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Examining the pH switch of copper-zinc superoxide dismutase mutant A4V

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Examining the pH Switch of Copper-Zinc Superoxide Dismutase mutant A4V

By

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Abstract

Amyotrophic lateral sclerosis(ALS) is a neurodegenerative disease that causes progressive loss to motor neurons. Buildup of superoxide anions (O_2^-) and aggregation of superoxide dismutase (SOD) is thought to be probable causes of damage to the neurons. This damage results in the progressive loss of function in the neurons resulting in flaccid paralysis. Superoxide anions were originally thought to cause irreparable damage to the motor neurons; however, recent studies have shown that the aggregation of SOD causes damage to the cell. Furthermore, the ability for the SOD1 to get metalated to dissuade aggregation has become an important topic of study in ALS research. In this study, human SOD1 A4V mutant was successfully expressed and purified and set for crystallization at various pH to understand the effect of minor pH changes on the stability of the protein structure. Future X-ray crystallography analysis could result in further understanding of how SOD1 aggregates which might help produce viable treatments for the familial form of ALS.

Introduction

Amyotrophic lateral sclerosis(ALS) is a disorder that results in the loss of motor function (Masrori, 2020). This neurodegenerative disorder targets the motor neurons within the motor cortex of the brain resulting in fatal flaccid paralysis. The average lifespan after the diagnosis of ALS is between two and five years due to the failure of respiratory muscles. Ten percent of all ALS cases are designated as familial ALS (fALS) while the remainder can be designated as sporadic ALS (sALS). fALS is thought to be inherited through autosomal dominant inheritance (Rosen et al, 1993).

In a large percentage of cases of fALS, there is a link to a mutated copper-zinc binding superoxide dismutase (SOD1). The varying rate occurrences of fALS can be observed differently in different populations globally (Mejzini, 2019). As seen in figure 1 below, SOD1 is found in higher proportions within Asian populations when compared to European populations.

Figure 1. Comparison of fALS to sALS in European and Asian populations (Mejzini, 2019).

SOD1 is classified as a homodimeric metalloenzyme that is responsible for catalyzing the reaction of a superoxide anion (O_2^-) into gaseous oxygen (O_2) and hydrogen peroxide (H_2O_2) (Klug et al, 1972). Aerobic respiration is the main source of the production of superoxide anions (Dröse, 2012). In the normal processes of ubiquinone oxidoreductase (complex I) and cytochrome c oxidoreductase (complex III), superoxide anions are produced as a natural byproduct. The presence of these superoxide anions is known to cause oxidative stress that can potentially damage DNA, lipids, and proteins (Davies, 2000).

As a defense mechanism to counteract the oxidative stress produced by aerobic respiration, many organisms have evolved enzymes such as SOD1. Human SOD1 (hSOD1), bovine SOD1 (bSOD1), tomato SOD1 (tSOD1), and yeast SOD1 (ySOD1) are all shown to be nearly identical in both structure and genetic sequence (Sea et al, 2021). A mature SOD1 protein consists of identical subunits folded as eight-stranded Greek key beta-barrels (Tainer, 1982). From these beta barrel structures protrude two long loops that participate in the binding of the metal ions.

The viability of SOD1 to catalyze the disproportionation of superoxide anions is dependent on two loops called the “zinc loop” and “electrostatic loop” (Hart, 1999; Tainer, 1982). The zinc loop is comprised of 24 amino acids; the amino acids H63, H71, H80, and D83 maintain the binding site for the zinc ion (Hart, 1999). This distorted tetrahedral zinc site is responsible for maintaining the stability of the enzyme during the conformational changes that occur during the disproportionation reaction. The electrostatic loop is 22 amino acids long; the amino acids H46, H48, H63, and H120 are responsible for guiding the superoxide anion to the active site containing the copper II ion. Throughout the disproportionation reaction, the geometry of the associated amino acids around the copper ion alternate. The site is a distorted square-

planar geometry when in the copper II oxidation state; however, when in the copper I oxidation state, the geometry of the active site was found to be trigonal planar as shown in Figure 2.

