Characterization of the oligomeric state
of amyloid proteins

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
In Biomedical Engineering

By

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ABSTRACT OF THE THESIS

Characterisation of the oligomeric state
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Master of Science in Biomedical Engineering
University of California, Los Angeles, 2012
Professor David Eisenberg, Co-Chair
Professor Matteo Pellegrini, Co-Chair

Conversion of soluble amyloid proteins into their fibrillar form has been postulated to progress through various intermediate stages which are only stable transiently, and the final fibrillar form is the most stable and thus the easiest to characterize. Oligomers have been characterized as two types namely soluble oligomers and the fibrillar oligomers[1]. The soluble oligomers are intermediates that form as the soluble monomers progress into mature fibrils. Previous attempts at characterizing these oligomers have been limited to indirect methods such as circular dichroism, FTIR and electron microscopy. But the recent characterization of an 11-residue peptide from alpha-B-crystallin by X-ray crystallography is the first detailed structure of a toxic amyloidogenic peptide segment in its oligomeric state[2]. We
hypothesize that this structure, termed cylindrin, is a common oligomeric state of other amyloid proteins. Using this structure as a model, we identified various peptide segments in more than five other amyloid forming proteins including alpha synuclein, tau, abeta and IAPP. In this work I characterized five segments from alpha synuclein and superoxide dismutase 1 (SOD) that were predicted to form cylindrins. The segments were found to have beta sheet structure by circular dichroism and a dimeric oligomeric state by static light scattering. They were also found to be toxic to cultured cells. This study provides preliminary evidence of cylindrin like oligomeric state of amyloid segments and further studies are required to validate our hypothesis.
The thesis of Smriti Sangwan is approved.

Hong Zhou

Matteo Pellegrini, Committee Co-Chair

David Eisenberg, Committee Co-Chair

University of California, Los Angeles

2012
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A special thanks to Angie and Anni, whom I look up to as my mentors in the lab.
**Introduction**

Produced as a linear, flexible, and initially disordered polypeptide chain, proteins need to be folded in a particular 3-dimensional form to be functionally active. This difference between the unfolded and folded state of a protein is often attributable to several diseases as well [3]. More than 20 different diseases including Alzheimer’s and Parkinson’s that are associated with ageing are often characterized by important well-folded proteins reverting back to the unfolded state or getting ‘Misfolded’ which leads to their aggregation and formation of fibrillar deposits.

The fibrillar form is also found in several functionally relevant states such as in bacterial biofilms. However, more often it is the found in a number of currently incurable diseases [4]. Alzheimer’s disease alone afflicts nearly 5.4 million people in America according to recent estimates and almost 27 million people worldwide. It is characterized by the formation and accumulation of toxic proteinaceous deposits in brain of Abeta protein [5, 6]. Parkinson’s is another deadly disease associated with old age and dementia. Currently nearly 10 million people are thought to be suffering from it worldwide. It is also characterized by toxic amyloid deposits in which the major component is alpha synuclein. The majority of neurodegenerative diseases are also caused by protein misfolding which leads to accumulation of aggregated proteins [7].

Amyloids by definition are insoluble protein aggregates, fibrillar in nature caused mainly by misfolding of the naturally occurring proteins [8]. The misfolding caused by either mutations or alternations in protein environment, causes protein molecules to form aggregates. In some cases the deposits are extracellular whereas in others it is intracellular[9]. These deposits in patients may be systemic (for eg. transthyretin and lysozyme) or organ specific (for eg. insulin and IAPP) [4].
The Nomenclature Committee of the International Society of Amyloidosis has currently designated 27 proteins to be amyloidogenic that are capable of forming amyloid deposits[4]. Some of the most extensively studied of these are alpha synuclein (Parkinson’s), abeta (Alzheimer’s), tau (Alzheimer’s), Superoxide Dismutase 1 (SOD1, Amyotrophic lateral sclerosis), PrP (transmissible spongiform encephalopathies) and lysozyme (systemic amyloidosis). Recently, some more proteins such as LECT2- a chemotactic factor made by liver cells and crystallin, the eye lens protein have been found in amyloid deposits thus increasing speculation that more proteins than initially thought are capable of misfolding and thus leading to disease [4, 10-12].

These amyloid deposits, regardless of protein sequence, length, organ affected or age, have several signature properties which are used by pathologists and biochemists alike for their identification. First, the amyloid deposits are isolated from tissue. Second, they bind to the dye Congo red and have a characteristic green birefringence when viewed under polarized light. Last, amyloid fibrils have a characteristic cross-beta diffraction pattern (Figure 1)[8]. The pattern consists of two perpendicular arcs- one diffuse, equatorial arc at 10 Å spacing (X axis) which corresponds to the distance between the two beta-sheets. There is also a sharper meridional arc at 4.7 Å (Y axis) which corresponds to the distance between the beta-strands forming the sheets. The "stacks" of beta sheet are short and traverse the breadth of the amyloid fibril; the length of the amyloid fibril is built by these strands aligned against each other [13, 14]. The fibrillation process can be followed by measuring fluorescence of Thioflavin T, a dye which is known to give a characteristic fluorescent signal when bound to amyloid fibrils. Changes in protein concentration, pH, temperature, can cause native proteins to form fibrils, whose progress can be monitored by measuring the Thioflavin T fluorescence. In general, protein fibril formation is a sigmoidal shaped curve with an initial lag phase (no dye binding) followed by a log phase in which the fluorescence increases and finally stabilizes.
Figure 1 Cross B diffraction pattern by amyloid fibers. Figure taken from ref. [5]

Previous work by several research groups has identified the core of the fibrils formed by different amyloid proteins. It has been found that the entire sequence is not required for fibrillation and generally a short segment with high propensity to form fibrils that can be four residues or longer can form the steric zipper structure and are sufficient to make the core of the amyloid fibril [13-15].

