

**ALTERED AMINO ACID SEQUENCE AFFECTS AMYLOID
FORMATION IN A β (25-35)**

A Senior Scholars Thesis

by

MORGAN CHATEAU

Submitted to the Office of Undergraduate Research
Texas A&M University
In partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2007

Majors: Biochemistry and Genetics

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Approved by:

Research Advisor:
Associate Dean for Undergraduate Research:

Dr. Martin Scholtz
Robert C. Webb

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ABSTRACT

Altered amino acid sequence affects amyloid formation in A β (25-35)

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Many neurodegenerative diseases are associated with protein misfolding where the protein forms β -sheet rich polymers called amyloid. Alzheimer's disease (AD) is an amyloid aggregation disease involving the A β (1-42) peptide fragment of the Amyloid Precursor Protein (APP) in the nervous tissue of the brain. Within the peptide there are regions thought to be more critical for amyloid formation; one of which is A β (25-35). This sequence has the ability to form amyloid at sufficient peptide concentrations. Within this region, residues 33-35 have been found by terminal deletions to be a core region (1), the residues necessary for amyloid formation. Using Cys-scanning mutagenesis the estimated core region (31-34) is slightly different. Previous papers have shown, the peptide loses the ability to form amyloid fibrils when the sequence of amino acids is changed (2). For this experiment, four different A β (25-35) sequences were tested, each having the same composition but a different sequence. FPLC was used in conjunction with Thioflavin-T fluorescence to monitor amyloid development. The results showed a definite effect on amyloid formation as compared to wild type.

Sequence S-N showed nearly no change in behavior. Sequence S-M appeared to lose all amyloid forming ability. The remaining two sequences were unable to remain in solution long enough to be accurately tested.

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Special thanks are due to Katherine Ridinger who provided constant guidance and emotional support throughout the research process. Also, many thanks go to Dr. Scholtz for editing my paper as well as letting me work in his lab. All members of the Scholtz and Pace Labs were very supportive and willing to answer questions at any time.

NOMENCLATURE

Th-T	Thioflavin-T
S-C	Carboxyl-terminal partial scramble mutant
S-N	Amino- terminal partial scramble mutant
S-CN	Carboxyl- and amino- biterminal partial scramble mutant
S-M	Middle partial scramble mutant
Wt	Wild type (non-mutated A β (25-35))
FPLC	Fast performance liquid chromatography

TABLE OF CONTENTS

	Page
ABSTRACT	iii
ACKNOWLEDGMENTS.....	v
NOMENCLATURE.....	vi
TABLE OF CONTENTS	vii
LIST OF FIGURES.....	viii
LIST OF TABLES	ix
CHAPTER	
I INTRODUCTION.....	1
II METHOD.....	3
Partial scramble mutant synthesis	3
Assay Methods.....	3
Thioflavin – T Confirmation Test.....	4
III RESULTS.....	5
IV SUMMARY AND CONCLUSIONS.....	7
Summary	7
Conclusions	7
REFERENCES.....	9
CONTACT INFORMATION.....	10

LIST OF FIGURES

FIGURE	Page
1 168 Hour Assay (Dual Trial) done at 37°C and pH 6	5
2 72 Hour assay done at 37°C and pH 6.....	6

LIST OF TABLES

TABLE	Page
1 Sequence of wild type and partially scrambled mutants tested.....	3

CHAPTER I

INTRODUCTION

AD is a neurodegenerative disease commonly associated with the formation of an ordered aggregate or plaque in the intracellular space between cells. These plaques are formed by a specific type of protein aggregate classified as amyloid, a β -sheet rich polymeric strand made of many misfolded proteins. The monomeric building blocks are known as prions (proteinaceous infectious particles) (3). Prions are infectious because they have the ability to misfold a properly folded protein into a similar prion, bind to it, and then misfold another (4). This unique ability to spread disease without any genetic material has created a new classification of disease, prion diseases. Fully mature amyloids also retain this infectivity.

Misfolded proteins (prions) are exported from the cell in monomer form and then bond with each other outside of the cell. Many fibrils may start to grow after a varying lag phase. After fibrils have started they behave like nucleation points and protofibril growth accelerates, much like crystals. These protofibrils then associate to form chains that will eventually grow long enough to be seen under the microscope.

This project further explored the importance of amino acid sequence in amyloid formation. This project tested the possibility that scrambling a part of the

This thesis follows the style of *Journal of Biological Chemistry*.

peptide sequence will result in the loss of amyloid forming ability of the peptide. By partially changing the sequence of amino acids, an amyloid core region of the peptide was indicated. Four partial scrambles were tested; an amino terminus scramble, a carboxyl terminus scramble, a center scramble, and a bi-terminus scramble (See Table1). Wild type was also tested with each assay as a positive control and as a basis of comparison.

