MECHANISTIC STUDIES OF BETA PROTEIN AND NAC AMYLOID FORMATION IN ALZHEIMER'S DISEASE AND BIOCHEMICAL PROBES OF NACP FUNCTION

by

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder which afflicts over 20 million individuals worldwide. AD is characterized by the presence of insoluble fibrillar deposits of protein in the brain, in what are known as "amyloid" plaques. The primary core protein found in these amyloid fibrils is the 39-43 amino acid β amyloid protein (Aβ). Aβ forms amyloid fibrils via a nucleation-dependent mechanism, resembling that of a crystallization. A structural model for the β34-42 amyloid fibril has been developed using solid state NMR. To test the critical features of this model, a series of β34-42 peptide mimetics were synthesized as chemical probes of hydrogen bonding and the proposed cis geometry of the Gly37-Gly38 amide bond. 1H NMR spectrometry was used to demonstrate that a hydrophobic cluster, formed by the residues flanking the Gly37-Gly38 amide bond, stabilizes the cis isomer in aqueous solution. A model is presented, based on these observations, in which hydrophobic cluster formation increases the rate of amyloid nucleation by stabilizing a key feature of the aggregating conformation.

A 35 amino acid peptide named non-Aβ component of AD amyloid (NAC), is a minor but intrinsic component of AD amyloid plaque and shares a region of high local sequence homology to Aβ. The ability of synthetic NAC to "seed" the aggregation of β1-40 was investigated. Seeding is a highly specific phenomenon and requires a significant degree of structural similarity. NAC was shown to seed fibril formation by Aβ and vice versa, suggesting that NAC aggregation may serve as a pathogenic "trigger" leading to disease. NAC is derived from a precursor protein of 14 kDa, called NACP, which is found in synapses and has been implicated in learning. We have found that NACP binds specifically to valosin-containing protein (VCP, also called p97), a cytosolic protein which has been implicated in protein trafficking, suggesting that NACP may have a function in regulating synaptic vesicle formation. This finding may have important implications for our understanding of the molecular basis of learning and its connection to AD.
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Acknowledgments

I realize that this is the first, and in many cases the only part of my thesis that people will read. Talk about pressure! It's hard to summarize nearly 5 years in just a few paragraphs, but here goes...

First and foremost, I would like to thank Professor Peter Lansbury, Jr., my graduate advisor. Peter's boundless enthusiasm and aggressive attitude toward science are contagious. I could always count on him to tell me when we were just about to perform "the key experiment"; of course, this seemed to happen every few days. I wish him the best of luck as he prepares to move the lab to the Center for Neurologic Disease at Harvard Medical School; I am certain that MIT will regret having let him get away.

As a member of Peter's fifth class of graduate students, I have had the opportunity to work with every PhD student and postdoc in the Lansbury group at MIT, many of whom have helped me directly with my research, and all of whom have contributed to my MIT experience.

Sharing this perspective with me has been Krista Evans. Krista is one of the most unselfish people I have ever met, and, although I will no longer have to hear every Friday about how I shouldn't have let her eat that donut at group meeting, I will miss working beside her.

Joe Jarrett and Beth Berger carried out experiments which elucidated the mechanistic issues surrounding $\beta$ amyloid formation. Joe also initiated the project which comprises Chapter III of this thesis. Someday I would love to have just half of Joe's imagination and scientific creativity, and Beth's work ethic and compassion.

Dr. Brian McGuinness made more analogs of $\beta$ amyloid C-terminal peptides than anyone would ever want to make, and the analogs described in Chapter II were
inspired by his work. Brian was also instrumental in putting together our Peptide Symposium poster entitled, "Multiple-Chimp Solid-Phase Peptide Synthesis (MCPPS), a Novel Method for the Construction of Random Peptide Libraries."

Dr. Hogyu Han synthesized NAC and worked with me on the project described in Chapter IV. Hogyu is a model of persistence, and will make a fine advisor himself someday.

The initial identification of an NACP binding protein was carried out by Anna Poon. Anna's efforts made the work described in Chapter V possible, and her sense of humor always livened up the lab.

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Jim "Wheels" Harper ran the AFMs shown in this thesis and refined the aggregation assay to a point where it actually became reproducible. I could always count on Jim for advice about computers, cars, and poker.

Dr. Cheon-Gyu Cho was always there when I needed advice from a "real" synthetic chemist; Cho has never been afraid to voice his opinion, and he never should be.

Jon Come synthesized the PrP peptides which I used (I told you I'd thank you for that, Jon) and is master of the charcoal grill; I look forward to his and Sandy's wedding (finally). Ed Licitra (the "Big Lemon") and I have gone through the entire MIT experience together, whether he was digging out the Volvo with a piece of his Caravan, trying (often in vain) to explain the basics of biology to me, or getting me free beer at the Muddy. Guys, I think this is the year for Slacks softball...

I have worked with several very talented undergraduates at MIT, including Stephen Chan, who synthesized one of the depsipeptide analogs, and Abhi Vaishnav, who made several of the sarcosine-containing peptides.

The Lansbury group has always had its own unique personality, and it always
will. I am sure that the current group members, Magdalena Anguiano, Chris May, Jim, Kelly, Weiguo, and Cho, will carry this style to the new lab, and I am just as sure that they will be successful in whatever they choose to do.

Several researchers outside of the lab have contributed to my work in invaluable ways. Phil Costa, working in Prof. Robert Griffin's lab, clarified the structural features of the Aβ amyloid fibril which serve as the basis for much of our mechanistic work. Dr. Adriana Ferreira, at Harvard Medical School, had the patience to try and teach a group of chemists about neurobiology. Dr. Michel Goedert at the MRC in Cambridge, England provided the expression vector for α-synuclein. Dr. David Clayton (University of Illinois) provided large quantities of anti-NACP antibodies. Dr. Lawrence Samelson (NIH, Bethesda, MD) provided us with both anti-VCP antibodies and the expression vector for GST-VCP. Andrew Rhomberg, a graduate student of Klaus Biemann's at MIT, spent a lot of time trying to get mass spectral data on my NACP samples, and eventually succeeded despite my best efforts to the contrary.

The majority of the published work on NAC and NACP to date has come from the laboratory of the late Dr. Tsunao Saitoh at UCSD. Dr. Saitoh's numerous contributions to the field of Alzheimer's neuropathology, and his willingness to share unpublished results with us, are greatly appreciated.

I have to thank my parents for encouraging my curiosity; I remember, at about age 7, sitting in my father's office and playing with molecular models. At the time I thought they were fun; I guess, for only slightly more mature reasons, I still do.

I've saved the best for last, of course. Carolyn (a.k.a. "Mrs. Dr. Weinreb"), marrying you was the smartest thing I did in graduate school. With you by my side, I have truly been able to enjoy my time at MIT, and as we move on together, I am certain that the best has yet to come...
List of Abbreviations

Aβ  β amyloid protein
AD  Alzheimer's disease
ApoE  apolipoprotein E
APP  β amyloid precursor protein
Boc  t-butoxycarbonyl
BOP  benzotriazol-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate
CD  circular dichroism
DIC  diisopropylcarbodiimide
DIEA  diisopropyylethylamine
DMAP  4-dimethylaminopyridine
DMF  dimethylformamide
DMSO  dimethylsulfoxide
DTT  dithiothreitil
EM  electron microscopy
FABMS  fast atom bombardment mass spectrometry
FAD  familial Alzheimer's disease
FTIR  Fourier-transform infrared spectroscopy
Fmoc  fluorenylmethoxycarbonyl
GST  glutathione-S-transferase
HFIP  hexafluoro-2-propanol
HMPB  4-hydroxymethyl-3-methoxyphenoxybutyric acid
LB  Luria-Bertani broth
MALDI-TOF  matrix-assisted laser desorption mass spectrometry
MBHA  methylbenzhydrylamine
NAC  non-Aβ component of AD amyloid
NACP  non-Aβ component of AD amyloid, precursor
NFT  neurofibrillary tangle
PAM  4-hydroxymethylphenylacetamidomethyl
PBS  phosphate-buffered saline
PDMS  plasma-desorption mass spectrometry
PHF  paired helical filament
PMSF  phenylmethylsulfonyl fluoride
PNP-14  phoshoneuroprotein 14
PrP  prion protein
PVDF  polyvinylidene difluoride
PyBOP  benzotriazol-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
PyBroP  bromo-tris-pyrrolidino-phosphonium hexafluorophosphate
R² SSNMR  rotational resonance solid state nuclear magnetic resonance
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFA  trifluoroacetic acid
TFMSA  trifluoromethanesulfonic acid
VCP  valosin-containing protein
Chapter I

Amyloid Proteins in Alzheimer's Disease

In 1906, a German physician described an unusual case of dementia in a female patient.\(^1\) The woman, Frau Auguste D., showed symptoms "which could not be classified under any well-known clinical patterns," beginning with "a strong feeling of jealousy toward her husband" and progressing to disorientation, severe memory loss, delirium, and an inability to understand and use language over the four and a half years until her death at age 55. During autopsy of Frau Auguste's brain, the doctor utilized a recently developed silver staining technique to discover the "deposition of a peculiar substance." He also noted significant loss of neurons, which appeared to have been replaced by "only a tangled bundle of fibrils." The physician, of course, was named Alois Alzheimer, and the disease which now bears his name remains nearly as cryptic today as it was to Dr. Alzheimer nearly a century ago.

Alzheimer's disease (AD) affects nearly 4 million individuals in the United States, and over 20 million worldwide.\(^2\) It is the most common form of progressive dementia in humans, and is increasing in prevalence in developed countries as lifetimes increase.\(^3\) The risk of acquiring AD increases dramatically in people over 65 years old and continues to increase with age.\(^2\) The prevalence of AD is difficult to determine precisely because of the current lack of definitive objective diagnostic
criteria; however, it is estimated that AD afflicts ~10% of the population over 65 and nearly 50% of the population over 85.

At present, there is no effective treatment or cure for AD. The inability of modern medicine to successfully combat this disease stems from a poor understanding of the factors responsible for causing the disease. Over the last decade, tremendous advances have been made in defining some of the possible biological processes and biochemical entities which may play key roles. Only through the detailed study of these factors will we be able to determine the molecular basis for AD and, eventually, develop effective strategies for the treatment of this devastating disease.

**AD Pathology**

Establishing diagnostic criteria for Alzheimer's disease is difficult because of the large number of other diseases with similar symptoms of dementia. Although genetic AD risk factors have been identified, there is no known biological marker which can be used to make a positive diagnosis. Instead, clinical diagnosis for probable AD is made by the exclusion of other potential causes. The only true distinction between AD and other related neurodegenerative disorders is in the pathology of the AD brain, which can only be determined upon autopsy.

The pathological hallmarks of AD are the presence of insoluble proteinaceous deposits in the brain, in the form of neurofibrillary tangles and senile plaques. In 1963, electron microscopy experiments first revealed the morphology of the neurofibrillary tangles (NFT) as ~10 nm twisted fibrillar structures which were called "paired helical filaments" (PHF). Immunocytochemical studies at first suggested that the PHF contained neurofilament proteins. However, subsequent experiments demonstrated that the primary component of the PHF is the microtubule-associated protein tau. The majority of tau found in the PHF is in a
highly phosphorylated state. There is no evidence, as yet, for a protein component other than hyperphosphorylated tau in the PHF.

The senile plaques, on the other hand, are complex lesions comprising a number of species. Plaques are found not only in AD, but also in patients with Down's Syndrome (trisomy 21), and, to a lesser extent, in normal aging. The plaques were named "amyloid" (meaning starch-like), because they could be stained with classic amyloid dyes, such as Congo Red. Despite the subsequent identification of the proteinaceous nature of amyloid deposits, the term has endured. Plaques consist of a proteinaceous core of amyloid fibrils, surrounded by dystrophic neurites, activated microglia, and fibrillary astrocytes. The amyloid fibrils of the plaque show morphology which is distinct from that of the PHF; they are unpaired and ~8 nm in diameter. The fibrils do, however, resemble those found in a number of unrelated systemic disorders, termed "amyloidoses".

To date, more than 15 normally nonfibrillar proteins have been identified as forming amyloid fibrils in a number of diverse clinical situations (Table 1.1). For

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<td>Transthyretin</td>
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<td>Pancreas</td>
<td>Islet Amyloid Polypeptide</td>
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<td>Musculoskeletal</td>
<td>β-2-microglobulin</td>
</tr>
<tr>
<td>Brain</td>
<td>β Amyloid Protein</td>
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<td>Prion Protein</td>
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<td></td>
<td>Cystatin C</td>
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<td>Skin</td>
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example, prion protein amyloid is a hallmark of Creutzfeldt-Jakob disease in humans and its counterpart bovine spongiform encephalopathy ("mad cow disease") in cattle. A number of systemic diseases are also characterized by extensive deposition of amyloid, suggestive of a universal pathogenicity of amyloid deposits. An understanding of the structure and mechanism of amyloid deposition in AD may therefore provide insight into a wide range of diseases.

The β Amyloid Protein and APP Processing

The discovery which has probably had the most profound effect on the modern era of AD research was the identification in 1984, by Glenner and Wong, of the primary protein found in the amyloid plaque core. Amyloid filaments were isolated from meningeal vessels and solubilized in 6M guanidine hydrochloride. The N-terminal 24 amino acids were sequenced, and the novel protein was termed the β amyloid protein (β protein, Aβ). The protein has a charged N-terminus and an extremely hydrophobic C-terminus, which was later shown to extend to 39-43 residues.

Cloning of Aβ cDNAs indicated that the peptide is derived proteolytically from an integral membrane protein called APP (Amyloid Protein Precursor), which is encoded by a gene on chromosome 21. APP is expressed as several transcripts of up to 770 amino acids; the most prevalent form contains 695 residues. APP has a 17-residue signal peptide at the N-terminus. Two alternatively spliced exons, of 56

![Figure 1.1 Amino acid sequence of the β amyloid protein (residues 1-42).](image-url)
and 19 amino acids, are inserted at position 289 (numbers correspond to APP770); the longer of these contains a serine protease inhibitor domain of the Kunitz type (KPI).\(^{18}\) APP has two sites of N-glycosylation (542 and 571), and a single putative membrane-spanning domain, at residues 700-723.\(^{19}\) Approximately 28 residues of Aβ are extracellular, while the last 11-15 amino acids are located within this putative transmembrane region of APP.

![Figure 1.2 The amyloid precursor protein (APP). The Aβ sequence is indicated by the dark box, and is located partially within the cell membrane. The positions of APP cleavage by the putative α-, β-, and γ-secretases are indicated.](image)

APP is normally processed in a constitutive secretory pathway in which cleavage occurs at Aβ16\(^{20}\) to produce a large secreted derivative and an ~8.7 kDa membrane-associated fragment, neither of which can produce amyloid because they do not contain the intact Aβ sequence. This cleavage is carried out by an unidentified enzyme designated "α-secretase." Based on this data, it was initially assumed that only aberrant processing under pathological conditions could lead to the formation of Aβ and its release from the membrane.
It has become evident, however, that only a minority of APP molecules are processed through this secretory pathway. Full length APP, as well as C-terminal fragments of APP which contain the complete Aβ sequence, have been found intracellularly within isolated clathrin-coated vesicles. Additionally, surface antibody and biotinylation experiments have shown that APP can be reinternalized from the cell surface. Together with other supporting experiments, these findings suggest that full-length APP, as well as some Aβ-containing fragments, are targeted to lysosomes for degradation.

The discovery that Aβ-containing fragments of APP are generated under normal conditions suggested that aberrant processing of APP might not be required for the production of Aβ. This concept was supported by the unexpected finding that Aβ is secreted into the blood and cerebrospinal fluid of normal, healthy individuals, and is secreted into the medium of a variety of cultured cells. APP proteolysis leads to a heterogeneous population of Aβ and closely related peptides. The majority of the peptides produced begin at the N-terminus of Aβ, Asp-1. Minor amounts of peptides which begin at Val-3, Ile-6, Glu-11, or Phe-4 have also been identified. The C-termini of the Aβ peptides produced also varies considerably. The primary plaque variants are the longer forms, β1-42 and, to a lesser extent, β1-43. The shorter variants are prevalent in the circulatory system; β1-39 is the primary component of cerebrovascular amyloid, and β1-40 is found in the blood and CSF. The putative enzymes responsible for N- and C-terminal cleavage at the Aβ sequence have been designated β- and γ-secretases, respectively.

**Aβ: AD Cause or Byproduct?**

A causative role for Aβ in AD has by no means been proven. However, there is growing support among AD researchers for a Aβ protein amyloid formation as a critical event in AD pathogenesis. The observation that patients with Down's
Syndrome, who have an extra copy of chromosome 21, invariably develop pathology resembling that of AD patients strongly supports the hypothesis that the metabolism of APP into Aβ may play a critical role in the pathogenesis of disease.31

One significant indication that Aβ amyloid deposition might be a primary event in AD is the effect of APP mutations which correlate to early-onset familial AD (FAD). One such mutation, a double-mutation of K670N/M671L found in a Swedish family results in a 6- to 15-fold increase in the secretion of Aβ in cell culture.32,33 This mutation is immediately N-terminal to the Aβ sequence, and apparently affects the proteolytic recognition of the β-secretase. This finding provides a clear link between a familial AD genotype and the generation of Aβ. The increased production of Aβ may lead to a greater extent of fibril formation and, in turn, an increased risk of AD.

![Figure 1.3 APP mutations in AD which affect Aβ production. Numbering corresponds to APP770. The Aβ sequence is indicated in bold.]

A second set of FAD mutations occurs at position 717, only a few residues C-terminal to the Aβ sequence. Unlike the Swedish mutation, these mutations have no effect on the total production of Aβ peptides.33 They do, however, lead to the production of longer, more readily aggregating peptides,33 lending support to the idea of Aβ aggregation as a pathogenic event.34 The significance of "long β" production is discussed in greater detail later in this chapter.
Support for a pathogenic role for Aβ has come from the direct demonstration of its neurotoxicity. Studies of the neurotoxicity of Aβ were initially plagued by inconsistent results. Aβ was shown to have both neurotrophic and neurotoxic properties depending on the concentration used. Moreover, different results were obtained depending on the specific preparation of peptide. Many of these problems may have stemmed from inconsistencies in the aggregation state of the peptide. Subsequent work has helped to clarify this issue, and it is now widely believed that fibrillar Aβ is toxic to cells.

Support for the amyloid hypothesis is growing rapidly, but it has by no means been universally accepted among AD researchers. Many alternative hypotheses have been presented. One school of thought, for example, holds that the neurofibrillary tangles show a more substantive correlation to cases of dementia. However, NFTs have also been found in several unrelated neurological disorders that are not characterized by amyloid deposition and are clinically and pathologically distinct from AD. Additionally, there are documented cases of AD brains with few tangles which still display plaques and neuronal loss. Most experts in the field, at least at the present time, agree that plaque formation precedes NFT formation, and that Aβ is a more likely candidate for a causal agent. Other hypotheses have focused on neurotransmitter release, inflammation, and alternate genetic risk factors (for example, apolipoprotein E) as possible pathogenic determinants. It is likely that no single pathway or event is solely responsible for disease, but that many factors, either singly or in combination, could have important roles in causing the complicated pathology of AD.

Structural Studies of Amyloid Fibrils

A primary research interest of this laboratory over the past several years has been the elucidation of the structure and mechanism of amyloid fibril formation.
Since amyloid fibrils are insoluble and non-crystalline, the traditional methods for protein structure determination, including X-ray and NMR techniques, have not been successful in determining amyloid structure. Because of the difficulty in obtaining information about amyloid structure, the cross-β fibril, a model proposed by Pauling in 1955 to describe the structure of *Bombyx mori* silk, has been adopted as a general model of amyloid structure (Fig. 1.4). Clearly this model is inadequate for describing individual amyloid structures on a molecular level. Our laboratory has developed new methods for amyloid structure determination which utilize our ability to specifically incorporate $^{13}$C atoms into the peptide backbone.

![Figure 1.4](image)

**Figure 1.4** Structural depiction of the cross-β fibril, as proposed by Pauling et al. The antiparallel peptide chains are arranged perpendicular to the direction of fibril growth to form a β-pleated sheet. Sheets are stacked in a parallel fashion, the intersheet distance being governed by the identity of the amino acid side chains. The alignment of the strands relative to each other is not specified.
The two techniques which have been used are isotope-edited Fourier transform infrared (FTIR) spectroscopy and rotational resonance solid state NMR spectroscopy (R$^2$ SSNMR).

In the course of developing a synthetic route to β1-42, it was noted that a C-terminal nonapeptide, β34-42, was highly insoluble in aqueous media, even in the presence of 6M guanidinium thiocyanate. The peptide formed amyloid fibrils with a morphology similar to that of the full-length Aβ found in amyloid plaque. X-ray diffraction and FTIR experiments revealed that the fibrils had a cross-β conformation. These initial experiments suggested that the C-terminus of Aβ might be important for amyloid formation and inspired structural studies of β34-42.

Isotope-edited FTIR of β34-42 indicated that Val36, Val39, and Val40 are located in a β-sheet region of the aggregate. The terminal residues, L34, I41, and A42, and the central residues, Gly37 and Gly38, on the other hand, did not appear to be part of an idealized β-sheet, and appeared to have an unusual conformation in the solid state. The conformation of this amide bond was investigated in greater detail through the use of novel solid state NMR methodology.

Rotational resonance (R$^2$) SSNMR, a technique developed in the laboratory of Professor Robert Griffin at M.I.T, allows the determination of the distance between members of a homonuclear spin pair based on rotation-enhanced transfer of Zeeman magnetization. This technique can be used to determine distances between specific carbon atoms in a given peptide by selective pairwise $^{13}$C labeling. These distances can be used to define each dihedral angle in the peptide backbone (ϕ, ψ, ω) to allow the systematic elucidation of protein structure. The application of R$^2$ to the structural determination of the β34-42 amyloid fibril has provided important information about the structure of this peptide, and of amyloid structure in general.

Two standard measurements determine the ϕ, ψ, and ω dihedral angles, and therefore the conformation of the peptide backbone. The distance between the α
carbon of one amino acid and the carbonyl carbon of the next (α1-2, e.g., α37-38 in Fig. 1.5) determines the amide bond configuration (ω1-2). If α1-2 ≤ 4.25 Å, then ω=0° (cis) and if α1-2 ≥ 4.4 Å, then ω=180° (trans). Otherwise, both cis and trans configurations must be considered. The φ2 dihedral angle can also be determined from the α1-2 distance. The distance between the carbonyl of amino acid 1 and the α carbon of amino acid 3 (1α3) depends on the angles φ2 and ψ2, and, in combination with the α1-2 distance, can be used to determine ψ2.

![Diagram of amino acid with distances measured](image)

**Figure 1.5** Distances measured using R² SSNMR. The possibility of the cis geometry for Gly37-Gly38 was suggested by α37-38, and the compact nature of the β-sheet was indicated by 37α39.⁴⁷

Initial R² experiments on β34-42 focused on defining the geometry at Gly37-Gly38, the region of the peptide which appeared to have an unusual structure by FTIR. In initial studies of β34-42, the α37-38 distance was determined to be 4.0 ± 0.2 Å, indicating that the Gly37-Gly38 amide bond has the unusual cis configuration.⁴⁶
Subsequent refinements to the SSNMR simulations, however, indicated that the effect of inhomogenous line broadening was not fully appreciated in the initial studies.\textsuperscript{47} Consequently, the $\alpha37$-$38$ distance was most likely underestimated in the original work. The improved simulation procedure led to the conclusion that either the cis or the trans configuration was possible.\textsuperscript{47} Cis amide bonds at other backbone positions were ruled out by the measured distances.

Using the $R^2$ distances in conjunction with molecular modeling, a library of possible structures was generated. A significant portion of this library comprised cis amide-containing structures. A working model for the $\beta34$-$42$ sheet was proposed, based on the common structural features of this library.\textsuperscript{47} This model defines certain critical features of the $\beta$ sheet. First, the sheet is well-defined with respect to interstrand orientation; this was not surprising, based on the importance of interstrand side-chain interactions in $\beta$-sheet formation. Second, the sheet appears to be more highly "pleated" than in the cross-$\beta$ fibril, a feature which may facilitate specific stabilizing side-chain interactions. Finally, the Gly-Gly amide backbone conformation, which may be cis, is another feature which could serve to stabilize the intermolecular packing interactions in the $\beta34$-$42$ amyloid fibril.

