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Synthesis and Evaluation of α-Synuclein (6-36)

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Synthesis and Evaluation of \(\alpha\)-Synuclein (6-36)

Madelyn Kallman

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of the
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Wellesley College
In conjunction with Dr. J. H. Miwa

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Parkinson’s disease is a common neurodegenerative disorder characterized by tremors and motors deficits, caused by the death of dopaminergic neurons and the resulting dopamine depletion in the brain. Examination of the substantia nigra of the brains of Parkinson’s patients reveals abnormal intraneuronal deposition of fibrillar aggregates of the protein α-synuclein. While Parkinson’s disease is generally idiopathic in origin, several mutations in α-synuclein have been shown to increase the likelihood of the disease. This further implicates α-synuclein in the pathogenesis of Parkinson’s disease. This report focuses on wild type α-synuclein. A 31-residue peptide corresponding to residues 6-36 of the N-terminus of α-synuclein was synthesized, purified, and its conformation examined using circular dichroism spectroscopy. The peptide showed conformational behavior that is similar to what has been found for this region in the full-length protein. Thus, the peptide can be used as a model system for the N-terminal region of α-synuclein. In future work, this peptide model and its A30P mutant counterpart can be compared to characterize the role of this mutation in Parkinson’s disease.
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Introduction

Parkinson’s Disease and Lewy Bodies

Parkinson’s disease is a devastating neurological condition causing uncontrollable shaking, muscle rigidity, and slowed movement. First described by James Parkinson in 1817, Parkinson’s disease is the second most common neurodegenerative condition after Alzheimer’s disease. Until recently, Parkinson’s disease had been viewed only as a movement disorder, but as of 2012, the clinical spectrum has come to include early non-motor features. In the pre-motor phase, usually before clinical diagnosis, common symptoms include reduced sense of smell, rapid-eye-movement behavior disorder, depression, and constipation. Later on, there is the characteristic debilitating motor impairment with tremors, difficulty initiating movement, and postural instability. Cognitive decline and dementia is common in advanced disease.

![Figure 1: The structures of a) dopamine and b) Levodopa.](image)

Parkinson’s disease is a consequence of the death of dopaminergic neurons in the substantia nigra, leading to reduced production of the neurotransmitter dopamine (Figure 1a). Currently, there is no treatment that can slow, stop, or reverse this cell death. The only therapeutic option is symptomatic treatment. The most common treatment is Levodopa (Figure 1b), a prodrug of dopamine, converted by decarboxylation, that can
cross the blood-brain barrier to replace the dopamine that the damaged neurons can no longer produce. A limitation of this drug is that dopaminergic neurons in the substantia nigra are needed to convert the Levadopa to dopamine. When too many neurons have died, the drug loses its efficacy.

When the substantia nigra of Parkinson’s patients are examined, these neurons are found to contain Lewy bodies - intracytoplasmic inclusions made up of fibrillar aggregates of the protein α-synuclein. As point mutations (A30P, E46K, A53T) and over expression of the gene coding for α-synuclein have been linked to Parkinson’s disease, it is clear that this protein is important to the pathogenesis of the disease. This work will establish the basis to characterize the chemical changes of the A30P mutation by experimentation on a synthetic peptide fragment of wild type α-synuclein.
Aggregating Proteins

For many proteins, such as hemoglobin, collagen, and actin, assembly into oligomers of a defined size is necessary for proper protein function. For other proteins, unnatural assembly, called aggregation, spells disaster. A schematic of the possible pathways of aggregation is shown below (Figure 2).

Figure 2: Schematic of the possible pathways of protein aggregation.

Aggregation is a generic property of peptides and proteins. It is generally an entropy driven process, believed to be a result of the water molecules released from the hydration shell upon assembly of the aggregate, due to a decrease in surface area. Therefore, there are more than 40 human diseases caused by the aggregation of at least 20 different proteins or peptides. For most of these species, aggregation is a consequence of misfolding of the protein or production of fragments of the protein that are unable to
fold properly. In this type of aggregation, the proteins form amyloid fibrils characterized by extensive β-sheet structure, with the β-strands perpendicular to the fibril axis. This structure is called a cross-β fibril. These fibrils accumulate to form insoluble amyloid plaques in the affected organ, such as the pancreas in type II diabetes, the heart, liver, and kidney in various forms of systemic amyloidosis, and the brain in neurodegenerative disorders. These deposits can be extracellular, cytoplasmic, or nuclear. Specific proteins have been identified in several of the most common neurodegenerative disorders, including amyloid-β (Alzheimer’s disease), polyglutamine (Huntington’s disease), prion proteins (prion diseases), and α-synuclein (Parkinson’s disease). These proteins have very little in common in their primary sequences, but they are very similar in their ability to undergo the conformational change to a cross-β fibril aggregated structure.