There is growing evidence that it is not the fibrillar form which is the toxic species and the root cause of disease but the intermediate states as the folded protein transitions into the unfolded state during the lag phase of ThT binding. These intermediate forms include oligomers, protofibrils and annular aggregates [16]. For Example, Abeta has been previously shown to exist in various oligomeric forms such as dimers, trimers and other higher multimeric forms. These oligomers were also shown to be more toxic than monomers or the mature fibrils [17].
With the recent structural characterization of the oligomeric species of Alpha b crystallin and Abeta to be cylindrin and beta barrel respectively, the structural features of oligomers needs to be further validated in other amyloid proteins [2, 19]. Cylindrin is the name given to the hexameric structure adopted by a segment of the Alpha B Crystallin, with amino acid sequence KVKVLGDVIEV, which binds to A11 antibody (oligomer specific antibody) and is toxic to cells. The structure of the eleven residue peptide has shed light on the possible toxicity mechanism of amyloid proteins. Cylindrin structure can be also used as a starting point for structural prediction of segments from other proteins that form oligomers with similar properties to the cylindrin oligomer.

The cylindrin structure has some commonalities as well as some differences as compared to the steric zipper structure of mature fibrils. Both the steric zipper and the cylindrin are formed of polypeptide chains in beta strand conformation. The difference comes in the alignment of these strands. The steric zipper structure is made up of two self-complementary beta sheets both of which are in register. The beta strands that stack onto each other to form one sheet mate with an identical strand in the opposing sheet, forming a very tight interface. In the cylindrin, the shape is very different as two beta strands form anti parallel dimers, which then assemble with two more dimers to form a cylindrical shaped

Figure 2 Progression of an amyloid protein from the folded state into a fibril. Figure taken from Ref. [18]
barrel having a three-fold axis. Both the steric zipper structure and the cylindrin have a dry interface. In
the zipper it is made up of the side chains of adjacent sheets which lock into each other. However, in
the cylindrin, it is the core of the barrel which has the apolar side chains protruding inwards. The shape
complementarity is high for both types of structures, 0.86 for the steric zipper of GNNQQNY from
Sup35 and 0.75 for the cylindrin [2], indicating the high stability of both types of structures with the
steric zipper perhaps being slightly more stable. Both structures have extensive hydrogen bonding
within one sheet for the steric zipper and with the adjacent strands for the cylindrin. The
characterization of the steric zipper structure of 6 residue sequence (G6V) from the 11 residue (K11V)
cylindrin also points towards the ability of the same sequence to form either type depending on the side
residues, the environment and other as yet unknown factors.

![Figure 3](image.png)

*Figure 3 Structure of the 11 residue K11V segment from alpha B crystalline (A) and the stretched out sheet
model (B). Figure taken from Ref [2]*

Since the steric zipper is a common structural motif of the mature fibrils, it may be reasonable to
hypothesize that the cylindrin is the basic structure adopted by the pre-fibrillar intermediates of other
amyloid proteins.
Native SOD1 is a 32kDa metalloenzyme involved in the catalysis of dismutation of superoxide radical to dioxygen and hydrogen peroxide [20]. It has been reported that wild type (WT) human SOD1, when lacking metal ions and in reduced state, forms amyloid-like aggregates in solutions which remain stable for a long time (months) even when exposed to air and at 37°C, pH 7, and 100 mM protein concentration that is physiological conditions [20]. Dimers were detected by mass spectrometry and higher molecular weight aggregates were detected by light scattering [20]. These oligomers have been predicted to have a critical role in the pathway to amyloidosis as they could bind thioflavin T and the non metallated protein could not. The SOD1 also has many segments in its sequence which were predicted to form fibrils (Figure 4) [21]. Thus, this protein was chosen for this study as oligomerization in the non metallated and reduced form seems to be a prerequisite for fibrillation. 

Figure 4 Fibrillation propensity profile of human SOD1. Vertical axis is the energy (in kcal/mol) of the segments and horizontal axis the sequence of SOD1. Red bars indicate the starting amino acid from where a six residue segment has less than -23 kcal/mol rosetta energy, hence higher fibrillation propensity.

Alpha-synuclein is another amyloid-forming protein, this one associated with Parkinson’s disease [22]. It is a 14kDa protein mainly found in brain tissues and involved in vesicle trafficking regulation where it associates with membranes and interacts with SNARE complexes[23]. It is the main component of amyloid deposits found in patients with Parkinson’s disease. As seen from Figure 5 there are several
segments in alpha-synuclein that are predicted to form fibrils. Many of these segments have been characterized structurally and have a characteristic steric zipper form [24].