**Occurrence of Cis Amide Bonds in Proteins**

Non-proline cis amide bonds have been identified in several crystalline proteins.\textsuperscript{51-54} The apparent frequency of their occurrence, however, is much lower than would be expected based solely on thermodynamics: An estimate of the free energy difference between cis and trans isomers of approximately 3 kcal/mol would suggest that 1 out of every 100 peptide bonds should be cis.\textsuperscript{55} This discrepancy may be due, at least partially, to the misassignment of peptide bond geometry in crystal structure determination.\textsuperscript{52} A 1990 survey of protein crystal structures found 17 non-
proline cis amides; of these, the only sequence to appear more than once was Gly-Gly.\textsuperscript{53}

In a globular protein, a cis amide must allow the protein to assume a conformation with stabilizing tertiary interactions to offset the energetic cost of amide isomerization. Similarly, β34-42, a short peptide which assumes an extended conformation in the fibrillar aggregate, would have to derive a significant amount of intermolecular interaction energy through isomerization. One structural feature, a hydrophobic cluster which could drive amide isomerization and subsequent aggregation, is described in Chapter 3. It is interesting to note that, in addition to the currently accepted model for the cross-β fibril, Pauling also presented two models with alternating cis and trans peptide bonds.\textsuperscript{56}

**Cis or Trans: A Matter of Angstroms**

Although the R\textsuperscript{2} technique is a very powerful tool for solid-state structure determination, its application to the determination of amyloid structure is still undergoing refinements. The experiments described in Chapters 2 and 3 of this work were carried out in parallel with the R\textsuperscript{2} work as an independent method for evaluating the feasibility and the functional consequences of the putative cis geometry of the Gly\textsubscript{37}Gly\textsubscript{38} amide. Following the completion of these experiments, a new set of R\textsuperscript{2} experiments were performed which better account for the effect of intermolecular interactions. These results\textsuperscript{57} have led to a reevaluation of the data and suggest that the α\textsubscript{37,38} distance is in the 4.6-4.8Å range, restricting the amide geometry to the trans isomer. Assuming that these values are now correct, this very recent result obviously affects the interpretation of some of the experiments described herein. Where possible, this has been incorporated into the appropriate discussion sections. This reevaluation of the NMR data stresses the importance of our ability to evaluate the mechanistic consequences of specific structural features.
Mechanism of Amyloid Formation: A Nucleation-Dependent Polymerization.

Amyloid formation in vitro occurs via a nucleation-dependent polymerization process (Fig. 1.6).\textsuperscript{58-60} A similar mechanism characterizes many common assembly processes, including protein crystallization, microtubule assembly, flagellum assembly, sickle-cell hemoglobin fibril formation, bacteriophage procapsid assembly, and actin polymerization.\textsuperscript{59} A series of unfavorable equilibria ($K_n$) involving intermolecular association events precede formation a "nucleus." Nucleation is an energetically unfavorable process because the resultant intermolecular interactions do not outweigh the entropic cost of association.\textsuperscript{61} In order for nucleation to occur, a critical concentration of the monomer must be exceeded. Following nucleation, addition of monomer to the nucleus becomes thermodynamically favorable, and fibril growth ($K_g$) occurs rapidly. The growth process slows and eventually reaches an equilibrium at the thermodynamic solubility of the amyloid species.

\textbf{Figure 1.6} A mechanistic scheme for nucleation-dependent polymerization of an A\textbeta amyloid fibril. Nucleation is thermodynamically unfavorable ($K_n<<1$), while growth is favorable ($K_g>>1$).

Experimentally, this process manifests itself as a sigmoidal curve, as illustrated in Fig 1.7, in which no detectable aggregates are observed during an initial lag time. During this period, trace amounts of dimer, trimer and eventually n-mer
(nuclei) are formed, but the monomer is still the predominant species. This phenomenon, referred to as "kinetic solubility," can lead to the illusion of an amyloid peptide being "soluble" when in fact it is simply slow to form nuclei.

As a consequence of this nucleation-dependent mechanism, the concentration of the aggregating species in solution is critical in controlling the rate of nucleus formation. The concentration-dependence of the lag time is dependent on the number (n) of molecules in the nucleus. In fact, the rate of nucleation is proportional to the concentration raised to the n\textsuperscript{th} power (C\textsuperscript{n}).\textsuperscript{62} For example, a 5% increase in the concentration of the aggregating species, with a nucleus size of 20, would be expected to lead to a increase in the nucleation rate of \((1.05)^{20}\), or \(\sim3\)-fold. Small changes in concentration can therefore cause disproportionately large increases in the rate of nucleation.

**Figure 1.7** Kinetics of nucleation-dependent amyloid formation. No aggregates are observable during nucleation (lag time). Following nucleation, growth is rapid. Addition of a preformed fibril ("seeding") eliminates the requirement for nucleation and leads to immediate growth.
A further consequence of the nucleation-dependent mechanism is that addition of a preformed nucleus will eliminate the need for a nucleation event. This "seeding" phenomenon is homologous to seeding a crystallization by addition of a seed crystal. However, unlike a crystallization, which can often be "seeded" by random particulate matter (e.g. dust particles), amyloid nucleation is a highly sequence-specific event. Only the most minimal sequence differences can be tolerated; for example, a single amino acid change (Met129 to Val) in PrP118-133 retained the ability to seed, but OsmA, a peptide in which the glycine in the GXXX repeat of OsmB28-44 was replaced with alanine, was ineffective at seeding OsmB. This sequence specificity is most likely due to a structural specificity; the structure of the seed must provide a complementary growth surface for addition of monomers.

This mechanism has ramifications for the treatment of AD and other amyloid-characterized diseases. Slowing the rate of amyloid formation, either by reducing the concentration of the aggregating monomer or by interfering with nucleation, could be one therapeutic strategy. Compounds that bind and stabilize the nucleus or its precursors may slow amyloid formation, or compounds which inhibit addition of monomers to the growing fibril could allow dissolution of the plaques by normal clearance mechanisms. Alternatively, interfering with the seeding of amyloid formation by endogenous molecules (vide infra) could be an effective strategy.

The Position of the C-Terminus of Aβ is Critical

The major physiological variants of Aβ are heterogeneous in their C-termini. Studies of the in vitro kinetics of amyloid formation by a series of model peptides corresponding to the different C-terminally truncated isoforms led to the discovery that the longer variants of Aβ (B26-42 and B26-43) formed fibrils with no lag time, whereas the shorter variants (B26-39 and B26-40) exhibited a lag
time of hours to days prior to the observation of any turbidity (Table 1.2). A similar pattern was observed with full-length Aβ (β1-39 and β1-42). Furthermore, fibril formation by "short β" could be seeded by the addition of "long β" fibrils, suggesting that production of long Aβ variants may be a pathogenic event.

Table 1.2 Measured lag times for Aβ amyloid fibril formation. Initial concentrations of peptide: 200 μM for β26-4x; 20 μM for 1-4x.

<table>
<thead>
<tr>
<th>peptide</th>
<th>nucleation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>β26-43</td>
<td>&lt;30 sec</td>
</tr>
<tr>
<td>β26-42</td>
<td>&lt;30 sec</td>
</tr>
<tr>
<td>β26-40</td>
<td>~12 h</td>
</tr>
<tr>
<td>β26-39</td>
<td>~60 h</td>
</tr>
<tr>
<td>β1-42</td>
<td>&lt;30 sec</td>
</tr>
<tr>
<td>β1-39</td>
<td>~96 h</td>
</tr>
</tbody>
</table>

These in vitro studies were later supported by cell-culture and pathology experiments. Younkin et al. demonstrated that mutations in APP (V717I and V717F) which cause early-onset familial AD affect the specificity of the proteolytic release of Aβ, resulting in a 1.5 to 1.9-fold increase in the percentage of "long β" relative to "short β" in cultured cells. Studies of pathology provided supporting evidence for long β as an initially deposited species in senile plaques, and β1-42 was found to be the primary constituent of "diffuse" amyloid, presumed to be the precursor to senile plaque. Furthermore, Younkin has recently demonstrated that a mutation on chromosome 14 (corresponding to the presenilin protein S182) also leads to an increased ratio of long to short Aβ. These studies support the idea of long β as a pathological species.

The effect of the C-terminus of Aβ on the thermodynamic stability of the amyloid fibril has also been studied. The C-terminus had little effect on the observed thermodynamic solubility. Differences in fibril structure were
demonstrated by FTIR, with β26-40 displaying a different β-sheet structure than β26-43. A mixture of long and short β formed segregated, rather than integrated fibrils, with thermodynamic properties equivalent to the sum of the individual fibrils. This suggests that short β, once formed, could dissolve more rapidly than long β, making its clearance feasible. Long β, on the other hand, may be difficult to remove by standard physiological clearance mechanisms, thereby accentuating its pathological effect.

**The Non-Aβ Component of AD Amyloid (NAC)**

Much of the focus of AD research over the last several years has revolved around β amyloid. This is primarily due to the presence of Aβ as the primary proteinaceous component of the amyloid plaques. A number of other molecules have also been reported to be associated with isolated plaque amyloid, including heparan sulfate proteoglycan, immunoglobulins, and many acute-phase proteins, such as α1-antichymotrypsin, apolipoprotein E, complements, serum amyloid P, gamma-trace peptide, and lysosomal proteinases. These components have been identified on the basis of immunohistochemical studies. There is, however, no biochemical data demonstrating the presence of these components in amyloid preparations, raising the possibility that these species might not be intrinsic components of amyloid, but instead are only peripherally associated with the plaque core.

In 1993, Uéda et al. identified a second intrinsic component of the plaque core. Human AD brain was homogenized in the presence of SDS, solubilized in formic acid, and digested sequentially with CNBr and Achromobacter lyticus protease I. The resultant peptides were analyzed by HPLC and amino acid sequencing. The majority of the HPLC peaks had sequences corresponding to Aβ. Two additional peaks were detected, and named peptide X (sequence:
EQVTNVGGAVVTGVTAVAQK) and peptide Y (TVEGAGSIAAXXGFV). These sequences were recovered in essentially identical concentrations and were present at about 1/10 the concentration of Aβ fragments. These peptides were hypothesized to be derived from a larger fragment, which was named NAC, signifying "non-Aβ component of AD amyloid" (Fig. 1.8).74

![Figure 1.8 The non-Aβ component of AD amyloid (NAC). Peptides "X" and "Y" copurified with Aβ from human AD amyloid plaque.](image)

Using a piece of cDNA encoding peptide X, a cDNA library was screened in order to isolate the NAC cDNA. Sequence analysis of the clone revealed a 420-bp open reading frame encoding a protein of 140 amino acid residues, which was named NACP (for "NAC precursor protein"). The deduced amino acid sequence showed that X- and Y-peptide sequences are localized immediately next to each other in the middle of NACP.74

Because NAC was isolated using a proteolytic enzyme, it was not possible to determine the N-terminal amino acid of NAC. However, because no other fragments of NACP were detected in the SDS-insoluble fraction, the 35 amino acid sequence was considered to be a minimal NAC sequence, although the full-length species in plaque might be longer by a few amino acids.74 The C-terminus of NAC is not at an endopeptidase cleavage site (C-terminal to lysine residues), and is therefore unambiguous. Presumably NAC is generated from NACP in vivo by proteolytic activity. The protease responsible for this cleavage has not been discovered.
Although the authors did not recognize it at the time, the NACP sequence corresponds to a protein, named α-synuclein, which is a member of a family of proteins that had previously been identified in *Torpedo* (electric eel)\textsuperscript{75} and rat,\textsuperscript{76} and was subsequently identified in humans.\textsuperscript{77} NACP is a presynaptic protein of unknown function. Recent studies, however, have suggested roles for NACP in learning and AD (Chapter 5).\textsuperscript{78,79} The biochemical basis for these putative functions, however, remains a mystery.

**Deciphering the Complex Molecular Logic of AD**

A growing body of scientific evidence indicates that amyloid deposition may play a primary role in AD. The first important step toward understanding amyloid formation is to determine the critical structural features which mediate this process. Only when the structure of the amyloid fibril can be successfully related to the mechanism of fibril formation *on a molecular level* will it become possible to design therapeutic agents which either bind to amyloid, and serve as diagnostics, or which disrupt amyloid formation, and thereby serve as treatments for disease. The initial goal of the experiments described in this work, therefore, was to decipher the molecular logic underlying Aβ amyloid fibril formation in AD.

It is becoming clearer with each new discovery related to AD that this disease is a complex pathophysiological phenomenon. It is therefore important to study the role of not only Aβ, but also of other physiological species whose pathological mechanisms may intersect, either directly or indirectly, with amyloid deposition. NAC and its precursor NACP are such species, and are the subjects of the latter part of the work described herein.

Despite the tremendous advances of modern medicine in fighting disease, AD remains among the most poorly understood, and among the most economically and emotionally costly, of all the diseases facing the world today. The ultimate goal
of AD research is, of course, the development of effective treatments or cures. The accomplishment of this goal requires a true understanding of the factors responsible for the disease.
References for Chapter I

64. Younkin, S.G., personal communication.
Chapter II

Synthesis and Aggregation of β Amyloid Peptide Mimetics: Chemical Probes of Amyloid Aggregation in AD.

Background

A primary goal of medicinal chemistry is to determine the effect of chemical structure on biological activity. In natural products chemistry, this has for many years entailed the systematic introduction of alternate substituents via organic synthesis. Likewise, the field of peptide chemistry has also succumbed to the grasp of medicinal chemistry.\textsuperscript{1-4} The systematic replacement of amino acid side-chains with natural or unnatural groups led to the first generation of such designed peptide variants, or "peptide mimetics". A natural continuation of this strategy was the design of peptide mimetics which alter the peptide backbone itself. An enormous amount of work has gone into the design of new peptide mimetics and the application of these novel structures as probes of biological activity.\textsuperscript{1-3} With the advent of biotechnology, molecular biology has become a driving force behind the design of therapeutic agents. Accordingly, peptides and proteins have become major targets for drug design, despite the significant problems associated with such compounds in terms of low bioavailability, rapid metabolism, and lack of oral activity. By replacing the polypeptide backbone with peptide mimetic structures, the medicinal chemist is able to develop biologically active compounds which
simultaneously probe the structural features responsible for binding to biologically relevant target molecules.

Many peptide bond replacements, or "isosteres", have been used in structure-activity investigations. Table 2.1 lists some of the more common amide replacements, which are designated by the symbol $\Psi$, followed by the formula for the amide replacement. For example, replacement of an amide (-CONH-) with an alkyl group (-CH$_2$-CH$_2$-) would be designated $\Psi[\text{CH}_2\text{CH}_2]$. Some of these replacements increase the flexibility of the linkage (e.g. alkyl, thioalkyl) whereas others lock the bond into a specific geometry (e.g. alkene). Several amide isosteres which specifically mimic the geometry of the cis amide isomer have been developed.$^{5-8}$

<table>
<thead>
<tr>
<th>amide modification</th>
<th>designation</th>
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<tbody>
<tr>
<td>-COCH$_2$-</td>
<td>$\Psi[\text{COCH}_2]$</td>
</tr>
<tr>
<td>-COO-</td>
<td>$\Psi[\text{COO}]$</td>
</tr>
<tr>
<td>-CH$_2$CH$_2$-</td>
<td>$\Psi[\text{CH}_2\text{CH}_2]$</td>
</tr>
<tr>
<td>-CH=CH-</td>
<td>$\Psi[(Z)-\text{CH}=\text{CH}]$ or $\Psi[(E)-\text{CH}=\text{CH}]$</td>
</tr>
<tr>
<td>-COS-</td>
<td>$\Psi[\text{COS}]$</td>
</tr>
</tbody>
</table>

Studies of model peptides have suggested some key structural features of the $\alpha\beta$ amyloid fibril.$^9,10$ One prominent feature of the model is the possibility of a cis amide bond at Gly37Gly38 in the solid state. The body of work on peptide bond isosteres provided us with a promising method to probe the ability of a cis amide to facilitate $\alpha\beta$ amyloid formation. To this end, the first part of this chapter describes the incorporation of cis and trans amide isosteres into the $\beta34-42$ fibril, and the
investigation of the effect of these substitutions on the kinetics and thermodynamics of amyloid fibril formation. Additionally, the general role of hydrophobicity is addressed through the introduction of alternate amino acids. In the latter part of this section, the role of specific hydrogen bonds in stabilizing the amyloid fibril is addressed by the use of amide to ester replacements.

Results and Discussion

Selection and Synthesis of Gly37-Gly38 Amide Isosteres

When selecting an amide bond replacement, it is important to consider the ability of the surrogate to accurately mimic the steric and electronic properties of the amide bond. While exact identity is only possible in the case of isotopic replacements, it is clearly advantageous to mimic the desired conformation as closely as possible. On the other hand, structural identity is not necessarily a requirement, and subtle changes could actually enhance the ability of an isostere to access a key conformation. With these considerations in mind, the isosteres shown in Fig. 2.1 (1 - 5) were chosen to test the conformational requirements for the glycylglycine dipeptide in β34-42 amyloid fibril formation.

In the case of β34-42, we required a compound with a fixed "cis" configuration which would only minimally affect the intermolecular packing interactions in the amyloid fibril. The trans alkene isostere, Ψ[(E)-CH=CH] has been successfully employed to mimic the trans amide configuration,6 but the corresponding cis alkene is difficult to prepare, due to the ease of cis/trans isomerization during synthesis.5 The 1,5-tetrazole ring Ψ[CN₄] has been developed as an alternative cis amide bond mimic, but can be difficult to prepare.11 A readily prepared, isosteric cis amide replacement is the 1,2-disubstituted pyrrole analog 7, reported in 1992.8
The pyrrole-based amino acid 7 was synthesized via a published procedure as shown in Figure 2.2.¹ Alkylation of pyrrole-2-carboxaldehyde with iodoacetic acid led to aldehyde 6. Reductive ammination of 6 in methanol led to the desired amino acid surrogate 7.

Amino terminal protection of 7 with a tert-butoxycarbonyl (Boc) group (8) has been reported.¹ This protection strategy has a fatal flaw, as the pyrrole analog is extremely sensitive to acid, and decomposes under the conditions required for Boc deprotection. Because of this, chain elongation could only be carried out in the direction of the C-terminus, leaving the pyrrole analog at the N-terminus of the resultant peptide.¹ We required a method to incorporate this analog within a polypeptide chain. To this end, 7 was protected with the base-labile protecting group fluorenylmethoxycarbonyl (Fmoc), and the resultant N-Fmoc amino acid 9 was purified by HPLC. The analog was incorporated into β34-42 in place of Gly38, using

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**Figure 2.1** Amide isosteres used in this work. These compounds were incorporated into β34-42 in place of the glycyglycine dipeptide moiety.
solid-phase peptide chemistry. The acid-lability of the analog dictated the use of the 4-hydroxymethyl-3-methoxyphenoxybutyric acid (HMPB) resin, which can be cleaved under mild acid conditions. The resultant peptide was purified by reversed phase HPLC.

In addition to the pyrrole analog, several other isosteres were prepared and incorporated into β34-42. These compounds, while readily synthesized, most likely bear less of a steric and electronic resemblance to the amide bond than does the pyrrole. The use of o-aminomethylphenylacetic acid (2) derivatives as cis amide surrogates has been reported. The synthesis of 2 was carried out by a simple, published procedure (Fig. 2.3). Addition of sodium azide to indanone 10 in sulfuric acid provided the lactam 11. Hydrolysis of the lactam with concentrated HCl followed by protection of the resultant quaternary amide salt of 12 with Fmoc-
succinimide led to Fmoc-protected amino acid 13. This analog was incorporated into β34-42 via solid-phase synthesis.

![Chemical reaction diagram]

**Figure 2.3** Synthesis and protection of 12.

The incorporation of *p*-aminobenzoic acid into peptides (i.e. 3) has been reported. This compound is a less conservative amide replacement; in addition to eliminating the peptide bond, when used as a replacement for Gly37-Gly38 it also restricts another degree of freedom at the glycine α position. This compound is commercially available, and was easily protected with an Fmoc group and incorporated into the peptide.

The final amide replacement 4 was chosen for its potential as a trans amide mimic. As with 3, this analog imposes multiple restrictions upon the peptide backbone. The parent amino acid 3-aminophenylacetic acid is commercially available, and, as with 3, was protected with an Fmoc group and incorporated into β34-42. The replacement of both glycine residues with α-aminoisobutyric acid (Aib, compound 5) is technically not an amide replacement; however, this peptide is restricted to the trans configuration due to the steric bulk of the α-methyl groups, and was therefore included in this study.
Fibril Formation by Amide Isosteres

Initially, the ability of 1-5 to form amyloid fibrils from a supersaturated aqueous solution was evaluated. Each of the β34-42 analogs formed amyloid fibrils as defined\textsuperscript{14} by 1) fibrillar structure when viewed under an electron microscope (EM); 2) staining with the amyloidophilic dye Congo Red; and 3) a FTIR spectrum dominated by β-sheet structure. EM suggested slight structural differences between the fibrils, but it is difficult to ascribe the macroscopic fibrillar structure to any specific molecular influences. The ability of all of these analogs to form amyloid indicates that the peptide backbone can be modified fairly substantially without eliminating the ability of the peptide to form fibrils.

Effect of Amide Isosteres on Solubility

The solubility of each analog following an aggregation experiment was measured by quantitative amino acid analysis (Table 2.2). In theory, assuming the solution has reached an equilibrium, these values would represent true "thermodynamic" solubilities. In practice, however, these values should be interpreted only as "effective" measured solubilities, and are meaningful only as relative values.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Solubility (μM)</th>
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<tbody>
<tr>
<td>β34-42</td>
<td>26 (±5)</td>
</tr>
<tr>
<td>1</td>
<td>8 (±1)</td>
</tr>
<tr>
<td>2</td>
<td>12 (±1)</td>
</tr>
<tr>
<td>3</td>
<td>4 (±2)</td>
</tr>
<tr>
<td>4</td>
<td>25 (±4)</td>
</tr>
<tr>
<td>5</td>
<td>30 (±5)</td>
</tr>
</tbody>
</table>
In general, the cis amide mimics were less soluble than the native sequence. Specifically, the pyrrole and p-aminobenzoic acid derivatives (1 and 3) were highly insoluble relative to β34-42 itself, as was 2, to a lesser extent. In interpreting these results, we considered the possibility that the addition of an aromatic ring to the peptide, as in 3, may alter the solubility not by conformational effects, but simply by adding hydrophobicity. However, 4, which also contains an aromatic ring, does not show the same decreased solubility. This "trans" derivative, as well as the all-trans Aib analog 5, both have comparable solubilities to the native peptide, indicating that specific molecular details, such as the amide configuration, and not hydrophobicity in general, are more likely to be responsible for the observed solubility properties.

Effect of Amide Isosteres on Kinetic Solubility

The kinetics of amyloid fibril formation by β34-42 and analogs 1 - 5 under identical conditions (375 μM, 25°C, 10% DMSO/phosphate-buffered saline (PBS)) were monitored using a turbidity assay. The results are shown in Fig. 2.4. All of the peptides showed kinetic aggregation profiles consistent with a nucleation-dependent polymerization process.15 β34-42 alone, as noted before, exhibits no lag time prior to the onset of aggregation. Cis analogs 1 and 3 also showed no significant lag time preceding aggregation. This is consistent with the hypothesis that a cis amide facilitates fibril formation. Ideally, one would expect to observe a decrease in the nucleation time upon constraining the amide into its aggregating conformation, however, as β34-42 itself does not exhibit a lag time, this cannot be determined. The p-aminobenzoic acid analog 2 and the Aib derivative 5 exhibited measurable lag times, and 5 had a much slower rate of growth than the other derivatives. It appears that these analogs are unable to access a rapidly aggregating conformation, possibly due to the limited degrees of freedom in these compounds. In fact, these compounds may be forming aggregates with totally different conformations, which
may not be energetically favorable in the native peptide, but which become accessible in the modified analogs. Obviously, the more changes made to the peptide backbone, the more difficult it will be to infer the underlying structural explanations for the observed results. It is therefore important not to over interpret these results; this is illustrated by the supposedly "trans" compound 4, which did not show a lag time.

**Figure 2.4** Kinetics of Amyloid Formation by β34-42 analogs (375 μM, stirred continuously). (Left) "cis" amide mimics: (o) β34-42, (●) 1, (△) 2, (●) 3. (Right) "trans" amide mimics: (●) 4, (△) 5.

**Effect of Amino Acid Hydrophobicity**

The general role of hydrophobicity of the amino acids in β34-42 was probed by replacing Val36 and/or Val39 with alanine (Fig. 2.5). The prior set of experiments suggested that increasing the hydrophobicity of the Gly-Gly amide did not enhance fibril formation either kinetically or thermodynamically. With the flanking residues, however, a clear dependence on hydrophobicity was observed, as reflected by the direct correlation between the number of valines and decreased
thermodynamic and kinetic solubilities of the peptides shown in Fig. 2.5. This effect may be due to the stabilizing effect of a hydrophobic cluster, such as that discussed in detail in Chapter 3.\textsuperscript{16}

![Graph showing solubility and aggregation kinetics of β34-42 hydrophobicity probes.]

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Solubility (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMAGGAVIA</td>
<td>104 (φ)</td>
</tr>
<tr>
<td>LMAGGVVIA</td>
<td>73 (ο)</td>
</tr>
<tr>
<td>LMVGGVVIA</td>
<td>26 (Δ)</td>
</tr>
</tbody>
</table>

**Figure 2.5** Solubility and aggregation kinetics of β34-42 hydrophobicity probes. (φ) LMAGGAVIA (lag time ~12,000 s), (ο) LMAGGVVIA (lag time ~3,000 s), (Δ) LMVGGVVIA (β34-42, no lag time).

**Depsipepptide Analogs of β34-42**

The cis amide peptide mimetics described in the first part of this chapter were designed to probe the importance of the Gly37-Gly38 peptide bond geometry. Initial efforts focused on this particular amide because of its unusual conformation, as indicated by FTIR and SSNMR. In addition to the amide configuration, we also
wanted to test the pattern of hydrogen bonds proposed in the model for the β34-42 amyloid fibril. In this model (Fig. 2.6), amide hydrogen bonds stabilize the intermolecular interactions, with the glycyglycine moiety serving as a flexible "hinge" to optimize the packing of the β-sheet.