Dimers and trimers of amyloid-β, aggregates of prion proteins, and protofibrils of α-synuclein have been shown to be toxic to cells in vitro. There are competing theories about the mechanism of toxicity. One theory is that the toxicity is caused by exposure of residues, typically buried in the interior of the protein in the correctly folded native state, that become exposed to and damage the cellular components in the diseased state. Another theory is that early amyloid aggregates organize into doughnut shaped assemblies, which form pores in the cellular membranes and disrupt homeostasis. Whatever the mechanism, we can inhibit the toxicity by inhibiting the conformational shift to amyloid cross β-sheet structure. This can be accomplished by stabilizing the native structure or by destabilizing the amyloid structure.
**α-Synuclein Function and Structure**

α-Synuclein is estimated to account for around 1% of brain protein.\(^{17}\) Its normal function is not fully understood, but postulated actions include the refilling and trafficking of synaptic vesicles,\(^{18}\) fatty acid binding,\(^{19}\) and regulation of dopamine.\(^{20}\) It is not essential to the neurotransmitter release processes, but is thought to contribute to the long-term maintenance of nerve terminal function.\(^{21}\)

α-Synuclein is a natively unstructured 140 amino acid protein lacking both cysteine and tryptophan,\(^{22}\) and containing an imperfectly repeated 11-residue consensus sequence with a highly conserved KTKEGV motif and linker residues.\(^{23}\) Its sequence is below (Figure 3).

<table>
<thead>
<tr>
<th>N-Terminal Repeat Domain:</th>
<th>1</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDVFMKGLS<strong>KAKEGV</strong>VAEEKTKQGVAAAGKTKEGVLVYGSKTKKEGVLYVGVATVAEKTKEQVTNV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NAC Domain:</th>
<th>70</th>
<th>80</th>
<th>90</th>
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</thead>
<tbody>
<tr>
<td>GGAVVTGVTAQA<strong>KTVEGA</strong>GSIAAATGFVI</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C-Terminal Acidic Domain:</th>
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<th>110</th>
<th>120</th>
<th>130</th>
<th>140</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KKDQLGKNEEGAPQEGILEDMVPVDPDNEAYEMPSEEGYQDYEPA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3:** The sequence of human α-synuclein, split into its three domains. The conserved motif is in bold.
α-Synuclein is made up of three domains with very different properties (Figure 4). The amino terminal domain (residues 1-65) is highly conserved among different species. As shown below, the first five of the six repeats are in the amino terminal repeat domain. NMR studies have shown that, upon membrane binding, this region adopts an α-helical conformation consisting of two helices interrupted by a short break around residues 43-44.

![Figure 4: Human α-synuclein domains. The conserved motif is shown as the darkest grey boxes.](image)

The central domain (residues 66-95) is known historically as the non-\(\beta\) component of plaque (NAC) because of its inclusion in the characteristic plaques of Alzheimer’s disease. It has been hypothesized to be responsible for the conformational change from random coil to β-sheet necessary to form fibrils. The carboxy terminal domain (residues 96-140) has a strong negative charge, and is highly variable between species. It has no recognized structural elements.

Experiments have shown that α-synuclein can adopt a number of conformations in vitro, though little is known about the states of the protein in vivo. These conformations may be stabilized by long-range interactions. From studies purifying α-synuclein from \(E.\ coli\), we know that it exists as an unfolded monomer under native conditions, but there are also a number of different conformations that have been
implicated in the pathogenesis of Parkinson’s disease, including oligomers, protofibrils, and fibrils. It is possible that, naturally, α-synuclein exists in equilibrium between these different conformational and oligomeric states. Of these states, partially folded and soluble oligomers have exhibited neurotoxicity \textit{in vitro}, whereas insoluble aggregates and fibrils are hypothesized to be neuroprotective, since their formation from the smaller aggregates has lowered toxicity.\textsuperscript{22} These conformational changes are likely to be important in the pathogenesis of Parkinson’s disease and Lewy body formation.

![Diagram of α-synuclein aggregation process](image)

**Figure 5:** The α-synuclein aggregation process from monomers to fibrillar aggregates.\textsuperscript{31}

The currently accepted model of α-synuclein aggregation is shown in Figure 5. These fibrillar aggregates are the main component of Lewy bodies.\textsuperscript{32} If the hypotheses about the toxicity of the intermediates and the neuroprotective nature of the insoluble aggregates are true, then the formation of Lewy bodies may be an attempt to sequester the oligomers and save the neuron.
Stabilizing α-Helices and Salt Bridges

The stability of an α-helix depends on its amino acid composition. The likelihood of an amino acid to be in an α-helix can be estimated based on the compositions of known helices, or determined experimentally using thermodynamic studies. Values based on both methods are shown below (Table 1).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>$P_\alpha^a$</th>
<th>$\Delta\Delta G_\alpha$ (kcal/mol)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>1.51</td>
<td>-0.27</td>
</tr>
<tr>
<td>Met</td>
<td>1.45</td>
<td>-0.50</td>
</tr>
<tr>
<td>Ala</td>
<td>1.42</td>
<td>-0.77</td>
</tr>
<tr>
<td>Leu</td>
<td>1.21</td>
<td>-0.62</td>
</tr>
<tr>
<td>Lys</td>
<td>1.16</td>
<td>-0.65</td>
</tr>
<tr>
<td>Phe</td>
<td>1.13</td>
<td>-0.41</td>
</tr>
<tr>
<td>Gln</td>
<td>1.11</td>
<td>-0.33</td>
</tr>
<tr>
<td>Trp</td>
<td>1.08</td>
<td>-0.45</td>
</tr>
<tr>
<td>Ile</td>
<td>1.08</td>
<td>-0.23</td>
</tr>
<tr>
<td>Val</td>
<td>1.06</td>
<td>-0.14</td>
</tr>
<tr>
<td>Asp</td>
<td>1.01</td>
<td>-0.15</td>
</tr>
<tr>
<td>His</td>
<td>1.00</td>
<td>-0.06</td>
</tr>
<tr>
<td>Arg</td>
<td>0.98</td>
<td>-0.68</td>
</tr>
<tr>
<td>Thr</td>
<td>0.83</td>
<td>-0.11</td>
</tr>
<tr>
<td>Ser</td>
<td>0.77</td>
<td>-0.35</td>
</tr>
<tr>
<td>Cys</td>
<td>0.70</td>
<td>-0.23</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.69</td>
<td>-0.17</td>
</tr>
<tr>
<td>Asn</td>
<td>0.67</td>
<td>-0.07</td>
</tr>
<tr>
<td>Gly</td>
<td>0.57</td>
<td>0.00</td>
</tr>
<tr>
<td>Pro</td>
<td>0.57</td>
<td>-3</td>
</tr>
</tbody>
</table>

