Stabilization of SOD1 A4V for identification of drug compounds for therapeutic treatment of Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS), known as Lou Gehrig's disease, is a fatal, progressive neurodegenerative disease with a median survival post diagnosis of only 3-5 years. This late on-set disease is characterized by the death of brain and spinal cord motor neurons. The only FDA-approved medication, Riluzole, has very limited effects on both patient survival and quality of life. Given this information, it is imperative to develop new strategies to fight the pathology of this debilitating disease. Mutations in the gene-encoding copper/zinc superoxide dismutase (SOD1) are responsible for approximately 20% of the inherited forms of ALS called fALS and about 2% of total cases. The cause of fALS is understood to be due to a toxic gain-of-function mutation in the SOD1 protein, which is supported by both dominant inheritance and lack of symptoms in SOD knock-out mice. This toxic gain-of-function suggests a decrease in protein stability and an increase in the likelihood that the fALS variant will aggregate and then lead to disease pathology. The goal of this study is to reduce aggregation of a fALS variant called SOD1 A4V by either preventing post translation modification of SOD1 or stabilizing the native SOD1 dimer through cross-linking two symmetric cysteine residues. It was found that compounds ZINC 27780 and ZINC95819 did not stabilize the SOD1 protein. However, it was found that a 3-fold molar excess of cross-linkers BMOE and DTME significantly increased the amount of dimer present. Also, the presence of twice as much BMOE to SOD1 A4V stabilized the protein by ~20 °C.

Introduction

Amyotrophic lateral sclerosis (ALS), known as Lou Gehrig's disease, is a fatal, progressive neurodegenerative disease with a median survival post diagnosis of only 3-5 years. This late on-set disease is characterized by the death of brain and spinal cord motor neurons. The only FDA-approved medication, Riluzole, has very limited effects on both patient survival and quality of life. Given this information, it is imperative to develop new strategies to fight the pathology of this debilitating disease.

Mutations in the gene-encoding copper/zinc superoxide dismutase (SOD1) are responsible for approximately 20% of the inherited forms of ALS called fALS and about 2% of total cases. Therefore, about 90% of cases are of unknown cause (sporadic ALS)[i-ii]. Mutations in the SOD1 gene are one of the most common forms of fALS and it is hoped that since sporadic and familial ALS affect the same neurons with similar disease pathology (indicative of degeneration and death of upper and lower motor neurons), that therapies effective in mutant fALS models will also be effective in sporadic ALS[iii]. In this study I focus on a fALS variant, SOD1 A4V, which is not only the most prevalent ALS causing mutation in North America but also the most common ALS causing mutation in the *sod1*.

The cause of fALS is generally attributed to a toxic gain-of-function mutation in the SOD1 protein, which is supported by both dominant inheritance and lack of symptoms in SOD knock-out mice[iv]. This toxic gain-of-function suggests a decrease in protein stability and an increase in the likelihood that the fALS variant will aggregate and then lead to disease pathology[v]. Although the effects of this gain-of-function are not well understood, there is evidence that even relatively minor changes in the primary structure of SOD1 can cause fALS[vi].

A possible explanation is that post-translational modifications of wild-type proteins affect structural changes[vii]. In fact, it has been demonstrated that oxidation of SOD1 occurs with the onset of aging[viii]. In addition to posttranslational modifications such as oxidation, dimer stability has also been implicated in SOD1 aggregation[ix]. A common hypothesis links the toxicity of fALS SOD1 variants to dimer destabilization and dissociation into monomers. That is, in order to achieve increased protein stability, it may be necessary to stabilize the SOD1 native dimer because dimer dissociation and subsequent monomer aggregation may be a direct gateway to fALS[x].

The general approach in this study is to reduce SOD1 aggregation by either preventing post translation modification of SOD1 or stabilizing the native SOD1 dimer. First, I looked at a modification-prone residue, tryptophan-32 (W32) in wild-type SOD1, which has been previously shown to be oxidized[xi]. The Agar lab changed this tryptophan residue to a phenylalanine (F) because of its tendency to less likely be oxidized. It was found that mutating W32 to phenylalanine slowed the rate of oxidative modification and decreased the neuron death and protein aggregation in the fALS mutation, SOD1 G93A[xii]. *Since W32F cannot be replicated in humans, small molecules that bind to the W32 site to prevent oxidative modification were tested in an attempt to mimic the success of the W32F mutant.* Docking was performed by Dr. Walter Novack on a ZINC database for the W32 region and the top two scoring molecules, ZINC 27780 and ZINC95819 were chosen to be further

investigated using the fALS mutant SOD1 A4V.

Second, I attempted to stabilize the native SOD1 dimer using two symmetric cysteine residues on opposite sides of the dimer interface located approximately 9 Angstroms apart. *These residues are not in close enough proximity to interact through an endogenous disulfide bond, therefore, I explored cross-linkers as small drug-like molecules with high binding affinity to these residues in an attempt to stabilize the fALS mutant SOD1 A4V and prevent aggregation*[xiiixiv]. The maleimide cross-linkers tested were Dithio-bismaleimidoethane (DTME, spacer arm 13.3Å) and bis(maleimido)ethane (BMOE, spacer arm 8.0Å). I hypothesize that stabilization of SOD1 will prevent aggregation, thus ameliorating the disease phenotype.

Materials and Methods

Protein expression and purification:

wtSOD1 and the A4V variant, cloned into the Yep351 yeast expression vector, were transformed into a *S. Cerevisiae* strain lacking SOD (EGy118(ΔSOD1)) and grown on –Leu selection plates for 5 days at 30 °C. Individual colonies were picked and incubated overnight in YPD media at 30 °C. These starter cultures were then used to inoculate larger 1 liter flasks of YPD media and were grown for 36-48 hours at 30 °C. The cells were pelleted and frozen at –80 °C until purified. Prior to purification cell pellets (approximately 8-12 liters worth) were thawed, resuspended in 200 mM Tris-HCl, pH 8.0, 50 mM sodium chloride, 0.1 mM EDTA, and 1 sigma protease inhibitor tablet, and lysed using 0.5 mm glass beads and a blender. The lysis mixture was then centrifuged at 10,000 RPM for one hour, the supernatant was subjected to a 60 % ammonium sulfate precipitation, and then spun down at 10,000 RPM for one hour. The supernatant was loaded onto a hydrophobic interaction column (HIC) and eluted with a 2M-0M ammonium sulfate gradient. Fractions containing SOD1 (as determined via SDS-PAGE gel) were pooled and excess salt was removed via Amicon Ultra-15 concentration and dilution into 10 mM Tris-HCl, pH 8.0 buffer. The desalted protein sample was then loaded onto an anion exchange column, MQ10, and eluted with a 0-30% NaCl gradient. Presence of SOD1 was confirmed via MALDI-TOF MS.