One strategy which allowed us to test the hydrogen bonding pattern in β34-42, as well as to further explore the GlyGly amide conformational requirements, was the replacement of specific peptide bonds with esters. Such "depsipeptides" are found in numerous naturally occurring linear and cyclic peptides, and have frequently been used in the study of structure-activity relationships in bioactive peptides. For example, an oxytocin depsipeptide derivative in which three supposed intramolecular hydrogen bonding amides were replaced with esters still

![Figure 2.6 Proposed model for hydrogen bonding in the β34-42 amyloid fibril.](image)

The intermolecular R² effects (indicated by arrows) constrain the interstrand alignment. These effects are consistent with a single β-sheet with the indicated hydrogen bonding pattern.
showed significant activity, suggesting that these hydrogen bonds are not absolute requirements for activity. The ester to amide replacement affects hydrogen bonding in two ways, by directly removing a hydrogen bond donor and by altering the hydrogen bond accepting ability of the carbonyl.

Three depsipeptide analogs of β34-42 were synthesized, in which either the Val36-Gly37 amide, the Gly37-Gly38 amide, or the Gly38-Val39 amide was replaced with an ester (denoted "VoG", "GoG", and "GoV", respectively). An examination of the thermodynamic solubility and kinetics of amyloid formation by these peptides were used to probe the individual role of each of these amide bonds.

Each of the three depsipeptide analogs formed fibrils from a supersaturated aqueous solution. In each case, the precipitate exhibited fibrillar structure when examined with an electron microscope, and could be stained with the amyloidophilic dye Congo Red. The FTIR spectra of all three depsipeptides are compared to β34-42 itself in Fig 2.7. All show, as expected, a significant degree of β-sheet structure. GoG was the most similar to β34-42, with a strong absorbance at ~1631 cm⁻¹, while the spectra of VoG and GoV showed a broad range of IR bands, indicative of significant differences in structure from β34-42.

The kinetics of amyloid formation by β34-42 and the three depsipeptide analogs were compared under identical conditions (375 µM, 25°C, 10% DMSO/PBS) (Fig. 2.8). Both β34-42 itself and the GoG analog exhibited no lag time, whereas the VoG depsipeptide had a short lag time (~ 140 sec) and the GoV peptide had a much longer delay prior to nucleation (~ 5500 sec).
Figure 2.7 FTIR spectra of β34-42 depsipeptide analogs.
At first we considered the implications of these results in terms of the putative cis amide at Gly37-Gly38. Peptide GoG formed fibrils with no lag time, similar to β34-42. The energetic barrier to rotation in an ester is about 10-15 kcal/mol, as opposed to ~20 kcal/mol for an amide. Thus, all else being equal, if amide isomerization is rate limiting, this ester replacement should aggregate more rapidly than β34-42. Since β34-42 itself has no lag time, this was of course impossible to determine. In any case, the replacement did not slow down the aggregation process, and is thus consistent with a requirement for an isomerization event.

### Table 2.8 Solubility and aggregation kinetics of depsipeptide analogs of β34-42 (375 µM, stirred continuously at 1550 rpm).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Solubility (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMVGGV VIA</td>
<td>25 (±5)</td>
</tr>
<tr>
<td>LMVoG V VIA</td>
<td>28 (±8)</td>
</tr>
<tr>
<td>LMVGoG V VIA</td>
<td>26 (±6)</td>
</tr>
<tr>
<td>LMVGGo V VIA</td>
<td>76 (±16)</td>
</tr>
</tbody>
</table>

Figure 2.8 Solubility and aggregation kinetics of depsipeptide analogs of β34-42 (375 µM, stirred continuously at 1550 rpm).
The aggregation kinetics (Fig. 2.8) can be more easily rationalized in terms of the putative hydrogen bonding pattern in \( \beta \)-34-42. Replacement of the Gly37-Gly38 amide with an ester has no apparent effect on the kinetic solubility of \( \beta \)-34-42, consistent with the proposed role of the glycylglycine as a flexible hinge. The \( \text{VoG} \) and \( \text{GoV} \) analogs, on the other hand, exhibited longer nucleation times. This may be due to the removal of key hydrogen bonding elements which stabilize the \( \beta \)-sheet. In any discussion of hydrogen bonding, it is important to keep in mind the concept of hydrogen bond "inventory"; that is, the difference between an inter- or intramolecular H-bond to a peptide versus an intermolecular H-bond to water. It is likely that introduction of a water molecule into the highly hydrophobic \( \beta \)-sheet is unfavorable, and that loss of an intermolecular peptide hydrogen bond would have a negative effect on the stability of the amyloid fibril.

The effect of the depsipeptide replacements on the measured solubility of the amyloid fibrils is consistent with the above interpretation. The \( \text{GoG} \) and \( \text{VoG} \) peptides had comparable solubilities to \( \beta \)-34-42, whereas the \( \text{GoV} \) peptide, which exhibited the longest nucleation time, also had the highest solubility. Thus, the structural features which affect the kinetics of amyloid formation probably have similar effects on the "thermodynamic" solubility as well.

The depsipeptide analogs provide a tool for the evaluation of the importance of specific interactions in stabilizing the \( \beta \)-34-42 amyloid fibril. Ongoing investigations into the effect of depsipeptides at the Val39-Val40 and Ile41-Ala42 amides (C. May, unpublished results) should provide further information about hydrogen bonding patterns in \( \beta \)-34-42 and may provide details about the structural effects of the C-terminal residues which distinguish long and short A\( \beta \) variants.
Experimental Section

Standard Procedures for Peptide Synthesis and Characterization

Peptides were synthesized using either N-Boc or N-Fmoc protected amino acids and either of the following general procedures, unless specified otherwise, on either the HMPB-BHA, Wang, 4-hydroxymethylphenylacetamidomethyl (PAM), or oxime resin. All peptides were prepared with a free amine at the N-terminus and a free acid at the C-terminus. Amino acid analysis was carried out by the MIT Biopolymers Laboratory on an Applied Biosystems Model 420 PTH amino acid analyzer. Peptides LMAGGAVIA and LMAGGVVIA were synthesized and characterized by J. Chiang, and β34-42 was prepared by T. Ashburn. Analytical HPLC was performed on a Waters 600E system equipped with a photodiode array detector using either a Waters (C₄, 300Å, 15µm, 3.9 x 300 mm) or a Vydac 214TP (C₄, 5 mm, 4.6 x 250 mm) reversed phase column. Semipreparative HPLC was performed using a YMC (C₄, 300Å, 10µm, 20 x 250 mm) column. Preparative HPLC was performed on a Waters PrepLC4000 system using a PrepPak C₄ cartridge.

_N-Boc amino acids._ (1) Wash resin with CH₂Cl₂ (2x) followed by iPrOH (2x), repeat 3x; (2) remove a small amount of resin and perform the Kaiser test for free amine (the test should be positive (blue)); (3) wash with DMF (3x); (4) add Boc-amino acid (3 eq, 0.1M in DMF), benzotriazol-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate (BOP) or an equivalent amount of benzotriazol-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (3 eq, 0.1M in DMF), and diisopropylethylamine (DIEA) (5.3 eq), shake resin for 30 sec, shake mixture for at least 40 min at room temperature (RT); (5) wash with DMF (3x); (6) wash with CH₂Cl₂ (2x), iPrOH (2x), CH₂Cl₂ (2x); (7) remove a small amount of resin and perform the Kaiser test for free amine. If positive (blue) then repeat steps
(3) - (7) until a negative (yellow) result is obtained; (8) add acetic anhydride (10 eq) and DIEA (5 eq) in CH$_2$Cl$_2$, and shake for >20 min; (9) wash with CH$_2$Cl$_2$ (3x); (10) add 25% trifluoroacetic acid (TFA) in CH$_2$Cl$_2$ and shake for 30 min. Repeat steps (1) - (10), ending with deprotection of the N-terminal amino acid. (11) Acetylate the N-terminal amino acid via steps (6) - (9).

**N-Fmoc amino acids.** (1) Wash resin with DMF (3x), CH$_3$OH (3x), CH$_2$Cl$_2$ (3x); (2) remove a small amount of resin and perform the Kaiser test for free amine (the test should be positive (blue)); (3) wash with DMF (3x); (4) add Fmoc-amino acid (3 eq, 0.1M in DMF), BOP reagent (3 eq, 0.1M in DMF) and DIEA (5.3 eq), shake resin for 30 sec, shake mixture for at least 40 min at RT; (5) wash with DMF (3x); (6) wash with CH$_2$Cl$_2$ (2x), CH$_3$OH (2x), CH$_2$Cl$_2$ (2x); (7) remove a small amount of resin and perform the Kaiser test for free amine. If positive (blue) then repeat steps (3) - (7) until a negative (yellow) result is obtained; (8) add acetic anhydride (10 eq) and DIEA (5 eq) in CH$_2$Cl$_2$, and shake for >20 min; (9) wash with CH$_2$Cl$_2$ (3x); (10) add 50% piperidine in DMF and shake for 30-45 min. Repeat steps (1) - (10), ending with deprotection of the N-terminal amino acid. (11) Acetylate the N-terminal amino acid via steps (6) - (9).

**Standard Procedure for Trifluoroacetic Acid (TFA) Deprotection**

Dried peptide-resin was placed in a flask under Ar. Ethanedithiol (300 µL/g resin) and thioanisole (200 µL/g resin) were added, and the reaction was stirred at 0°C for 10 min. TFA (9.5 µL/g resin) was added, and the reaction was stirred at room temperature for 2h. The resin was removed by filtration, and the TFA solution was added to cold ether, resulting in precipitation. The pellet was collected by centrifugation (2000 x g, 10 min), washed 3x with ether and lyophilized to dryness.
Synthesis and Characterization of Glycylglycine Dipeptide Isosteres

2-Formyl-1-pyrrolylacetic acid (6). To a solution of 0.92 g (23 mmol, 2.2 eq) of NaOH in H₂O (5 mL) was added 1.0 g (10.5 mmol, 1 eq) of pyrrole-2-carboxaldehyde, and the reaction was stirred at 0°C until the solute had dissolved. Iodoacetic acid (2.15 g, 11.6 mmol, 1.1 eq) was added and the reaction was warmed to RT and stirred for 15.5 h. The aqueous solution was washed with CHCl₃, acidified to pH 3 with 6M HCl, and extracted with EtOAc. The organic layer was dried over MgSO₄, filtered, and concentrated in vacuo. The product was purified by flash column chromatography on silica gel (30% EtOAc/hexane, 1% acetic acid) to give 581 mg (36%) of an off-white solid. ¹H NMR (250 MHz, CDCl₃) 8 9.55 (s, 1H, -CHO), 7.22 (dd, 1H, J= 8Hz, 2Hz, pyrrole -CH), 6.96 (m, 1H, pyrrole -CH), 6.33 (dd, 1H, J = 8Hz, 2Hz, pyrrole -CH), 5.10 (s, 2H, -CH₂-).

2-Aminomethyl-1-pyrrolylacetic acid (7). To a solution of 6 (581 mg, 3.80 mmol, 1eq) in CH₃OH (12 mL) was added ammonium acetate (2.34 g, 30.4 mmol, 8 eq) followed by sodium cyanoborohydride (191 mg, 3.04 mmol, 0.8 eq). The reaction was stirred under N₂ at room temperature for 18 h, when a precipitate had formed. The solid was isolated via vacuum filtration. The product was lyophilized repeatedly to remove excess NH₄OAc, and used without further purification. ¹H NMR (250 MHz, D₂O) 8 6.78 (m, 1H, pyrrole -CH), 6.33 (m, 1H, pyrrole -CH), 6.12 (m, 1H, pyrrole -CH), 4.50 (s, 2H, -CH₂-NH₂), 4.13 (s, 2H, -CH₂-CO₂H). FABMS (m/z): calc’d for C₇H₁₀N₂O₂, 154.16; found 155 (M+H)⁺, 177 (M+Na)⁺.

N-Fmoc-Gly-Ψ[C₄N]-Gly-CO₂H (9). To a solution of 7 (0.59 g, 3.25 mmol, 1 eq) in 10% aqueous Na₂CO₃ (12 mL) was added N-(9-fluorenylmethoxycarbonyloxy) succinimide (1.10 g, 3.45 mmol, 0.9 eq) in dioxane (6 mL), and the reaction was
stirred at ambient temperature for 2 h, when a milky white precipitate had formed. The mixture was diluted with water and washed 3x with ether. The aqueous layer was acidified to pH 3 and the product was extracted with ethyl acetate. The organic layer was dried over MgSO$_4$, filtered, and concentrated in vacuo to provide 360 mg (0.96 mmol, 25% crude yield) of a white solid. The product was purified by reversed phase HPLC. Analytical HPLC (C$_4$, 2.0 mL/min): 60/40 H$_2$O/CH$_3$CN, (0.1% TFA), linear gradient to 30/70 (16 min), $R_v$ = 13 mL. Semipreparative HPLC (C$_4$, 15 mL/min): 70/30, linear gradient to 20/80 (16 min), $R_v$ = 170.4 mL. $^1$H NMR (250 MHz, CDC$_3$) $\delta$ 7.75 (d, 2H, J = 9 Hz, Fmoc aromatic -CH), 7.58 (d, 2H, J = 9 Hz, Fmoc aromatic -CH), 7.40 (d, 2H, J = 9 Hz, Fmoc aromatic -CH), 7.32 (d, 2H, J = 9 Hz, Fmoc aromatic -CH), 6.54 (m, 1H, pyrrole -CH), 6.11 (m, 2H, pyrrole -CH), 4.62 (m, 2H, -CH$_2$-), 4.35 (m, 4H, -CH$_2$- & Fmoc -CH$_2$-), 4.20 (m, 1H, Fmoc -CH-). PDMS (m/z): calc'd for C$_{22}$H$_{20}$N$_2$O$_4$, 376.4; found 375.7 (M+H)$^+$.  

**$o$-Aminomethylphenylacetic acid $\delta$-lactam (11).** Lactam 11 was synthesized by a published procedure.$^7$ $^1$H NMR (250 MHz, CDC$_3$) $\delta$ 7.28 (m, 2H, aromatic -CH), 7.19 (m, 2H, aromatic -CH), 6.73 (broad s, 1H, -NH-), 4.51 (s, 2H, -CH$_2$NH-), 3.59 (s, 2H, -CH$_2$C(O)NH-).  

**N-Fmoc-$o$-Aminomethylphenylacetic acid (13).** Amino acid 12 (unprotected) was synthesized by a published procedure.$^7$ To a solution of 12 (170 mg, 0.846 mmol, 1 eq) in 10% aqueous Na$_2$CO$_3$ (4 mL) was added N-(9-fluorenylmethoxycarbonyloxy)succinimide (314 mg, 0.93 mmol, 1.1 eq) in dioxane (2 mL). The reaction was diluted with water and washed with ether. The aqueous layer was acidified to pH 3, extracted with EtOAc, dried over MgSO$_4$, filtered and concentrated in vacuo, to yield 375 mg of a white solid, which was used without further purification. $^1$H NMR (250 MHz, DMSO-d$_6$) $\delta$ 7.88 (d, 2H, J = 8 Hz, Fmoc
aromatic -CH), 7.78 (dd, 1H, J = 8 Hz, 8Hz, -NH), 7.70 (d, 2H, J = 8 Hz, Fmoc aromatic -CH), 7.42 (t, 2H, J = 8 Hz, Fmoc aromatic -CH), 7.32 (t, 2H, J = 8 Hz, Fmoc aromatic -CH), 7.20 (m, 4H, aromatic -CH), 4.32 (d, 2H, J = 7 Hz, Fmoc -CH2-), 4.22 (t, 1H, J = 7 Hz, Fmoc -CH-), 3.65 (s, 2H, -CH2NH-), 3.34 (s, 2H, -CH2CO2H).

Synthesis and Characterization of β34-42 Analogs

LMVG-Ψ[C4N]G-VVIA (1). Fmoc-Ala was coupled to the HMPB-BHA resin using diisopropylcarbodiimide (5 eq) and 4-dimethylaminopyridine (DMAP) (0.3 eq) in DMF for 24 h. Fmoc-VVIA was synthesized using the standard procedure for Fmoc synthesis. Protected amino acid 9 (1.1 eq) was coupled using 2 eq BOP reagent and 3 eq DIEA in DMF overnight. The remaining amino acids were coupled using the standard procedure, and the N-terminus was deprotected prior to cleavage. Peptide-resin was incubated with 5 mL of 1% TFA/CH2Cl2 for 2 min. The reaction was immediately filtered into 1 mL CH3OH containing 100 µL pyridine to neutralize. The procedure was repeated until the resin turned pink in color (8 x). The washes were precipitated into ether to yield a brown solid (49 mg from 429 mg peptide-resin), and the product was purified by reversed-phase HPLC. Analytical HPLC (C4, 2.0 mL/min): 80/20 H2O/CH3CN, (0.1% TFA), linear gradient to 20/80 (20 min), Rv = 21.8 mL. Semipreparative HPLC (C4, 15 mL/min): 80/20, linear gradient to 20/80 (20 min), Rv = 192 mL. Amino acid analysis: Ala 1.0 (1), Val 2.6 (3), Met 0.7 (1), Ile 0.9 (1), Leu 1.0 (1). PDMS (m/z): calc'd for C42H73N9O9S, 880.2; found, 880.8 (M+H)+.

LMVG-Ψ[C6H4]G-VVIA (2). Fmoc-VVIA was synthesized using the standard Fmoc protocol beginning with Fmoc-Ala coupled to the Wang resin (Novabiochem). Protected amino acid 13 (1.05 eq) was coupled using 2 eq BOP reagent and 3 eq DIEA in DMF for 2h. The remaining amino acids were coupled
using the standard procedure, and the N-terminus was deprotected prior to cleavage. Peptide was removed from the resin using the standard TFA cleavage procedure, and purified by reversed-phase HPLC. Analytical HPLC (C₄, 2.0 mL/min): 85/15 H₂O/CH₃CN, (0.1% TFA), linear gradient to 35/65 (16 min), Rᵥ = 26 mL. Preparative HPLC (C₄, 65 mL/min): 95/5 isocratic for 4 min, followed by a linear gradient to 5/95 (14 min), Rᵥ = 1040 mL. Amino acid analysis: Ala 1.0 (1), Val 2.7 (3), Met 0.8 (1), Ile 0.8 (1), Leu 1.0 (1). PDMS (m/z): calc'd for C₄₄H₇₄N₈O₉S, 891.2; found, 891.9 (M+H)+.

LMV-[NHPhCO]-VVIA (3) Fmoc-VVIA was synthesized using the standard Fmoc protocol beginning with Fmoc-Ala coupled to the Wang resin (Novabiochem). N-Fmoc-p-aminobenzoic acid (synthesized by L. Dong) (3 eq) was coupled using 3 eq BOP reagent and 3 eq DIEA in DMF for 1.5 h. The remaining amino acids were coupled using the standard procedure, and the N-terminus was deprotected prior to cleavage. Peptide was removed from the resin using the standard TFA cleavage procedure, and purified by reversed-phase HPLC. Analytical HPLC (C₄, 2.0 mL/min): 80/20 H₂O/CH₃CN, (0.1% TFA), linear gradient to 30/70 (16 min), Rᵥ = 22.6 mL. Semipreparative HPLC (C₄, 15 mL/min), 80/20, linear gradient to 30/70 (20 min), Rᵥ = 204 mL. Amino acid analysis: Ala 1.0 (1), Val 2.1 (3), Met 0.5 (1), Ile 0.7 (1), Leu 1.0 (1). PDMS (m/z): calc'd for C₄₂H₇₀N₈O₉S, 863.13; found, 864.0 (M+H)+.

LMV-[NHPhCH₂CO]-VVIA (4). Boc-Ala was coupled to the PAM resin using the standard BOP coupling procedure. Boc-VVIA was synthesized using the standard procedure for Boc synthesis. N-Fmoc-3-aminophenylacetic acid (synthesized by L. Dong) (1 eq) was coupled using 2 eq BOP reagent and 3 eq DIEA in DMF for 1.5 h. The remaining amino acids were coupled using the standard Boc
coupling procedure, and the N-terminus was deprotected prior to cleavage, using the "low-high" trifluoromethanesulfonic acid (TFMSA) cleavage protocol.\textsuperscript{22} Low: Peptide resin (520 mg) was added to a flask under Ar. Dimethylsulfide (1.2 mL), m-cresol (0.4 mL), TFA (2 mL), and TFMSA (0.4 mL) were added and the reaction was stirred at 0°C for 2h. The resin was filtered and washed with TFA. The filtrate was added to cold ether, resulting in precipitation. The pellet was collected by centrifugation (2000 x g, 10 min), washed 3x with ether, and dried \textit{in vacuo} to yield 21 mg crude solid. High: To the remaining resin was added thioanisole (0.8 mL), TFA (5.3 mL), and TFMSA (0.53 mL), and the reaction was stirred for 1 h at 0°C. The resin was removed by filtration and washed with TFA. The filtrate was precipitated into cold ether, washed 3x with ether, and dried \textit{in vacuo} to yield 48 mg crude solid. Both deprotection steps led to products with similar HPLC traces; the "high" sample was purified by reversed-phase HPLC. Analytical HPLC (C\textsubscript{4}, 2.0 mL/min): 80/20 H\textsubscript{2}O/CH\textsubscript{3}CN, (0.1% TFA), linear gradient to 15/85 (16 min), \(R_v = 19\) mL. Semipreparative HPLC (C\textsubscript{4}, 15 mL/min): 80/20, linear gradient to 30/70 (20 min), \(R_v = 179\) mL. Amino acid analysis: Ala 1.0 (1), Val 3.1 (3), Met 0.5 (1), Ile 0.9 (1), Leu 0.8 (1). PDMS (m/z): calc'd for C\textsubscript{43}H\textsubscript{72}N\textsubscript{8}O\textsubscript{9}S, 877.2; found, 877.8 (M+H\textsuperscript{+}).

\textbf{LMV-Aib-Aib-VVIA (5).} Fmoc-VVIA was synthesized using the standard Fmoc protocol beginning with Fmoc-Ala coupled to the Wang resin (Novabiochem). Fmoc-Aib residues were coupled using O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (3 eq)\textsuperscript{23} and DIEA (5.3 eq) in DMF. The remaining amino acids were coupled using the standard procedure, and the N-terminus was deprotected prior to cleavage. Peptide was removed from the resin using the standard TFA cleavage procedure, and purified by reversed-phase HPLC. Analytical HPLC (C\textsubscript{4}, 2.0 mL/min): 95/5 H\textsubscript{2}O/CH\textsubscript{3}CN, (0.1% TFA), linear gradient to 5/95 (15 min), \(R_v = 29\) mL. Semipreparative HPLC (C\textsubscript{4}, 15
mL/min): 85/15, linear gradient to 5/95 (16 min), \( R_v = 172.5 \) mL. Amino acid analysis: Ala 1.0 (1), Val 2.4 (3), Met 0.7 (1), Ile 0.9 (1), Leu 1.0 (1). PDMS (m/z): calc'd for \( C_{43}H_{79}N_9O_{10}S \), 914.2; found, 915.1 (M+H)+.

**LMV-Ψ[CO₂]-GGV VIA (V₀G)** Boc-Ala was coupled to the hydroxymethyl (Merrifield) resin using 3 eq of amino acid, 3 eq DIC, and 0.5 eq of DMAP in CH₂Cl₂ for 30 h. Boc-GVVIA was synthesized on using the standard procedure for Boc-protected amino acids. The N-terminus was deprotected, and bromoacetic acid (3 eq) was coupled with 3 eq PyBOP and 5.3 eq DIEA. Boc-Val-CO₂-Cs⁺ was prepared by dissolving Boc-Val-CO₂H (1.74 g, 8 mmol, 10 eq) in 16 mL EtOH/H₂O (4:1). The solution was titrated to pH 7 with 20% CsCO₃. The solution was dried in vacuo, DMF was added, and the suspension was dried in vacuo 2x further. The product was added to the reaction vessel as a suspension in a minimum volume of DMF, and the suspension was shaken for 18 h. The resin was washed 2x each with: DMF, DMF·H₂O (1:1), H₂O, THF·H₂O (1:1), THF, CH₃OH, and CH₂Cl₂. The remaining amino acids were coupled using the standard procedure, and the N-terminus was deprotected prior to cleavage. Peptide was removed from the resin using the "low-high" TFMSA cleavage procedure, and purified by reversed-phase HPLC. Analytical HPLC (C₄, 2.0 mL/min): 85/15 H₂O/CH₃CN, (0.1% TFA), linear gradient to 20/80 (16 min), \( R_v = 28 \) mL. Preparative HPLC (C₄, 65 mL/min), 80/20, linear gradient to 60/40 (18 min), \( R_v = 249 \) mL. Amino acid analysis: Gly 1.0 (1), Ala 1.0 (1), Val 2.3 (3), Met 0.6 (1), Ile 0.9 (1), Leu 0.8 (1). PDMS (m/z): calc'd for \( C_{39}H_{70}N_8O_{11}S \), 859.1; found, 860.5 (M+H)+.

**LMVG-Ψ[CO₂]-GV VIA (G₀G)** This peptide was synthesized and HPLC purified by Dr. B. McGuinness on the PAM resin by a parallel procedure to that described for
V_{oG}. Amino acid analysis: Gly 1.9 (2), Ala 1.0 (1), Val 1.8 (2), Met 0.7 (1), Ile 0.9 (1), Leu 0.9 (1). PDMS (m/z): calc'd for C_{39}H_{70}N_{8}O_{11}S, 859.1; found, 859.8 (M+H)^+.