a. $P_\alpha$ is a measure of the frequency of each residue in naturally occurring α-helices.$^{33}$

b. $\Delta\Delta G_\alpha$ is the difference in free energy of helix formation of each residue compared to that of glycine.$^{34}$

From these values, we see that it is favorable for alanine to be in a helix, and unfavorable for proline to be in a helix. Though it is often a helix breaker, and despite its high $\Delta\Delta G$ value, proline is sometimes found in α-helices. When this occurs, the ring of the proline
pushes away from the preceding turn of the $\alpha$-helix by about one angstrom, producing a bend of about $30^\circ$ in the helix axis, and breaking the hydrogen bond of the following amino acid.\(^{35}\) This kink is shown in Figure 6.

Figure 6: An $\alpha$-helix containing a proline, showing the missing hydrogen bond and the $30^\circ$ kink.\(^{35}\)

The charged amino acids glutamine and lysine are commonly found in $\alpha$-helices. If appropriately spaced, these amino acids can stabilize an $\alpha$-helix by forming a salt bridge. When two oppositely charged amino acids are four residues apart, their side chains are optimally aligned for an ionic interaction. Since the helix has a dipole that is positive at the N terminus and negative at the C terminus resulting from the polarity of the carbonyls, the electrostatic effects of this ionic interaction are favorable if the negative ion is closer to the N terminus and the positive ion is closer to the C terminus.\(^{36}\)
Figure 7: Helical wheel projection of residues 6-36 of α-synuclein, showing the salt bridge between E28 and K32.

We can examine the putative α-helix that would be formed by residues 6-36 of α-synuclein by plotting the peptide on a helical wheel projection (Figure 7). From this projection, we can see that there is likely to be a salt bridge between E28 and K32, and that this interaction is in the correct alignment with the helix dipole. This salt bridge may be broken by the A30P mutation, as the residues will be farther apart on a bent helix. From this, we can see how disruptive this mutation will be on the stability of the α-helix of the N-terminal repeat region.
Ubiquitination of α-Synuclein

α-Synuclein can be modified by several post-translational modifications including serine and tyrosine phosphorylation, nitration, enzymatic crosslinking, C-terminal truncation, and ubiquitination. The only one affecting the N-terminal repeat domain is ubiquitination.

Ubiquitin is a 76-residue protein which is attached to another protein by the sequential action of three enzymes. This process is summarized in Figure 8.

**Figure 8:** In the first step, the C-terminal glycine residue of ubiquitin is activated by an ATP-dependent specific activating enzyme (E1), where ubiquitin forms a thiolester linkage to a cysteine residue of E1. In step two, the activated ubiquitin is then transferred to a cysteine residue of a ubiquitin-carrier protein (E2). In the third step, a ubiquitin-protein ligase (E3) catalyzes the linkage of ubiquitin to the substrate protein.
The two proteins are attached by an isopeptide bond between the C-terminus of ubiquitin and the ε-amino group of the substrate’s lysine residue (Figure 9).

![Diagram of isopeptide bond between ubiquitin and lysine](image)

**Figure 9:** The isopeptide bond (bolded) between ubiquitin’s C-terminal G76 to one of the substrate’s lysine residues.

Ubiquitin has seven lysine residues, all of which can be ubiquitinated, forming polyubiquitin chains, which regulate different processes, such as DNA repair, and protein degradation by the autophagy and proteasome systems. Alternately, monoubiquitination regulates the activity of proteins located at membranes, and can target them for degradation in the lysosome.

α-Synuclein is lysine rich, containing 15 lysine residues, and is modified at different sites by three different ubiquitin-protein ligases: parkin, UCH-L1, and SIAH. Mutations in parkin and UCH-L1 have been linked to familial Parkinson’s disease, and Parkinson’s disease susceptibility, respectively, indicating that ubiquitination is an important process in preventing the disease. Additionally, ubiquitin is a common inclusion in the core of Lewy bodies, but absent from the periphery, which implicates the ubiquitination of α-synuclein in the early stages of Lewy body formation.