Cross-linking:

In initial experiments, wtSOD1 (5 μM) and SOD1 A4V (5 μM) were incubated with either Dimethyl sulfoxide (DMSO), 5 μM Dithio- bismaleimidoethane (DTME) or 5 μM bis(maleimido)ethane (BMOE). DMSO was used as a control because the cross-linking reagents were resuspended in DMSO were the final DMSO concentration was about 2-4%. The cross-linking reaction was incubated at room temperature for one hour. In subsequent experiments the ratio of protein to cross-linker was varied (1:2, 1:3, etc.). Results of cross-linking were determined with Matrix Assisted Laser Desorption Ionization- Time of Flight (MALDI-TOF) mass spectrometry where the data was acquired in linear mode with 20 mg/mL sinipinic acid as the matrix. The cross-linked samples of both wtSOD1 and SOD1 A4V were also characterized using a thermo stability assay (see below).

Stabilization:
wtSOD1 (10 μM) and SOD1 A4V (10 μM) were incubated with 10 μM of each chemical compound ZINC27780 and ZINC95819, respectively. These compounds were previously identified in an in silico screen as binding to the W32 region (data not shown), and I wanted to test in vitro binding and stabilization of these compounds. Therefore, stabilization of wtSOD1 or SOD1 A4V was determined by measuring relative fluorescent using Sypro Orange and a Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) machine. Briefly, Sypro Orange binds to hydrophobic patches in proteins, which are usually buried in the core of a protein's structure. As the protein denatures, or melts, more of these hydrophobic patches become exposed and allow the Sypro dye to bind, thus allowing me to monitor the relative fluorescence of the protein related to its melting temperature.

Results

wtSOD1 and SOD1 A4V were purified from yeast
Approximately 5.4 mgs of wtSOD1 and 1 mg of SOD1 A4V were purified (Figure 1A and1B). In both cases some residual dimer is present (Figure 1A and 2B), however it is interesting to note that in the A4V purification other higher order oligomeric forms appear to be present (Figure 1B). There are significantly more higher order oligomers seen in SOD1 A4V; these higher order species however may be an artifact of using high laser powers on the MALDI-TOF.

Figure 1A: MALD-TOF of purified wtSOD1. Mostly monomeric protein (expected molecular weight ~16,000) is present. The wtSOD1 concentration was found to be 113μM, ~ 2 mg/mL, as determined by Pierce BCA assay.

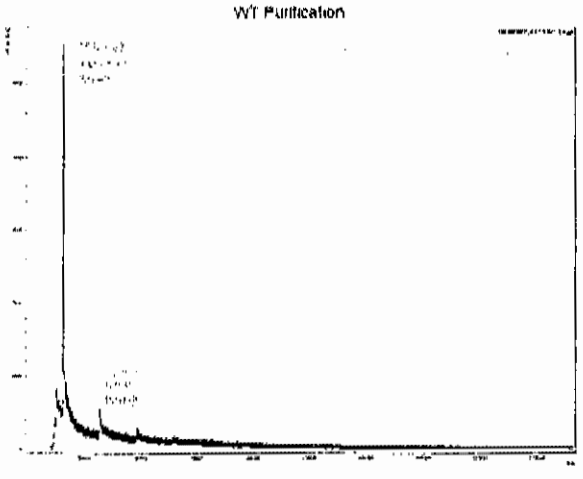
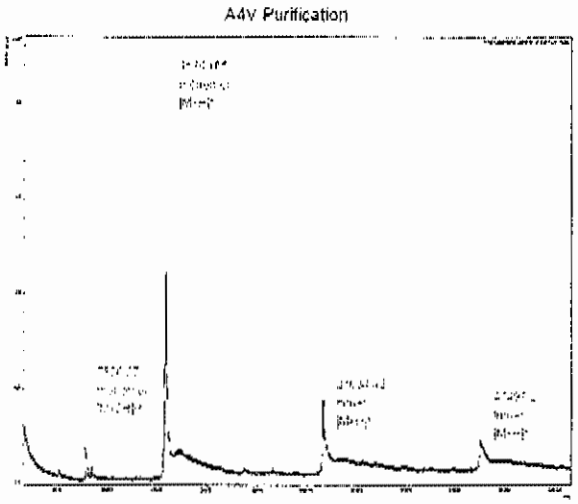


Figure 1B: MALDI-TOF of purified SOD1 A4V. Mostly monomeric protein is observed but also with some dimeric and trimeric protein; these higher order species may be an artifact of using high laser powers on the MALDI-TOF. Concentration of SOD1 A4V was found to be 55μM, ~1mg/mL, as determined by Pierce BCA assay.



ZINC 27780, ZINC 95819 Did Not Stabilize wtSOD1 or SOD1 A4V

Binding of chemical compounds ZINC27780, ZINC95819 and DMSO (as a control) to SOD1 were determined by a melting curve assay using a fluorescent dye, SYPRO orange, and an RT-PCR machine. Neither ZINC27780 nor ZINC95819 appeared to produce a significant increase in the melting temperature of wtSOD1 or SOD1 A4V from 65ºC, (Figure 2A and 2B), however further experiments need to be performed to investigate the compounds ability to stabilize SOD1. In addition, wtSOD1 appears to be more stable than A4V based on the shape of the curve.

Figure 2A: Binding of DMSO, 22780 and 95819 to wtSOD1 in equimolar amounts (1:1) did not appear to increase the melting temperature. The melting temperature of wtSOD1 is approximately 65ºC.