![Fibrillation propensity profile of Alpha Synuclein](image)

*Figure 5 Fibrillation propensity profile of Alpha Synuclein. Vertical axis is the energy (in kcal/mol) of the segments and horizontal axis the sequence of alpha synuclein. Red bars indicate the starting amino acid from where a six residue segment has less than -23kcal/mol rosetta energy, hence higher fibrillation propensity.*

Recently, the structures of different segments from alpha-synuclein were characterized [24]. These segments were found to structurally exist in coils very different from the alpha-helical form of the native protein [25]. Various studies using atomic force microscopy and electron microscopy have shown that alpha-synuclein forms globular soluble oligomers with approximately 30% beta sheet content which further increases to 60% in the fibril form [26]. Furthermore, alpha-synuclein oligomers have been shown to be toxic in vivo [27]. In a separate study it was shown that oligomers from recombinant protein were less toxic than those in vivo [28]. It thus becomes imperative to study this protein and its intermediate oligomeric state.

The goal of this project is to find and characterize segments in alpha synuclein and SOD1 that form cylindrin like oligomeric species and determine whether these predicted cylindrin-like segments adopt a similar structure to the alpha B crystallin oligomer.
Materials and Methods:

Identification of potential Cylindrin forming segments:

To find possible cylindrin-forming sequence segments, an approach analogous to the Rosetta profile method [29] was used by Dr. Rebecca Nelson and Dr. Lukasz Goldschimdt. The sequences of the selected toxic amyloid proteins were threaded onto the backbone structure of the cylindrin using the modeling program PyRosetta[30], and the energy of each of the resulting model structures was calculated. Various parameters were strained or relaxed to get the best overall fitting sequences. In total, 15 segments from A-beta, alpha-synuclein, PrP, IAPP, and SOD1 were identified and 6 were further characterized in this work.

Cloning and Expression:

The tandem repeat cylindrin peptide sequences were codon optimized for E. coli and designed using DNAWorks by Bobby Sahachartsiri from our lab. They were constructed using PCR-based gene synthesis as described previously[31]. The synthetic gene was PCR amplified with Platinum Pfx polymerase (Invitrogen, Carlsbad, CA) with the N-terminal primer containing a SacI restriction and TEV protease site, and a C-terminal primer containing a stop codon and XhoI restriction site. Agarose gel purified PCR product was extracted using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Gel purified PCR product and custom vector, p15-MBP (made previously in our lab which is a custom vector constructed from the NdeI and XhoI digestion products pET15b (Novagen, Gibbstown, NJ), and the maltose binding protein (MBP) gene from pMALC2X (New England Biolabs, Ipswich,
MA). This results in an N-terminal His-tag MBP fusion vector, which was digested with SacI and XhoI according to manufacturer’s protocol (New England Biolabs, Ipswich, MA). Digested vector products were gel purified and extracted. DNA concentrations were determined using BioPhotometer UV/VIS Photometer (Eppendorf, Westbury, NY). A ligation mixture was performed using a Quick Ligation kit (New England Biolabs, Ipswich, MA) according to manufacturer protocol and transformed into E. coli cell line TOP10 (Invitrogen, Carlsbad, CA). Several colonies were grown overnight, and plasmids containing the synthetic gene were purified using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The final construct - p15-MBP-K11V-TR was sequenced prior to transformation into E. coli expression cell line BL21 (DE3) gold cells (Agilent Technologies, Santa Clara, CA).

**Purification:**

A similar purification strategy was used for all the targeted peptides and is described below:

**Expression**

I grew the primary culture by inoculating a single colony in 100 ml of autoclaved LB miller broth (fisher Scientific) supplemented with 100μg/ml ampicillin and growing it overnight at 37°C with shaking at 225 rpm. I then inoculated 1L of autoclaved LB supplemented with ampicillin at 100μg/ml at 3% with the primary culture and grown at 37°C. The culture was induced with 0.5mM IPTG after it reached an OD₆₀₀ of 0.5-0.6 and grown at 32°C for 4 hours. The OD₆₀₀ was measured using a BioPhotometer UV/VIS spectrophotometer. The cells were harvested by centrifugation at 5000 x g for 15 minutes at 4°C. The pellet was stored at -80°C until purification.
Purification Protocol

A two-step purification protocol was used by me including initial purification using Ion Metal Affinity Chromatography followed by Reversed-Phase (RP) HPLC and finally lyophilization.

The cell pellet was thawed on ice and resuspended in 4ml per gram of Buffer A (50mM Sodium Phosphate, 300mM Sodium chloride, 20mM Imidazole pH 8.0) supplemented with 100µl per L of cell pellet of Protease Inhibitor Cocktail (Halt EDTA free Thermo Scientific). Resuspended cells were disrupted by sonication at 85% amplitude with 6 sec on 6 sec off pulses for 4 minutes maintaining the temperature at less than 15°C. The cell debris was removed by centrifugation at 15000X g for 30minutes at 4°C.

The supernatant was filtered using a 0.2µm filter and loaded onto 5ml HisTrap-HP column (GE Healthcare) equilibrated with Buffer A. After binding, the His-Trap column was washed with 5 column volumes of Buffer A to remove the weakly-bound impurities. The protein was eluted by running a linear gradient (0%-100%) over 40ml of Buffer B (50mM Sodium Phosphate, 300mM Sodium chloride, 500mM Imidazole pH 8.0). The Protein eluted at 50% Buffer B and peak fractions were pooled and dialyzed using a 10-MWCO- dialysis cassette (Thermo fisher Scientific) overnight at 4°C in Buffer C (25mM Sodium Phosphate, 200mM Sodium Chloride and 20mM Imidazole pH 8.0).