The cellular basis for α-synuclein ubiquitination is not well understood, especially since as a natively unfolded protein it is likely to be degraded in a ubiquitin-independent
Two popular theories are that the modification is an attempt to (1) unfold or degrade misfolded α-synuclein, or (2) block interactions of inappropriately exposed residues. Whatever the reason, this modification backfires, as ubiquitinated α-synuclein inhibits the proteasome system by partly entering the proteasome and causing steric occlusion. Alone, proteasome inhibition does not generally cause cell toxicity, but it has been shown to strongly enhance the toxicity of α-synuclein species. In summary, ubiquitin plays a supporting role in the pathogenesis of Parkinson’s disease, as a lack of ubiquitination can increase susceptibility to contract the disease, but proper ubiquitination can increase the toxicity of the α-synuclein oligomeric species.
The A30P Mutation

In 1998, a missense mutation was discovered in a German family that passed down early onset familial Parkinson’s disease as an autosomal dominant trait – the replacement of an alanine at residue 30 with a proline (A30P). The amino acids alanine and proline (Figure 10) have very different chemical properties.

![Figure 10: Structures of a) alanine and b) proline.](image)

Alanine is small, nonpolar, and slightly hydrophobic. Since its side chain is so small, there is very little steric hindrance, and therefore it can be a part of many conformations. Proline is also a small amino acid, but it is unique in having a secondary rather than a primary amine. When proline is in a peptide chain, it has no hydrogen available for hydrogen bonding. This means that proline creates kinks in helices, and is likely to be found in tight turns. The effects of this mutation on secondary structure have been studied for full-length α-synuclein.

Circular dichroism and FTIR data have shown that under physiological conditions, A30P α-synuclein has the same natively unfolded conformation as wild type α-synuclein. Additionally, similar to wild type α-synuclein, the mutant undergoes a reversible transformation to a partially folded state under acidic or heated conditions. Amino acid level studies show the effects of the mutation on the secondary and tertiary
structure of the protein. The α-helicity of mutant α-synuclein has been studied experimentally by NMR α-carbon chemical shifts and predicted computationally by the heirarchical neural network method, resulting in agreement that the helical propensity of residues 18-31 present in wild type α-synuclein is absent in the mutant. Specifically, the helicity is most perturbed at E28. As was stated earlier, E28 likely forms a salt bridge with K32. Disrupted helicity at E28 will also disrupt the salt bridge, further affecting the stability of the helix in this region. The same computations predict that the mutant is more likely to form β-structure than wild type α-synuclein. Circular dichroism data confirms this prediction. Additionally, FRET data suggests that proline significantly affects the three-dimensional conformation of α-synuclein, bringing the N and C termini closer together than in the wild-type protein. NMR residual dipolar couplings and paramagnetic relaxation enhancement studies corroborate that the mutation perturbs the native conformation, and makes the backbone more flexible. These secondary and tertiary structure effects will affect the behavior of A30P α-synuclein in its membrane binding and aggregation.

There has been conflicting evidence on the effects of the A30P mutation on α-synuclein lipid interactions. Certain studies have shown a complete lack of membrane binding by the mutant. This result may be caused not by the mutation, but by the biotin used in the assay, which has since been shown to eliminate α-synuclein membrane binding. Other studies have shown that the mutation has no effect on protein binding to the membranes of intact cells. Others have shown decreased or defective binding to phospholipid vesicles. Additionally, circular dichroism has shown that the A30P mutant does not undergo the normal transition from random coil to α-helix in the
presence of lipids.\textsuperscript{55} The lack of lipid membrane binding may be important for the pathogenesis of Parkinson’s disease, as there will be a higher population of free α-synuclein, which may increase the kinetics of aggregation.

We know that when α-synuclein aggregates, it undergoes a secondary structure change from its initial random coil formation to an anti-parallel β-sheet.\textsuperscript{46} Therefore, the greater propensity for β-sheets of the A30P mutant is expected to affect its aggregation. SDS-PAGE, thioflavine-S staining, electron microscopy and circular dichroism studies all show that the mutant protein aggregates at a higher rate than the wild type.\textsuperscript{51,46} Conway et al. clarify that while the soluble A30P monomer forms oligomers more rapidly than the wild type, its fibrils form more slowly.\textsuperscript{56} This means that the mutant α-synuclein’s aggregation kinetics favor the prefibrillar intermediate. Aggregation is also enhanced by the increased flexibility of mutant α-synuclein, as it can more easily overcome the barrier for self association.\textsuperscript{29} Electron micrographs show that the mature fibrils formed from the mutant and wild type α-synuclein are indistinguishable.\textsuperscript{57}

In summary, the studies comparing wild type and A30P α-synuclein have found that the mutant’s decreased propensity for α-helical structure and increased likelihood for β-sheet structure lead to decreased lipid binding, increased oligomerization, and decreased fibrillization, but do not seem to affect the mechanism of aggregation. All of these studies have been done on the full-length protein. The effects of the mutation can be determined more specifically and definitively if we examine only the region surrounding the mutation.