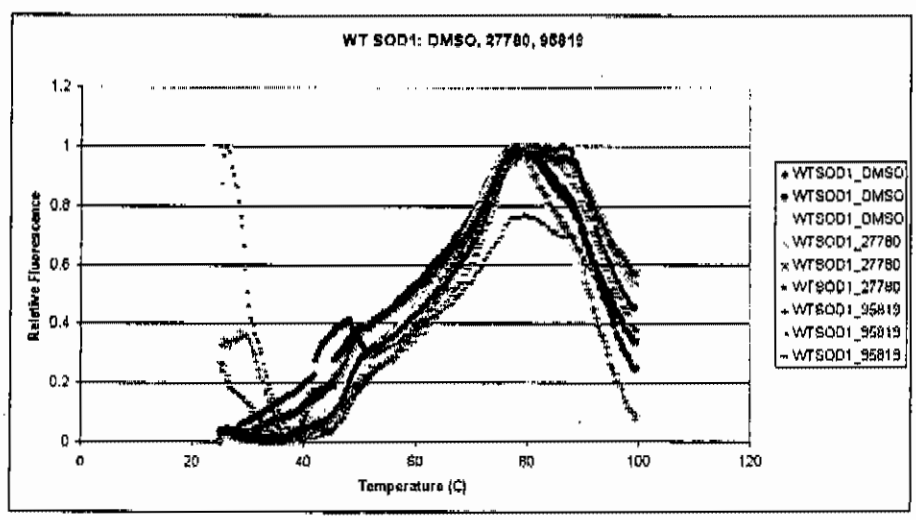
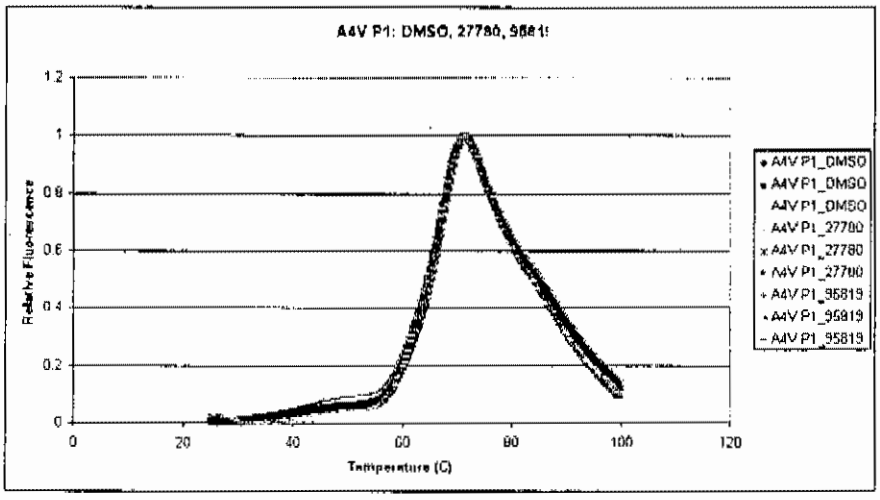


Figure 2B: Binding of DMSO, 22780 and 95819 to SOD1 A4V did not appear to increase the melting point of SOD1 A4V. The melting point of SOD1 A4V is approximately 65ºC.



Cross-linking of wtSOD1 in Equimolar Concentrations Resulted in Minimal Dimer Formation

Cross-linking of two cysteine residues (approximately 9 Angstroms apart) in the SOD1 dimer was performed by incubating wtSOD1 with either DTME or BMOE in equimolar concentrations. The cross-linking was performed in duplicate for each cross-linker and the results were identical. The DMSO control shows the presence of mostly monomeric wtSOD1 (Figure 3A), whereas wtSOD1 incubated in the presence of either BMOE (Figure 3B) or DTME (Figure 3C) showed mostly monomer with a slight increase in dimer. This analysis is complicated by the fact that DMSO masks some of the MALDI signal.

Cross-linking and dimer formation of wtSOD1 has been observed previously using both DTME and BMOE. Therefore, I would have expected dimer formation of wtSOD1, however that is not what I observed. It is likely that the cross-linking reagents were degraded and the experiments should be repeated with fresh cross-linkers.

Figure 3A: wtSOD1 incubated with DMSO in equimolar concentrations for one hour at room temperature shows mostly monomeric protein with some residual dimer present.

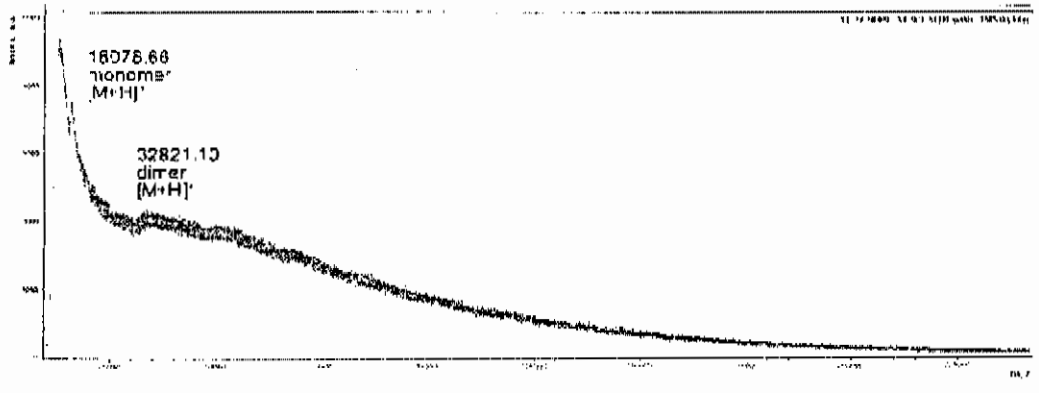


Figure 3B: wtSOD incubated with BMOE in equimolar concentrations for one hour at room temperature shows mainly monomeric protein with a slight increase in dimeric protein.

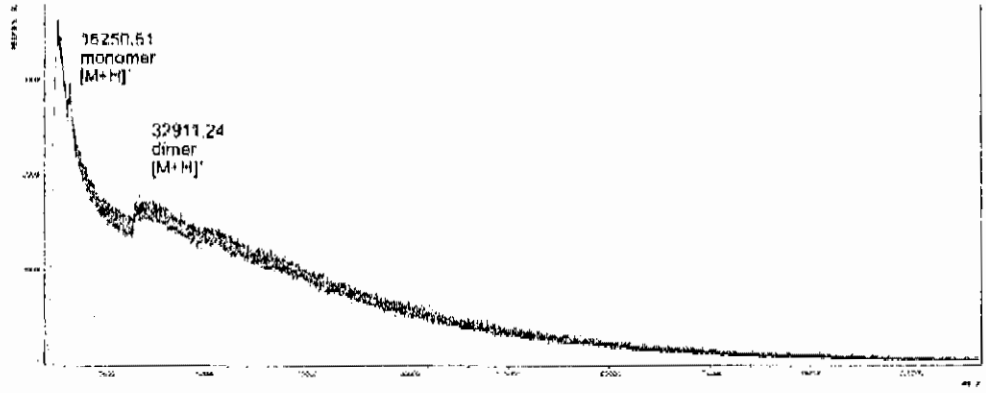
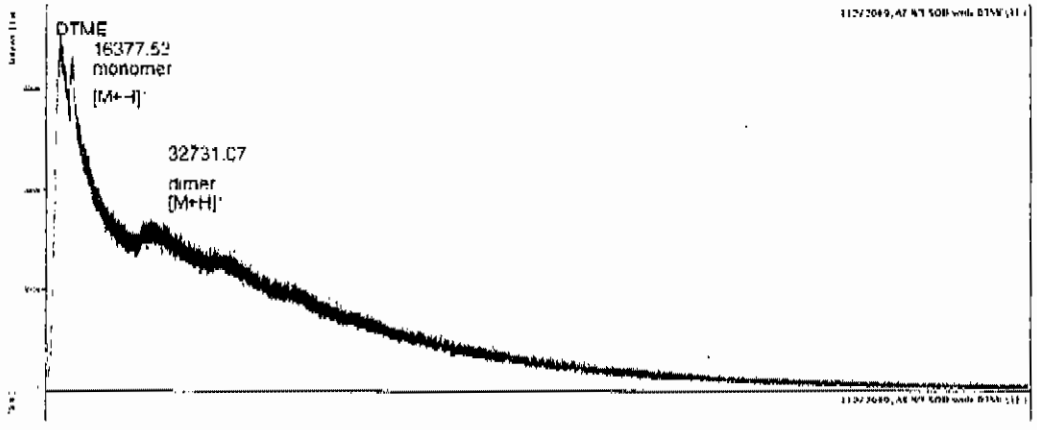