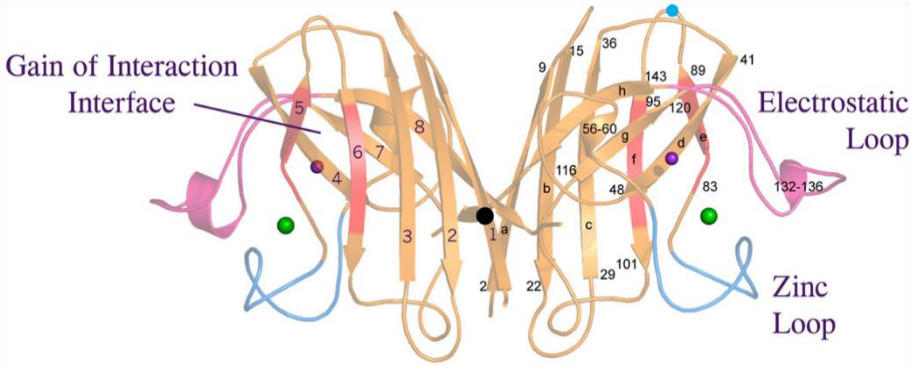


Figure 2. Three-dimensional structure of hSOD1.

The enzymatic activity of SOD1 relies on the ability of the enzyme to obtain copper within the active site (Valentine, 1981). Even though superoxide dismutase obtains the copper ion needed for the disproportionation of a superoxide anion, it is still susceptible to misfolding in both the dimer and monomer forms as shown in figure 3.

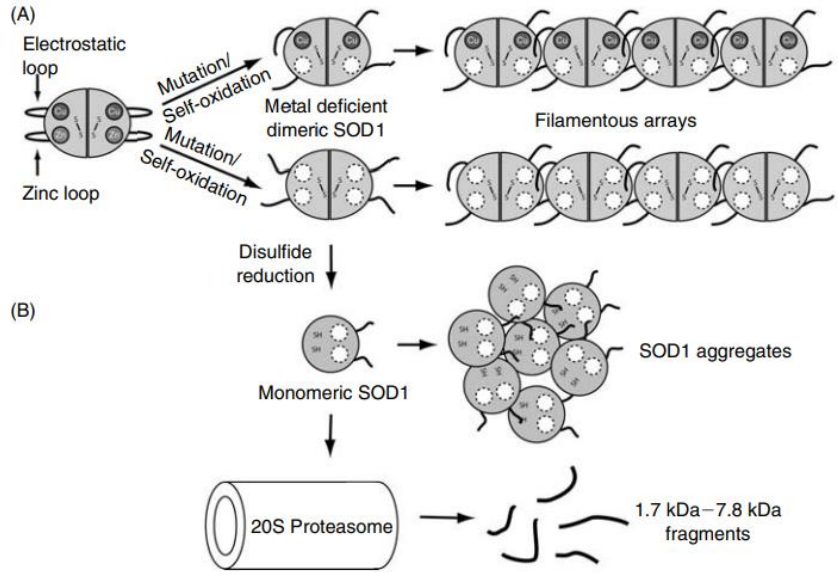


Figure 3. The possible aggregation pathways for copper-bound dimers, dimers, and monomers thought to potentially cause harm in neurons (Galaleldeen, 2007).

When hSOD1 A4V is in the pathogenic monomeric form, there is approximately sixty-five percent of the proteins remaining intact after being paired with the 20 S proteasome after ninety minutes of incubation at thirty-seven degrees Celsius (Galaleldeen, 2007). The mutation caused by the A4V mutation makes the protein less susceptible to be attached to the proteosome. The percent degradation of the mutated protein increases with an increase in temperature.

ALS has many pathways in theory that allow for it to continually degrade the neurons until flaccid paralysis occurs. The two main drugs used to prolong the life of ALS affected patients are Riluzole and Edaravone. Riluzole has been an FDA approved drug for twenty years that focuses on the inhibition of glutamate release (Blyufer et al., 2021). The reuptake of glutamate within the synapses of the central nervous system is also enhanced through the use of Riluzole. There has also been indications that Riluzole can reduce the production of reactive oxygen species(ROS). ALS patients using Riluzole have been found to have a longer life span post-diagnosis of three months on average (Miller et al., 2012). Edaravone has been found to reduce the effects of ROS on cells found within the central nervous system (Park et al., 2020). Effectiveness of Edaravone treatment was measured using a functioning scale produced for ALS patients called the Revised Amyotrophic Lateral Sclerosis Functioning Rate Scale (ALSFRS-R). Through the use of this scale, a multitude of tests are used to track the decline of function of people diagnosed with ALS. Improvement of ALSFRS-R scores have been recorded across numbers of patients being treated with Edaravone (Okada et al., 2018). The improvement of scores using the ALSFRS-R can be viewed as a higher quality of life for those who are suffering from ALS.