This work examines the secondary structure of a small synthetic fragment of residues 6-36 of wild type α-synuclein with circular dichroism spectroscopy under
varying conditions. This fragment was synthesized using solid phase peptide synthesis. In future work, these tests can be repeated for the same residues of the mutant protein to elucidate the changes caused by the mutation. These results will further our understanding of the conformational changes inherent to the pathogenesis of Parkinson’s disease, and will be valuable to those working to develop therapies that interrupt the aggregation process.
Solid Phase Peptide Synthesis

Developed in the 1960’s, solid phase peptide synthesis is a process for making peptides, commonly used when the protein cannot be expressed with bacteria. In this novel approach, the C-terminal amino acid is anchored to an insoluble resin bead (Figure 11), which acts as a solid support for the growing peptide chain.\textsuperscript{58, 59, 60}

![Figure 11: Resin with Fmoc protected Rink linker. The circled P is polystyrene.](image)

Amino acids are added stepwise to the free end of the growing polypeptide chain. The resin bead prevents the chain from passing through a filter during the synthesis, allowing for easy separation of the chain from the solvent and reagents. This allows one to run the synthesis with excess reagents, producing higher yields after each step than traditional organic synthesis, as the excess drives reactions to completion. Additionally, since the polypeptide chain is purified after synthesis rather than between each addition of amino acid, solid phase peptide synthesis minimizes loss of product during purification.

In solid phase peptide synthesis, the peptide is bound to the resin by the C-terminus. During the synthesis, new amino acids are attached to the N-terminal amino acid. It is important that only the amino terminus of the peptide is able to react with the
carboxylic acid group of the new amino acid. If the side chain of an amino acid is able to react, it must be protected to prevent any undesired reactions. Additionally, the amine of the amino acid being added must be protected to prevent it from reacting with itself. Therefore we need a “temporary” protecting group for the α-amino groups that can be removed during each coupling cycle, and a “semi-permanent” one to protect the side chains that will be removed only after the synthesis is complete.

In our synthesis, we use fluorenyl-methoxy-carbonyl (Fmoc), for the “temporary” protecting group, and tert-butoxycarbonyl (t-Boc), for the “semi-permanent” protecting group (Figure 12).

**Figure 12:** Structure of a) Fmoc and b) t-Boc protecting groups.

These two are orthogonal protecting groups, as Fmoc is labile in base and t-Boc is labile in acid. The Fmoc group can be removed in the mild base piperidine. The t-Boc groups will be removed after synthesis using trifluoroacetic acid (TFA).
Figure 13 outlines the key steps of solid phase peptide synthesis. Details for each step will follow.

Figure 13: Overview of the steps of solid phase peptide synthesis.
First the Fmoc protecting group is removed under basic conditions using piperidine to reveal a free amine at the N-terminus of the peptide chain (Figure 14).

Figure 14: Mechanism of Fmoc deprotection of the amino group.
Then the new amino acid must be activated using diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBT), which converts the carboxylic acid into an ester. This ester then undergoes nucleophilic attack from the nitrogen of the free amino group, forming a peptide bond between the new amino acid and the growing peptide chain (Figure 15).

**Figure 15:** Carboxylic acid activation and amino acid coupling mechanism.
In every coupling step, fewer than 2% of the peptide chains do not successfully react with the new amino acid. As these chains have been deprotected and have a free amino group, they are reactive. They may couple with other amino acids in later steps of the synthesis, creating unwanted peptides with deletions. To avoid this, we can cap the free amino groups by acetyling them using acetic anhydride (Figure 16).

**Figure 16:** Mechanism for the acetylation reaction.
Once the synthesis is complete, the side chain protecting groups can be removed in the same step as cleaving the peptide from the resin solid support using TFA (Figure 17).

**Figure 17**: a) cleavage of peptide from rink linker and b) deprotection of t-Boc group using strong acid.

The crude peptide can then be purified using high pressure liquid chromatography and analyzed using circular dichroism spectroscopy.
**Reversed Phase High Pressure Liquid Chromatography (HPLC)**

Chromatographic separations are based on the interactions of different compounds with a liquid mobile phase moving through the solid stationary phase of a column. HPLC is named for the high-pressure system that pushes the mobile phase through the column. A reversed phase system separates components based on their hydrophobic nature with a hydrophobic stationary phase and a polar mobile phase. In this research the separation is done on a C18 column, which has silica derivatized with hydrocarbon chains that average 18 carbons in length as the stationary phase. The mobile phase is a mixture of acetonitrile and water, the composition of which changes over time according to a linear gradient. As we increase the amount of acetonitrile, we in turn decrease the polarity of the mobile phase. In the column, each component of our crude peptide mixture is in equilibrium between adsorption on the solid phase surface and dissolution in the mobile phase. Since this equilibrium is based on hydrophobic interactions, as we increase the concentration of acetonitrile in the mobile phase, we shift the equilibrium to dissolution. The equilibria for the different peptides will shift at different rates, so they will have different rates of movement through the column and elute at separate times. We can monitor this process with UV absorbance measurements of the eluant.

In practice, the difficulty of HPLC is in optimizing the gradient, or solvent program. Changing the concentrations of the mobile phase and the rate at which they change will affect when components elute. An optimized system has the desired product elute from the column quickly and with high purity. Once a system is optimized, it can be used to purify the desired peptide.
Circular Dichroism Spectroscopy

The secondary structure of a peptide is determined by the dihedral angles (Figure 18) of its backbone, and certain hydrogen bonds.

**Figure 18:** The dihedral angles of a peptide backbone. The $\phi$ angle is along the $\alpha$-carbon to nitrogen bond, and the $\psi$ angle is along the $\alpha$-carbon to carbonyl bond.