Figure 3C: wtSOD1 incubated with DTME in equimolar concentrations for one hour at room temperature shows mainly monomeric protein with a slight increase in dimeric protein.



Cross-linking of SOD1 A4V in Equimolar Concentrations Resulted in Minimal Dimer Formation

Cross-linking was also performed with SOD1 A4V using DTME and BMOE in equimolar concentrations. The cross-linking was performed twice for each cross-linker and the results were identical. SOD1 A4V incubated with DMSO (control) shows mostly monomeric product with residual higher order oligomers (Figure 4A). Cross-linking of SOD1 A4V with either BMOE (Figure 4B) or DTME (Figure 4C) shows mostly monomer with a slight increase in other oligomeric forms. The increase in other oligomeric forms may be unique to the SOD1 A4V variant as lower amounts of these same oligomers are present in the uncross-linked sample and not seen in the wtSOD1 sample.

Figure 4A: SOD1 A4V incubated with DMSO in equimolar concentrations for one hour at room temperature shows mostly monomeric protein with some residual oligomers.

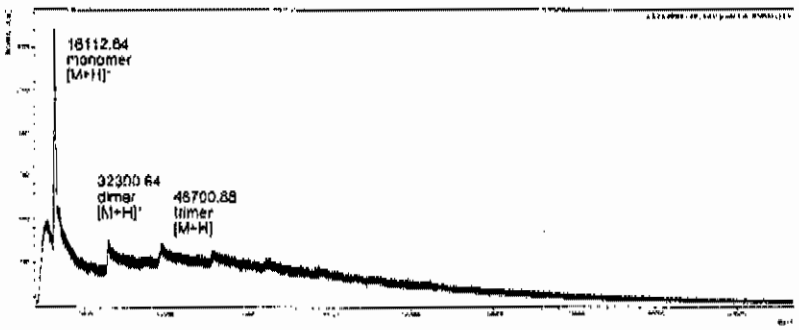


Figure 4B: SOD1 A4V incubated with BMOE at equimolar concentrations for one hour at room temperature shows mostly monomeric protein with an increase in dimer and other oligomeric states such as trimer.

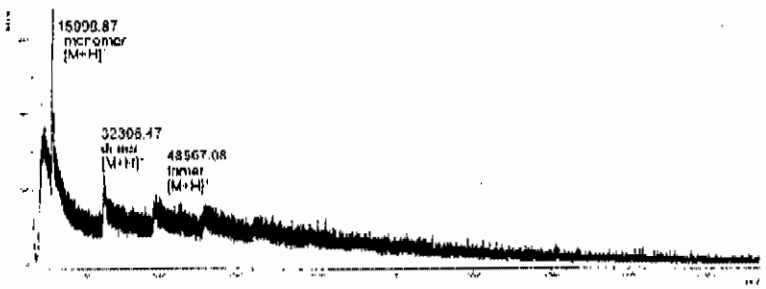
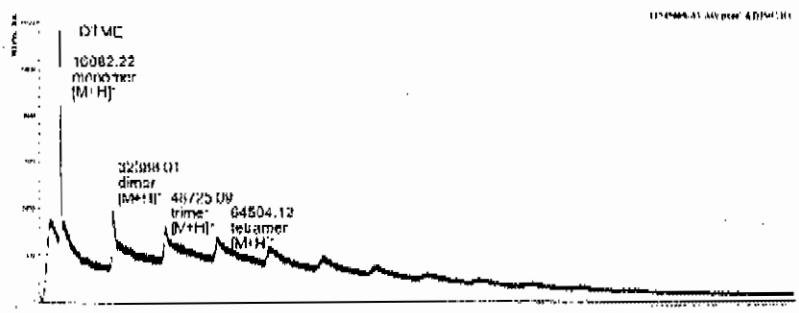


Figure 4C: SOD1 A4V incubated with DTME in equimolar concentrations for one hour at room temperature shows monomeric protein with an increase in dimer, trimer and other higher order oligomers.



Cross-linking of SOD1 A4V at Low Concentrations of Cross-linker Resulted in no Dimer Formation, Higher Concentrations Resulted in Significant Dimer Formation

As a control, when SOD1 A4V was treated with DMSO (.5:1 DMSO to Protein) there was no dimer formed. At a higher concentration of cross-linker (2:1 cross-linker to protein), it was observed that SOD1 A4V treated with BMOE showed an increased dimer formation compared to the DMSO control. Treating SOD1 A4V with DTME at the same cross-linker concentration (2:1), did not increase dimer formation. At even higher concentrations, such as when the cross-linker to protein ratio was tripled and quadrupled, SOD1 A4V treated with both BMOE and DTME showed significant dimer formation. A 5-fold molar excess of cross-linker to protein ratio showed that when SOD1 A4V was treated with BMOE and DTME there was a decrease in dimer formation from the amount seen in both the 3 and 4-fold molar excess cross-linker concentrations. These experiments need to be repeated under the identical conditions used above. Cross-linking was performed twice for each cross-linker and the results were identical.

Figure 5A: SOD1 A4V incubated with DMSO (0.5:1 protein to DMSO) for one hour at room temperature shows monomeric protein.