The dialyzed protein sample was pooled and cleaved with a TEV protease stock at 1:100 OD280nm ratio for 4hrs at room temperature. The digested protein was loaded onto a 5ml Histrap-HP column equilibrated with buffer A to remove the His-tagged MBP. I then collected the peptide of interest in the flow through and filtered with a 0.22µm filter.
In the last step of purification, I purified the peptide to 95% purity by reverse phase HPLC (RP-HPLC) by loading on a 2.2 x 25 cm Vydac 214TP101522 column equilibrated with 8% buffer Y (Acetonitrile/0.1% TFA). The peptide was eluted by running a linear gradient of Buffer Y (8%-100%) over 40mins at a flow rate of 9ml/min. The peptide eluted at 34% buffer Y and was detected by measuring 220nm Absorbance using a Waters 2487 dual λ absorbance detector. The pooled fractions were checked for purity using MALDI-TOF mass spectrometry. Fractions containing the peptide of interest were lyophilized and stored at -20°C. The yield was about 5 mgs per/ L of cell culture.

Purification of Alpha Synuclein (68-78) (18)

The protocol described above was used initially but the peptide is very prone to aggregation and forms insoluble aggregates. The purification protocol was further optimized by adding 20% glycerol in Buffer A and B and C. Glycerol helped in increasing peptide solubility but it was not suitable for HPLC. The protocol is further being optimized by dissolving the aggregated peptide in 6M Guanidinium Hydrochloride.

Purification of Alpha-Synuclein (63-73) (17)

The general protocol described above was optimized with the RP-HPLC being done using a vydac column maintained at 80 °C and immediately lyophilized as the peptide aggregated in pH 6 at room temperature.
Crystallization:

The lyophilized peptides (construct 14 asyn (46-73), construct 16 asyn (68-98), construct SOD 19 (31-41), SOD 20 (33-43) were resuspended in 50mM Tris pH 8.0 at 7.5mg/ml and filtered using a 0.1 μm Centrex MF filter. Filtered peptide was used for setting up crystal trays. Initial screening was done using six commercially available screens namely Crystal Screen, Index, Salt and wizard, PACT. Initial hits were obtained in Index (Hampton Research) #E9 (0.05 M Ammonium sulfate, 0.05 M BIS-TRIS pH 6.5, 30% v/v Pentaerythritol ethoxylate (15/4 EO/OH)) and #C9 (1.1 M Sodium malonate pH 7.0, 0.1 M HEPES pH 7.0, 0.5% v/v Jeffamine ® ED-2001 pH 7.0) and are currently being optimized.

Oligomer characterization by Static Light Scattering and Circular Dichroism:

Secondary structure determinations were done using a JASCO J-715 Circular Dichroism spectrophotometer. 200ul of peptide dissolved at 0.2mg/ml in water or 10mM LiOH and measured in a 1mm cuvette. The data obtained was corrected for the buffer (50mM Tris pH 8.0). The following parameters were for the measurements: scanning range- 260nm-190nm, 800 data points collected at 20 nm/min and 4 scans per sample.

ThT Fiber Assay conditions:

Lyophilized peptides were dissolved in Buffer K (5mM Lithium Hydroxide, 20mM Sodium Phosphate, 100mM Sodium Chloride) and filtered through 0.1um filter. 10μM Thioflavin T was added to the
filtered peptides and immediately assayed. Samples (200 μL) with ThT were incubated at 37 oC in black clear 96-microwell plates that were sealed to prevent evaporation. The ThT fluorescence of each sample was recorded every 5 min using a VarioSkan microplate reader (Thermo Scientific.) with 444nm excitation. Emission was measured at 482nm.

**Fibril Formation and Electron Microscopy:**

Grids for EM were prepared from peptide samples which were either freshly solubilized or incubated at 37C with shaking for 5 days. 5 μL were spotted directly on freshly glow-discharged carbon-coated electron microscopy grids (Ted Pella, Redding, CA). After 3 min incubation, grids were rinsed twice with 5-μL distilled water and stained with 1% uranyl acetate for 1 min. Specimens were examined in a T-12 electron microscope at an accelerating voltage of 80 kV. Images were recorded digitally by wide angle (top mount) BioScan 600W 1 × 1K digital camera (Gatan, Pleasanton, CA).

**Cell Culture and Viability Assays:**

Cell viability was investigated using a Cell Titer 96 aqueous non-radioactive cell proliferation assay kit (MTT) (Promega cat. #G4100). HeLa and PC12 were used to assess the toxic effect of cylindrin peptides. HeLa cells were cultured in DMEM medium with 10% fetal bovine serum. PC-12 cells were cultured in ATCC-formulated RPMI 1640 medium (ATCC; cat.# 30-2001) with 10% heat-inactivated horse serum and 5% fetal bovine serum. Cells were maintained at 37 °C in 5% CO2. For all toxicity experiments, 96-well plates (Costar cat. # 3596) was used. HeLa and PC-12 cells were plated at 10,000 cells per well. Cells were cultured for 20h at 37 °C in 5% CO2 prior to addition of peptide samples. 10 μl of sample was added to each well containing 90 μL medium, and allowed to incubate for 24h prior to the addition of 15 μl Dye solution (Promega. cat. #G4102) into each well, followed by incubation for
4h at 37°C in 5% CO2. After incubation, 100 µl solubilization Solution/Stop Mix (Promega cat. #G4101) was added to each well. After 12h incubation at room temperature, the absorbance was measured at 570nm. Background absorbance was recorded at 700nm. Each of the experiments was repeated 2 times with 4 replicates per sample per concentration.
Results