**LMVG-G-Ψ[CO_{2}]VVIA (G_{o}V)** Boc-Ala was coupled to the Kaiser oxime resin using DIC (1.5 eq) in CH_{2}Cl_{2} for 24 h. Boc-VIA was synthesized using the standard procedure, and the N-terminus was deprotected. 2-Hydroxy-3-methylbutyric acid (3 eq) was coupled to the peptide-resin by the standard coupling procedure. Boc-Gly was coupled to form the depsipeptide linkage using DIC (3 eq) and DMAP (0.5 eq) in CH_{2}Cl_{2} for 20 h. The remaining amino acids were coupled using the standard procedure, and the N-terminus was deprotected prior to cleavage. Cleavage was carried out by addition of solid N-hydroxypiperidine (4 eq) to the resin-bound peptide in CH_{2}Cl_{2}. The suspension was shaken for 12 h at 23°C and filtered to remove resin. The resin was washed with CH_{2}Cl_{2}, DMF, and CH_{3}OH, and the filtrate was concentrated. Zn dust (10 eq) and 90% acetic acid (10 mL/g resin) were added, and the reaction was stirred for 4 h. The Zn was removed by filtration, the solution was concentrated to remove excess acid, and the peptide was added to H_{2}O, resulting in precipitation. The pellet was collected by centrifugation (2000 x g, 10 min), dried *in vacuo*, and purified by reversed-phase HPLC. Analytical HPLC (C_{4}, 2.0 mL/min): 85/15 H_{2}O/CH_{3}CN, (0.1% TFA), linear gradient to 15/85 (16 min), R_{v} = 25.6 mL. Semipreparative HPLC (C_{4}, 15 mL/min), 95/5, 5 min isocratic, followed by a linear gradient to 5/95 (20 min), R_{v} = 353 mL. Amino acid analysis: Gly 1.2 (2), Ala 1.0 (1), Val 2.1 (2), Met 0.7 (1), Ile 1.1 (1), Leu 1.0 (1). PDMS (m/z): calc'd for C_{39}H_{70}N_{8}O_{11}S, 859.1; found, 860.2 (M+H)^+.

**Aggregation Kinetics**

A stock solutions of each β34-42 peptide analog was prepared by dissolving lyophilized peptide in DMSO. Stock solutions were filtered through Millex FGS 0.2
μm filters (for non-aqueous samples), and the peptide concentration was
determined by quantitative amino acid analysis. Prior to each run, an aliquot of
stock solution was diluted to 4.2 mM in 90 μL total DMSO, and added to 910 μL of
buffer (10 mM sodium phosphate, 100 mM NaCl, pH 7.4), to a final peptide
concentration of 375 μM. The supersaturated solution was stirred at 1550 rpm and
the absorbance at 400 nm was measured every 15 s. Each set of data shown
corresponds to a representative experiment; averaging multiple runs led to
distortion of the data.

**Solubility Measurements**

Following a kinetic aggregation experiment, the solution was stirred for at
least 24 h and filtered through a Millex-GV 0.22 μm aqueous filter (Millipore).
Quantitative amino acid analysis was used to calculate solubility. Data shown is an
average of at least three separate measurements.

**FTIR Spectroscopy**

Aggregated peptides were centrifuged at 4000 rpm for 10 min and spread on a
CaF2 plate. After air drying, the infrared spectra were recorded on a Perkin-Elmer
1600 series FTIR spectrometer.
References for Chapter II

22. Stewart, J.M. & Young, J.D. Solid Phase Peptide Synthesis; 1984, Pierce Chemical Co., Rockford, IL.
Chapter III

Peptide Models of a Hydrophobic Cluster at the C-Terminus of the β Amyloid Protein.

There is mounting evidence that production of long Aβ (primarily β1-42) may be a pathological event.\textsuperscript{1} In vitro aggregation studies from this laboratory have demonstrated that long Aβ nucleates much more rapidly than short Aβ.\textsuperscript{2} The conclusions drawn from these studies were supported by cell culture experiments, which demonstrated increased production of long Aβ by APP mutants which correlate to AD,\textsuperscript{3} and pathology experiments which implicated deposition of long Aβ as a primary event in amyloid plaque formation.\textsuperscript{4-10} We are interested in determining the structural and mechanistic basis for the observed differences in rates of fibril formation. Specifically, we are interested in determining why the addition of just two amino acids to the C-terminus of Aβ (i.e. β1-40 to β1-42) can have such a dramatic effect on the rate of nucleation.

The development of new methodology has provided a means for the determination of amyloid structure on a molecular level.\textsuperscript{11} As described in Chapter 1, SSNMR and FTIR techniques have demonstrated that β34-42 has an unusual conformation in fibrils. Initial R\textsuperscript{2} SSNMR experiments indicated that the Gly37Gly38 amide bond in this peptide might be in the cis configuration. Subsequent refinement to the R\textsuperscript{2} SSNMR has recently suggested that this amide bond is in fact most likely in the trans configuration. The discrepancy between the
former and latter NMR results stresses the importance of alternative methods to evaluate the functional significance (in this case, in terms of promoting amyloidogenesis) of putative structural features. The experiments described in this chapter are one such method, and were designed to test the feasibility of a cis amide as an important structural feature for amyloid fibril formation.

Trans to Cis Isomerization may be a Prerequisite for Nucleation.

In order for the Aβ amyloid fibril to contain a cis amide bond, this intrinsically unfavorable structural feature must allow the fibril to assume a structure which contains a compensating interaction(s). Conversely, if a cis peptide bond is a requirement for amyloid formation, then structural features which stabilize the cis amide in solution should accelerate amyloidogenesis. This reasoning can be incorporated into the kinetic model for Aβ amyloid formation, as shown in Figure 3.1. If a cis amide bond is a prerequisite to nucleation, then stabilization of the cis amide should lead to an increased nucleation rate by increasing the effective concentration of the aggregating species in solution. By the nature of the nucleation-dependent polymerization mechanism, the effect of even very small alterations in $K_{CT}$ will be magnified exponentially (recall that the nucleation rate is proportional to the concentration raised to the nth power, where n is the number of molecules in the nucleus).

With Figure 3.1 as a model, one can propose a structural basis for the increased nucleation rate observed for the longer variants of Aβ. The amino acids surrounding a glycylglycine dipeptide moiety will obviously influence the conformational preference of the peptide, and specifically of the GlyGly amide bond. If the residues found in β34-42 are particularly effective at stabilizing the cis isomer, this will provide a driving force for aggregation. The amino acids which distinguish long Aβ from short Aβ (residues 41, 42, and 43) may have the most pronounced
effects on $K_{CT}$. The resultant differences in the propensity for the cis isomer in solution would affect the relative rates of nucleation for long $A\beta$ and short $A\beta$, perhaps even to the extent observed in *in vitro* aggregation experiments. To test this proposal, we have prepared a series of synthetic peptides with a glycylglycine amide flanked by either the $A\beta$ sequence or alternate amino acids, and determined by $^1H$ NMR spectroscopy the effect of these amino acids on the preference of the glycylglycine amide for the cis isomer in solution. These experiments have allowed us to evaluate the feasibility of a cis amide as a critical structural feature of the $\beta$ protein aggregate.

![Proposed mechanism in which amide isomerization is a prerequisite to nucleation. If $K_{CT}$ is slow relative to the subsequent intermolecular association steps, then this equilibrium will dictate the nucleation rate. Small changes in $K_{CT}$ could have large effects on the rate of fibril formation.](image)

**Figure 3.1** Proposed mechanism in which amide isomerization is a prerequisite to nucleation. If $K_{CT}$ is slow relative to the subsequent intermolecular association steps, then this equilibrium will dictate the nucleation rate. Small changes in $K_{CT}$ could have large effects on the rate of fibril formation.

**Results and Discussion**

**The Glycine to Sarcosine Replacement Leads to Population of the Cis Isomer.**

In a sample of glycylglycine in aqueous buffer, no significant portion of the cis isomer is detected (<2%, $\Delta G > 2$ kcal/mol), typical of nonproline peptide bonds in solution. Even upon addition of valine residues both N- and C-terminally, the cis isomer is not detected in the $^1H$ NMR spectrum. A difference between the $K_{CT}$ values for these compounds (for example, 1% vs. 1.25% cis isomer) would not be measurable. To solve this problem, the Gly37-Gly38 amide bond was replaced with an N-methylamide (Gly38 = sarcosine, Sar), which affords comparable populations

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of cis (E) and trans (Z) conformers in solution (Figure 3.2). Additionally, the cis and trans N-methyl groups of sarcosine are well-resolved singlets in the $^1$H NMR spectrum, the integration of which provides an accurate measure of the cis/trans ratio ($K_{CT}$), which can be used to calculate the free energy ($\Delta G$) for the isomerization.

The validity of this substitution relies on the assumption that the added N-methyl group does not interact directly with the flanking sequence. Examination of models suggests that the Sar N-methyl group cannot directly interact with neighboring sidechains in a manner that stabilizes the cis conformation. In addition, NOE difference and ROESY spectra of compound 2 showed no evidence for an interaction between the N-methyl group and any of the Val36 or Val39 protons. Assuming this to be the case, the ratio of cis to trans amide in the Gly-Sar peptides should be proportional to the effect on the actual Gly-Gly ratio (e.g. an increase from 20 to 25% in Gly-Sar would correspond to an increase from 1% to 1.25% in Gly-Gly).

![Figure 3.2](image)

*Figure 3.2* N-methylation leads to population of the cis isomer in solution. Glycylglycine (upper) has a negligible amount of cis isomer, whereas glycylsarcosine (lower) has $\sim22\%$ cis isomer.
The C-Terminal Sequence Stabilizes the Cis Isomer

A series of peptides, based on the β protein C-terminus but containing a sarcosine residue in place of Gly38, were synthesized in order to probe the effect of the hydrophobic flanking sequence on the amide cis/trans ratio. Peptides were synthesized as N-acetyl carboxamides in order to most effectively mimic an internal peptide sequence, and to minimize a possible charge interaction between the N- and C-termini. The relative percentages of cis and trans isomers were determined to within 0.4% by integration of the well-resolved N-methyl singlets in the 500 MHz $^1$H NMR spectrum of a sample in aqueous buffer. The equilibrium constant ($K_{CT}$) and the free energy ($\Delta G$) of isomerization in each case were calculated based on the percent cis.

Table 3.1 % Cis Amide in β-Amyloid C-Terminal Model Peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>% cis (±s.d.)</th>
<th>$K_{CT}$</th>
<th>$\Delta G^*$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-GX-CONH$_2$</td>
<td>22.3 (±0.37)</td>
<td>0.29</td>
<td>0.73</td>
</tr>
<tr>
<td>Ac-VGXV-CONH$_2$</td>
<td>25.0 (±0.03)</td>
<td>0.33</td>
<td>0.66</td>
</tr>
<tr>
<td>Ac-VGXVV-CONH$_2$</td>
<td>26.6 (±0.32)</td>
<td>0.36</td>
<td>0.61</td>
</tr>
<tr>
<td>Ac-VGXVVI-CONH$_2$</td>
<td>26.6 (±0.32)</td>
<td>0.36</td>
<td>0.61</td>
</tr>
<tr>
<td>Ac-MVGXV-CONH$_2$</td>
<td>25.3 (±0.28)</td>
<td>0.34</td>
<td>0.64</td>
</tr>
<tr>
<td>Ac-MVGXVV-CONH$_2$</td>
<td>26.9 (±0.41)</td>
<td>0.37</td>
<td>0.59</td>
</tr>
<tr>
<td>Ac-EVGXVE-CONH$_2$</td>
<td>24.9 (±0.14)</td>
<td>0.33</td>
<td>0.66</td>
</tr>
<tr>
<td>Ac-LEVGXVE-CONH$_2$</td>
<td>24.9 (±0.42)</td>
<td>0.33</td>
<td>0.66</td>
</tr>
<tr>
<td>Ac-EVGXVEI-CONH$_2$</td>
<td>25.7 (±0.33)</td>
<td>0.34</td>
<td>0.63</td>
</tr>
<tr>
<td>Ac-LEVGXVEI-CONH$_2$</td>
<td>26.4 (±0.31)</td>
<td>0.36</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Dipeptide 1 (22.3% cis) was considered a control for interactions of the N-methyl group with the adjacent glycine backbone, and was used as a baseline level to assess the affect of adding flanking residues. Addition of Val36 and Val39 (1→2) led to an increase in the amount of cis isomer to 25% (an overall increase of 12%
relative to the control). Addition of Val40 (2→3 and 5→6) increased the amount of cis isomer by 6%, but addition of Met35 (2→5 and 3→6) had little effect in this context. No cooperative effect between Met35 and Val40 was observed (e.g. 2→3 = 5→6 and 2→5 = 3→6).

Hydrophobic Residues Stabilize the Cis Isomer

In addition to Aβ peptides, a series of model peptides containing alternate amino acids were synthesized to investigate the importance of hydrophobicity in determining the cis/trans ratio. In general, increasing the hydrophobicity of the residues flanking the N-methylamide led to an increased percentage of cis isomer (Table 3.2). At one extreme, the Ala containing peptide 12 showed no additional stabilization relative to the GlySar dipeptide 1. At the other extreme, the tetrapeptide 17 containing phenylalanine, among the most hydrophobic of amino acids, showed a significant increase in the % cis. Peptide 11, which contains terminal residues with opposite charges, showed no stabilization of the cis amide, suggesting that the effect of ionic interactions is minimal.

<table>
<thead>
<tr>
<th>Table 3.2</th>
<th>% Cis Amide in Hydrophobic Model Peptides (X = sarcosine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>peptide</td>
<td>% cis (± s.d.)</td>
</tr>
<tr>
<td>1</td>
<td>Ac-GX-CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>11</td>
<td>Ac-KGXE-CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>12</td>
<td>Ac-AGXA-CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>13</td>
<td>Ac-AAGXAA-CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>14</td>
<td>Ac-AGXV-CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>15</td>
<td>Ac-VGX-CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Ac-VGXV-CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>16</td>
<td>Ac-IGXI-CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>17</td>
<td>Ac-FGXF-CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
</tbody>
</table>
This data suggests that the cis amide is stabilized by formation of a "hydrophobic cluster" involving the adjacent residues. Manual examination of space-filling models of 2 indicates that, with the amide in the cis configuration, residues 36 and 39 are able to form a compact, globular structure which minimizes the exposed hydrophobic surface (Fig. 3.3). Similar hydrophobic clusters have been implicated in intramolecular protein folding processes.\textsuperscript{16-27} The development of sensitive NMR methods has permitted the detection of folded structures in short peptide fragments under conditions where native proteins fold.\textsuperscript{18} Residual folded structure due to hydrophobic clustering has been detected in the urea-denatured states of the 434-repressor\textsuperscript{25} and lysozyme.\textsuperscript{28} These clusters have been postulated to nucleate the intramolecular process of protein folding. One could imagine that, in A\textsubscript{β}, formation of a hydrophobic cluster serves to nucleate the intermolecular process of aggregation. The mechanistic implications of cluster formation in amyloid nucleation are discussed in more detail later in this chapter.

\textbf{Figure 3.3} (following page) Space-filling models of Ac-Val-Gly-Sar-Val-CONH\textsubscript{2} (2) with the glycylsarcosine in either the cis (upper) or trans (lower) configuration. The lower model is depicted as an idealized extended β-strand. The upper model illustrates the formation of a hydrophobic cluster involving the valine residues.
Because of the tendency of hydrophobic Aβ peptides to form aggregates, we considered the possibility that these peptides could be forming soluble oligomers. Intermolecular interactions could then be responsible for stabilizing the cis isomer in solution, just as similar intermolecular interactions apparently stabilize the cis amide in the solid state.\textsuperscript{29,30} This issue was addressed by recording $^1$H NMR spectra at varying concentrations. Peptide 2 showed no concentration-dependence of the $^1$H NMR spectrum or of $K_{CT}$ over a range of 3 mM to 27 mM (Table 3.3), indicating that oligomerization is not responsible for the observed effects.

**Table 3.3 Concentration-dependence of $K_{CT}$ in peptide 2.**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Concentration</th>
<th>% Cis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Ac-VGXV-CONH$_2$</td>
<td>3mM</td>
<td>24.7</td>
</tr>
<tr>
<td>2 Ac-VGXV-CONH$_2$</td>
<td>10mM</td>
<td>25.0</td>
</tr>
<tr>
<td>2 Ac-VGXV-CONH$_2$</td>
<td>27mM</td>
<td>24.9</td>
</tr>
</tbody>
</table>

**Effect of Salt and Denaturant on $K_{CT}$**

The influence of hydrophobic effects on the observed stabilization was evaluated by recording NMR spectra under different solvent conditions. Hydrophobic effects should be accentuated in the presence of a high concentration of salt, and minimized in the presence of denaturant.\textsuperscript{31} This was found to be the case (Table 3.4): 1, which lacks the hydrophobic flanking sequence, showed little effect, but peptides 2 and 3, both of which contain flanking valine residues, show an increase in $K_{CT}$ in the presence of saturated NaCl, and a decrease in $K_{CT}$, essentially to the levels of 1, in the presence of 6M GdmCl. Peptide 7 was affected in a similar manner, although not as dramatically. These findings support the idea of a hydrophobic cluster as a stabilizing force for the cis amide isomer.
Table 3.4 Effect of High Concentrations of Salt and Denaturant on Cis-Trans Equilibrium

<table>
<thead>
<tr>
<th>Peptide</th>
<th>phys. buffer</th>
<th>sat'd NaCl</th>
<th>GdnSCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-GX-CONH₂</td>
<td>22.3</td>
<td>23.3 (±0.26)</td>
<td>22.3 (±0.25)</td>
</tr>
<tr>
<td>Ac-VGXV-CONH₂</td>
<td>25.0</td>
<td>27.8 (±0.24)</td>
<td>22.7 (±0.95)</td>
</tr>
<tr>
<td>Ac-VGXVV-CONH₂</td>
<td>26.6</td>
<td>29.1 (±0.17)</td>
<td>23.8 (±0.19)</td>
</tr>
<tr>
<td>Ac-EVGXVE-CONH₂</td>
<td>24.9</td>
<td>27.1 (±0.15)</td>
<td>25.1 (±0.17)</td>
</tr>
</tbody>
</table>
Table 3.5 Effect of Temperature on Cis-Trans Equilibrium

<table>
<thead>
<tr>
<th>Peptide</th>
<th>10°C ± s.d.</th>
<th>25°C ± s.d.</th>
<th>50°C ± s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-VGXV-CONH₂</td>
<td>24.9 (±0.17)</td>
<td>25.0 (±0.09)</td>
<td>26.1 (±0.26)</td>
</tr>
<tr>
<td>Ac-MVGXVV-CONH₂</td>
<td>27.0 (+0.3)</td>
<td>27.6 (+0.3)</td>
<td>28.3* (+0.3)</td>
</tr>
</tbody>
</table>

* measurement taken at 45°C

One thermodynamic parameter which is considered a hallmark of the hydrophobic effect is a positive change in heat capacity ($ΔC_p$ $>$ 0). $ΔC_p$ is defined as the change in reaction enthalpy ($ΔH$) with temperature. A positive $ΔC_p$ is observed upon unfolding of proteins, and is attributed to the ordering of water on exposed, nonpolar surfaces which are buried in the native conformation. Microcalorimetric methods have been developed to measure $ΔC_p$ for the folded and unfolded states of globular proteins. However, in the case of the small, multiconformational peptides described here, the expected differences in $ΔC_p$ would be infinitesimal, and would not be expected to provide interpretable information.

An Interaction Between Leu34 and Ile41 Stabilizes the Cis Isomer

We were particularly interested in assessing the effect of the C-terminal residues which distinguish the long and short variants of Aβ. Addition of Ile41 to peptide 3 (3→4) did not affect the cis/trans ratio. This suggested that in order for Ile41 to participate in a hydrophobic cluster, it may require an interaction with residues N-terminal to the Gly-Gly amide, and that this residue does not interact with residues 36, 39, and 40. Addition of Leu34 and Ile41 to the models resulted in peptides which were not soluble enough to be accurately analyzed. To increase the solubility of the peptide, Met35 and Val40 were replaced with glutamic acid, and the incremental change upon addition of Leu34, Ile41, and both residues together was
measured. The increase in % cis upon addition of both residues (7→10 = +6%) was greater than the sum of the individual contributions (7→8 = +0% and 7→9 = +3%). Ile41, therefore, may interact with Leu34 in a cooperative manner to stabilize the putative hydrophobic cluster.

Mechanistic Implications of Hydrophobic Cluster Formation

A model for amyloid fibril formation which incorporates a hydrophobic cluster is presented in Fig. 3.4. This model suggests the following scenario: Formation of a hydrophobic cluster stabilizes conformers which contain the cis isomer in solution. This stabilizing effect leads to a shift in the cis-trans equilibrium ($K_{CT}$) toward the cis isomer, effectively increasing the concentration of the aggregating species in solution, and leading to a corresponding increase in the rate of nucleation.

Figure 3.4 One possible mechanism for AD amyloid fibril formation. Initial trans to cis isomerization is slow relative to cluster formation and the subsequent intermolecular association processes. Therefore, $K_{CT}$ is a critical determinant of the rate of nucleus formation. The cis amide is stabilized by formation of a hydrophobic cluster. The side-chain interactions indicated were inferred from the model studies and may be direct or indirect. They do not necessarily persist in the amyloid fibril.
This proposed mechanism is consistent with the observed effect of the residues surrounding the Gly37-Gly38 amide bond in the Aβ sequence. In particular, the C-terminal residues which play such a critical role in determining the rate of amyloid formation\(^2\) may exert their effect by adding stabilization to the hydrophobic cluster. This increase in the concentration of the aggregating species could have a dramatic effect on the rate of nucleus formation. Given a nucleus size of 25, for example, the increase observed for addition of Val40 and Ile41 (~6%) would lead to a greater than 4-fold \((1.06)^{25}\) acceleration of nucleation, providing one possible explanation for the measured difference in nucleation times between long Aβ and short Aβ (β26-39, 60 h; β26-40, 12 h; β26-42, <30 sec). The cluster does not necessarily have to persist in the final amyloid fibril. In fact, SSNMR experiments have ruled out the presence of a folded hairpin structure centered around the cis amide bond\(^2^9\). Instead, the cluster serves to stabilize one key structural feature of the aggregate.

One assumption implicit to this model is that isomerization is a slow process relative to the subsequent intermolecular association steps. If isomerization were rapid, then K\(_{CT}\) would be irrelevant to the rate of aggregation; this is an application of the Curtin-Hammett principle. At the peptide concentration used in our aggregation assay (40 μM), representative rates of ~10 s\(^{-1}\) for interprotein association \((k = 10^5 - 10^6 \text{ M}^{-1}\text{s}^{-1})\)\(^{33}\) and ~10\(^{-2}\) s\(^{-1}\) for amide isomerization\(^{34}\) would be expected.

This mechanistic interpretation of the data assumes that the glycylglycine cis amide is a prerequisite for fibril formation. The discovery that hydrophobic cluster formation stabilizes the cis amide geometry in solution supports structural models of the β34-42 fibril which contain a cis amide bond\(^{29,30}\). Although this was originally thought to be the case, recent evidence suggests that this bond may actually be in the trans configuration\(^{35}\). If this is the case, then stabilization of the cis amide configuration will be irrelevant to the rate of fibril formation. However, formation
of a hydrophobic cluster may still accelerate amyloidogenesis by reducing the entropic barrier to aggregation. In this scenario, the cis-trans ratio in the model peptides would serve as a indicator of the extent of cluster formation, rather than as a critical parameter for aggregation.

The observations recorded here may be of interest in the field of protein folding in a more general sense. Much current interest within this field is focused on defining the scope of hydrophobic effects, and in particular on elucidating the specific forces which mediate hydrophobic interactions in protein folding processes. The observed correlation between hydrophobicity and $K_{CT}$ presented here suggests that this system may provide a useful method for determining the strength of specific hydrophobic interactions between two amino acid residues. A more exhaustive examination of the effect of amino acids with varying degrees of hydrophobicity might provide useful insight into the conformational features which influence protein structure.
Experimental Section

Standard Procedures for Peptide Synthesis

Peptides were synthesized on either the 4-methylbenzhydrylamine (MBHA) resin or the Rink amide resin using either N-Boc or N-Fmoc protected amino acids and either of the following general procedures. For coupling glycine to sarcosine, BOP reagent was replaced with an equivalent amount of PyBrOP.37

N-Boc amino acids. (1) Wash resin with CH₂Cl₂ (2x) followed by iPrOH (2x), repeat 3x; (2) remove a small amount of resin and perform the Kaiser test for free amine (the test should be positive (blue)); (3) wash with DMF (3x); (4) add Boc-amino acid (3 eq, 0.1M in DMF), BOP reagent (3 eq, 0.1M in DMF), and DIEA (5.3 eq), shake resin for 30 sec, shake mixture for at least 40 min at RT; (5) wash with DMF (3x); (6) wash with CH₂Cl₂ (2x), iPrOH (2x), CH₂Cl₂ (2x); (7) remove a small amount of resin and perform the Kaiser test for free amine. If positive (blue) then repeat steps (3) - (7) until a negative (yellow) result is obtained; (8) add acetic anhydride (10 eq) and DIEA (5 eq) in CH₂Cl₂, and shake for >20 min; (9) wash with CH₂Cl₂ (3x); (10) add 25% trifluoroacetic acid (TFA) in CH₂Cl₂ and shake for 30 min. Repeat steps (1) - (10), ending with deprotection of the N-terminal amino acid. (11) Acetylate the N-terminal amino acid via steps (6) - (9).