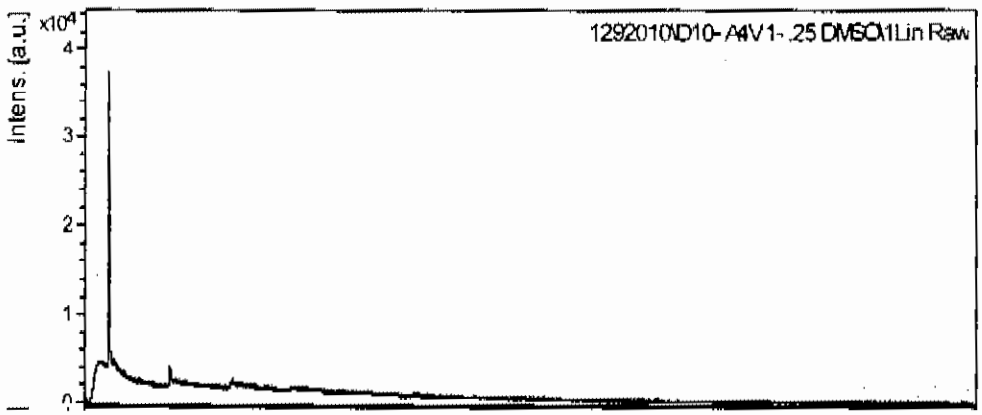


Figure 5B: SOD1 A4V incubated with BMOE (2:1 cross-linker to protein) concentrations for one hour at room temperature shows increased dimer protein.

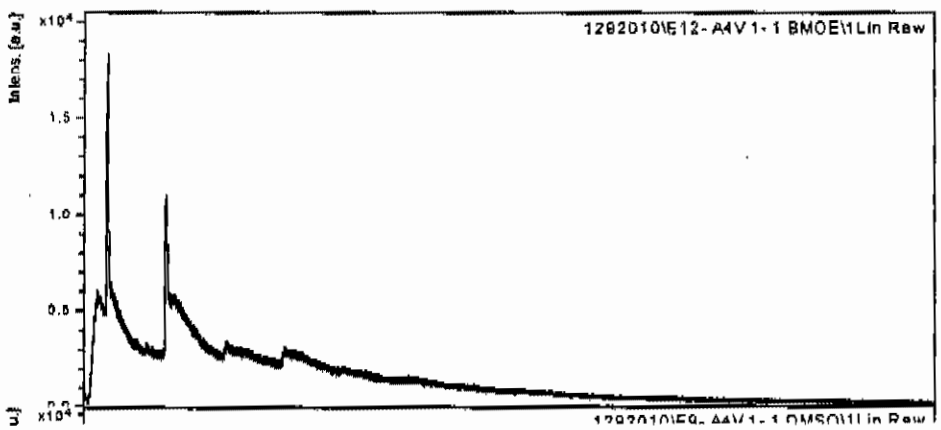


Figure 5C: SOD1 A4V incubated with DTME (2:1 cross-linker to protein) concentrations for one hour at room temperature shows monomeric protein.

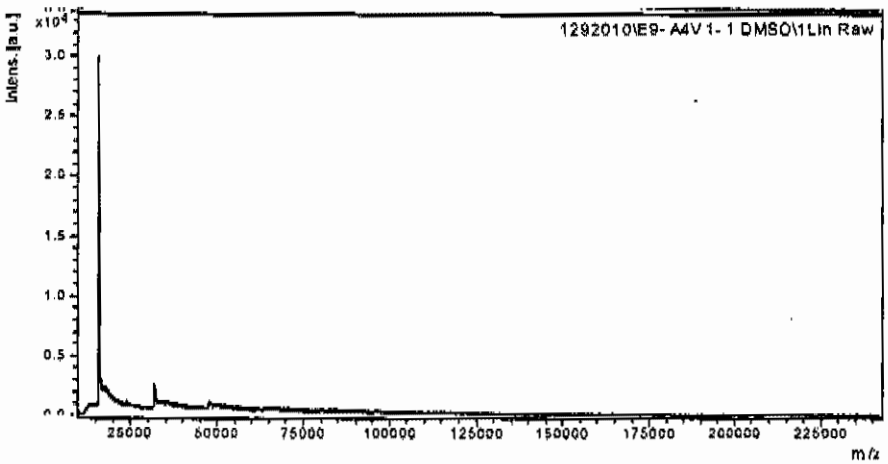


Figure 5D: SOD1 A4V incubated with BMOE (3:1 cross-linker to protein) concentrations for one hour at room temperature shows significant dimer increase and some higher order oligomers.

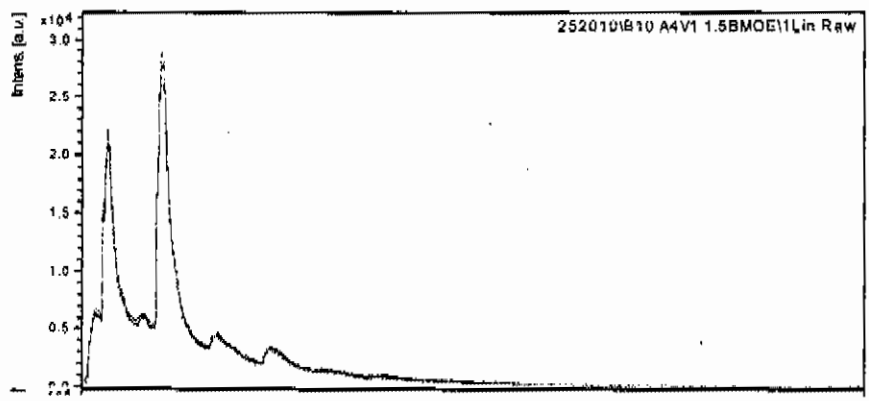


Figure 5D: SOD1 A4V incubated with DTME (3:1 cross-linker to protein) concentrations for one hour at room temperature shows significant dimer increase and increased higher order oligomers.

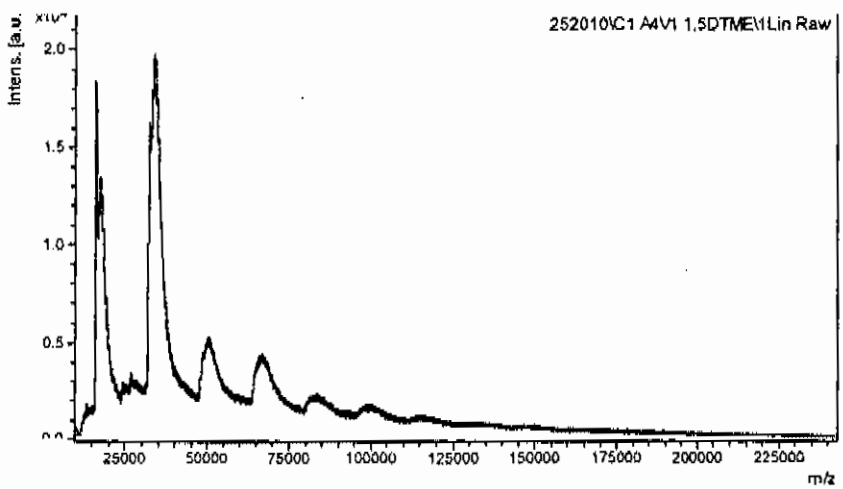


Figure 5E: SOD1 A4V incubated with BMOE (4:1 cross-linker to protein) concentrations for one hour at room temperature shows significant dimer increase and some higher order oligomers.