A4V mutation is located in strand 1 at the dimer interface. A4V dimers are similar to that seen in the “metal binding region” (MBR) mutants S134N and apo-H46R. Both copper and zinc

sites should be occupied as the mutation site is far from the metal sites. The short helical segment in the electrostatic loop displays relatively large atomic displacement parameters which may be correlated to static disorder of copper in its binding site and in turn portray its vulnerability for aggregation. In A4V, both electrostatic and zinc loops are disordered. These observations suggest that disordered or mobile electrostatic and zinc loops may share a similar mechanism of aggregation proposed previously for metal deficient mutants S134N and H46R. We were not able to get crystals to finish the structural studies but the work is continuing in the lab in pursuit of these structures at low pH.

With the lack of more effective drugs, it is essential for research to be done to help produce longer lifespans and higher qualities of life for those suffering from ALS. Through research on the proteins associated with the potential cause of neurodegenerative disease, one can only hope to find a potential breakthrough in ALS research.

Methods and Results

Plasmid

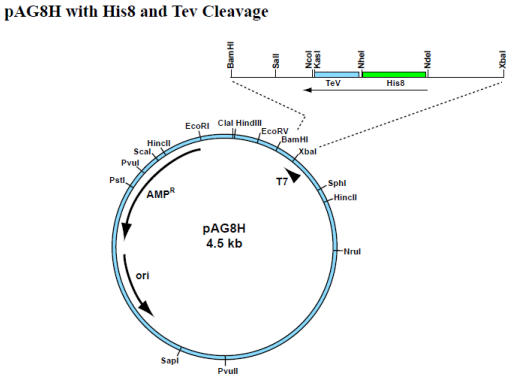


Figure 4. Map of pAG8H plasmid used for cloning.

To allow for the expression of hSOD1 A4V in *E. coli* DE3 cells, the DNA encoded hSOD1 A4V gene with an attached eight-time Histidine tag and ampicillin resistant gene is cloned into the expression vector pAG8H. The eight-time Histidine tag at the N-terminal of the RNA transcript is essential in the purification steps of the protein. The ampicillin resistant gene is important to isolate the expression of the plasmid within the cultures to ensure the target protein is expressed.

Transformation

To express the hSOD1 A4V within *Escherichia coli* BL21 DE3 cells, the plasmid must be transformed into these cells. After thawing the cells, five microliters of the plasmid was added at a concentration of five nanograms per microliter, and the cells were heat shocked to allow the plasmid entrance into the cell. Next, five hundred microliters of Terrific Broth was added to the *E. coli* cells containing the plasmid. The cells were incubated for thirty minutes at thirty-seven degrees Celsius to allow for small scale expression. The cells were then centrifuged, and five hundred microliters were removed before resuspension of the pellet.

After transformation of the pAG8H into *E. coli* BL21 DE3 cells, the cells were spread on solidified media in Petri dishes. The media was composed of 47.57mM agar, .286mM ampicillin, and terrific broth. The presence of ampicillin allows for the plasmid expressing cells to have a better chance of survival due to the ampicillin resistance gene present in the plasmid. The Petri dishes were then placed in a thirty-seven-degree Celsius warm room for sixteen hours to allow for the formation of colonies expressing the plasmid DNA.

SOD1 expression

A one hundred mL culture of terrific broth was prepared and autoclaved. Ampicillin was also present within the small culture at a concentration of .286 mM to allow for a higher chance of

expression of the hSOD1 A4V gene. After the sixteen-hour incubation period of the plated *E. coli* cells, a colony was removed from the plate and inoculated into the one hundred milliliter culture. The small liquid culture was incubated at thirty-seven degrees Celsius for sixteen hours while being shaken at two hundred twenty revolutions per minute.

Large Culture

Six, one-liter cultures were prepared containing terrific broth and .286mM ampicillin. After the sixteen-hour incubation of the one hundred milliliter culture, ten milliliters of the small culture were inoculated into each of the one-liter large cultures under sterile technique to allow for large scale expression of the hSOD1 A4V protein. These cultures were incubated at thirty-seven degrees Celsius and two hundred twenty revolutions per minute for two hours. After the initial two hours, the cultures were checked for A_{600} of 0.6 to 0.8 at every thirty-minute interval. After three hours, the targeted A_{600} was reached. One milliliter of Isopropyl β-D-1-thiogalactopyranoside(IPTG) was added with a one molar concentration. The large cultures were incubated overnight at seventeen degrees Celsius. The next day the culture was centrifuged, pellet collected, and frozen.