N-Fmoc amino acids. (1) Wash resin with DMF (3x), CH₃OH (3x), CH₂Cl₂ (3x); (2) remove a small amount of resin and perform the Kaiser test for free amine (the test should be positive (blue)); (3) wash with DMF (3x); (4) add Fmoc-amino acid (3 eq, 0.1M in DMF), BOP reagent (3 eq, 0.1M in DMF) and DIEA (5.3 eq), shake resin for 30 sec, shake mixture for at least 40 min at RT; (5) wash with DMF (3x); (6) wash with CH₂Cl₂ (2x), CH₃OH (2x), CH₂Cl₂ (2x); (7) remove a small amount of resin and
perform the Kaiser test for free amine. If positive (blue) then repeat steps (3) - (7) until a negative (yellow) result is obtained; (8) add acetic anhydride (10 eq) and DIEA (5 eq) in CH₂Cl₂, and shake for >20 min; (9) wash with CH₂Cl₂ (3x); (10) add 50% piperidine in DMF and shake for 30-45 min. Repeat steps (1) - (10), ending with deprotection of the N-terminal amino acid. (11) Acetylate the N-terminal amino acid via steps (6) - (9).

**Standard Procedure for TFMSA Cleavage of Peptide-Resin**

Peptide-resin was placed in a flask under Ar. Thioanisole (2 mL/g resin) was added, and the flask was cooled to 0°C. TFA (20 mL/g) was added, followed after 10 min by trifluoromethanesulfonic acid (TFMSA, 2 mL/g). The reaction was stirred at 0°C for 30 min, then at RT for an additional 2 h. The reaction was filtered to remove resin, and the resin was washed with a minimum amount of TFA. The solution was added dropwise to excess cold diethyl ether, resulting in precipitation. The precipitate was collected by centrifugation (2000 x g, 10 min), washed 3x with ether, and dried *in vacuo*.

**NMR Methods**

Deuterated PBS was prepared by repeated lyophilization of PBS (100 mM NaCl, 10 mM phosphate, pH 7.4) and reconstitution with D₂O. NMR samples were prepared by dissolving ~3 mg of purified, lyophilized peptide in 1 mL of deuterated buffer. ¹H NMR spectra were recorded on a Varian Unity 500 MHz spectrometer. Cis/trans ratios shown are an average of at least ten separate integrations of the N-methyl proton singlets.
Synthesis of Sarcosine-Containing Peptides

Unless otherwise noted, peptides were synthesized on the 4-methylbenzhydrylamine (MBHA) resin using the standard procedures given above, and characterized by plasma desorption mass spectrometry (PDMS). All peptides were judged to be >95% pure by analytical HPLC. The synthesis and full characterization of the following peptides was performed by J. Jarrett, and has been reported elsewhere:14 Ac-Gly-Sar-CONH₂, Ac-Ala-Gly-Sar-Ala-CONH₂, Ac-Val-Gly-Sar-Val-CONH₂, Ac-Met-Val-Gly-Sar-Val-Val-CONH₂, and Ac-Met-Val-Gly-Sar-Val-Val-CO₂H.

Ac-AAGSarAA-CONH₂ Ac-Ala-Ala-Gly-Sar-Ala-Ala-CONH₂ was synthesized and HPLC purified by A. Vaishnav. 1H NMR (100 mM NaCl, 10 mM sodium phosphate, pH 7.4 in D₂O) δ 3.95-4.30 (m, 8H, αH), 3.01 (s, 2.3H, Sar trans CH₃), 2.83 (s, 0.7H, Sar cis CH₃), 1.93 (s, 3H, acetyl), 1.24-1.35 (m, 12H, Ala CH₃).

Ac-AGSarV-CONH₂ Ac-Ala-Gly-Sar-Val-resin was synthesized and cleaved using the standard procedures. Analytical HPLC (C₄, 2.0mL/min): 100/0 H₂O/CH₃CN, (0.1% TFA) 10 min isocratic followed by a linear gradient to 50/50 (10 min), Rᵥ = 11 mL. Semipreparative HPLC (C₄, 15 mL/min): 100/0 isocratic, Rᵥ = 132 mL. 1H NMR (100 mM NaCl, 10 mM sodium phosphate, pH 7.4 in D₂O) δ 3.98-4.31 (m, 6H, αH), 3.06 (s, 2.2H, Sar trans CH₃), 2.88 (s, 0.8H, Sar cis CH₃), 2.06 (m, 1H, Val βH), 1.98 (s, 3H, acetyl), 1.36 (d, 3H, J = 9 Hz, Ala CH₃), 0.93 (m, 6H, Val CH₃).

Ac-VGSarA-CONH₂ Ac-Val-Gly-Sar-Ala-CONH₂ was synthesized and HPLC purified by A. Vaishnav. 1H NMR (100 mM NaCl, 10 mM sodium phosphate, pH 7.4 in D₂O) δ 3.95-4.24 (m, 6H, αH), 3.02 (s, 2.2H, Sar trans CH₃), 2.84 (s, 0.8H, Sar cis
Ac-KGSarE-CONH$_2$  Ac-Lys-Gly-Sar-Glu-CONH$_2$ was synthesized and HPLC purified by A. Vaishnav. $^1$H NMR (100 mM NaCl, 10 mM sodium phosphate, pH 7.4 in D$_2$O) δ 3.91-4.37 (m, 6H, αH), 3.05 (s, 2.2H, Sar trans CH$_3$), 2.87 (s, 0.8H, Sar cis CH$_3$), 2.42 (m, 2H, Glu γH), 2.09 (m, 1H, Glu βH), 1.97 (s, 3H, acetyl), 1.93 (m, 1H, Glu βH), 1.80 (m, 1H, Lys βH), 1.69 (m, 1H, Lys βH), 1.63 (m, 2H, Lys δH), 1.41 (m, 2H, Lys γH).

Ac-FGSarF-CONH$_2$  Ac-Phe-Gly-Sar-Phe-CONH$_2$ was synthesized and HPLC purified by A. Vaishnav. $^1$H NMR (100 mM NaCl, 10 mM sodium phosphate, pH 7.4 in D$_2$O) δ 7.2-7.4 (m, 10H, Phe aromatic H), 4.60 (m, 2H, Phe αH), 3.90 (m, 4H, Gly αH), 3.20 (m, 2H, Phe βH), 2.91 (m, 2H, Phe βH), 2.82 (s, 2H, Sar trans CH$_3$), 2.71 (s, 1H, Sar cis CH$_3$), 1.94 (s, 1H, cis acetyl), 1.93 (s, 2H, trans acetyl).

Ac-IGSarI-CONH$_2$  Ac-Ile-Gly-Sar-Ile-resin was synthesized and cleaved using the standard procedures. Analytical HPLC (C$_4$, 2.0 mL/min): 95/5 H$_2$O/CH$_3$CN, (0.1% TFA), linear gradient to 85/15 (20 min), $R_v$ = 27.6 mL. Semipreparative HPLC (C$_4$, 15 mL/min): 90/10, linear gradient to 75/25 (20 min), $R_v$ = 240 mL. $^1$H NMR (100 mM NaCl, 10 mM sodium phosphate, pH 7.4 in D$_2$O) δ 3.97-4.22 (m, 6H, αH), 3.06 (s, 2.2H, Sar trans CH$_3$), 2.89 (s, 0.8H, Sar cis CH$_3$), 2.01 (s, 3H, acetyl), 1.83 (m, 1H, Ile βH), 1.42 (m, 1H, Ile γCH$_2$), 1.19 (m, 1H, Ile γCH$_2$), 0.92 (m, 3H, Ile γCH$_3$), 0.84 (m, 3H, Ile δCH$_3$).

Ac-MVGSarV-CONH$_2$  Ac-Met-Val-Gly-Sar-Val-resin was synthesized using the standard procedure, and cleaved using the "low-high" TFMSA cleavage.$^{38}$ Low: Peptide resin (1.1 g) was added to a flask under Ar. Dimethylsulfide (3.3mL), m-cresol (1.1 mL), TFA (5.5 mL), and TFMSA (1.1 mL) were added and the reaction was
stirred at 0°C for 2h. The resin was filtered and washed with TFA. The filtrate was added to cold ether, resulting in precipitation. The precipitate was collected by centrifugation (2000 x g, 10 min), washed 3x with ether, and dried in vacuo. High: To the remaining resin was added thioanisole (2 mL), TFA (11 mL), and TFMSA (1.1 mL), and the reaction was stirred for 30 min at 0°C. The resin was removed by filtration and washed with TFA. The filtrate was added to cold ether, resulting in precipitation. The precipitate was collected by centrifugation (2000 x g, 10 min), washed 3x with ether, and dried in vacuo. Both deprotection steps led to products with similar HPLC traces. Analytical HPLC (C₄, 2.0 mL/min): 95/5 H₂O/CH₃CN, (0.1% TFA), linear gradient to 20/80 (16 min), Rᵥ = 22.4 mL. Semipreparative HPLC (C₄, 15 mL/min): 95/5, linear gradient to 20/80 (20 min), Rᵥ = 210 mL. ¹H NMR (100 mM NaCl, 10 mM sodium phosphate, pH 7.4 in D₂O) δ 4.39 (m, 1H, αH), 4.12 (m, 6H, αH), 3.05 (s, 2.2H, Sar trans CH₃), 2.88 (s, 0.8H, Sar cis CH₃), 2.50 (m, 2H, Met γH), 2.07 (s, 3H, Met SCH₃), 2.06 (m, 4H, Met & Val βH), 1.96 (s, 3H, acetyl), 0.91 (m, 12H, Val CH₃).

**Ac-VGSarVV-CONH₂** Ac-Val-Gly-Sar-Val-Val-resin was synthesized and cleaved using the standard procedures. Analytical HPLC (C₄, 2.0 mL/min): 100/0 H₂O/CH₃CN, (0.1% TFA), linear gradient to 75/25 (25 min), Rᵥ = 32 mL. Semipreparative HPLC (C₄ Deltaprep, 65 mL/min): 100/0, 6 min isocratic, followed by a linear gradient to 75/25 (16 min), Rᵥ = 1.53 L. ¹H NMR (100 mM NaCl, 10 mM sodium phosphate, pH 7.4 in D₂O) δ 3.9-4.2 (m, 7H, αH), 3.00 (s, 2.2H, Sar trans CH₃), 2.83 (s, 0.8H, Sar cis CH₃), 2.04 (m, 3H, Val βH), 1.97 (s, 3H, acetyl), 0.88 (m, 18H, Val CH₃).

**Ac-VGSarVVI-CONH₂** Ac-Val-Gly-Sar-Val-Val-Ile-resin was synthesized and cleaved using the standard procedures. Analytical HPLC (C₄, 2.0 mL/min): 95/5
H₂O/CH₃CN, (0.1% TFA), linear gradient to 5/95 (16 min), Rᵥ = 23 mL. Semipreparative HPLC (C₄, 15 mL/min): 100/0, 4 min isocratic followed by a linear gradient to 30/70 (18 min), Rᵥ = 208.5 mL. ¹H NMR (100 mM NaCl, 10 mM sodium phosphate, pH 7.4 in D₂O) δ 3.65-4.15 (m, 8H, αH), 3.04 (s, 2.2H, Sar trans CH₃), 2.87 (s, 0.8H, Sar cis CH₃), 2.10 (m, 3H, Val βH), 2.01 (s, 3H, acetyl), 1.80 (m, 1H, Ile βH), 1.49 (m, 1H, Ile γCH₂), 1.23 (m, 1H, Ile γCH₂), 0.81-0.95 (m, 24H, Ile & Val CH₃).

Ac-EVGSarVE-CONH₂ Ac-Glu-Val-Gly-Sar-Val-Glu-Rink amide resin was synthesized according to the standard procedure. The peptide-resin (1.2 g) was added to a flask under Ar. Ethanedithiol (300 μL, 3%), thioanisole (200 μL, 2%), and TFA (9.5 mL, 95%) were added, and the reaction was stirred under Ar for 3 h at RT. The resin was removed by filtration and washed with TFA. The filtrate was added to cold ether, resulting in precipitation. The precipitate was collected by centrifugation (2000 x g, 10 min), washed 3x with ether, and dried in vacuo. Analytical HPLC (C₄, 2.0 mL/min): 95/5 H₂O/CH₃CN, (0.1% TFA), linear gradient to 5/95 (16 min), Rᵥ = 13.2 mL. Semipreparative HPLC (C₄, 15 mL/min): 95/5, linear gradient to 5/95 (20 min), Rᵥ = 146.1 mL. ¹H NMR (100 mM NaCl, 10 mM sodium phosphate, pH 7.4 in D₂O) δ 3.95-4.30 (m, 8H, αH), 3.05 (s, 2.2H, Sar trans CH₃), 2.87 (s, 0.8H, Sar cis CH₃), 2.31 (m, 4H, Glu γH), 2.04 (m, 4H, Glu & Val βH), 1.97 (s, 3H, acetyl), 1.90 (m, 2H, Glu βH), 0.90 (m, 12H, Val CH₃).

Ac-LEVGSarVE-CONH₂ Ac-Leu-Glu-Val-Gly-Sar-Val-Glu-resin was synthesized and cleaved according to the standard procedures. Analytical HPLC (C₄, 2.0 mL/min): 95/5 H₂O/CH₃CN, (0.1% TFA), linear gradient to 70/30 (20 min), Rᵥ = 32 mL. Semipreparative HPLC (C₄, 15 mL/min): 95/5, linear gradient to 70/30 (20 min), Rᵥ = 274.5 mL. ¹H NMR (100 mM NaCl, 10 mM sodium phosphate, pH 7.4 in D₂O) δ 3.98-4.33 (m, 9H, αH), 3.07 (s, 2.2H, Sar trans CH₃), 2.90 (s, 0.8H, Sar cis CH₃),
2.25 (m, 4H, Glu γH), 2.08 (m, 4H, Val & Glu βH), 1.99 (s, 3H, acetyl), 1.92 (m, 2H, Glu γH), 1.57 (m, 3H, Leu βH & γH), 0.87-0.95 (m, 18H, Val & Leu CH₃).

**Ac-EVGSarVEI-CONH₂**  Ac-Glu-Val-Gly-Sar-Val-Glu-Ile-resin was synthesized and cleaved according to the standard procedures. Analytical HPLC (C₄, 2.0 mL/min): 95/5 H₂O/CH₃CN, (0.1% TFA), linear gradient to 75/25 (20 min), Rᵥ = 36 mL. Semipreparative HPLC (C₄, 15 mL/min): 90/10, linear gradient to 60/40 (18 min), Rᵥ = 187.5 mL. ¹H NMR (100 mM NaCl, 10 mM sodium phosphate, pH 7.4 in D₂O) δ 3.94-4.30 (m, 9H, αH), 3.04 (s, 2.2H, Sar trans CH₃), 2.88 (s, 0.8H, Sar cis CH₃), 2.20 (m, 4H, Glu γH), 2.06 (m, 4H, Val & Glu βH), 1.96 (s, 3H, acetyl), 1.87 (m, 3H, Ile & Glu βH), 1.43 (m, 1H, Ile γH), 1.13 (m, 1H, Ile γH), 0.58-0.93 (m, 18H, Ile & Val CH₃).

**Ac-LEVGSarVEI-CONH₂**  Ac-Leu-Glu-Val-Gly-Sar-Val-Glu-Ile-resin was synthesized and cleaved according to the standard procedures. Analytical HPLC (C₄, 2.0 mL/min): 85/15 H₂O/CH₃CN, (0.1% TFA), linear gradient to 50/50 (16 min), Rᵥ = 21.6 mL. Semipreparative HPLC (C₄, 15 mL/min): 95/5, linear gradient to 5/95 (20 min), Rᵥ = 169.5 mL. ¹H NMR (100 mM NaCl, 10 mM sodium phosphate, pH 7.4 in D₂O) δ 3.96-4.30 (m, 8H, αH), 3.03 (s, 2.2H, Sar trans CH₃), 2.87 (s, 0.8H, Sar cis CH₃), 2.20 (m, 4H, Glu γH), 2.04 (m, 4H, Val & Glu βH), 1.97 (s, 3H, acetyl), 1.92 (m, 3H, Ile & Glu βH), 1.54 (m, 3H, Leu βH and Leu γH), 1.43 (m, 1H, Ile γCH₂), 1.13 (m, 1H, Ile γCH₂), 0.79-0.91 (m, 24H, Ile γCH₃, Ile δCH₃, Val CH₃, Leu CH₃).
References for Chapter III

38. Stewart, J.M. & Young, J.D. *Solid Phase Peptide Synthesis;* 1984, Pierce Chemical Co., Rockford, IL.
Chapter IV

The Non-\(A\beta\) Component of AD Amyloid: A Common Trigger or Target in Alzheimer's Disease?

Background

In 1993, Uéda et al. identified the "non-\(A\beta\) component of AD amyloid" (NAC) as a second intrinsic component of the plaque core, based on its copurification with \(A\beta\) in the SDS-insoluble fraction of brain homogenate and immunocytochemical localization to amyloid plaques.\(^1\) The minimal 35 amino acid NAC sequence is shown in Fig. 4.1; the exact length of NAC found \textit{in vivo} has not been determined.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{The non-\(A\beta\) component of AD amyloid (NAC).}
\end{figure}

\begin{center}
\textbf{H}_2\text{N-}\text{EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFV-}\text{CO}_2\text{H} \\
\end{center}

We noted that the primary sequence of an amino-terminal portion of NAC (residues 66-73) resembles the amyloidogenic carboxyl terminus of \(A\beta\) (Fig. 4.2).\(^2\) This is a critical region of \(A\beta\): as discussed in Chapter 1, truncated variants of \(A\beta\), which form amyloid more slowly \textit{in vitro},\(^3\) are the primary circulating proteins while the longer, more rapidly aggregating variants are the primary plaque

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proteins. A similar sequence is also found in a region of the prion protein (PrP, residues 117-124, Fig. 4.2). The substitution of Val for Ala at codon 117 of PrP is found in Gerstmann-Straussler-Scheinker syndrome (GSS). A peptide containing this region (PrP106-126) has been shown to form amyloid fibrils and is neurotoxic, suggesting that it is an important region for PrP amyloid formation. These sequence similarities suggested that specific hydrophobic interactions between NAC (or NACP), PrP, and Aβ could occur in vivo.

<table>
<thead>
<tr>
<th>β1-43</th>
<th>GQPHGGG/GWGGGGTTSSWKimKPSKPKTMKHMAGAA</th>
<th>VAGAVVGG</th>
<th>LGYMLGSMSRPHIFGSDYEDRYY</th>
<th>A117V</th>
</tr>
</thead>
<tbody>
<tr>
<td>NACP</td>
<td>GKTKEGVL/VGSK/TEKVEVHGAT/AEKTEQYTN</td>
<td>VGGAWTG</td>
<td>VTAVAQKTVEGAGIAATGFVKKDQ</td>
<td></td>
</tr>
<tr>
<td>PrP</td>
<td>(82-150)</td>
<td>VAGAVVGG</td>
<td>NACP GKTKEGVLYVGSKTEKVEVHGATVAEKTKEQYTN</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NACP GKTKEGVLYVGSKTEKVEVHGATVAEKTKEQYTN</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.2 A comparison of sequences from β1-43, NACP, and PrP. Sequence identities are highlighted. The box indicates the eight-residue hydrophobic region with local sequence homology. The A117V mutation (*) segregates with the human prion disease Gerstmann-Straussler-Scheinker syndrome.

Results and Discussion

NAC Peptide Aggregates from Supersaturated Solution to Form Amyloid Fibrils

A 35 amino acid peptide corresponding to the minimal NAC sequence was synthesized on the solid phase and purified by HPLC. The peptide was characterized by plasma desorption mass spectrometry, amino acid analysis, and N-terminal amino acid sequencing. A supersaturated aqueous solution of NAC was prepared by addition of a concentrated stock solution (in DMSO) to PBS. Aggregation was monitored by turbidity measurements. The precipitate was isolated by centrifugation and characterized.
Electron microscopy of NAC showed that it formed unbranched, twisted fibrils of ~50 Å in diameter. In comparison to β1-40, the NAC fibrils appeared narrower and often were shorter in length. This observation was confirmed by using atomic force microscopy to visualize the NAC fibrils, which demonstrated the same relative morphology (Fig. 4.3). A film of NAC fibrils could be stained with Congo Red, and, under a polarizing light microscope, exhibited the birefringence characteristic of amyloid. The Fourier transform infrared (FTIR) spectrum of fibrillar NAC (Fig. 4.4) was dominated by two low frequency absorption amide I bands, at 1633 and 1627 cm⁻¹, indicative of antiparallel β-sheet structure. These experiments were the first to demonstrate the ability of purified NAC to form fibrillar structures which meet the criteria to be called amyloid.

Figure 4.3 (following page) Atomic force micrographs of NAC and β1-40 amyloid fibrils. NAC forms unbranched amyloid fibrils (upper). A magnification of an NAC fibril (lower right, corresponds to the fibril at the top of upper panel) reveals that NAC is slightly narrower than β1-40 (lower left). Note that the scales on the lower panels are identical. Similar morphologies are observed using electron microscopy.
Figure 4.4 Upper trace: FTIR spectrum of NAC amyloid fibrils; lower trace: Fourier-deconvoluted spectrum. Maxima are at 1633 cm\(^{-1}\) and 1627 cm\(^{-1}\) with a shoulder at ~1690 cm\(^{-1}\), indicative of antiparallel β-sheet structure.
The circular dichroism (CD) spectrum of a sample of saturated soluble NAC (centrifuged following an aggregation experiment) is shown in Fig. 4.5. This CD spectrum indicates a high percentage of β-sheet, the characteristic structure of amyloid fibrils. A similar CD spectrum was observed for aqueous solutions of β10-43 and β1-42.\(^{11,12,13}\) It is unlikely that NAC is monomeric under these conditions; β10-43 is dimeric in water.\(^11\) Rather, it is probable that the NAC in this sample is a dimeric or oligomeric β-sheet. Because the solution is stable at equilibrium, any oligomer present must be smaller than the NAC nucleus. Further investigation into the size of such "soluble aggregates", for example using dynamic light scattering, may shed light on the solution conformation of NAC.

**Figure 4.5** CD spectrum of NAC peptide [20 μM peptide in physiological buffer (100 mM NaCl, 10 mM phosphate, pH 7.4)].

**Solubility of Aβ and PrP Peptides in the Presence of NAC**

The colocalization of NAC and Aβ in amyloid plaque could be due to the formation of highly insoluble coaggregates of these two species. Integrated fibrils would be expected to have properties which differ from those of the two
homogeneous, or segregated, fibrils. To test this hypothesis, we compared the solubility of each isolated peptide to the solubility of that peptide in the presence of one other peptide (Table 4.1). Fibrils were prepared by stirring two supersaturated mixtures, containing one peptide (40 μM) or equimolar in two peptides (40 μM each). After aggregation, the concentration of each peptide in the soluble phase was compared using quantitative amino acid analysis. As well as β1-40 and PrP106-126, we also tested several shortened forms of the Aβ (β26-40, β26-42, and β26-43) and a mutant form of the PrP peptide (106-126, Ala117Val).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Measured Solubility (± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β26-40</td>
<td>6.2 (1.2)</td>
</tr>
<tr>
<td>β26-42</td>
<td>3.4 (0.3)</td>
</tr>
<tr>
<td>β26-43</td>
<td>3.1 (0.2)</td>
</tr>
<tr>
<td>PrP106-126</td>
<td>15.9 (2.5)</td>
</tr>
<tr>
<td>PrP106-126 A117V</td>
<td>8.7 (1.0)</td>
</tr>
<tr>
<td>NAC</td>
<td>10.9 (0.8)</td>
</tr>
</tbody>
</table>

The individual measured solubilities in mixtures of Aβ and PrP-derived peptides were indistinguishable, within the experimental error, from the solubilities of the isolated peptides. In contrast, mixtures of NAC with Aβ or PrP peptides produced anomalous effects; although the solubility of NAC was not significantly changed by the presence of other peptides (10.9 ± 0.8 μM), some of the β and PrP peptides showed increased solubility in the presence of 40 μM NAC (Table 4.2). This phenomenon is not easily interpreted, and an effect on solubility does not necessarily correlate with the ability to serve as a seed. These experiments revealed no evidence for the formation of a stable coaggregated species.
Table 4.2 Thermodynamic solubilities of peptide mixtures (40 μM each).