For example, the $\alpha$-helix has dihedral angles $\phi=60^\circ$ and $\psi=45^\circ$, and hydrogen bonds from the carbonyl group of the $i^{th}$ residue to the NH of the $i+4^{th}$ residue. The $\beta$-sheet has dihedral angles $\phi=130^\circ$ and $\psi=20^\circ$, and hydrogen bonds between $\beta$-strands, which can be arranged parallel or antiparallel to each other.\(^6\) These structures are shown in Figure 19.

**Figure 19:** Illustrations of $\alpha$-helix and $\beta$-sheet protein secondary structure.\(^6\)}
A beam of light has associated time dependent electric and magnetic fields, and can be polarized differently using suitable prisms and filters. For circular dichroism spectroscopy, we use circularly polarized light, which is made up of clockwise (E<sub>R</sub>) and counterclockwise (E<sub>L</sub>) components. When asymmetric molecules interact with circularly polarized light, they absorb the two components differently, resulting in E<sub>R</sub> and E<sub>L</sub> vectors of different intensities that sum to an ellipse. This light is said to be elliptically polarized. For a detailed and animated explanation of circularly polarized light and its interaction with matter, see Szilágyi’s website.

Figure 20: Circular dichroism spectra of a) pure and b) composite secondary structure.

In a circular dichroism spectrometer, the chirality of bonds can be elucidated based on the absorbance of the two components of circularly polarized light, and the resulting ellipticity, defined as the angle whose tangent is the ratio of the minor to the major axis of the ellipse. For proteins, we can see the amide of a peptide bond at
wavelengths below 250 nm. Experimental data have produced model curves of the ellipticities at different wavelengths for each secondary structure. These curves are shown in Figure 20a. Additionally, it is possible to determine the secondary structure composition of a protein by adding the standard curves to fit the experimental curve, as shown in Figure 20b. For illustrations and detailed mathematics of this process, see Beychok.  

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Results and Discussion

In the first phase of this project, a peptide following the sequence of residues 6-36 of α-synuclein was synthesized using solid phase peptide synthesis. After synthesis, the crude peptide was analyzed using reversed phase high performance liquid chromatography (HPLC). The crude peptide displayed three peaks (Figure 21).

![Figure 21: Absorbance at 225 nm of eluant from the analytical HPLC of crude peptide.](image)

Note that the large peak at the end of the separation is due to a wash of the column with acetonitrile. The peptide isolated from each peak was analyzed using MALDI-TOF mass spectroscopy. The spectra for peaks A, B, and C are shown in appendices 1, 2, and 3 respectively. The calculated mass for residues 6-36 of α-synuclein is 3042 Da, so it is expected to have an [M+H] peak at 3043 M/Z. The mass spectra show that this peptide is the major component of peak A in high purity.
The HPLC analysis was then scaled up to preparative scale, and optimized to baseline resolve peaks A and B, and minimize the time of each run (Figure 22). The optimization process is the key to a good purification. Baseline resolution is necessary to ensure high purity. Short run time is optimal for two reasons. First, it reduces cost in labor and materials. Secondly, shorter retention times yield narrower peaks, and therefore more concentrated eluant, which makes isolation of the pure peptide easier.

Figure 22: Absorbance at 225 nm of eluant from the preparative HPLC of crude peptide.

As we can see from the new absorbance spectra of the preparative eluant, the new solvent profile has allowed for narrower, resolved peaks, and has shortened the retention time of
peak A from 30 minutes to 21 minutes. Run time could not be shortened further, as it interfered with the resolution of the two peaks.

The peptide purified by HPLC was isolated from solution, and then examined using circular dichroism spectroscopy. Though residues 6-36 of α-synuclein form an α-helix upon membrane binding in the full protein, a fragment of these residues may act differently. The rules governing small peptides’ secondary structures are different from those of large proteins. For example, if a secondary structure is stabilized by long-range interactions, the absence of these distant residues means the protein may not fold correctly. Since α-synuclein is intrinsically unstructured, we do not expect much tertiary structure or long-range interactions. Based on this, we expect that a fragment of α-synuclein will act as a model of its respective region of the full protein. Additionally, peptides that are too short will not adopt secondary structure, regardless of any other factor, because their conformational change is not entropically favorable. By synthesizing a 31-residue peptide, we have avoided this issue. If our peptide does undergo the expected conformational shift from random coil to α-helix upon membrane interactions, then we will have confirmed that it is a good model system for the region of α-synuclein surrounding the A30P mutation.

2-(4-Morpholino)-ethane sulfonic acid (MES) buffer is commonly used in peptide analysis since it has a pKa of 6.15 at 20°C and minimal change at other temperatures, and therefore a good buffering capacity at physiological pH. It is also chemically and enzymatically stable, highly water soluble, and has minimal absorption in the UV range. All these characteristics combined make it a good buffer for examining the CD spectra of
peptides. In 10 mM MES buffer in water at pH 7, the peptide showed random coil secondary structure (Figure 23).

![Circular dichroism spectra](image)

**Figure 23:** Circular dichroism spectra of residues 6-36 of α-synuclein in pH 7 10 mM MES buffer in water. The peptide had random coil secondary structure at room and body temperatures (25°C and 37°C respectively).