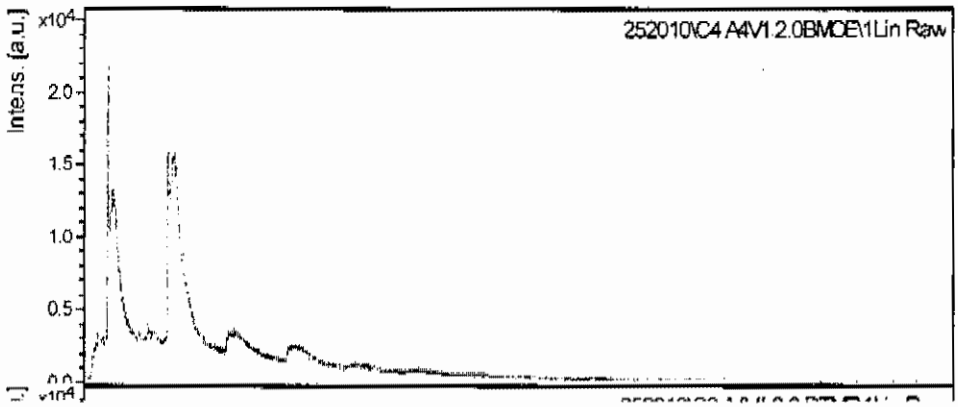


Figure 5F: SOD1 A4V incubated with DTME (4:1 cross-linker to protein) concentrations for one hour at room temperature shows significant dimer increase and some higher order oligomers.

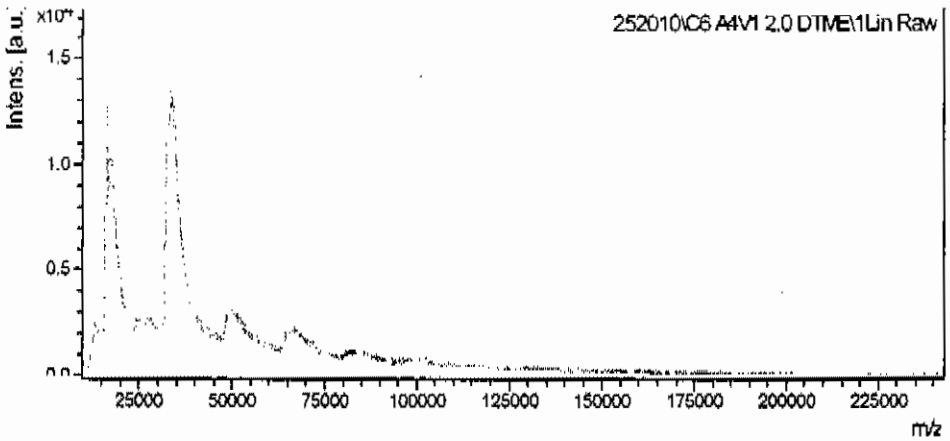


Figure 5G: SOD1 A4V incubated with BMOE (5:1 cross-linker to protein) concentrations for one hour at room temperature shows a decrease in dimer and a decrease of higher order oligomers

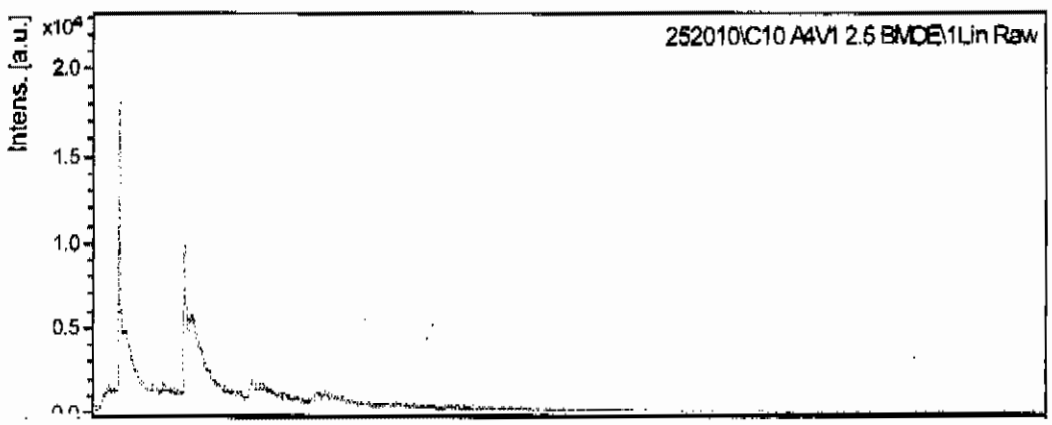
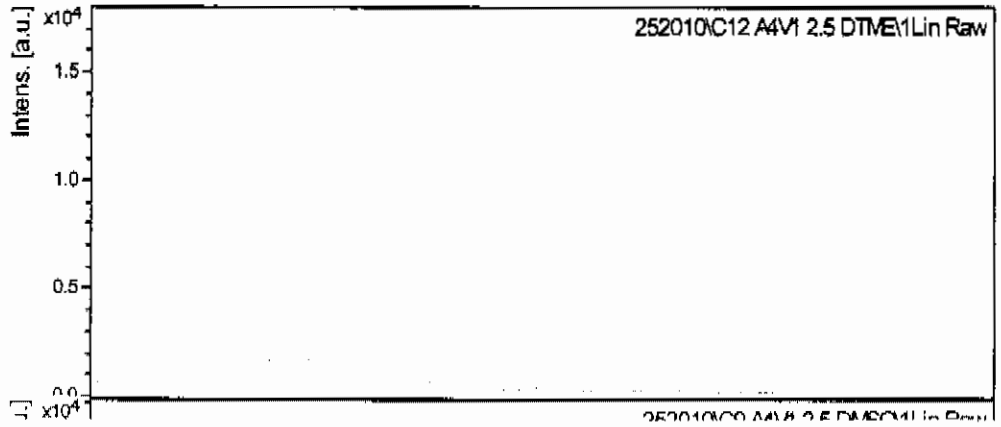


Figure 5H: SOD1 A4V incubated with DTME (5:1 cross-linker to protein) concentrations for one hour at room temperature shows decreased dimer.