<table>
<thead>
<tr>
<th>peptide measured solubility</th>
<th>peptide measured solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>β26-40 11.8 (1.2)</td>
<td>NAC 10.0 (1.1)</td>
</tr>
<tr>
<td>β26-42 7.2 (0.3)</td>
<td>NAC 11.5 (0.7)</td>
</tr>
<tr>
<td>β26-43 6.5 (0.5)</td>
<td>NAC 8.9 (0.9)</td>
</tr>
<tr>
<td>β1-40 0.5 (0.1)</td>
<td>NAC 11.0 (2.9)</td>
</tr>
<tr>
<td>PrP106-126 24.1 (3.0)</td>
<td>NAC 12.1 (0.4)</td>
</tr>
<tr>
<td>PrP106-126 A117V 16.4 (2.5)</td>
<td>NAC 11.5 (0.1)</td>
</tr>
<tr>
<td>β26-40 9.6 (0.7)</td>
<td>PrP106-126 16.5 (7.9)</td>
</tr>
<tr>
<td>β26-42 5.1 (0.6)</td>
<td>PrP106-126 15.1 (8.4)</td>
</tr>
<tr>
<td>β26-43 4.7 (0.3)</td>
<td>PrP106-126 16.9 (7.1)</td>
</tr>
<tr>
<td>β1-40 0.4 (0.1)</td>
<td>PrP106-126 20.1 (3.1)</td>
</tr>
<tr>
<td>β26-40 9.1 (0.6)</td>
<td>PrP106-126 A117V 7.3 (0.2)</td>
</tr>
<tr>
<td>β26-42 6.4 (1.5)</td>
<td>PrP106-126 A117V 7.8 (2.3)</td>
</tr>
<tr>
<td>β26-43 5.1 (2.2)</td>
<td>PrP106-126 A117V 5.1 (0.8)</td>
</tr>
<tr>
<td>β1-40 0.5 (0.2)</td>
<td>PrP106-126 A117V 4.5 (0.3)</td>
</tr>
</tbody>
</table>

NAC Amyloid Formation is Nucleation-Dependent and can be Seeded by NAC Fibrils

Supersaturated solutions of NAC were metastable. Amyloid formation by NAC at 80 μM did not proceed immediately but exhibited a lag time (666 ± 88 min) characteristic of nucleation-dependent polymerization (Fig. 4.6). This is about 3 to 4 times longer than the lag time observed for β1-40 at the same concentration.³ A similar mechanism best describes amyloid formation by a number of amyloidogenic species.³,⁸,¹⁵-¹⁷ One feature of this mechanism is that addition of a preformed fibril (seed) eliminates the need for nucleation. Thus, addition of NAC fibrils to a supersaturated NAC solution eliminated the lag time completely, and immediate seeded growth was observed (Fig. 4.6).
β1-40 Amyloid Formation can be Seeded by NAC Fibrils

The rates of amyloid formation from a supersaturated solution of β1-40 (80 μM) in the presence and the absence of NAC seed fibrils (20 μM) were compared by monitoring the increase in turbidity (Fig. 4.7). The turbidity due to the presence of the seed peptide was subtracted from all data points. β1-40 alone exhibited characteristic nucleation-dependent kinetics of aggregation, with a lag time of ~8000 sec. In the presence of NAC, the length of the nucleation phase preceding amyloid formation decreased by more than 50% (Table 4.3, g). A similar effect was observed for model peptide β26-40 seeded with NAC (Table 4.3, h). Seeded growth does not occur immediately, as it does in self-seeding experiments; seeding in this case manifests itself as a reduction, rather than an elimination, of the observed lag time. This observation is consistent with the idea that a greater degree of primary sequence homology leads to more efficient seeding. Seeding is a chemically discriminating event; small changes in primary sequence can eliminate the ability of
a peptide to serve as a seed.\textsuperscript{17} This property was demonstrated using peptides derived from the OsmB protein\textsuperscript{8} and from PrP.\textsuperscript{18} The prion peptides showed no significant ability to seed β26-40 (Table 1, i, j).

In contrast, PrP106-126 and Prp106-126 A117V were not seeded by NAC. In fact, under certain conditions amyloid formation by the PrP peptides was inhibited by NAC fibrils. It is possible that soluble NAC formed by disaggregation of the fibril is responsible for this inhibitory effect. An intriguing corollary to this result is the observation that addition of NAC fibrils significantly inhibits the conversion of PrP\textsuperscript{c} to PrP\textsuperscript{Sc} in a cell-free system (D. Kocisko, personal communication). Further exploration of the biochemistry of NAC inhibition of PrP amyloid formation may therefore be warranted.

\textbf{Figure 4.7} Seeding of β1-40 by NAC peptide. •, β1-40 unseeded (80 μM); ○, β1-40 (80 μM) seeded with NAC fibrils (20 μM).
Table 4.3  Nucleation (lag) times measured by turbidity.

<table>
<thead>
<tr>
<th>Solution (80 μM)</th>
<th>Seed (20 μM)</th>
<th>Lag time relative to unseeded*</th>
</tr>
</thead>
<tbody>
<tr>
<td>a NAC</td>
<td>β1-40</td>
<td>0.70 (0.02)</td>
</tr>
<tr>
<td>b NAC</td>
<td>β26-40</td>
<td>0.79 (0.19)</td>
</tr>
<tr>
<td>c NAC</td>
<td>β26-42</td>
<td>0.47 (0.09)</td>
</tr>
<tr>
<td>d NAC</td>
<td>β26-43</td>
<td>0.44 (0.14)</td>
</tr>
<tr>
<td>e NAC</td>
<td>PrP106-126</td>
<td>0.71 (0.22)</td>
</tr>
<tr>
<td>f NAC</td>
<td>PrP106-126A117V</td>
<td>0.60 (0.12)</td>
</tr>
<tr>
<td>g β1-40</td>
<td>NAC</td>
<td>0.4 (0.1)</td>
</tr>
<tr>
<td>h β26-40</td>
<td>NAC</td>
<td>0.60 (0.04)</td>
</tr>
<tr>
<td>i β26-40</td>
<td>PrP106-126</td>
<td>0.9 (0.2)</td>
</tr>
<tr>
<td>j β26-40</td>
<td>PrP106-126A117V</td>
<td>1.1 (0.3)</td>
</tr>
</tbody>
</table>

* Presented as a fraction relative to unseeded lag times [in min (±S.D.): NAC, 666 (88); β1-40, 119 (25); β26-40, 115 (3)].

**NAC Fibril Formation can be Seeded by β and PrP Peptide Fibrils**

The β protein analogs shown in Table 4.3 seeded NAC amyloid formation, albeit less effectively than did NAC itself (Fig. 4.8). Short β (β1-40 and the model peptide β26-40), which does not contain the entire homologous sequence, was a less effective seed than models of long β (β26-42, β26-43) (Table 4.3, a - d). Addition of fibrils comprising PrP106-126 and PrP106-126 A117V (a mutation which segregates with GSS) also led to a decrease in the observed nucleation time (Fig 4.8, Table 4.3 i, j). No significant difference between the abilities of the Ala- and Val-containing variants to seed NAC was observed (Table 4.3 e, f). Neither NAC nor β was an effective seed for either PrP analog.
Figure 4.8 (a) Seeding NAC amyloid formation with Aβ. ✶, NAC unseeded (80 μM); ○, NAC (80 μM) seeded with β1-40 (20 μM); •, NAC (80 μM) seeded with β26-43 (20 μM); †, NAC (80 μM) seeded with β26-42 (20 μM). (b) Seeding NAC amyloid formation with PrP peptides. ✶, NAC unseeded (80 μM); •, NAC (80 μM) seeded with PrP106-126 (20 μM); ○, NAC (80 μM) seeded with PrP106-126 A117V (20 μM).
NAC May be a Common Trigger or Target in AD

Our experimental data support the theory that NAC, which comprises 10% of the total protein concentration in the plaque, could promote Aβ amyloid formation in vivo (Fig. 4.9). One could modify this proposal to suggest that NACP, the precursor to the NAC peptide, could seed Aβ aggregation. While we have observed no evidence for amyloid formation by NACP (up to millimolar concentrations), a tendency of recombinant NACP to precipitate has been reported by another laboratory. If, under physiological conditions, NACP could form fibrils, it could potentially serve as a seed in a parallel manner as NAC.

Support for the idea of NAC (or NACP) as a "trigger" for Aβ amyloid formation comes from subsequent work by Saitoh and coworkers, who confirmed our finding that synthetic NAC forms amyloid fibrils in vitro, and found that soluble NACP can induce fibril formation by β1-39. The conditions used in this study vary significantly from ours: NACP was used as a soluble factor, which precludes any seeding phenomenon; and the formation of any insoluble material by β1-39, which is soluble in the absence of NACP, was monitored, rather than differences in the kinetics of aggregation. Both results, however, point to NAC as a possible pathogenic "trigger"; production of NAC at high local concentration at the synapse (via proteolysis of NACP) could seed amyloid formation and have a neurotoxic effect.

Figure 4.9 (following page) A general mechanism for nucleation-dependent amyloid fibril formation by Aβ and NAC. A similar mechanism is applicable for PrP. Heterogeneous seeding was demonstrated to be effective, albeit less effective than homogeneous seeding.
An alternative theory is that the promotion of NAC amyloid formation by β or prion proteins could be pathogenic (Fig. 4.9). We find that peptides derived from both Aβ and PrP sequences exhibited the ability to seed NAC amyloid formation in our in vitro assay. The 'long β' models, which have a greater sequence homology to NAC, were more efficient seeds, once again supporting the importance of the sequence homology. Thus, aggregation of NAC itself, or the synaptic precursor protein NACP, may be promoted by other amyloid proteins in vivo.

This possibility leads back to the role of NACP/α-synuclein, the synaptic protein from which NACP is derived. Although the function of NACP is unknown, it is found primarily in brain tissue, and is localized within neurons at the presynaptic nerve terminal. Synaptic amyloid deposition may be a common theme among amyloid-characterized neurodegenerative disorders. This premise inspired further biochemical studies, described in the next chapter, designed to investigate the structure and function of NACP.
Experimental Section

Peptide Synthesis, Purification, and Characterization

NAC was synthesized and purified by Dr. Hogyu Han using the standard Fmoc protocol (Chapter II) on the Wang resin. Analytical HPLC (C4, 2.0 mL/min): 70/30 H2O/CH3CN (0.1% TFA) isocratic (Rv = 18 mL). Amino acid analysis (from a DMSO solution): Asx 0.6 (1), Glx 3.0 (4), Ser 1.0 (1), Gly 5.5 (6), Thr 3.9 (5), Ala 7.0 (7), Val 5.7 (8), Ile 1.1 (1), Phe 1.1 (1), Lys 0.9 (1). N-terminal amino acid sequencing (MIT Biopolymers Laboratory) of the first 26 residues of the peptide was consistent with the expected sequence; a trace amount (0.5% to 1%) of an impurity beginning at Ala16 of the NAC sequence was also detected. PDMS (M + H+): calc'd 3262.7, found 3258.4.

Peptides β26-40, β26-42, β26-43, PrP106-126, and PrP106-126 A117V were prepared by methods reported previously.14,16 β1-40 was purchased from Bachem (Torrance, CA). Stock solutions were prepared by dissolving lyophilized peptides in DMSO. The solutions were filtered through Millex FGS 0.2 μm non-aqueous filters, and concentrations were determined by quantitative amino acid analysis.

Electron Microscopy, Atomic Force Microscopy, and Congo Red Staining

Samples for EM were prepared by stirring supersaturated solutions of peptide (80 μM) in 100 mM NaCl/8.2 mM Na2HPO4/1.8 mM NaH2PO4, pH 7.4/10% dimethylsulfoxide (PBS/10% DMSO). EM samples were placed upon carbon-coated copper grids, negatively stained with 2% uranyl acetate, and viewed at 60K-120K on a JEOL 1200 CX EM at 80 kV.

CR staining was performed by the following procedure:8 A suspension of peptide fibrils in buffer was dried on a glass microscope slide. The peptide was immersed in a solution containing 1 mM Congo Red, 100 mM NaCl, and 10 mM
phosphate (pH 7.4) for 1 min and dried. Birefringence was determined with a Wild Leitz M3Z light microscope equipped with a polarizing stage.

Atomic force microscopy was performed with the assistance of James Harper. A suspension of aggregated NAC (initial concentration, 80 μM) was prepared as described in "Aggregation Kinetics." A portion of this suspension (20 μL) was placed onto a piece of freshly cleaved mica. The sample was incubated for 1 h at RT with high humidity and was rinsed 2 x 50 μL with ddH2O to remove unbound material. The sample was imaged immediately under ambient conditions using a NanoScope IIIa MultiMode scanning probe workstation (Digital Instruments, Santa Barbara, CA) operating in the TappingMode™, using an "E"-type scanner and etched silicon TESP nanoprobe (Digital Instruments).

**Circular Dichroism Spectroscopy**

Following an aggregation experiment, a suspension of NAC was centrifuged to remove large aggregates, and filtered through a Millex-GV 0.22 μm aqueous filter (Millipore). Spectra were recorded on a Jasco J-500 Spectropolarimeter, in a 0.1 cm quartz cell. The following parameters were used: Band width = 1.0 nm; slit width = "auto"; time constant = 16 sec; step resolution = 0.4 nm; scan speed = 10 nm/min.

**FTIR spectroscopy**

Aggregated peptides were centrifuged at 4000 rpm for 10 min and spread on a CaF2 plate. After air drying, the infrared spectra were recorded on a Perkin-Elmer 1600 series FTIR spectrometer.

**Solubility**

A supersaturated solution of peptide (40 μM) or a mixture of two peptides (40 μM each) in PBS/10% DMSO was prepared by addition of 20 μL of a DMSO solution
of peptide to 180 µL of PBS, and stirred at 1800 rpm for 72 h. The suspension was filtered through a Millex-GV 0.22-µm aqueous filter (Millipore). Total peptide concentrations were determined by quantitative amino acid analysis (MIT Biopolymers Laboratory), and the relative ratios were determined based on unshared amino acids. Data shown represent an average of three trials.

**Aggregation Kinetics and Seeding Experiments**

A supersaturated solution of peptide (80 µM) in standard buffer was prepared by addition of peptide dissolved in DMSO (100 µL; 50 µL for β1-40 and β26-40) to PBS (900 µL; 450 µL for β1-40 and β26-40) and stirred continuously at 1800 rpm (1550 rpm for β1-40 and β26-40). Samples were vortexed briefly (except for β1-40 and β26-40) and aggregation was measured by turbidity at 400 nm versus a buffer blank. Seeding experiments were initiated by addition of a suspension of fibrils (250 µL; 125 µL for seeding β1-40 and β26-40; 20 µM final) from a previous aggregation trial. The turbidity due to the seed peptide was accounted for by blanking on the suspension of seeds in buffer immediately before addition of the DMSO stock solution. Lag times were determined by solving the best fit line to the growth phase of the aggregation curve for y = 0.
References for Chapter IV

Chapter V

Biochemical Probes of NACP Structure and Function: Interaction of NACP, a "Natively Denatured" Protein, with the NSF Homolog VCP (p97)

Background

The NACP/Synuclein Family of Proteins

NAC was identified in 1993 as an intrinsic component of AD amyloid plaque. The gene encoding the protein precursor to NAC was cloned, and the protein was named "NACP". The human NACP gene is on chromosome 4 and encodes a highly acidic 140 amino acid protein with a predicted molecular weight of 14,460 (Fig. 5.1, 5.2). NACP isolated from human brain tissue has a molecular mass of 14,681, suggesting that NACP may be post-translationally modified. The N-terminal amino acid of isolated NACP is not amenable to Edman degradation, suggesting that this modification may occur at the amino terminus. There are no consensus glycosylation sites, signal sequences, or phosphorylation motifs in the NACP sequence. However, a homolog of NACP (PNP-14) is phosphorylated in vitro. Cysteine, arginine, and tryptophan residues are all noticeably absent from the NACP sequence.

NACP, also called α-synuclein, is a member of a highly conserved family of proteins encoded by equivalent genes in a number of different species. This family
includes Torpedo synuclein,\textsuperscript{9} rat synucleins 1, 2, and 3,\textsuperscript{10} bovine phospho-
neuroprotein 14,\textsuperscript{6,11-13} canary synelfin,\textsuperscript{7} and human \( \beta \)-synuclein\textsuperscript{5} (Fig. 5.2). There are
two major isoforms in this family, which differ in their C-terminal amino acid
composition. \( \alpha \)-synuclein, rat synuclein 1, and canary synelfin share the
same acidic C-terminus. These proteins can be considered interspecies homologs;
that is, proteins with equivalent functions in different species. Human and rat
\( \alpha \)-synuclein share ca. 95\% sequence homology,\textsuperscript{14} while canary \( \alpha \)-synuclein (synelfin) is ca. 85\%
homologous to both sequences.\textsuperscript{7} The second set of isoforms includes bovine PNP-14
and the human homolog \( \beta \)-synuclein, which do not contain the intact NAC
sequence. An alternatively spliced form of human \( \alpha \)-synuclein (NACP112) has been
discovered\textsuperscript{8} which lacks amino acids 103 to 130, but contains the intact NAC
sequence.

The N-terminal sequence is highly conserved among all members of the
NACP/synuclein/synelfin/PNP-14 family. This sequence is composed almost
entirely of variants of an 11 amino acid consensus motif. This type of motif has also
been described in the exchangeable apolipoproteins (AI, AII, AIV, CI, CII, CIII, and E).
<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
<th>Start</th>
<th>End</th>
</tr>
</thead>
<tbody>
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<td>43</td>
</tr>
<tr>
<td>rat synuclein 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rat synuclein 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rat synuclein 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Torpedo syn</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>canary synelfin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bovine PNP14</td>
<td>M T E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>human β-syn</td>
<td>M T E</td>
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</tr>
<tr>
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<td>rat synuclein 3</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Torpedo syn</td>
<td>QS NT ANV A NT S VENV SV L</td>
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<tr>
<td>canary synelfin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bovine PNP14</td>
<td>Q S ASHL FS--------------N L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>human β-syn</td>
<td>R Q S ASHL FS--------------N L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>human NACP</td>
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<td>140</td>
</tr>
<tr>
<td>rat synuclein 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rat synuclein 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rat synuclein 3</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Torpedo syn</td>
<td>EH REIPAEQVAEKGQTQ------E LV T ATE T ----K----</td>
<td></td>
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</tr>
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<td></td>
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</tr>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 5.2** Sequences of NACP in various species. Amino acids that differ from the human NACP sequence are indicated.

The apolipoproteins have α-helical structures, and the 11 residue repeat has been suggested to provide an amphipathic motif responsible for lipid binding.\(^{15}\) Helical wheel analysis indicates that the NACP amino terminal sequence (residues 9-26) also shows clear segregation of polar and nonpolar residues on opposite faces of the predicted helix.\(^{7}\) Furthermore, as in the apolipoproteins, there is a basic residue at each polar-apolar interface; such residues have been proposed to be responsible for a "snorkeling" effect to maximize lipid binding.\(^{16}\) Direct NACP binding to lipid, therefore, may be responsible for its observed association with membranes (*vide infra*). Alternatively this amphipathic helix may mediate interactions of NACP with other proteins. Specifically, the similarity to apoE suggests that this region may
mediate interactions between NACP and Aβ; apoE has been shown to bind Aβ in vivo\textsuperscript{17-19} and alter the kinetics of amyloid fibril formation \textit{in vitro}.\textsuperscript{20}

\textbf{Other NACP Homologies}

In addition to the strong resemblance of the N-terminus of NACP to the amphipathic helix of the apolipoproteins, some other possible sequence homologies have been noted, which may provide valuable clues as to the function of NACP. The KTKEGV sequence, which is a part of the conserved 11 amino acid repeat, is found in the carboxy terminal portion of the \textit{rho} gene product, a protein implicated in signal transduction,\textsuperscript{9} and in the low molecular weight heat shock protein HS27.\textsuperscript{10} A possible tyrosine phosphorylation consensus sequence (KTKEGVLY) is found at the end of the third repeat,\textsuperscript{10} and the threonine residue in the KTKEGV may constitute a favorable protein kinase C target.\textsuperscript{1} Clusters of acidic and basic amino acids, such as in the KTKEGV repeat sequence, are known to mediate binding between structural proteins.\textsuperscript{5}

The C-terminus of NACP showed limited homology to the calmodulin dependent-kinase, type II, and is somewhat reminiscent of the YGP/QQG repeat of the synaptophysin tail.\textsuperscript{10} This region also bears some homology to the CD3ζ subunit of the T-cell antigen receptor.\textsuperscript{8} The C-terminus of β-synuclein contains a conserved motif found at the beginning of the coil region of the intermediate filament and in the clathrin light chain.\textsuperscript{10}

\textbf{NACP is a Presynaptic Protein}

The tissue distribution and intracellular localization of the NACP/synuclein family of proteins has been studied extensively. Scheller and colleagues used an antiserum against cholinergic synaptic vesicles in their initial discovery of α-synuclein in Torpedo and rat.\textsuperscript{9} The synuclein gene was shown to be expressed only
in neurons, and immunoreactivity was localized to a region of the nucleus as well as the presynaptic nerve terminal (hence the name, "synuclein"). Using in situ hybridization in rat brain tissue, the same group later demonstrated very high levels of synuclein expression in cerebral cortex, hippocampus, and dentate gyrus, and showed that the protein is reversibly associated with synaptosomal membranes. A similar tissue distribution and intracellular localization was observed for human NACP (α-synuclein). Immunochemical studies with an affinity purified NACP antibody suggested that the protein is localized only to the synapse, and not to nuclei as proposed previously. The immunoreactivity in the synapse was associated with synaptic vesicles; however, as the protein was recovered primarily in cytosolic fractions, it was postulated to be only loosely associated with the membrane or with membrane binding proteins. This antibody was also used to confirm preferential NACP expression in the hippocampus, striatum, frontal cortex, and olfactory bulb. β-synuclein, similarly, is expressed predominantly in brain, and associates with synaptosomal fractions, but not with purified synaptic vesicles. Differences in the intracellular distribution of human α and β synuclein have been suggested by immunochemical studies. The alternatively spliced NACP transcript NACP112 shows a different tissue distribution from NACP, suggesting tissue-dependent alternative splicing.

The conclusions from these studies are as follows. The synuclein family of proteins is predominantly expressed in brain tissue, and specifically in the regions of the brain which are most affected by AD. The proteins are reversibly associated with synaptosomal membranes and synaptic vesicles. Within neuronal cells, NACP and homologs are specifically localized to the presynaptic nerve terminal. This convergence of evidence suggested that NACP may play a role in synaptic transmission.
Canary NACP (Synelfin) is Upregulated During Songbird Learning

Perhaps the most exciting clue as to the function of NACP came from a seemingly unrelated research project. A group led by David Clayton, at the University of Illinois, was studying the molecular mechanisms which control songbird learning. Using differential hybridization techniques, this group identified a gene which is a target of dynamic regulation during the critical period for song learning. The gene was cloned and sequenced, and was found to encode a protein, dubbed "synelfin," which is the canary homolog of NACP (Canary NACP and rat NACP share ca. 85% sequence identity). It was suggested that synelfin is responsible for regulating "synaptic plasticity"; that is, in establishing and maintaining synaptic interconnections. This report provided a link between NACP expression and learning/memory, and raised the possibility of a broader connection between AD and the regulation of neural plasticity.

An NACP Promoter Polymorphism Correlates to AD

Recent attempts have been made to discover a direct link between NACP and AD. Analysis of the NACP/synuclein gene in a group of 26 patients with familial AD revealed no mutations in the coding sequence. However, a mutation in the promoter region of the NACP gene has been shown to correlate to disease. Additionally, NACP concentrations in various regions of the brain were slightly altered in AD. This results provide the first evidence for a link between NACP expression and Alzheimer's disease.

NACP: A Genetic Connection Between Learning and AD?

NACP, a presynaptic protein associated with synaptic vesicles, is expressed primarily in the regions of the brain which are most affected by AD. A fragment of NACP is deposited in AD amyloid plaques. NACP expression, which is regulated
during a critical period for songbird learning, is altered in AD. Taken together, these findings suggest that NACP may play a role in connecting the processes of AD and learning. The molecular basis for this connection, however, remains unclear. The results presented in this chapter describe the use of biochemical methods to elucidate the molecular details of NACP structure and function, and suggest a molecular connection between learning and AD.

Results and Discussion

Structural Studies of Recombinant NACP

Expression and Purification of Recombinant NACP

A pRK172 plasmid encoding the human α-synuclein sequence (kindly provided by R. Jakes and M. Goedert, MRC) was used to transfect E coli BL21(DE3) cells. Bacteria were grown and induced with isopropyl-1-thio-β-galactoside as described in the Experimental Section. The soluble fraction of the cell lysate was treated with streptomycin sulfate to precipitate nucleic acids. NACP was purified by boiling the cell homogenate for 10 min, which precipitates most cellular proteins, leaving NACP as the major component of the soluble fraction. Final purification of NACP was accomplished using size-exclusion chromatography. The protein eluted from a Biogel P-10 column in the void volume, but was deemed sufficiently pure to use in structural studies by SDS-PAGE and analytical reversed-phase HPLC. Subsequent purifications have utilized a Sephacryl S-300 column to achieve greater purity (W. Zhen, unpublished results). NACP elutes from an S-300 column with an apparent Mₐ of ~55 kDa relative to globular protein standards, suggesting an elongated or oligomeric structure (vide infra).

This purification procedure takes advantage of the heat-stability of α-
synuclein. We were concerned that, although it does not lead to precipitation, boiling of the protein could lead to loss of the native folded structure. We therefore developed a milder, conventional purification of recombinant NACP which did not involve boiling (Fig 5.3). Following the streptomycin sulfate precipitation, NACP was precipitated using 47% ammonium sulfate. The precipitate was further purified using size-exclusion and ion-exchange chromatography. This purification method should retain the native folded structure, if any, of NACP.

Figure 5.3 Parallel methods for the purification of recombinant NACP. Scheme (a) involves the use of a boiling step, and may not retain the native protein structure. Scheme (b) utilizes conventional purification methods which retain native structure.