The progress of amyloid formation was monitored by watching changes in monomer concentrations in solution over time followed by a Thioflavin-T binding test to ensure the decreasing levels of monomer was because of amyloid formation and not from amorphous aggregate. The wild type protein reached a stable equilibrium within hours. Modification of the sequence may only have slowed amyloid formation instead of preventing it. Therefore, a long time scale assay was done to ensure loss of amyloid formation. Then a series of assays were done focusing on the first twenty hours of amyloid formation.

CHAPTER II

METHODS

Partial scramble mutant synthesis

The five eleven residue peptides were synthesized using solid-state syntheses on Wang resin and purified by fast performance liquid chromatography (FPLC) based on hydrophobicity with 15RPC resin (5). Mass spectroscopy was employed to confirm peptide identity. The resulting lyophilized peptide was dissolved in 88% formic acid to maintain a monomeric state and stored at -80°C (6).

TABLE 1
Sequence of wild type and partially scrambled mutants tested

Peptide	Sequence
Wt A β (25-35)	N ¹ -GSNKGAIIGLM-C'
S-N	N ¹ -NGSKGAIIGLM-C'
S-C	N ¹ -GSNKGAIILMG-C'
S-NC	N ¹ -NGSKGAIILMG-C'
S-M	N ¹ -GSNIAIKGGLM-C'

Since amyloid fibrils grow similar to the way crystals form, it was important for all starting materials to be monomeric, otherwise, the peptides may have had nucleation centers already and each assay would have different kinetics, due to varying nucleation numbers.

Assay Methods

Assays were done in 11X34 mm ultra centrifuge tubes using parafilm to prevent evaporation. Each assay involved diluting a quantity of stock solution in water and adjusting the pH to the pI(=6) to increase amyloid formation (7). The tubes were then

incubated in a rotor-spinner at 37°C. Time points were taken by centrifuging the tubes at 70K rpm for 25min, taking a 50µL sample of the supernatant, and resuspending the pellet by vortexing before returning tubes to incubator. The much heavier amyloid fibrils and protofibrils pelleted, leaving only monomer in the supernatant.

The 50µL samples were placed in a -80°C freezer until they could be analyzed by FPLC so as to prevent further amyloid formation. Each sample remained frozen until immediately before injection into the FPLC. This was to ensure the samples did not continue reacting while waiting for analysis as even a small amount of time can affect the results greatly. The absorbance at 220nm was recorded for the monomeric peak.

Thioflavin – T Confirmation Test

A confirmation Thioflavin-T (Th-T) test was done at the end of each assay to ensure the presence of amyloid as opposed to an amorphous aggregate (8,9). Th-T is an amyloid specific fluorescent dye thought to associate with the protofibrils, a precursor to mature amyloid. When the dye is bound, it fluoresces at a different wavelength than when it is not bound, and thus the enhancement of fluorescence is related to the extent of amyloid formation (9).

CHAPTER III

RESULTS

Figure 1 shows the long term assay for the four mutants and the wild type protein (Wt)

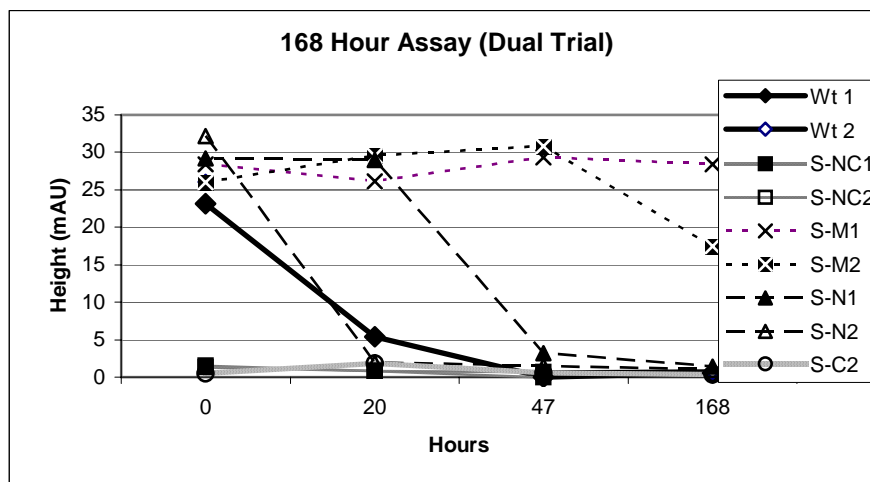


Figure 1. **168 Hour Assay (Dual Trial)** done at 37°C and pH 6. Two trials were done at the same time at the same pH and temperature.

performed with dual trials, as notated by the 1 or 2 following the mutant abbreviation.