Melting Curve Assay shows BMOE and DTME Shift Melting Temperature of SOD1 A4V by ~20 ℃

A melting point assay was performed for certain ratios of cross-linker to protein. It was found that when SOD1 A4V was treated with increasing concentrations of cross-linker (BMOE), there was a noticeable shift in melting temperature. The largest shift in melting temperature was observed when SOD1 A4V was treated with twice as much cross-linker as protein. The temperature appears to shift approximately ~20 ℃ (Figure 6A).

However, when SOD1 A4V was treated with increasing concentrations of DTME, there was no significant shift in melting temperature observed. It can be seen that there is a small shift produced when there was twice as much cross-linker as protein (Figure 6B), but not as dramatic as the shift seen at the same BMOE concentrations.

Figure 6A: SOD1 A4V incubated with increasing concentrations of BMOE shows a significant shift in melting temperature

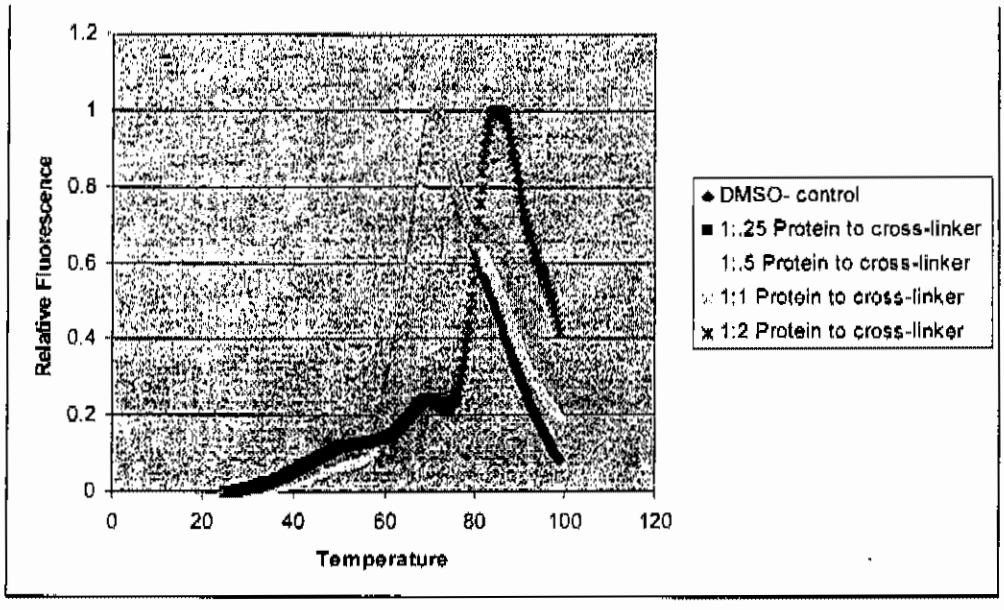
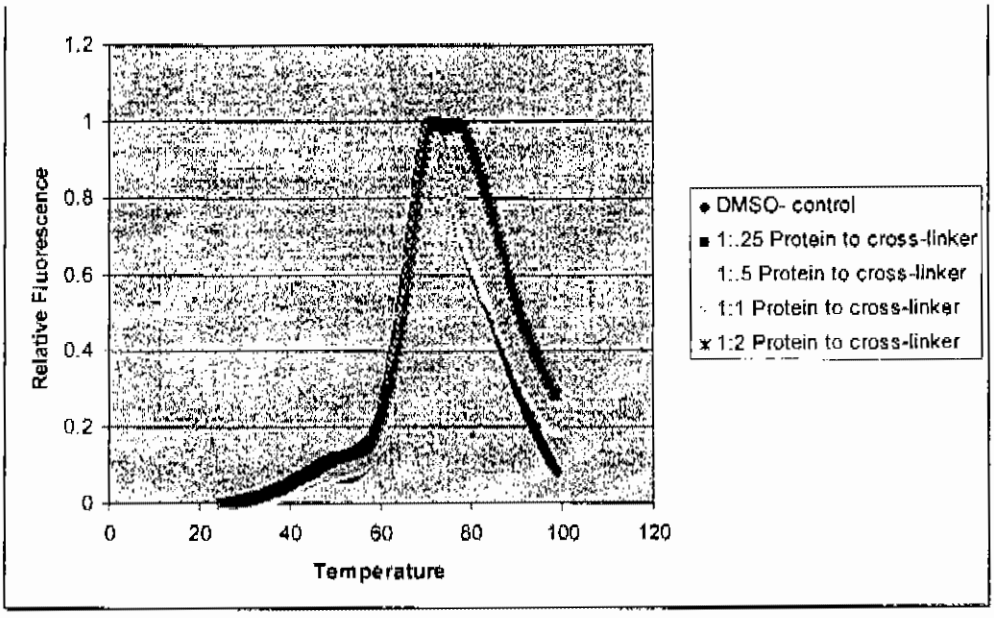


Figure 6B: SOD1 A4V incubated with increasing concentrations of DTME shows a minimal significant shift in melting temperature

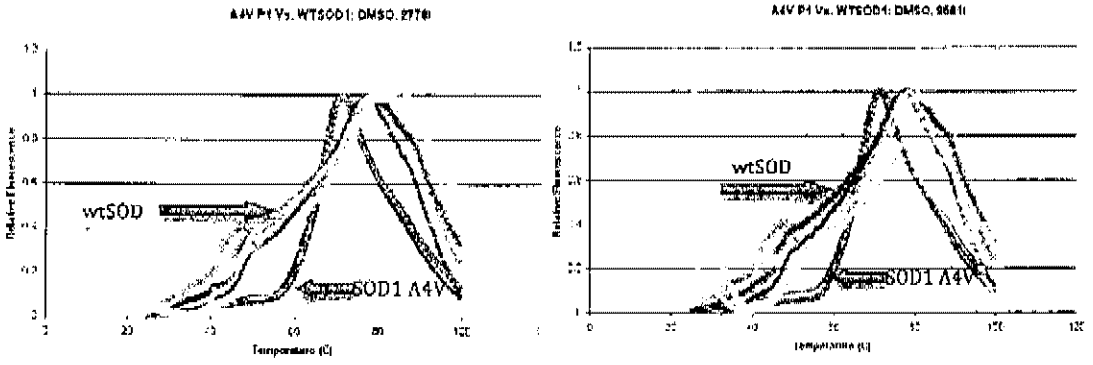


Discussion

Stabilization of SOD1 has been implicated as a method in which to prevent protein aggregation and thus alleviate the ALS disease phenotype. To begin to understand the stability of wtSOD1 and the most prevalent fALS mutant in North America, SOD1 A4V, two experiments were designed to target two distinct regions of SOD1. The first goal was aimed to prevent oxidative post-translational modification by binding chemical compounds (ZINC27780, ZINC51890) to a previously determined target region, W32. The second experiment, focused on increasing stability of the protein by preventing dimer dissociation through cross-linking chemicals (DTME, BMOE) and thereby identifying drug compounds for therapeutic treatment.