Recombinant NACP was characterized by several independent methods. MALDI-TOF mass spectrometry confirmed the predicted mass of 14,461, and amino acid analysis was consistent with the expected sequence. Proteolytic digestion with trypsin followed by plasma desorption mass spectrometry (PDMS) of the resultant peptide mixture led to the positive identification of several NACP fragments. Finally, Western blotting of recombinant NACP with a monoclonal antibody to canary synelfin confirmed NACP immunoreactivity.
Secondary Structure Prediction

Analysis of the predicted secondary structure of NACP was performed using the classical Chou-Fasman algorithm (kindly performed by Dr. G.D. Fasman, Brandeis University). Predictions such as this can often be helpful in locating well-defined structural motifs and providing an initial sense of protein structure, although by no means should such predictions be equated with experimental methods for structure determination. The result is shown in Fig. 5.4. The N-terminus of NACP is predicted to be largely $\alpha$-helical, particularly a stretch between amino acids 9 and 32. This is consistent with helical wheel analysis (see Background section), which suggests an amphipathic helix motif. The hydrophobic center of the protein, which contains the NAC sequence, is predicted to have primarily $\beta$ structure. The C-terminus is predicted to have a heterogeneous structure; however, the preference is not particularly strong in this portion of the molecule for any of the possible structural types.

![Secondary structure prediction of NACP](image)

Figure 5.4 Secondary structure prediction of NACP, using the Chou-Fasman algorithm. $\alpha$ = $\alpha$-helix; $\beta$ = $\beta$-sheet, $\beta_t$ = $\beta$-turn. The NAC sequence is underlined.
NACP is Monomeric in Solution

Recombinant NACP exhibited unusual mobilities on both SDS-PAGE and size-exclusion chromatography. NACP, with a molecular weight of 14.4 kDa, runs as an Mr~19kDa protein on denaturing gel electrophoresis. This effect has been reported previously,\(^5\) and may be attributed to abnormally low binding of SDS by the highly acidic NACP C-terminal sequence. A similar effect has been reported for other highly acidic proteins.\(^{26}\)

Upon size exclusion chromatography, NACP elutes in the void volume of both Biogel P-10 and P-100 columns. On a Sephacryl S-300 column, the protein elutes with an apparent molecular weight of \(~55\) kDa, relative to globular protein standards. \(\beta\)-Synuclein reportedly exhibited a similar mobility (Mr \(~56\) kDa).\(^{11}\) This was interpreted to mean that the protein exists as a tetramer in solution. However, an alternative explanation is possible: Elongated, non-globular structures have been reported to exhibit unusually high Stokes' radii relative to compact, globular proteins. We used native gel electrophoresis to clarify whether NACP is oligomeric or monomeric in solution.

Recombinant NACP and several globular protein standards were run on three different percentage non-dissociating ("native") polyacrylamide gels (6%, 10%, and 14%). A plot of the relative mobility \((R_f)\) of each species versus the gel concentration (% acrylamide) (Ferguson plot\(^{27}\)) was used to calculate retardation coefficients (Fig. 5.6). The slope of the Ferguson plot is defined as \(K_r\), the retardation coefficient. Since the Ferguson plot relates to mobility during electrophoresis when only the gel pore size (as determined by % acrylamide) is varying, then \(K_r\) is a measure of the retardation of the protein by the gel, and is directly related to molecular size. A plot of \(K_r\) vs. molecular weight allows the estimation of a molecular weight of \(~20\) kD for NACP. (Fig. 5.5) This clearly corresponds to a monomer. It is likely that this type of analysis would reveal a similar monomeric
nature for β-synuclein, as opposed to the reported tetramer. This analysis, along with CD and UV denaturation experiments and urea gel-shift experiments (vide infra) confirms that NACP is clearly monomeric in solution.

![Graph](image)

**Figure 5.5** Determination of NACP molecular weight by non-dissociating polyacrylamide gel electrophoresis. A retardation coefficient ($K_r$) was calculated for each protein, based on relative mobilities in gels with different percentages of acrylamide. A plot of $K_r$ vs. molecular weight indicates NACP is monomeric.

**Circular Dichroism Indicates NACP is Predominantly Random Coil**

With recombinant NACP in hand, we initially chose to examine the structure of the protein using circular dichroism (CD) spectroscopy. CD is a useful tool for determining the dominant secondary structural elements for a given protein. The CD spectrum of NACP purified by boiling (20 μM in PBS, Fig. 5.6) has a negative ellipticity at ~200 nm, and is characteristic of random coil structure. The method of Greenfield and Fasman was used to estimate the relative abundance of each
secondary structural element (Table 5.1). This method is fairly effective at calculating helix content; the \( \beta \)-sheet content obtained using these calculations is much less reliable. Using this method, NACP is calculated to have only about 2% helical structure, with the remainder primarily random coil.

![Figure 5.6 CD spectrum of NACP. The negative ellipticity at about 200 nm is characteristic of "random coil" structure. A similar spectrum has been observed for denatured proteins.](image)

Table 5.1 Calculated percentages of secondary structural elements in NACP under varying conditions.

<table>
<thead>
<tr>
<th>[NACP] (( \mu )M)</th>
<th>conditions</th>
<th>% ( \alpha )</th>
<th>% ( \beta )</th>
<th>% coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>heat-treated</td>
<td>4</td>
<td>29</td>
<td>67</td>
</tr>
<tr>
<td>20</td>
<td>&quot;</td>
<td>2</td>
<td>30</td>
<td>68</td>
</tr>
<tr>
<td>40</td>
<td>&quot;</td>
<td>2</td>
<td>29</td>
<td>69</td>
</tr>
<tr>
<td>60</td>
<td>&quot;</td>
<td>1</td>
<td>31</td>
<td>68</td>
</tr>
<tr>
<td>80</td>
<td>&quot;</td>
<td>1</td>
<td>32</td>
<td>67</td>
</tr>
<tr>
<td>20</td>
<td>&quot;native&quot;</td>
<td>2</td>
<td>30</td>
<td>68</td>
</tr>
<tr>
<td>20</td>
<td>10% (v/v) HFIP</td>
<td>52</td>
<td>1</td>
<td>47</td>
</tr>
<tr>
<td>&quot;</td>
<td>10 mM SDS</td>
<td>27</td>
<td>13</td>
<td>60</td>
</tr>
<tr>
<td>&quot;</td>
<td>10 mM CaCl(_2)</td>
<td>0</td>
<td>33</td>
<td>67</td>
</tr>
<tr>
<td>&quot;</td>
<td>120 ( \mu )M ATP</td>
<td>1</td>
<td>28</td>
<td>71</td>
</tr>
<tr>
<td>&quot;</td>
<td>pH 4.5</td>
<td>2</td>
<td>30</td>
<td>68</td>
</tr>
<tr>
<td>&quot;</td>
<td>pH 10</td>
<td>2</td>
<td>30</td>
<td>68</td>
</tr>
</tbody>
</table>
The material used in these initial CD studies was purified using a heat treatment step. We hypothesized that the apparent lack of structure observed in the NACP CD spectrum was due to denaturation of the protein by the boiling step. The experiment was repeated, this time with a sample of NACP which had been purified by conventional methods designed to retain native protein structure. The CD spectrum of this preparation was identical to that of the boiled sample (Fig. 5.7). This was confirmed by boiling the "native" CD sample, which led to an identical CD profile. Thus, the heat treatment step does not appear to affect the structure of the protein, and may be safe to use in future NACP purification schemes.

![Figure 5.7 CD spectra of "boiled" (solid line) and "native" (dashed line) NACP.](image)

We investigated the possibility that oligomerization of NACP could induce secondary structure, by monitoring the concentration dependence of the CD spectrum. Over a range of 10 μM to 620 μM, the spectrum was independent of concentration. This indicated that NACP is monomeric over this concentration range.
We next considered the possibility that exogenous or endogenous molecules could induce structure in NACP. Addition of 10-20% hexafluoroisopropanol (HFIP) induced α-helical structure (52%). This was expected based on the documented ability of HFIP to promote helical structure.\textsuperscript{30} Addition of SDS micelles (10 mM) also induced helical structure, though not as effectively as HFIP (Figure 5.8). This effect is not as straightforward to interpret. SDS has been shown in some cases to increase helical structure, presumably by providing a less polar solvent environment, and in other cases to decrease helical structure, due to denaturation effects.\textsuperscript{31} The effect of SDS in this case may be attributed to a direct binding interaction between the amphipathic helix of NACP and the SDS micelle.

Figure 5.8 CD spectrum of NACP in buffer (solid line), with 10% HFIP (dotted line), and with 10 mM SDS (dashed line).

Other potential mediators of structure had no effect on the CD of NACP. The spectrum was independent of pH over a range of 4.5 to 10. Addition of Ca\textsuperscript{2+} ions (10 mM CaCl\textsubscript{2}), at both pH 4.5 and 7, had no measurable effect, nor did addition of ATP, another ubiquitous intracellular species. A CD spectrum such as that observed for
NACP does not in and of itself preclude the presence of any folded protein structure. For example, the spectrum of the polyproline II helix is very similar to the classical random coil spectrum, despite the highly ordered structure of the molecule. It would therefore be unwise to conclude that NACP is unstructured solely on the basis of a "random coil" CD spectrum. To clarify this issue, we performed several independent methods of structural characterization which confirm the unstructured nature of NACP.

**Fourier Transform Infrared (FTIR) Spectroscopy of NACP**

In addition to its traditional use in characterizing functional groups in organic molecules, infrared spectroscopy can be used to determine secondary structures in proteins. The position of the amide I band of proteins, specifically, is indicative of the predominant type of secondary structure. α-Helix generally shows an absorbance in the range of 1658-1650 cm\(^{-1}\); β-sheet absorbs at 1695-1690 cm\(^{-1}\), and random coil proteins generally absorb near 1648 cm\(^{-1}\). The FTIR solution spectrum of NACP in buffer (Fig. 5.9) shows a broad absorbance at 1642 cm\(^{-1}\), characteristic of a denatured or random coil protein.

**Non-Dissociating Gel Electrophoresis and UV Spectroscopy of NACP in Denaturants**

The UV absorption spectrum of an aromatic amino acid depends on the nature of the molecular neighborhood of the chromophore. In folded proteins, some of the aromatic amino acids are buried within the hydrophobic core of the molecule. Denaturation of a globular protein leads to exposure of amino acids to a more polar aqueous environment. This, in turn, leads to a shift in the wavelength of the aromatic amino acids to a lower wavelength (blue shift). Such shifts are generally small, but can easily be detected using difference spectroscopy. The spectrum of NACP in the presence and absence of 6 M guanidine hydrochloride...
Figure 5.9 FTIR solution spectrum of NACP. Maximum absorbance is at 1642 cm\(^{-1}\), indicative of random coil.
(GdmCl) was measured (Fig. 5.10). The difference spectrum (folded - unfolded) shows no significant peaks in the 285-288 nm range, where differences in tyrosine residue environment would be detected. NACP contains 4 tyrosine residues, 3 of which are in the acidic C-terminus. This experiment indicates that these residues are not buried within a folded, hydrophobic core, consistent with the idea of a protein with minimal folded structure.

![UV spectra of "native" (dotted line) and "denatured" (dashed line) NACP. The spectrum of NACP in 6M guanidine hydrochloride was subtracted from the spectrum of NACP in buffer to obtain the difference spectrum (solid line). The absence of a peak at ~280 nm in the difference spectrum is consistent with a lack of buried aromatic residues.](image)

Fig. 5.10 UV spectra of "native" (dotted line) and "denatured" (dashed line) NACP. The spectrum of NACP in 6M guanidine hydrochloride was subtracted from the spectrum of NACP in buffer to obtain the difference spectrum (solid line). The absence of a peak at ~280 nm in the difference spectrum is consistent with a lack of buried aromatic residues.

A parallel experiment was performed by examining the aromatic contribution to the CD spectrum (W. Zhen, unpublished results). As in the UV experiment, changes in the environment of the aromatic residues should be detectable in the
"folded - unfolded" difference spectrum. No significant peaks were detected in the aromatic region of the CD difference spectrum of NACP.

Addition of denaturants to the sample loading buffer has been shown to alter the mobility of globular proteins on non-dissociating ("native") polyacrylamide gel electrophoresis. Addition of saturated urea to a sample of NACP did not affect the mobility of the protein. This confirms both the monomeric nature of NACP and the lack of any significant folded structure.

**NACP May Define a Family of Natively Denatured Proteins**

The results presented in the preceding sections provide overwhelming evidence that, in solution, NACP is a monomer with no detectable folded structure. The monomeric nature of NACP was demonstrated by Ferguson analysis, and is consistent with a concentration-independent CD spectrum and a native gel mobility which is unaltered in the presence of urea. The random coil nature of the protein was demonstrated by CD, FTIR, UV, and native gel electrophoresis, as well as an unusually high Stokes' radius on size-exclusion chromatography.

These features allow us to characterize NACP as a "natively denatured" protein. This term was coined by Mandelkow and coworkers to describe another AD-related protein, tau, and can be defined as a protein which, under physiological conditions, does not adopt a folded, globular structure. This property has only been noted in a limited number of proteins. Our research on NACP has led us to investigate this issue in more depth, and we have now identified a family of proteins with similar physical properties, all of which are likely to be natively denatured (Table 5.2). The proteins classified into this family have one or more of the following properties: 1) stability to heat denaturation; 2) an abnormally high Stokes radius upon size exclusion chromatography; 3) a highly charged (often acidic) amino acid sequence; 4) abnormal SDS binding leading to unusual mobility on SDS-
PAGE; and 5) random coil structure as demonstrated by CD. These properties are consistent with a "natively denatured" structure: A highly charged amino acid sequence interferes with SDS binding and allows the protein to be soluble in a "denatured" state, which is, by definition, resistant to heat-induced denaturation. The elongated nature of the molecule alters its mobility on a size-exclusion column in comparison with globular standards. Table 5.2 presents a putative list of these proteins based solely on a survey of existing literature.

Table 5.2 Proposed family of "natively denatured" proteins. These proteins are characterized by one or more of the following properties: heat-stability to denaturation; an abnormally large Stokes' radius upon size-exclusion chromatography; an acidic amino acid sequence; or direct structural evidence, for example CD spectroscopy, which indicates a lack of folded structure.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NACP/α-synuclein</td>
<td>YES</td>
<td>YES</td>
<td>6.1</td>
<td>YES</td>
<td>found in synapse</td>
<td>--</td>
</tr>
<tr>
<td>β-synuclein</td>
<td>YES</td>
<td>YES</td>
<td>5.5</td>
<td>NA</td>
<td>found in synapse</td>
<td>11</td>
</tr>
<tr>
<td>Tau</td>
<td>YES</td>
<td>NO</td>
<td>10.4</td>
<td>NA</td>
<td>promotes microtubule assembly</td>
<td></td>
</tr>
<tr>
<td>Chromogranin A</td>
<td>YES</td>
<td>YES</td>
<td>5.0</td>
<td>YES</td>
<td>found in chromaffin granules</td>
<td>38</td>
</tr>
<tr>
<td>MAP2</td>
<td>YES</td>
<td>NO</td>
<td>4.7</td>
<td>YES</td>
<td>phosphatase inhibitor</td>
<td>39</td>
</tr>
<tr>
<td>DARPP-32</td>
<td>YES</td>
<td>NA</td>
<td>&lt;7</td>
<td>NA</td>
<td>phosphatase inhibitors</td>
<td>40</td>
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<tr>
<td>I1PPI, I2PPI</td>
<td>YES</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>phosphatase inhibitors</td>
<td>41</td>
</tr>
<tr>
<td>I1PPIA, I2PPIA</td>
<td>YES</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>phosphatase inhibitors</td>
<td></td>
</tr>
<tr>
<td>I1PTP, I2PTP</td>
<td>YES</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>tyrosine phosphatase inhibitors</td>
<td>42</td>
</tr>
<tr>
<td>PKA-I</td>
<td>YES</td>
<td>YES</td>
<td>NA</td>
<td>YES</td>
<td>c-AMP dependent kinase inhibitor</td>
<td>43</td>
</tr>
<tr>
<td>Prothymosin α</td>
<td>NO</td>
<td>YES</td>
<td>3.5</td>
<td>YES</td>
<td>found in thymus</td>
<td>44</td>
</tr>
<tr>
<td>81K CBP</td>
<td>YES</td>
<td>YES</td>
<td>4.3</td>
<td>NA</td>
<td>calmodulin binding protein</td>
<td>45</td>
</tr>
<tr>
<td>32K inhibitor</td>
<td>YES</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>c-AMP phosphodiesterase inhibitor</td>
<td>46</td>
</tr>
</tbody>
</table>

Of this group, the structure of prothymosin α has been the most thoroughly characterized. CD, gel filtration, x-ray scattering, and dynamic light scattering were all used to establish that this biologically active protein has a random coil conformation. Several other proteins included in this family are heat-stable phosphatase inhibitors, which have not been well-characterized structurally. It is of interest to note that β-synuclein and tau share this structural similarity, as well as
chromogranin A, a protein involved in secretory granule formation. Table 5.2 is by no means comprehensive; more detailed structural studies of these and other proteins are required to fully define the physical properties of natively denatured proteins. It is likely that this common structural property (or lack thereof) has a functional significance, possibly in mediating protein-protein interactions.

Identification of an NACP Binding Protein

**A Natively Denatured Structure Suggested Protein-Protein Interactions.**

The observed flexibility of NACP suggested that it may function via complexation with another protein, rather than as a monomer. It is possible that either the protein adopts a specific three-dimensional structure upon interaction with membranes or receptor molecules, or that its nonfolded conformation might be advantageous for interactions with other biological macromolecules. A similar proposal has been made for the natively denatured protein prothymosin α.44 We therefore initiated a search for endogenous NACP binding partners.

Initial efforts to identify NACP binding molecules were carried out using affinity chromatography.24 Recombinant NACP was immobilized by covalent attachment of lysine residues to a polymeric matrix. Rat brain cytosolic extracts were then passed over the beads, and SDS-PAGE was used to identify bound proteins. This method met with limited success, and suffers from several potential drawbacks. For example, the nonspecific attachment of lysine residues to the column may block potential binding sites, especially binding to the lysine-rich N-terminus of NACP. Additionally, native NACP (and not the recombinant protein) may have a posttranslational modification which mediates binding. Affinity chromatography, therefore, may not be the optimal method for isolating NACP binding partners.
Immunoprecipitation was selected as an alternative to affinity methods. Coprecipitation is a classical method of detecting protein-protein interactions. The basic experiment involves the addition of an antibody to cell lysate or homogenate, followed by precipitation of the antibody-antigen complex, generally using an immunoglobulin-binding molecule immobilized on a solid support. Analysis by SDS-PAGE allows the identification of proteins bound to the antigen. The method has a number of distinct advantages over other methods. First, native proteins are used in the midst of all the competing proteins found in a crude lysate, providing an inherent control. Second, both the antigen and binding protein are present in the same concentrations as in a cell, eliminating artifacts due to abnormally high levels of test protein. Third, elaborate complexes, which may be difficult to reassemble after they are dissociated, are already in their natural state and can be readily precipitated. One disadvantage of the method is that coprecipitation is not as sensitive as protein affinity chromatography, because the concentration of antigen is lower than it is in protein affinity chromatography. Also, coprecipitating proteins do not necessarily interact directly, since they can be part of a larger complex; this can be accounted for by the use of binding experiments with purified proteins. Finally, proteins can coprecipitate as a result of cell lysis and not reflect a relevant physiological interaction; this can be addressed by demonstrating colocalization of the two proteins in vivo. For the initial identification of potential NACP binding partners, therefore, coimmunoprecipitation experiments were undertaken.

**Immunoprecipitation of NACP Leads to an Immunoreactive NACP Doublet.**

A mouse monoclonal antibody to NACP was obtained from D. Clayton (University of Illinois). This monoclonal antibody (H3C) is a mouse IgG1 antibody which was obtained in the form of reconstituted mouse ascites. It was raised to a
peptide from the canary synelfin C-terminal 16 amino acid sequence. The canary sequence differs from the rat sequence by only 2 amino acids in this region (Fig. 5.2), and the antibody has been shown to cross-react with a rat species of the correct mass. Monoclonal antibodies are generally less desirable for immunoprecipitation experiments, since only one antibody molecule is able to bind to one molecule of antigen. With polyclonal antibodies, overlapping immunoreactivity leads to more effective precipitation. However, an immobilized antibody binding reagent such as Protein G does allow the use of monoclonals.

Initially, we confirmed that we could use the H3C antibody to precipitate NACP from the cytosolic fraction of crude rat brain homogenate. NACP comprises as much as 0.5% to 1% of the total protein concentration in rat frontal cortex. Stripped rat brains were homogenized and separated into cytosolic and membrane fractions. Cytosol was incubated with antibody, followed by addition of Protein G immobilized on sepharose beads. Protein G binds tightly and specifically to the Fc chain of IgG1. Centrifugation followed by extensive washing of the beads was used to isolate the immobilized NACP-antibody complex.

This procedure resulted in the identification of two bands in the ~19 kDa range, as identified by 7% SDS-PAGE. The observation of an immunoreactive NACP doublet has been reported elsewhere. Western blotting indicated that both bands correspond to immunoreactive forms of NACP. The lower band of the doublet comigrates with recombinant NACP. N-terminal sequencing of both bands was unsuccessful, suggesting blocked N-termini. The bands were blotted to a PVDF membrane and eluted individually, and submitted for MALDI-TOF mass spectrometry. MS of the lower band was unsuccessful using this technique. The upper band showed a mass of 14,270.3. This mass may correspond to a truncated protein comprising residues 3-140 (Calc'd mass = 14,270.0). However, any posttranslational modifications, including acylation of the N-terminus, would affect
this calculation. Determination of the identity of these two immunoreactive bands, therefore, requires further experimental investigation.

A Mr~96 kDa Protein Coprecipitates with NACP

Examination of the anti-NACP immunoprecipitate on a 7% polyacrylamide gel reveals a band with an apparent molecular weight of approximately 96 kDa. (Fig. 5.11, lane 1). A number of control experiments were run in order to confirm the specificity of this interaction. Cytosol was incubated with just Protein G Sepharose to control for nonspecific binding to the Protein G beads (Fig 5.11, lane 2). To control for nonspecific binding to the constant regions of IgG and to control for proteins contained in mouse ascites, cytosol was incubated with mouse monoclonal antibodies to neurofilament 200, a common brain protein. The 96 kDa band appeared in none of the above control lanes, nor was it present in the antibody preparation itself (Fig 5.11, lane 3).

Identification of the 96 kDa band was achieved via standard microsequencing procedures. The immunoprecipitate was run on a 7% SDS gel and blotted to a PVDF membrane, which was stained with the reversible dye Ponceau S. Ponceau S is less sensitive than Coomassie Blue, which has a detection limit of ~0.2-0.5 μg, but it does not require fixing the protein to the blot. The band of interest was excised from the membrane and submitted for N-terminal sequencing. This procedure was unsuccessful, indicating that this protein, as well as NACP, has a blocked amino

Figure 5.11 (following page) Coimmunoprecipitation of VCP with NACP. Cytosolic fraction of rat brain homogenate was immunoprecipitated with antibodies to NACP and stained with Coomassie Blue R-250 (lane 1), immunoblotted with anti-NACP (lane 4), or immunoblotted with anti-VCP (lane 5). As controls, Protein G-Sepharose was incubated with cytosol (lane 2) or with anti-NACP (lane 3) and stained with Coomassie Blue R-250.
terminus. The protein was digested directly from the membrane using endopeptidase Lys-C. Fragments were collected as HPLC peaks, and three of these peaks were arbitrarily submitted for sequencing. The three HPLC peaks each contained two peptides. BLAST searches of the NIH protein sequence database were used to identify the proteins from which these peaks were derived. Two of the peptides corresponded to the heavy chain of IgG, and are most likely due to antibody contamination of the 96 kDa band. The remaining sequences are an exact match to rat valosin-containing protein (VCP) (Figure 5.12).

| RELQELVQYPVEHPXK | peptide 1 | VCP 487-502 |
| RELQELVQYPVEHPDK |          |             |
| RREAVXIVLSDDT    | peptide 2 | VCP 64-76   |
| RREAVCIVLSDDT    |          |             |
| GDIFLVRGMRAVEF    | peptide 3 | VCP 149-163 |
| GDIFLVRGMRAVEF    |          |             |
| EMVELPLRHPA      | peptide 4 | VCP 218-228 |
| EMVELPLRHPA      |          |             |

**Figure 5.12** Peptide sequences from proteolytic digest of 96 kDa NACP-binding protein. The corresponding amino acid sequences from valosin-containing protein are indicated. X indicates an unidentified amino acid.

**VCP is a Member of a Family of ATPases**

Valosin-containing protein (VCP) was first identified as the precursor to valosin, a 25 amino acid peptide thought to act as a digestive hormone. Koller and Brownstein identified and cloned the putative precursor, and concluded that valosin is an artifact of purification and does not occur in vivo. VCP became the first member of a family of ATPases named the AAA family, for ATPases Associated to a variety of cellular Activities. The common feature of the AAA family is an ATP binding module of about 230 amino acids. These proteins have been
implicated in a variety of cellular processes, including cell cycle regulation,\textsuperscript{52} HIV-1 gene expression,\textsuperscript{53} peroxisome assembly,\textsuperscript{54} proteasome function,\textsuperscript{55} and vesicle-mediated transport.\textsuperscript{14,56-59}

VCP has also been called "p97 ATPase" (in \textit{Xenopus laevis} oocytes)\textsuperscript{60} and "TER ATPase" (in rat endoplasmic reticulum);\textsuperscript{14} these monikers all refer to nearly identical proteins which are considered to be interspecies homologs (Table 5.3). These proteins all exist as homooligomeric ring-shaped structures reminiscent of chaperones. ATPase activity is detected only in the intact hexameric complex and not in dissociated molecules.\textsuperscript{60} The yeast homolog, cdc48p, which is about 70\% identical to the mammalian species, is involved in cell cycle control.\textsuperscript{52} Murine VCP was found to be tyrosine phosphorylated in response to CD4 receptor activation in lymphocytes, also supporting a possible role in cell division.\textsuperscript{61,62}

\textbf{Table 5.3} Interspecies homologs of valosin-containing protein (VCP). These proteins are considered functionally equivalent. A large number of other ATPases also share significant sequence homology to VCP.