Cell Pellet Lysis

After the cells were defrosted, one hundred milliliters of buffer A was added to allow for sonification. The cells were sonicated for seven minutes pulsing at four second intervals with sixty percent amplitude. After sonification, the cells were centrifuged at sixteen thousand revolutions a minute for fifty minutes. The soluble portion of the result containing hSOD1 A4V was removed. After removal of the soluble portion, the liquid is passed through a .45 micrometer

filter to clear of any sizeable contaminants. Buffer A contains 10mM Imidazole, 400mM sodium chloride, and 25mM Tris at a pH of 8.0.

Affinity Chromatography

To purify the targeted protein, a nickel His-Trap column was used. When passed through the column using buffer A, the attached 8x histidine tag of the hSOD1 A4V remained within the column due to its affinity to nickel. Proteins bound loosely were removed by washing the column with a ten percent elution buffer B. The target protein was removed by washing the column with a gradient of buffer A and buffer B from 10mM to 500mM concentration of imidazole as shown in figure 4. Buffer B contains 500mM Imidazole, 400mM sodium chloride, and 25mM Tris at a pH of 8.0.

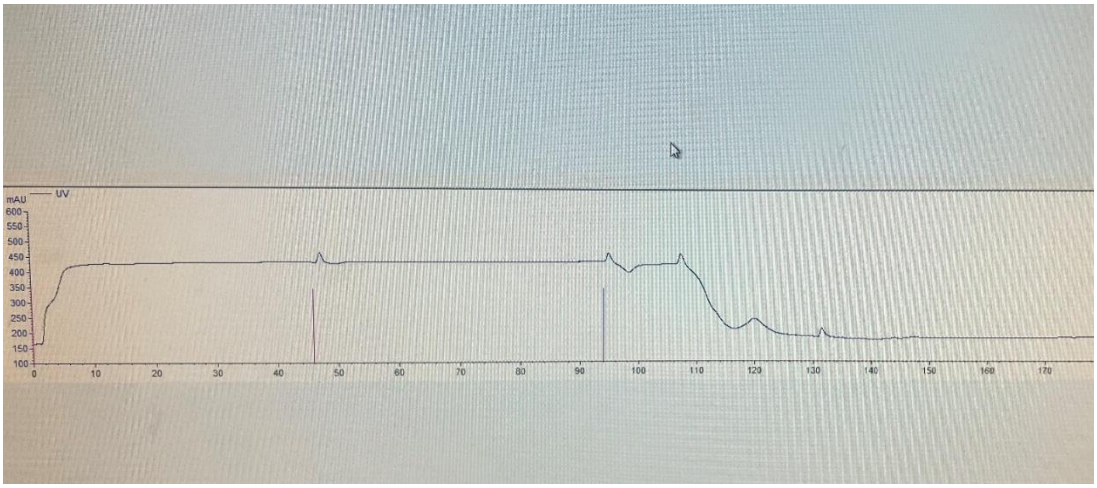
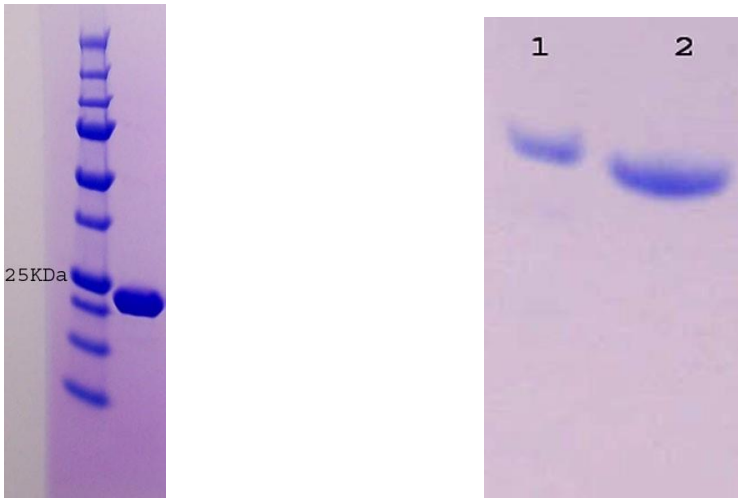


Figure 5. The UV reading of the Affinity Chromatography during the gradient imidazole wash.