Identification of Cylindrin like segments in other amyloid proteins:

Using the PyRosetta software, various targets in different amyloid proteins such as Amyloid-beta, alpha synuclein, SOD, tau and PrP were identified. Several unique features of the cylindrin structures were used to improve outcome of the computational prediction. The core of the cylindrin is made up of aliphatic amino acid side chains. A glycine at position 6 is required for cylindrin formation. Based on these analyses, the sequences (Table 1) from alpha synuclein and SOD1 were identified to potentially form a cylindrin like structure. For purification, the cylindrin-like segments were expressed as tandem repeats with different linkers. For the SOD1 sequences, a short double glycine was added between the repeats as done for the original cylindrin K11V sequence. For the alpha-synuclein sequence, longer linkers different in sequences were chosen.

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Amino Acid Sequence</th>
<th>No.of residues</th>
<th>Mol Wt.</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD1(31-41)-TR Construct 19</td>
<td>gKVWGSIKGLTEggKVWGSIKGLTE</td>
<td>25</td>
<td>2588.00</td>
<td>9.53</td>
</tr>
<tr>
<td>SOD1(33-43)-TR Construct 20</td>
<td>gWGSIKGLTEGLggWGSIKGLTEGL</td>
<td>25</td>
<td>2473.81</td>
<td>6.14</td>
</tr>
<tr>
<td>α-Syn(63-73)-TR Construct 17</td>
<td>gVTNVGGA VVTGggVTNVGGA VVTG</td>
<td>25</td>
<td>2099.33</td>
<td>5.52</td>
</tr>
<tr>
<td>α-Syn(68-78)-TR Construct 18</td>
<td>GAVVTGVTA VAggGAVVTGVTA V</td>
<td>24</td>
<td>1984.28</td>
<td>5.52</td>
</tr>
<tr>
<td>α-Syn(46-73)-TR Construct 14</td>
<td>gEGVVHVGVATVAektkeqVTNVGGA VVTG</td>
<td>29</td>
<td>2794.11</td>
<td>5.50</td>
</tr>
<tr>
<td>α-Syn(63-91)-TR construct 15</td>
<td>gVTNVGGA VVTGvtavaqqtVEGAGSIAAA</td>
<td>30</td>
<td>2655.99</td>
<td>6.00</td>
</tr>
<tr>
<td>Construct 16</td>
<td>GAVVTGVTAVAqktvegagsIAAATGFVKKD</td>
<td>31</td>
<td>2904.31</td>
<td>8.50</td>
</tr>
</tbody>
</table>

*Table 1: List of cylindrin targets. Lower case amino acids in the sequences refer to extra aminoacids or linker residues. Upper case refer to the sequence from amyloid proteins. Molecular Weights and pI were calculated using PROTPARAM tool from Expasy.*

Secondary Structure by Circular Dichroism:

The secondary structure measurements showed that the constructs had significant beta sheet content in solution at room temperature and in 50mM Tris pH 8(Figure 6). All the constructs consisted of approximately 50% beta sheet using the selcon software (Table 2). In comparison to the K11V cylindrin the spectrum was very similar in solution but the measurements in the crystal form are much higher with more than 80% beta sheets.

![Circular Dichroism measurement of different constructs at 0.2mg/ml at Room Temperature in 50mM Tris pH8.0. The vertical axis measures the ellipticity at different wavelengths plotted on the horizontal axis.14asyn is syn (46-73), 17asyn is syn (63-73), 15asyn is syn (63-91), 20SOD is SOD (33-43)](image)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Alpha Helices (in %)</th>
<th>Beta Sheets (in %)</th>
<th>Random coil (in %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 (α-Syn(46-73))</td>
<td>8</td>
<td>48</td>
<td>44</td>
</tr>
<tr>
<td>17 (α-syn(63-73))</td>
<td>4</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>15 (α-syn(63-91))</td>
<td>8</td>
<td>49</td>
<td>43</td>
</tr>
<tr>
<td>16 (α-syn(68-98))</td>
<td>1.2</td>
<td>28</td>
<td>70</td>
</tr>
<tr>
<td>20 (SOD1(33-43))</td>
<td>8</td>
<td>48</td>
<td>44</td>
</tr>
<tr>
<td>K11V-TR*</td>
<td>0</td>
<td>81</td>
<td>19</td>
</tr>
</tbody>
</table>

Table 2: Secondary structure determination in different sequences by CD (using Selcon software).* K11V secondary structure measurements taken from crystal structure in ref [2].
**Crystallization:**

All the purified constructs are currently in crystallization trials. Solubility tests were done using 5 different buffers namely water, 50mM tris pH 8.0, 10% glycerol, 20% glycerol, 10mM lithium hydroxide and at different concentrations.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Buffer used for dissolving</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A syn (46-73)</td>
<td>50mM Tris pH 8.0</td>
<td>5mg/ml</td>
</tr>
<tr>
<td>A syn (68-98)</td>
<td>10mM LiOH</td>
<td>5mg/ml</td>
</tr>
<tr>
<td>A syn (63-73)</td>
<td>20%glycerol</td>
<td>3mg/ml</td>
</tr>
<tr>
<td>SOD (31-41)</td>
<td>Water</td>
<td>5mg/ml</td>
</tr>
<tr>
<td>SOD (33-43)</td>
<td>Water</td>
<td>5mg/ml</td>
</tr>
</tbody>
</table>

*Table 3: Buffers used to solubilize different constructs after testing for various buffers.*

I obtained some initial crystallization hits with construct Asyn (46-73) in Index E9 and Index C9. These crystals shown in figure 7 and 8 are currently being optimized.

