugal filtration. Sample solutions were prepared by addition of SOD1, Tris(2-carboxyethyl) phosphine (TCEP), and ThT in aggregation buffer (10mM potassium phosphate, 5mM EDTA, pH 7.4). Final concentrations were as follows: 15μM or 30μM SOD1(dimer); 10mM TCEP; 20μM ThT. Aggregation buffer, SOD1, and TCEP were mixed and allowed to incubate at room temperature for thirty minutes. After the incubation period, ThT was added and solutions were immediately filtered using 0.2 μm syringe filter. Sample solution (200μL per well) was distributed to the wells of a black polystyrene microplate, which was cleared of any dust or similar particles using nitrogen gas. Each well contained a single 1/8" (3.13mm) white Teflon bead, and only perimeter wells were used (as to avoid evaporation). The microplate was sealed using an adhesive seal and inserted into a Fluoroskan Ascent. The Fluoroskan was run for 7 days for unseeded assays, shaking and measuring every 15 seconds. At the end of the 7-day period, the microplate was removed and unsealed. Solution still remaining in each well was collected, frozen, and stored at -80°C for later use.

Seeded assays were performed by adding 180uL of soluble SOD1 solution (with TCEP and ThT) prepared similar to above, followed by 20uL of fibril 'seed' solution to each well of the microplate. This seed solution was prepared from previous unseeded assays, which was washed of excess ThT by centrifuging at 18,000 g for 20 minutes, removing 500 μL of supernatant, resuspending the pellet, then replacing 500 μL of fresh aggregation buffer (10mM potassium phosphate, 5mM EDTA, pH 7.4). This process was repeated until the solution was mostly clear and no longer tinted. Seeded assays were measured using the Fluoroskan over 5 days, after which solution still remaining in each well was collected and stored similar to unseeded assays.

CHAPTER THREE

Results and Discussion

D101N Aggregation Occurs in the Presence of WT SOD1

Analysis of the aggregation assays done on the D101N mutant revealed that although the mutant did not aggregate on its own, introduction of soluble WT protein caused aggregation to

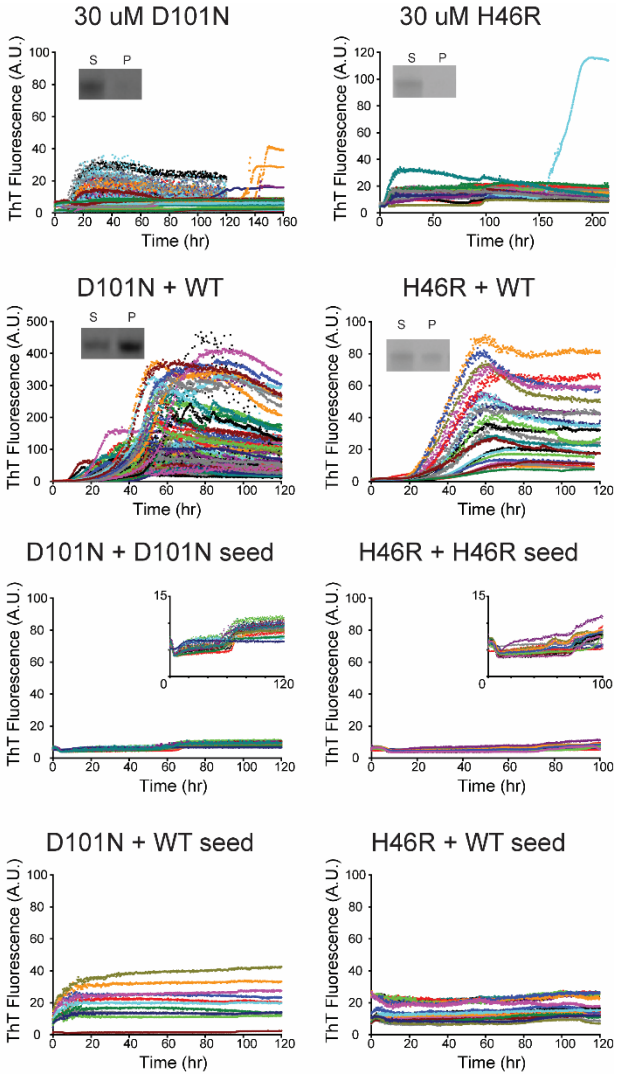

Figure 2. ThT analysis of H46R and D101N aggregation by itself and in the presence of WT protein, seeded and unseeded. Gel electrophoresis inserts show the ratio of soluble protein to insoluble protein after the assay.

occur *in vitro*. Figure 1 shows that very little aggregation occurred at all when D101N was the only soluble protein in solution. Upon introduction of soluble WT protein at a 1:1 molar ratio, a large increase in aggregation occurred. Gel electrophoresis of the remaining solution showed protein primarily in the aggregate form (indicated by the "P" on the gel insert) and little protein left in solution (indicated by the "S" on the gel insert). In both scenarios where soluble D101N was mixed with a fibril 'seed,' the protein did not aggregate, indicating that D101N does not co-aggregate with previously fibrillized protein.

H46R Does Not Aggregate In Vitro Within 10 Days

The assays performed using H46R show that its low aggregation propensity makes it difficult to study over a 7-day period like described in this research. It is unable to be determined whether H46R will aggregate at all, since there was not a significant portion of protein that formed aggregates in any of the assays using only soluble H46R, with or without fibril seeds. Mixing soluble H46R with soluble WT causes aggregation to take place, but gel electrophoresis does not conclusively show that more protein is found in the aggregates than in the solution as in the case of D101N, and so it is unclear whether H46R will co-aggregate with WT SOD1. One possible explanation of the H46R + WT results is that the soluble WT aggregated on its own, ignoring the present H46R protein and contributing to the aggregation that was observed through homodimerization, not heterodimerization.

CHAPTER 4

Conclusion

Studying heterodimerization of SOD1 is likely to be one of the more important avenues of fALS and protein aggregation research in the near future, and the fluorescence aggregation assay demonstrated in this paper serves as a proof-of-concept for how information can be gathered from these experiments. Because of the many ways SOD1 can be destabilized, systematic study of heterodimerization involving different metalation states or net charges could serve as valuable information in determining why SOD1 acts the way it does.

Identifying the nuances of how WT and mutant SOD1 interact could also identify certain mutants which necessitate further study. In this case, H46R is an especially puzzling mutation, as it does not behave intuitively for such an unstable mutant. As such, further studies could determine whether first, H46R aggregates at all, and second, if H46R could be co-aggregating with WT SOD1 albeit at a very slow rate.

Further studies could use alternative means of observing aggregates after aggregation assays, such as transmission electron microscopy or confocal microscopy. Direct observation of amyloidogenesis has already been described, and a similar technique could be used with SOD1 to determine how interwoven heterodimers are when fibrils are forming *in vitro*.[37] Determining whether fibrils form as a primary or secondary nucleation process could give further insight into the unique nature of SOD1 and how it affects those with fALS.

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