The x-axis represents the number of milliliters passed through the column containing the nickel. The first ninety milliliters represent the loading sequence in which the sample is passed through the machine. The ten percent elution wash was run from approximately ninety milliliters to one hundred and twenty-five milliliters. This wash is essential to clean the column of loosely bound

proteins. After this point, the remainder of the UV reading is due to the gradient wash. As seen on the figure, the spike around approximately one hundred and thirty milliliters may represent the presence of the target protein.



A B

Figure 6. SDS-PAGE results from the affinity chromatography gradient elution using imidazole.

In gel A, the first lane on the left represents a ladder to allow for an accurate comparison of the size of the proteins found within the SDS-PAGE. Lane 2 shows purified SOD1 mutant. Gel B shows mutant SOD1 before and after TEV treatment confirming the removal of the his tag.

Dialysis and Removal of the His-Tag

Next, the samples found to contain the targeted protein using the SDS-PAGE were purified by dialysis within a 25mM Tris solution at a pH of 8.0. After overnight dialysis in the 25mM Tris solution, one to two mg of tobacco etch virus protease was added to the dialysis bag to allow for cleavage of the eight-time histidine tag. After being maintained at room temperature overnight,

the sample was removed and tested for the eight-time histidine tag removal using another SDS-PAGE as shown in figure 6.

In the later lanes of the SDS-PAGE, the presence of two closely relevant lines represents the partial removal of the eight-time histidine tag from the target protein. The less noticeable readings in the earlier lanes of the SDS-PAGE are caused by the lower level of concentration of solution from the dialysis used. To allow for complete cleavage of the eight-time histidine tag from the target protein, one to two milligrams of tobacco etch virus protease was added again to the sample.

The protein was unstable and multiple trials have resulted in small amounts of purified protein that was concentrated and set in crystal trays, but no crystals were obtained.

Discussion

Through the steps until protein insolubility, the results of the experiment represent that the target protein hSOD1 A4V can be expressed through *E. coli* BL21 DE3 cells using transformation. After the transformation, the protein could be purified through the lysing of the cells and affinity chromatography. Based on multiple trials using luteinizing broth and terrific broth, terrific broth produced the trial detailed in this account with a large amount of expression which is represented in the darkness of the bands of the SDS-PAGE in figure four.

The detriment to the experiment occurred when the hSOD1 A4V was no longer soluble within the dialysis and following the cleavage steps within the experiment. Figure six shows the success seen with the cleavage of the eight-time histidine tag using the tobacco etch virus protease; however, a change in pH of the sample likely resulted in the protein becoming insoluble within the dialysis process.

With successful cleavage of the eight-time histidine tag, the sample would have been subjected to a second trial of affinity chromatography to purify the hSOD1 A4V further. Using a similar process as seen in the initial affinity chromatography, the exploitation of the eight-time histidine tag to the nickel column would result in the flow through containing only the target protein with the eight-time histidine tag remaining within the column due to its affinity to nickel. The successfulness of this step could be reviewed by using another SDS-PAGE to represent a lack of eight-time histidine tag from the protein based on the travel distance of the protein.

After a complete purification of the hSOD1 A4V, the pH of the sample solution can be manipulated using an acetate and EDTA buffer to allow for the release of the metals found on the hSOD1 A4V. This is an essential step to remove metals from the protein since the protein is known to be a metalloenzyme. To reload the copper onto the electrostatic loop of the protein, the sample can be dialyzed using a Tris and copper (II) chloride buffer. This would allow for the copper ions to be integrated back into the correct binding locations with the lack of the stabilizing zinc ion being within the zinc loop of hSOD1 A4V. A final dialysis using Tris can allow for removal of any unbound copper from the solution from the previous step.

Lastly, to achieve potential protein crystallization, the protein could once again be passed through a .45 micrometer filter. Next, concentration of the protein is essential before using the sitting-drop technique. The protein can be resuspended with an ammonium sulfate grid and incubated. The plate should be monitored for the formation of protein crystals to be analyzed with electron microscopy. With successful purification and crystallization of hSOD1 A4V, electron microscopy can allow for the examination of the structure of the protein with the lack of zinc being bound within the zinc loop. Through this examination of the protein, the goal is analyze and hypothesize the reasoning behind any conformational changes seen with the

presence of copper within the electrostatic loop and absence of zinc from the zinc loop. Future trials will attempt to produce sizable and purified crystals capable of examination.

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