In an attempt to prevent oxidative post-translational modification at the W32 site, wtSOD1 and SOD1 A4V were treated with chemical compounds ZINC27780 and ZINC95819 and their interactions with SOD1 were monitored using a melting curve assay. An increase in melting temperature (indicated by a shift to the right) would suggest increased stabilization of the protein. As shown in Figure 2A, treatment of wtSOD1 with DMSO, ZINC27780 and ZINC95819 did not appear to shift the melting temperature from its normal melting temperature of ~65 °C. Similarly, treatment of SOD1 A4V with the same chemicals also did not produce a shift in melting temperature of the fALS varient as seen in Figure 2B. The figures below illustrate that although compounds ZINC 27780 and ZINC95819 did not appear to stabilize wtSOD1 and SOD1 A4V (no shift in melting temperature), they do suggest that wtSOD1 appears to be slightly more stable than SOD1 A4V as shown by the shape of

the melting curve. In the future, this experiment should be repeated multiple times for accuracy and different protein to compound ratios should be investigated. Further, there are approximately 30 additional compounds that should be screened with potential affinity for the W32 region.



The second experiment attempted to stabilize the native SOD1 dimer to prevent dimer dissociation and aggregation. As seen in Figure 2A, the accurate molecular weight of wtSOD1 was not attainable due to the low peak resolution of MALD-TOF. However, it is approximately that of the accepted value, which shows that mainly monomer was present in this control experiment. Incubating wtSOD1 with DMSO in equimolar concentrations yielded mainly monomeric product with an insignificant amount of residual dimer. Treatment of wtSOD1 with BMOE (Figure 3B) and DTME (Figure 3C) with the same concentrations (1:1) showed mainly monomeric protein but with a small increase in dimeric protein. These data suggest that BMOE and DTME promote a minimal increase of dimer formation in wtSOD1 under the conditions (1:1 concentrations) tested.

SOD1 A4V incubated with DMSO in equimolar concentrations (Figure 4A) showed mainly monomeric product, with residual dimeric, trimeric and other higher order oligomers. These higher order oligomers are associated with an increased laser power on the MALDI-TOF, and are not an intrinsic property of the SOD1 A4V mutant. Incubation with DMSO does not cause higher order oligomers according to a previous experiment conducted by Dr. Jared Auclair. Similarily, treatment of SOD1 A4V in a 1:1 ratio of cross-linker to protein with BMOE and DTME also showed mainly monomeric protein with residual higher order oligomers.

Next, I tested other concentrations of BMOE and DTME to determine whether or not these compounds were worth pursuing; both lower and higher concentrations of cross-linker to protein ratios were tested. At low cross-linker to protein ratios were there was half as much cross-linker as protein, only monomeric protein was observed (Figure 5A). Conversely, at certain higher cross-linker concentrations, such as when there was twice as much BMOE as protein, it can be seen that there was a noticeable amount of dimer product (Figure 5B).

Interestingly, this trend did not follow for DTME as no dimer product was observed for the 2:1 cross-linker to protein concentration (Figure 5C). The most significant increase in dimer product was observed when there was a 3-fold molar excess of cross-linker to protein for both BMOE (Figure 5D) and DTME (Figure 5E). At a 4-fold molar excess of cross-linker, there still appeared to be significant dimer formation for both BMOE (Figure 5F) and DTME (Figure 5H), although noticeably less than the amount seen at a 3-fold molar excess. At the highest concentration of cross-linker to protein tested (5:1), it is evident that there was a significant decrease in dimer product for both BMOE (Figure 5G) and DTME (Figure 5H). This decrease can be attributed to the fact that such a high concentration of cross-linker may be denaturing the protein.

To determine whether an increase in dimer formation correlates to an overall increase in protein stability, a melting point assay was performed using RT-PCR. The data shows that at a 2-fold molar excess of cross-linker to protein ratio, BMOE stabilized the protein as indicated by a shift in the melting temperature by almost 20 C°. At a 2-fold molar excess of DTME, there was no such melting temperature shift observed.

These results begin to shed light on the amount of specificity that is required to understand fALS mutants. For example, while it was observed that a 2:1 BMOE to protein ratio increased dimerization, this was not the case for the same concentration of DTME. These observations point to the fact that potential treatment of ALS can be increasingly complicated by both compound and concentration specificity.

Further, the role of DMSO complicates the results of these data. Although DMSO is used as a control (because the cross-linkers are dissolved in it) it dulls the MALDI-TOF signal, which forces us to increase the laser power to be able to read the sample. At a high enough laser power, the laser causes the protein to aggregate which is observed in the data as residual higher order oligomers. This cause and effect of DMSO is necessary to take into consideration when evaluating the results.

In future experiments, it could be worth monitoring the dimerization of SOD1 using an SDS-page gel. In addition to the cross-linking data presented in this study, further testing should be performed using new cross-linking reagents. It may be worth exploring new cross-linkers since it was observed that the maximum dimerization occurred at a 3-fold molar excess of cross-linker (for both BMOE and DMTE) and minimal dimer was observed at both the lower concentration (.5:1 cross-linker to protein) and the highest concentration (5:1 cross-linker to protein). This indicates that it may be very complicated to further optimize these concentrations, if it is possible at all. That being said, BMOE and DTME should be further investigated as potential new drug compounds since they did produce such a profound increased dimer product at a 3-fold molar excess of cross-linker concentration.

In this study, two experiments were designed to target two distinct regions of SOD1. The first goal was aimed to prevent oxidative post-translational modification by binding chemical compounds (ZINC27780, ZINC51890) to a previously determined target region, W32. It was found that neither of those

compounds appeared to stabilize the protein. The second experiment, attempted to increase the stability of the protein by preventing dimer dissociation through cross-linking chemicals (DTME, BMOE) and thereby identifying drug compounds for therapeutic treatment. It was found that the greatest dimer product was observed when there was a 3-fold molar excess of cross-linker to protein for both BMOE and DTME. Finally, it was shown that a 2:1 BMOE to protein concentration stabilized the protein by ~20 °C.

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