*Figure 7 Crystals of a syn 14 obtained in Index E9 approximately 500µm in size.*

*Figure 8 Crystals obtained of asyn 14 in Index C9 approximately 200µm size.*
Cell Culture and Viability Assays:

Toxicity of the constructs was tested on PC12 and HeLa cells at different concentrations. Abeta protein was used as positive control at 0.5µM. As compared to Abeta, Asyn (63-73) (syn17) and Asyn (68-98) (syn16) were found to be toxic in the freshly solubilized form as well as the fibrillar form at 10µM and 50µM in a dose dependent manner as higher toxicity was seen with higher peptide concentration. Asyn (46-73) (syn14) was found to be non-toxic in freshly solubilized form but the fibrillar form was toxic.

Figure 9 Toxicity of the peptides on HeLa cells at various concentrations. Orange bars indicate freshly solubilized peptides and blue bars indicate fibrillized peptides. Abeta was used as positive control at 0.5µM.
Figure 10 Toxicity of the peptides on PC12 cells at various concentrations. Orange bars indicate freshly solubilized peptides and blue bars indicate fibrillized peptides.
ThT Fiber Assay:

Construct 14 A Syn (46-73)

Figure 11 shows the ThT binding curve for Asyn14 (46-73) at 0.5mM and 0.25mM. At 0.25mM there is a lag time of 2 hours after which the fluorescence increases logarithmically and finally reaches a stationary phase in about 10 hours. At 0.50mM the lag time could not be ascertained with accuracy.

Figure 11 ThT fiber assay on construct 14 A syn (46-73). Vertical axis measures the fluorescence by thioflavinT upon binding to fibrils. And horizontal axis is the time. This construct tested at two different concentrations of 0.50mM and 0.25mM showed a sigmoidal curve with a lag time of 20 minutes noticeable only at 0.25mM and an instantaneous increase the fluorescence at 0.5mM.
Construct 19 SOD (31-41)

SOD 19 fibrillation was monitored by ThT assay. In figure 12, thioflavin T binding was tested at two concentrations. Both the concentrations tested showed a typical sigmoidal curve with a lag time of 2 hours after which the fluorescence increased exponentially and finally stabilized after 5 hours.

Figure 1 ThT fiber assay on construct 19 SOD (31-41). Vertical axis measures the fluorescence by thioflavin T upon binding to fibril and horizontal axis is the time. A typical sigmoidal curve is seen at both the concentrations tested that is 1mM and 0.5mM with an initial lag time of 2 hours after which the fluorescence increases and finally stabilizes after 5 hours.
Construct 17 (Asyn 63-73):

Construct 17 (Asyn 63-73) showed a lag time of 20mins at 0.25mM and 0.5mM (Figure 13). At higher concentrations the lag time was too short to be ascertained.

Figure 13 ThT fiber binding assay for Construct 17 (Asyn 63-73) at 0.25mM and 0.5mM. Vertical axis gives the fluorescence which increases after thioflavin T binding to mature fibrils and the horizontal axis gives the time. As is typically seen for amyloid proteins or peptides, the fibrillation process is a sigmoidal curve with a lag time of 30 minutes in this case after which the fluorescence increases logarithmically for 6 hours and finally stabilizes.
Construct 16 (Asyn 68-98):

Figure 14 shows the fibrillation kinetics as measured by Thioflavin T binding for construct 16 (asyn 68-98). A lag time was 20mins was noticed before the fluorescence started increasing. Interestingly, the fluorescence had a local minima at 5hours after which it again started increasing. This feature is often attributable to either the presence of air bubbles or some protofibrils.

Figure 24 ThT binding of construct 16 Asyn (68-98) at two concentrations of 0.25mM and 0.50mM. The horizontal axis gives the time and the vertical axis indicates the intensity of fluorescence of thioflavin T binding to fibrils. A lag time of 20minutes can be seen at both concentrations after which the fluorescence increases exponentially and shows a local minima at 5 hours after which it again increases. This can be attributable to protofibrils slowly converting to mature fibrils.
Electron Microscopy

Figure 15. Transmission electron microscopy on different constructs in oligomeric and fibrillar form. Notice Construct 14 (A), Construct 17 (B), Construct 20 (E) and Construct 19 (F) show fibrils upon shaking at 37°C. Interestingly Construct 20(E) and Construct 19 (F) show the same oligomeric forms after fibrillation along with the mature fibrils. The freshly solubilized SOD 19 (C and D) show some fibrils and round objects as well which points to the heterogeneity of the sample [16].
Static Light Scattering:

Construct SOD 19 (31-41)

The oligomeric state of the constructs was estimated using static light scattering. Figure 16 shows the index of refraction calculated over an elution time of 40 minutes on a SEC-SLS column for Construct SOD 19 (31-41). The peak at 32 minutes elution time which the software calculated to 4 monomeric units.