This assay showed that the peptides achieved their final state by the third time point.

Amyloid formation or lack of formation was evident in less than 47 hours and the mutants remained in one state for the duration of the assay. Only part of the reaction mixture for the final time point of S-M2 was analyzed by FPLC due to an accident, which would explain the lower reading. Also, S-C1 was lost within the first reaction hour because the parafilm lid was not attached securely and the mixture evaporated.

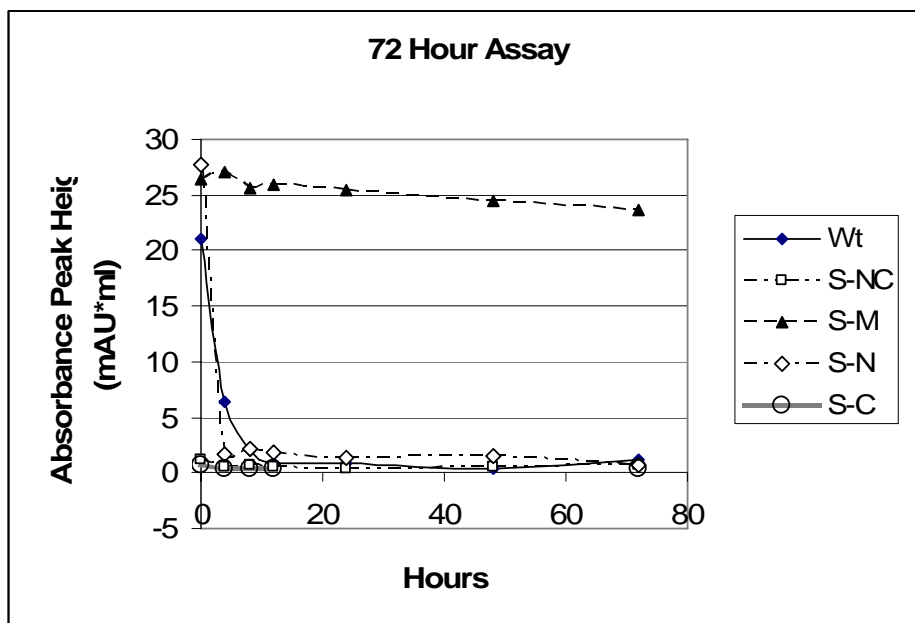


Figure 2. 72 Hour assay done at 37°C and pH 6.

The assay was repeated with more frequent time points to help narrow down when the peptides reach equilibrium (Figure 2). As seen in the graph, all strains of mutants achieved their final state within twenty hours. Mutant S-M showed nearly no decrease in monomer state peptide concentrations in solution for the duration of the testing time period. Th-T fluorescence confirmed a lack of amyloid presence. The wild type protein showed a characteristic shape seen in previous articles. The S-N mutant showed a trend nearly identical to Wt, suggesting again the order of amino residues in the 25-27 region is not essential for amyloid formation. Th-T confirmed the presence of amyloid formation. S-CN and S-N showed minimal absorbance immediately after mixing the initial reaction solutions and maintained these low levels for duration of the assay. Th-T testing for S-CN and S-N was inconclusive of whether aggregate was amyloid or amorphous aggregate.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Neurodegenerative diseases like Alzheimer's Disease are commonly associated with protein aggregates classified as amyloid, a β -sheet rich polymeric strand made of many monomeric building blocks. Prions are infectious because they have the ability to misfold a properly folded protein, bind to it, and then misfold another (4). This project further explored the importance of amino acid sequence in amyloid formation.

Scrambling a part of the peptide sequence can affect the amyloid forming ability of the peptide.

The wild type peptide from A β (25-35) reached a stable equilibrium within hours.

Modification of the sequence had three different results: no change in amyloid forming ability, loss of amyloid forming ability, and instantaneous precipitation out of solution.

A 168 hour assay (Figure 1) showed that these results were constant after the initial twenty hours of reaction, and remained so for the time period tested. From Figure 2 the S-M mutant lost amyloid forming ability and remained in the monomer state in solution. This suggests residues 28-32 are part of the core region, which supports the Cys-scanning core region as described earlier. The S-N mutant exhibited behavior like the wild type peptide. This supports both of the core regions found by deletions and by Cys-scanning.

One point of interest was S-NC's and S-C's dramatic decrease in solubility in water when there was a small change in the order of residues' 33-35. Changing their order doesn't change the net hydrophobicity or β -strand propensities of the peptide. These mutants were soluble in formic acid in the stock solutions. A possible explanation is that putting the hydrophobic residues closer together created a hydrophobic center which pulled the peptide out of solution.

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