2,2,2-Trifluoroethanol (TFE) solutions can mimic membrane environments by minimizing peptide-water interactions and stabilizing hydrogen bonds between the amino acids. It can be thought of as a secondary structure enhancer. In 10 mM MES buffer in 50% v/v TFE in water at pH 7, the peptide adopted an α-helical structure (Figure 24).
Figure 24: Circular dichroism spectra of residues 6-36 of α-synuclein in pH 7 10 mM MES buffer in 50% v/v TFE in water. The peptide had α-helical secondary structure at all scanned temperature, with slight denaturation as temperature increased.

These studies show that the peptide of residue 6-36 of α-synuclein acts similarly to the corresponding residues in the full-length protein. The peptide has random coil secondary structure natively, but adopts an α-helical structure in a membrane mimetic solution. This confirms that we will be able to use this peptide as a model system for the region of α-synuclein surrounding the A30P mutation in all future experiments.
Future Work

Now that we have confirmed that a small peptide fragment containing residues 6-36 of \(\alpha\)-synuclein acts as a model of its region in the full protein, we can use it to probe the chemical changes caused by the A30P mutation. This will be a two-step process.

In step one, we will further characterize the secondary structure of the wild type peptide using CD spectroscopy and varying temperature, pH, and buffer environments. So far, we have examined the protein at 25°C and 37°C. We can study the stability of the helix in the TFE, MES buffer by doing temperature denaturation studies. By examining the peptide at higher temperatures, we can determine the thermodynamics of \(\alpha\)-synuclein folding around residue 30. We have also only studied the peptide at pH 7. Future studies will examine the peptide at other pH values. By changing the pH, we will change which amino acid side chains are protonated or deprotonated. This can change the nature of the E28-K32 salt bridge and any other ionic interactions. These studies will show to what extent ionic interactions are necessary to the stability of the \(\alpha\)-helix. Lastly, we have studied the secondary structure shift from a 0% to a 50% TFE buffer environment. We can examine this shift further by performing a TFE titration on our buffer environment. In this way, we can determine how much TFE is required to promote the conformational change and determine how hydrophobic interactions influence this change.

In step two, we will characterize the chemistry of the A30P mutation. By repeating all studies from step one on the mutant peptide, we can determine how the mutation changes the secondary structure of the peptide, and \(\alpha\)-synuclein as a whole. A comparison of the data collected in the two steps will further our understanding of \(\alpha\)-
synuclein’s conformational changes, and can be used by those working to develop therapies that stop α-synuclein aggregation.
Experimental Procedures

**Solid Phase Peptide Synthesis**

Synthesis was carried out using 2 g of Rink amide resin as the solid support.

*Deprotection of the N-Terminal Amino Acid*

To remove the fluoroenylmethyloxycarbonyl (Fmoc) protecting group from the N-terminal amino acid on the peptide chain, the resin was mixed for 2 minutes with 15 mL of 25% piperidine in dimethylformamide (DMF), and then for 15 minutes with 15 mL of the same solution. The resin was then washed four times for 1 minute each with 15 mL of DMF. The deprotected amino group was then ready for coupling to another amino acid.

*Amino Acid Coupling*

To add a new amino acid to the growing peptide chain, first 2.7 mmol of the amino acid was dissolved in 4 mL of DMF, 4.5 mL of 0.65 M hydroxybenzotriazole in DMF, and 4.5 mL of 0.65 M N,N'-diisopropylcarbodiimide in DMF. This solution was then added to the resin and mixed for 60 minutes. The resin was then washed three times with 15 mL of DMF. This whole process was repeated twice with each amino acid to increase the proportion of successful couplings.
**Acetylation of Unreacted Peptide Chains**

After a coupling, the free amino groups on the unreacted peptide chains were capped by acetylation. To cap, the resin was mixed for 12 minutes with 15mL of 0.3 M acetic anhydride in DMF. The resin was then washed 4 times with 15 mL of DMF.

The deprotection, coupling, and capping were repeated until the chain was complete. This process was automated, using an Advanced ChemTech Model 90 Automated Peptide Synthesizer.

**UV-Vis assay**

The progress of the synthesis was monitored by determining the substitution level of Fmoc on the peptide. This was done by removing these protecting groups from the chain and determining their concentration in solution. A drastic change in substitution level is indicative of poor yield in a step of the synthesis. A 5-10 mg sample of the resin-bound peptide was washed with dichloromethane, and then dried under vacuum. This sample was then dissolved in 1 mL of 20% piperidine in DMF and mixed for 20-25 minutes to remove the Fmoc groups. 0.2 mL of this solution was diluted to a total volume of 4.0 mL with acetonitrile in a quartz cuvette. The absorbance at 300 nm was recorded on a Cary 500 Scan UV-Vis-NIR Spectrophotometer. The extinction coefficient (ε) of Fmoc at 300 nm is 7040 M⁻¹cm⁻¹. Using Beer’s Law, for X g of resin with an absorbance of A, we can calculate the substitution level of Fmoc in the peptide chain by

\[
SL(Fmoc) = \frac{20 \times A}{7040 \times X}.
\]

This assay was done at chain length 9, 14, and 18 amino acids to
ensure that the solid phase peptide synthesizer was functioning correctly and that the synthesis was proceeding as expected.