Figure 16 Construct 19 SOD (31-41) static light scattering. The horizontal axis measures the index of refraction over an elution time of 40 minutes given on the horizontal axis. The peak at 32 minutes was calculated to have a molecular weight of 11000g/mol and the weight of each monomer is 2533g/mol thus the oligomers formed in solution have 4 monomer units.
**Construct Asyn14 (46-73)**

Construct Asyn14 (46-73) has a positive peak at 32 minutes which corresponds to a dimeric form. The negative peak at 37 minutes corresponds to the sample buffer elution. Figure 17b is a zoom in of the peak 31.8 minutes to 33 minutes elution time. The DLS software calculates the molecular weight based on the index of refraction which calculated the molecular weight at 5467 g/mol. Asyn has a molecular weight of 2794 which corresponds to 2 monomeric units.

![Syn14](image)

**Figure 17 Construct 14 asyn (46-73) Static light scattering chromatogram.** The vertical axis measures the index of refraction and the horizontal axis gives the elution time. The positive peak seen at 31.8 minutes was calculated to have a molecular weight of 5467 g/mol which corresponds to 2 monomeric units. B. is a zoom in of the peak at 31.8 minutes. The negative peak at 35 minutes is due to the buffer.
Construct Asyn 16 (68-98)

Construct Asyn14 (68-98) has a positive peak at 32 minutes which corresponds to a dimeric form. The negative peak at 37 minutes corresponds to the sample buffer elution. Figure 18b. is a zoom in of the peak 31.8 minutes to 33 minutes elution time. The DLS software calculates the weight average molecular weight based on the index of refraction to be 11140 g/mol and since the weight of a monomer is 2904 g/mol, there are approximately four monomers forming one oligomer.

Figure 18 Construct 16 Asyn (68-98) Static light scattering chromatogram. The horizontal axis is the elution time and the vertical axis gives the index of refraction. The positive peak seen at 30 minutes was calculated to have a molecular weight of 11140g/mol which corresponds to 4 monomeric molecules. B.is a zoom in of the peak at 30 minutes. The negative peak at 33minutes is attributable to the buffer.
Discussion

Oligomers and toxicity

Mature amyloid fibrils are the most intensely studied species in amyloidogenic diseases. Initially thought to be the main mediators of disease, it is now being hypothesized that the intermediate species are the main cause for toxicity as the amyloid fibrils form from the monomers in various neurodegenerative diseases [32]. Still, the characterization of the intermediate species has proved to be extremely difficult because of their transient nature and heterogeneity. However, using several indirect methods such as microscopy and size exclusion chromatography, oligomers have been found to exist [17, 33, 34]. The most extensively characterized of these is the oligomers from Abeta, the precursor protein of amyloid deposits found in Alzheimers disease. This 40 or 42 residue-long peptide from Abeta has been found in dimeric form in its oligomeric form before it progresses into fibrils that have a standard steric zipper structure [15, 35]. Electron microscopy of the oligomers has shown them to be pore shaped. However, there are no functional studies done on these oligomers[16].

An intriguing case in point is the fibrillization of β2-microglobulin. The protein found as a component of Class 1 Major histocompatibility complex is prone to aggregation but only when the native structure is perturbed. It has a tendency to form amyloid fibers in patients undergoing hemodialysis. However, it has been found through various computational methods that even though 60% of the protein sequence is prone to aggregation, yet it only aggregates in exceptional conditions [21, 36-38]. It was found that the hydrophobic residues which can potentially cause aggregation are buried in the structure. Thus, for this protein to fibrillize the protein needs to take an off pathway and become unfolded. How this
unfolding occurs and what is the structure of the protein in this transient state are questions as yet unanswered but it does point towards the idea of the intermediate species being the cause of toxicity and not the mature fibrils themselves.

Another example is provided by the studies done by Arrasate et al. who show that cell death is lower in cells harboring large aggregates of polyglutamine rich huntington protein as compared to cells harboring soluble protein [36]. This study also supports the idea that the aggregates are not the main toxic species.

Studies on Abeta and its toxicity mechanism have shown that insoluble aggregates can indeed cause cell death but a general consensus is that the aggregates are often accompanied by some soluble protein as well [34].

The two proteins studied here have also been found to have toxic oligomers. The study by Winner et al. showed that oligomers of alpha synuclein are toxic in vivo [30]. They showed that alpha synuclein mutants which form fibrils quickly are less toxic than mutants which form oligomers. Another group has identified 30-50nm annular oligomers to be the potential toxic species as they nucleate the fibril formation [28]. They further showed that these annular oligomers were present only in vivo and in vitro spherical oligomers were formed. However, there have not been any structural studies on these oligomers and if there is a particular segment which can form a toxic oligomeric state in vitro. In case of SOD1, Banci et al. have shown that oligomers are the intermediate species as the protein progresses into fibrillation. Thus, the intermediate species and specifically oligomers have an important role in mediating toxicity.
There are many problems faced by researchers in investigating these intermediate states. Firstly, the conversion of monomers into mature fibrils is a highly dynamic process involving many different species and the overall heterogeneity of the population makes it challenging to get the oligomeric state in isolation. Electron microscopy can been used to get snapshots of these oligomers but the atomic resolution is lacking. Other techniques such as live cell imaging and fluorescence microscopy have provided important clues about the toxicity mechanism but the structural details are still obscure. The oligomeric species could be an on pathway or off pathway species, toxic or non-toxic, having a unique structure for each protein or having a common structural form, transient or long lasting. Some of these questions have been answered by the cylindrin model recently [2]. The cylindrin structure is considered to be an off pathway oligomeric state. It was found to be toxic to cells even though it does not cause membrane lysis, thus pointing towards a different mode of toxicity than membrane lysis. It is thus very important as it is the first piece of the puzzle that is the structure of oligomers and their role in mediating the diseases.

**Alpha Synuclein and SOD1**

Alpha-synuclein is an amyloid-forming protein that is found in the fibrillar deposits found in brain neurons in the case of Parkinson’s disease. Pathologically the disease is characterized by a loss of dopaminergic neurons from the substantia nigra. Alpha-synuclein fibrillization has been previously known to progress through the formation of discrete oligomeric intermediates which disappear after fibril formation. In such a case, a cylindrin like oligomeric state could be the answer to questions about this intermediate state as it has all the features for a classical oligomeric state that is it can fibrillize upon shaking into steric zipper form, it is more toxic in its oligomeric form than the monomeric or the fibrillar form and finally it binds to the oligomer specific A11 antibody. Out of the four peptide
segments identified to be cylin-drin-like in alpha synuclein, Asyn (46-73) was found to exist in dimeric form by static light scattering. Construct asyn (68-98) was also found to exist in oligomeric form with approximately 4 monomers. However further experiments are needed to validate as there may be high instrumentation error. Construct A syn (46-73) was found to be non-toxic in freshly solubilized form but the fibrillar form was toxic. Construct A syn (63-73) was found to be toxic in the freshly solubilized form also which can be correlated with its very high propensity to aggregate at even very low concentrations, thus already proceeding into the fibrillar form before the effect of the oligomeric form can be seen. An interesting case was presented by Asyn (68-98). This construct was found to be toxic in the oligomeric state as well as the fibrillar form. Thus, it may be the best potential target to form a toxic oligomeric species. Circular dichroism showed that the secondary structure for all these cylin-drin-like segments is nearly 50% beta sheets. Hence, from this study, it may be concluded that all segments of the alpha synuclein form beta sheet rich oligomers but the entire protein may not form cylindrin like structure before it progresses into fibrillation and quite possibly only a short stretch may form a cylindrin. It may contribute to the off pathway fibril formation as seen in beta microglobulin. This hypothesis of course, can only be validated if a structure of a toxic oligomer like Alpha Syn (68-98) can be obtained.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that is characterized by muscular paralysis caused by the death of motor neurons. The disease has been linked to neuron death which is associated with mutations in Superoxide Dismutase 1 (SOD1). Oligomerization of this protein has been known to be mutation dependent and in the presence of metals and physiological conditions of temperature and pH[39]. The two segments isolated from this protein formed oligomers as seen in electron micrographs and were found to have similar (i.e. 50%) beta sheet secondary structure. Construct 19, SOD (31-41) was found to non-toxic in oligomeric state but toxic in fibrillar form. The
Fibrilliation assay (ThT binding assay) also showed a typical sigmoidal curve with a 2 hour lag time before any thioflavin binding could be seen. Thus, these segments provide evidence of the existence of the oligomeric state in SOD1 which is similar to that seen in alpha synuclein but the segments studied here do not cause toxicity in the oligomeric state. Recently, Banci et al compared the structure of WT SOD and ALS associated SOD mutants in solution and its crystal structure and found two loops (68-78 and 125-140) to be missing electron density in the crystal in the mutant form[40]. The two loops were found to be disordered in solution in the non metalated form only [20, 40, 41]. Interestingly, these sequences have very low fibrillation propensity according to the rossetta energy profile. Further efforts are needed to look for more targets in this protein which can be tested for being the toxic oligomeric species.

**Further validation of Current Oligomeric model:**

Recent findings by Neudecker et al. have also confirmed the idea of an anti-parallel beta barrel as the intermediate species. In their studies on SH3 domains by NMR, they found a beta sheet structure as the protein progresses into fibrillization[42]. They also pointed out the low prevalence of this intermediate species in solution (approx. 2%) which also tells us about the heterogeneity of the fibrillation process. Together with the cylindrin model, this study further bolsters evidence in support of an anti-parallel beta barrel structure for the intermediate species. The presence and the relevance of these intermediate species, their mechanism of cytotoxicity and the structure of the next intermediate in the fibril formation pathway are some of the questions that need to be answered in this respect.
Summary and Outlook:

Even though research on amyloids- their causative oligomeric states, their reaction intermediates, the designing of inhibitors, methods for preventing aggregation and designing disaggregates- has been going on for the last 50 years, it is only in the last decade that structural studies on aggregates and their intermediates have made significant progress. With the structure of the aggregates being extensively studied and found to have a characteristic steric zipper structure, efforts are now being directed towards the intermediate oligomeric states. If they are indeed found to have a common structure, the cytotoxic mechanism might be similar in all amyloid proteins and thus therapeutic strategies can be directed towards the prevention of these common structures thus helping in treating different diseases with the same approach. The present study serves as a starting point after the foundation given by Laganowsky et al. [2] in elucidating the structure of amyloid precursors/ oligomers. It highlights the commonalities of the different segments from two proteins that are completely different at the sequence level.
References