Table 2: Substitution levels

<table>
<thead>
<tr>
<th>Length of peptide (amino acids)</th>
<th>Substitution Level (mmol peptide/ g resin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>0.187</td>
</tr>
<tr>
<td>14</td>
<td>0.144</td>
</tr>
<tr>
<td>18</td>
<td>0.129</td>
</tr>
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</table>

Cleavage

After the addition of the final amino acid, lysine, the N-terminal Fmoc was removed following the above procedures, then the resin was washed 3 times with 20 mL of dichloroethane. Note that the N-terminus of this peptide was not acetylated. The peptide-resin was then dried overnight under vacuum. Meanwhile, 20 mL of the cleavage cocktail [95% trifluoroacetic acid (TFA), 2.5% H₂O, 2.5% triisopropyl silane] was prepared. The cocktail was added to the dried peptide-resin, which turned dark red. This mixture was left at room temperature for 90 minutes and swirled occasionally. The resin was filtered out, and the light yellow filtrate containing the dissolved peptide was collected. This peptide solution was evaporated to an oil using a Buchi Rotovapor R-114 rotary evaporator and Buchi Waterbath B-480 combination. This oil was then added drop wise to cold ethyl ether while stirring. The peptide precipitated as a white solid. The ethyl ether/peptide mixture was stored in the freezer overnight to ensure full precipitation. Then the mixture was filtered to remove the solid peptide from the ether. The crude peptide was then dried under vacuum, and stored in a freezer.
Analytical HPLC of crude peptide

Analytical HPLC of the crude peptide was performed with a C-18 (VYDAC 218TP104) reversed phase column and a flow rate of 1.5 mL/min. This was used to get a sense of what products resulted from the synthesis. Two solvents were used in this process:

Solvent A: 0.1% TFA in H₂O
Solvent B: 0.1% TFA, 10% H₂O, 90% Acetonitrile

A 1 mg/mL solution of the crude peptide in 5% acetic acid, 0.1% TFA in H₂O was prepared. This was injected 100 µL at a time into the column. The gradient profile was adjusted to optimize the program. The optimized analytical program was as follows:

Table 3: Optimized analytical solvent program

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
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<td>15</td>
</tr>
<tr>
<td>4</td>
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</tr>
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<tr>
<td>50</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>52</td>
<td>85</td>
<td>15</td>
</tr>
</tbody>
</table>

Preparative HPLC of Crude Peptide

Preparative HPLC of the crude peptide was performed with a C-18 (Alltima 250mm) reversed phase column and a flow rate of 7 mL/min. The program was optimized to isolate peak A and reduce run time. The same solvents were used for this process as for the analytical HPLC. A 20 mg/mL solution of the crude peptide in 5% acetic acid, 0.1% TFA in H₂O was prepared. This was injected 100 µL at a time into the
column. The gradient profile was adjusted to optimize the program. The optimized preparative program was as follows:

**Table 4: Optimized preparative solvent program**

<table>
<thead>
<tr>
<th>Time (m)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
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<td>100</td>
</tr>
<tr>
<td>30</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

**Isolation of Pure Peptide**

The pure peptide was isolated from solution in two steps. First the HPLC eluant was spun on a Labconco centrivap concentrator with cold trap for approximately one hour to evaporate the acetonitrile. Then the aqueous solution was frozen in liquid nitrogen and put under vacuum on a Virtis Bechtop 3L Sentry lyophilizer to sublimate the water, leaving behind pure, dry peptide. This peptide was stored in a freezer to reduce degradation.

**MALDI-TOF Mass Spectroscopy**

The matrix solution was made by combining 10 mg of α-cyano-4-hydroxy cinnamic acid with 500 μL acetonitrile and 500 μL 0.2% trifluoroacetic acid in H₂O, mixing frequently over a 15-minute period to allow the crystals to dissolve. Only the clear supernatant was used in the next steps. Calibration standard and peptide solutions were prepared by dissolving 1 mg of sample in 62.5 μL acetonitrile and 62.5 μL 0.2%
TFA in H$_2$O. The standard and peptide solutions were mixed with the matrix solution in a one to one ratio, and spotted on a Bruker MTP 394 target plate in 1 $\mu$L duplicate applications, and allowed to dry thoroughly. The mass spectroscopy was then performed on a Bruker Autoflex Speed MALDI-TOF mass spectrometer.

**Circular Dichroism Spectroscopy**

A 10 mM solution of 2-(4-morpholino)-ethane sulfonic acid buffer in deionized water was made and adjusted to pH 7 with 1 M NaOH. This buffer was mixed 50% v/v with 2,2,2-trifluoroethanol. Pure peptide was added to each solution at 1 mg/mL. The spectra were obtained on an Olis DSM CD spectrophotometer. Ellipticity was measured in triplicate at 1nm intervals from 195 nm-250 nm with an integration time of 60 seconds.
References


Appendix 1: MALDI-TOF Mass Spectroscopy of HPLC peak A
Zoom in on peaks
Appendix 1: MALDI-TOF Mass Spectroscopy of HPLC peak B
Zoom in on peak region 1.
Zoom in on peak region 2
Appendix 1: MALDI-TOF Mass Spectroscopy of HPLC peak C
Zoom in on peaks