<table>
<thead>
<tr>
<th>species</th>
<th>name of protein</th>
<th>homology to rat VCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat</td>
<td>TER ATPase</td>
<td>100%</td>
</tr>
<tr>
<td>mouse</td>
<td>VCP</td>
<td>99%</td>
</tr>
<tr>
<td>pig</td>
<td>VCP</td>
<td>99%</td>
</tr>
<tr>
<td>\textit{X. laevis} (clawed frog)</td>
<td>p97</td>
<td>95%</td>
</tr>
<tr>
<td>yeast</td>
<td>cdc48p</td>
<td>68%</td>
</tr>
</tbody>
</table>

The subcellular location of neuronal VCP has not been reported. In other cell types, the protein is generally associated with the membrane. A soybean variant of VCP is associated with the plasma membrane.\textsuperscript{63} The \textit{Xenopus} homolog, p97, exhibits diffuse cytoplasmic expression in oocytes.\textsuperscript{60} Cdc48p partially localizes to the ER membrane in yeast.\textsuperscript{57}

Recently, a large number of laboratories have reported findings implicating VCP (p97) in vesicle fusion processes. Antibodies directed against rat VCP (TER
ATPase) blocked cell-free transfer in a system derived from rat liver endoplasmic reticulum and prevented the ATP-induced cell-free formation of transition vesicles.\textsuperscript{14} This result was supported recently with three consecutive papers in the journal Cell, which demonstrated the ability of p97 to mediate regrowth of Golgi cisternae and fusion of ER membranes.\textsuperscript{57-59} Additionally, VCP binds to clathrin, the structural protein associated with coated vesicles involved in receptor-mediated endocytosis.\textsuperscript{64} NSF (N-ethylmaleimide sensitive fusion protein),\textsuperscript{65} a mediator of membrane fusion through a well-defined pathway, is also a member of the AAA family, and shares a significant degree of homology with VCP (36\% over residues 206-605; 49\% over the ATP binding site, residues 234-414).\textsuperscript{60} These findings led to the proposal that VCP may participate in membrane fusion via interaction with components that may be entirely distinct from those required for NSF-mediated events (\textit{vide infra}).\textsuperscript{56}

**Confirmation of VCP as the 96 kDa species**

VCP has a calculated molecular weight of 88,706 daltons, but has been observed to migrate in the 95-100 kDa range on SDS-PAGE,\textsuperscript{14} consistent with our observation of a band at \textasciitilde96 kDa. To confirm the identity of the protein, we immunoblotted with antibodies to VCP. A rabbit polyclonal antibody (serum 725) was obtained from L. Samelson (National Institutes of Health).\textsuperscript{61} This antiserum specifically stained a band in both crude cytosol and the anti-NACP immunoprecipitate which comigrated with the observed 96 kDa species (Fig. 5.11, lane 5).

ATPase activity staining\textsuperscript{66} of non-dissociating polyacrylamide gels was used as another method for confirming the identity of VCP. The 96 kDa band was eluted from the immunoprecipitate with 0.5 M NaCl, and the buffer was exchanged to remove salt. The elute, which contained nearly homogeneous 96 kDa protein,
run on a 6% non-dissociating polyacrylamide gel. The gel was incubated with 2 mM MgCl₂ and 0.05% Pb(OAc)₂ overnight, and then was washed and treated with sodium sulfide. ATPase activity manifested itself as a dark spot of lead sulfide in the gel. This spot comigrated with the major Coomassie Blue-stained band, confirming that the 96 kDa species has ATPase activity.

**In Vitro Reconstitution of the NACP-VCP Interaction with Purified Proteins**

The observed coprecipitation of NACP with VCP could either be due to a direct binding interaction or could be mediated by a third species which contacts both proteins. This issue was addressed by attempting to reconstitute the binding interaction using purified proteins. A culture of competent *E. coli* transfected with the expression vector for a GST fusion protein of VCP was obtained from L. Samelson (NIH). This construct also includes a myc epitope tag at the C-terminus of the VCP sequence. This culture was used to inoculate a large-scale culture, which was induced as described. The cells were lysed using a French press. Purification of the GST-VCP-myc fusion protein was achieved by immobilization of the protein on glutathione-Sepharose 4B beads, followed by glutathione elution.

In addition to providing a simple method for protein purification, the GST fusion protein contains a handle with which to perform *in vitro* binding experiments. The fusion protein was immobilized on glutathione-Sepharose beads, and washed thoroughly to remove non-specific binders. The immobilized VCP was then incubated with a solution of recombinant NACP. The complex was again washed thoroughly, and the proteins bound to the beads were analyzed by SDS-PAGE (Fig. 5.13). As a control experiment for non-specific binding to the beads, recombinant NACP was incubated with glutathione-Sepharose under identical conditions. As shown in Fig. 5.13, VCP formed a stable complex with NACP. A small amount of non-specific binding to the beads was unavoidable (Fig. 5.13, lane
3), but was minor relative to the binding to VCP. No additives were required to reconstitute the binding interaction, indicating that NACP and VCP interact directly.

We attempted to localize the region of NACP responsible for binding VCP by competing for binding with peptides derived from the NACP sequence. The immobilized GST-VCP fusion protein was incubated with NACP, and one of the following peptides in a large excess relative to NACP (50:1 except for NAC): NACP19-48, NACP111-125, NACP96-125, and NACP61-95 (NAC, only a 1.5:1 excess due to solubility limitations). If any of these peptides contains the VCP binding site it should efficiently compete with NACP, and the beads should show a large decrease in the amount of bound NACP. In fact, none of the peptides showed a significant ability to compete with NACP for binding. This may indicate that the VCP binding site includes residues from distal regions of the NACP sequence. Alternatively, the binding site may simply overlap two of the peptides which we chose to examine. A more constructive method for localizing the binding regions of these proteins may be to use truncated and/or deletion constructs of the two proteins in the in vitro binding experiment. A genetic screen, such as the yeast II hybrid system, would be an alternative method for answering these types of questions.

Figure 5.13 (following page) In vitro interaction between NACP and GST-VCP fusion protein. Lane 1, NACP bound to GST-VCP immobilized on glutathione-Sepharose; Lane 2, immobilized GST-VCP; Lane 3, glutathione-Sepharose incubated with NACP (control).
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Coimmunofluorescence Indicates Colocalization of NACP and VCP in Cultured Neurons

The experiments discussed to this point have demonstrated the presence of a NACP-VCP complex in cell-free in vitro systems. To show that this interaction could have biological relevance, it is important to demonstrate that the proteins have the opportunity to interact in vivo, and that the interaction does not take place as a result of cell lysis. Two common techniques are used to determine if two proteins share an intracellular location. The first method is to use gradient ultracentrifugation experiments to show that the two species cosediment in a common fraction. A more direct method for showing colocalization, and the experiment which we carried out, is to visualize the proteins within cells using immunofluorescence microscopy. These experiments were performed in collaboration with Dr. Adriana Ferreira at the Harvard Medical School Center for Neurologic Disease.

Cultured E18 hippocampal neurons grown in culture for 14 days were immobilized, and double stained with mouse anti-NACP and rabbit anti-VCP antibodies (Fig. 5.14). In this culture, axons appear as thin, tapering "processes" which often run alongside of dendrites and extend far from cell bodies. After treatment with the primary antibodies, the cultures were rinsed and treated with secondary antibodies (goat anti-mouse IgG rhodamine conjugate and goat anti-rabbit FITC conjugate). Neurons were then visualized under a fluorescence microscope; the rhodamine appears red, the fluorescein appears green, and, using a dual FITC-rhodamine filter, the areas of costaining appear yellow (Fig. 5.15).

A diffuse pattern of NACP and VCP immunoreactivity was observed throughout the cell bodies, in contrast to punctate staining along the axonal processes. NACP and VCP seem to be colocalized at these spots. Since NACP has been shown to be highly concentrated at synapses in brain tissue sections, cultures
were double-stained with antibodies to the synaptic markers synapsin I\textsuperscript{69} or synaptophysin. Some, but not all, of the immunoreactive spots for NACP and VCP colocalized with these synaptic markers, suggesting that NACP-VCP is enriched at synaptic sites. To distinguish axonal and dendritic processes, we also double-labeled with antibodies to MAP2, a somatodendritic marker,\textsuperscript{70} and NACP. MAP2-positive processes were not stained with NACP, confirming that NACP is not present in dendrites.

\textbf{Figure 5.14} (following page) Immunofluorescence microscopy of NACP and VCP in cultured hippocampal neurons. NACP was visualized using rhodamine fluorescence (panels A and D), and VCP was visualized using FITC (panels B and E). Micrographs obtained using a dual FITC-Rhodamine filter show the colocalization of NACP and VCP in cell bodies and along the axons (panels C and F).
Implications of the NACP-VCP Complex for Vesicle-Mediated Transport, Learning, and AD

We have identified, using coimmunoprecipitation, an interaction of NACP with VCP. Several criteria are essential to substantiate the validity of a coimmunoprecipitation experiment. First, it has to be established that the coprecipitated protein is precipitated by the antibody itself and not by a contaminating antibody in the preparation. This problem was avoided by the use of a monoclonal antibody. Second, it has to be established that the antibody does not itself recognize the coprecipitated protein. We demonstrated that VCP does not stain with anti-NACP in a Western blot. Third, it is important to determine if the interaction is direct or proceeds through a third party. An in vitro binding experiment with purified recombinant proteins indicated that the interaction is specific and direct. Finally, it is critical to determine that the interaction occurs in the cell and not as a consequence of cell lysis. Coimmunofluorescence experiments demonstrated clear colocalization of NACP and VCP in cultured hippocampal neurons. We have therefore demonstrated that the NACP-VCP interaction is relevant and worthy of further investigation.

The function of the NACP-VCP complex, on the other hand, remains a matter of speculation. A great deal of circumstantial evidence, however, indicates a potential role of the complex in vesicle-mediated transport processes. Membrane fusion events during intracellular transport are mediated by large complexes of proteins. The first such protein, N-ethylmaleimide sensitive factor (NSF), was isolated as a mediator of membrane fusion which could be inactivated by the addition of N-ethylmaleimide. Using this protein as a starting point, the basic components of NSF-mediated membrane fusion have been elucidated. These include the SNAPs (soluble NSF attachment proteins), soluble proteins required for
NSF attachment to membranes, as well as the membrane-bound receptors for the putative NSF-SNAP complex, the "SNAREs" (Fig. 5.15). Several other soluble and membrane-bound proteins (e.g. synaptotagmin, rab3, and p115) have also been implicated in the NSF-mediated fusion pathway.

![Figure 5.15](image)

**Figure 5.15** (a) The molecular components of NSF-mediated membrane fusion. (b) A parallel pathway for membrane fusion proposed for VCP. NACP may be a SNAP equivalent. Adapted from reference 56.

Recently, it was noted that fusion of transport vesicles from the Golgi complex with the plasma membrane involved a different mechanism, one that was independent of NSF and SNAPs. The absence of an effect of NSF and SNAPs led to a search for alternative mediators of membrane fusion. Specifically, VCP was investigated based on its homology to NSF. Three independent reports indicated that VCP (actually, either *Xenopus* p97 or the yeast homolog cdc48) can reconstitute Golgi or endoplasmic reticulum transport. An alternative pathway was proposed, in which VCP plays a similar role to that proposed for NSF, but interacts with a completely independent set of proteins (Fig. 5.15). A review of these papers stated, "The search is now on to identify the likely accessory molecules with which
p97/cdc48p interacts, the functional analogs of SNAPs and SNAREs. We have shown that NACP interacts with VCP, and that the two proteins colocalize in synapses. It seems likely, therefore, that NACP is the first of such SNAP analogs to be discovered. Confirmation of this proposal will require the direct experimental demonstration that NACP has the ability to mediate membrane fusion.

Based on the proposal that the NACP-VCP complex plays a role in membrane fusion events in neurons, we can begin to speculate on the relationship of these proteins to learning and AD. The brain of a learning animal is characterized by upregulation of NACP, consistent with the idea that NACP-VCP dependent vesicle trafficking may be involved in synapse formation or stabilization. A function of NACP in mediating "synaptic plasticity" has already been proposed. The AD brain, in contrast, is characterized by a significant loss of synapses in proportion to the severity of cognitive impairment. The slightly reduced expression of NACP could confer susceptibility to AD, by decreasing the stability of synapses or by increasing the sensitivity of synapses to neurotoxic agents. Alternatively, the NACP-VCP complex could directly affect an AD pathogenic pathway, such as the intracellular production or nucleation of Aβ. The results presented here suggest future experiments which may define more clearly the molecular events underlying learning and AD.
Experimental Section

Protein Expression

*NACP Expression and Purification.* A pRK172 plasmid containing the human NACP (α-synuclein) sequence (provided by R. Jakes and M. Goedert, MRC, Cambridge, UK) was dissolved in TE buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA) and added to 25 μL of competent BL21(DE3) E. coli cells. The cells were incubated on ice for 30 min, heat shocked at 42°C for 30 s, and then incubated at RT for 2 min. To the cells was added 500 μL of sterile Luria-Bertani Broth (LB) containing 0.2% glucose which had been prewarmed to 37°C. After incubation at 37°C for 1 h, 200 μL of the bacteria was plated onto ampicillin positive agar plates. The bacteria was grown for 17 h at 37°C. A control plate (competent cells lacking the plasmid) did not grow colonies. Two colonies were chosen, and each was used to inoculate a stab culture, which was grown for 36 h and stored at -80°C. All cultures were henceforth grown from only one of these stab cultures.

The stab culture was used to inoculate 10 mL of sterile Luria-Bertani Broth (LB) containing ampicillin (50-100 μg/mL), which was incubated at 37°C with shaking overnight (12 h). A portion of this culture (5 mL) was used to inoculate 500 mL of LB-ampicillin, which was shaken at 37°C until the O.D. (600nm) reached 0.6 to 1.0 (~3 h). 120 μL of Isopropyl-1-thio-β-galactoside (1.68 M in H2O) was added (final concentration = 0.4 mM) to induce protein expression. After shaking for 4 h at 37°C (induction levels were monitored by SDS-PAGE), the cells were collected by centrifugation (30 min at 24,000 x g), resuspended in 20 mL of lysis buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM DTT, 0.1 mM phenylmethysulfonyl fluoride (PMSF)), and lysed using a French press at 16,000 p.s.i. The lysate was centrifuged at 24,000 x g for 30 min, and the pellet was discarded. 6 mL of 5% streptomycin sulfate (0.2 volumes) in lysis buffer was added, and the solution was stirred on ice. The resultant
suspension was centrifuged at 24,000 x g for 30 min, the supernatant was transferred
to a plastic centrifuge tube, and the pellet was discarded. The supernatant was then
immersed in a boiling water bath for 10 minutes, and the resultant suspension was
cooled to RT and centrifuged at 24,000 x g for 30 min. The supernatant was loaded
onto 25 g of Biogel P-10 (100-200 µm wet), eluted with lysis buffer (Rv=65 mL),
dialedyzed with several changes against ddH2O overnight (Spectrapor 1 tubing, MW
cut-off = 6,000-8,000), and lyophilized to a white solid. HPLC and SDS-PAGE
indicated the protein was >90% pure. Analytical HPLC (C4 reversed-phase, Vydac):
2.0 mL/min, 70/30 H2O/CH3CN, (0.1% TFA), linear gradient to 60/40 (20 min),
Rv=31mL. MALDI-TOFMS: (M + H)+ calcd for C627H1013N166O216S4 14461.2, found
14463.0 (+ 4.6). Amino acid analysis: Asx 10.1 (9), Glx 24.9 (24), Ser 4.6 (4), Gly 18.2
(18), His 1.8 (1), Thr 10.4 (10), Ala 19 (19), Tyr 3.5 (4), Val 15.4 (19), Met 3.6 (4), Ile 2.4
(2), Leu 4.7 (4), Phe 5.2 (5), Lys 16.6 (15). Tryptic digest: To ~1 mg of NACP
(determined by absorbance, using an estimated ε274 of 5600 M⁻¹cm⁻¹ based on the
tyrosine content of the protein) was added 500 µL of trypsin (0.04 mg/mL in 0.1M
NH4HCO3). The solution was incubated at 37°C for 80 min, and loaded directly onto
a PDMS target. PDMS: 688.6 (residues 97-102), 773.0 (1-6), 830.8 (24-32, 874.0 (13-
21),968.3, 1156.7 (1-10), 1252.2, 1296.9 (46-58), 1523.9 (46-60 and 44-58), 1930.0 (61-80),
2158.1 (59-80). Additional minor peaks were also detected. Alternate Purification
Method. NACP was purified without the use of a boiling step via a documented
procedure.24

GST-VCP-myc Expression. An E. coli culture (DH5α strain competent cells)
transformed with a plasmid containing the murine VCPmyc cDNA subcloned into
the BamHI/SmaI sites of pGEX-3X (Pharmacia LKB Biotechnology Inc.) was obtained
from L. Samelson (NIH, Bethesda, MD).62 100 µL of this culture was used to
inoculate 50 mL of sterile LB-amp medium (100 µg/mL ampicillin) in a 250 mL
Erlenmeyer flask, which was shaken overnight (18 h). Four aliquots of 0.5 mL were
removed and added to 0.5 mL of 30% glycerol; these tubes were frozen and stored at -80°C for future use. Additionally, 1.5 mL was removed for DNA isolation. 5 mL of the remainder was used to inoculate 500 mL of LB-amp, which was shaken in a 2L flask at 37°C until the O.D. (600 nm) reached 0.6 - 1.0. 500 μL of IPTG (24 mg/mL in H₂O) was added, and the cells were shaken at 37°C for 70 min, and centrifuged for 10 min at 6000 x g. The supernatant was discarded, and the cells were resuspended in 20 mL of "GST lysis buffer" (50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 2 mM EDTA, 2 mM MgCl₂, 0.5 mM DTT, 0.5 mM ATP, 0.5% Triton X-100, 10 μg/mL leupeptin, 5 μg/mL aprotinin, 0.1 mM PMSF) and lysed using a French press at 16,000 p.s.i. The lysate was centrifuged at 24,000 x g for 30 min. Glycerol (10% final concentration) was added to the supernatant, which was aliquotted into 1 to 1.5 mL portions and stored at -80°C until further use.

General Procedures for Polyacrylamide Gel Electrophoresis

Novex minigels (1.0 or 1.5 mm) were used throughout this work. All single percentage gels were poured with a 4% stacking gel (SDS or native) and run at 100-140V. All 1.0 mm gradient gels were purchased from Novex. Bis-acrylamide (40% solution, 29:1 acrylamide/bis) was purchased from BioRad. Running buffer: 24 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. Sample loading buffer: 125 mM Tris-HCl, pH 6.8, 15 mM EDTA, 20% glycerol, 10% w/v SDS, 4% β-mercaptoethanol, 1 mg/mL bromophenol blue.

Western blotting. Gels were transferred to PVDF membranes (BioRad) using a semi-dry transfer apparatus (Hoefer) at 100 mA for 45-50 min. The membrane was transferred to a plastic centrifuge tube, blocked with 5% nonfat dry milk in blotting buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) (RT, 20 min with gentle rotation). The blocking buffer was removed without washing, and primary antibody was diluted in buffer (1:100,000 for anti-NACP; 1:250 for anti-VCP) and incubated with
the blot (RT, 2 h). The blot was washed (1 x 1 min, 3 x 3 min) with transfer buffer, and incubated with secondary antibody (goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma), 1:4000 dilution for NACP; goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma), 1:15,000 for VCP) (RT, 1 h). The blot was washed again with buffer (1 x 1 min, 3 x 5 min) and incubated with 5-bromo-4-chloro-3-indolyl-phosphate/Nitro blue tetrazolium (Sigma FAST\textsuperscript{TM} BCIP/NBT, 1 tablet in 10 mL transfer buffer) until color development was complete.

*Preparation of Samples for Amino Acid Sequencing.* The anti-NACP immunoprecipitate was run on SDS-PAGE, blotted to PVDF, and stained with Ponceau S. Bands were excised using a clean razor blade, and submitted directly for either N-terminal sequencing or proteolytic digestion followed by sequencing.

*Coomassie Blue Staining (gels).* Gels were immersed in Coomassie Blue stain (2.5 g/L Coomassie Blue R-250, 40% MeOH, 7% AcOH) for at least 20 min, followed by fast destain (5% AcOH, 25% MeOH) for 30 to 120 min and then slow destain (14% AcOH, 10% MeOH) overnight.

*Coomassie Blue Staining (blots).* Blots were immersed in stain (0.025% Coomassie Blue R-250, 60% AcOH, 40% MeOH) for 1 min, followed by destain (50% MeOH, 10% AcOH) for ~1 min or until bands became visible.

*Ponceau S Staining (blots).* Blots were incubated with 0.2 % Ponceau S in 1% AcOH for 1 min, and destained in H\textsubscript{2}O for ~1 min or until bands became visible.

*Silver Staining (gels).* Gels were 1) Fixed for at least 1 h (50% MeOH, 12% AcOH, 0.5 mL 37% formaldehyde/L); 2) Washed 3 x 20 min with 50% EtOH; 3) Pretreated for 1 min with Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}•5H\textsubscript{2}O (0.2 g/L); 4) Rinsed with H\textsubscript{2}O 3 x 20 sec; 5) Impregnated for 20 min (2 g/L AgNO\textsubscript{3}, 0.75 mL 37% formaldehyde/L); 6) Rinsed with H\textsubscript{2}O 2 x 20 sec; 7) Developed for ~10 min (or until bands became visible) (60 g/L Na\textsubscript{2}CO\textsubscript{3}, 0.5 mL 37% formaldehyde/L, 4 mg/L Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}•5H\textsubscript{2}O); 8) Washed 2 x 2 min with H\textsubscript{2}O; and 9) Stopped with 50% MeOH, 12% AcOH for 10 min. Gels were stored in gel drying
solution.

**Ferguson Plot**

The following proteins were run on 6%, 10%, and 14% non-dissociating polyacrylamide gels: NACP, BSA (MW = 68,000), pepsin (35,000), insulin (5733). Rf (6%, 10%, 14%): BSA 0.71, 0.27, 0.13; pepsin 0.69, 0.32, 0.16; insulin 0.86, NA, 0.40; NACP 0.52, 0.29, 0.17. For each protein, K_f was calculated as the slope of a plot of logRf vs. % acrylamide: BSA, -0.0938; pepsin, -0.0800; insulin, -0.0417; NACP, -0.0608. A plot of K_f vs. MW was used to extrapolate an approximate MW of 19,000 for NACP.

**Circular Dichroism Spectroscopy**

Samples for CD were prepared by dissolving recombinant NACP in 300 μL of either Tris buffer (10 mM Tris-HCl, pH 7.5), PBS (10 mM sodium phosphate, 100 mM NaCl, pH 7.4), or acetate buffer (50 mM NaOAc, 100 mM NaCl, pH 4.5). Spectra were recorded on a Jasco J-500 Spectropolarimeter, in a 0.1 cm quartz cell. The following parameters were used: Band width = 1.0 nm; slit width = "auto"; time constant = 16 sec; step resolution = 0.4 nm; scan speed = 10 nm/min. The sensitivity was varied according to the observed intensity of each spectrum. Observed ellipticity (θ_obs) was converted to mean residue ellipticity (θ_MRW, in deg-cm²/dmole) by the following equation:

$$\theta_{MRW} = (\theta_{obs} \times 100,000)/(c \times l \times n)$$

where c = concentration (in μM), l = path length (in cm), and n = number of residues in the protein. The percentages of secondary structural elements were estimated based on the method of Greenfield and Fasman²⁸ using the following formulae:
\[
\% \alpha \text{ helix} = \frac{[-\theta_{MRW}(@208 \text{ nm}) - 4000]}{290}
\]
\[
\% \beta \text{ sheet} = \frac{[-\theta_{MRW}(@222 \text{ nm}) + 396 \%\alpha - 3900]}{177}
\]
\[
\% \text{ coil} = 100 - \%\alpha - \%\beta
\]

Data shown is an average of at least three runs.

**Other Spectroscopic Methods**

FTIR was performed on a Perkin-Elmer 1600 series spectrometer. Solution spectra were recorded using a solution cell with CaF\(_2\) plates (0.05 mm Teflon spacer). The FTIR spectrum of recombinant NACP (4 mg/mL in D\(_2\)O) was recorded, and the spectrum of D\(_2\)O alone was subtracted. UV spectra were recorded on a HP8452A UV/vis spectrophotometer.

**Preparation of Rat Brain Cytosolic Extract**

Ten stripped rat brains (PelFreez, Rogers, AR) were homogenized in 80 mL of lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF) using 2 x 30 second bursts on a Polytron at setting 5. The homogenate was centrifuged at 800 x g for 10 min, and the supernatant was centrifuged at 100,000 x g for 1 h to separate cytosolic and membrane fractions. Total protein concentration in the cytosolic fraction was 6.8 mg/mL, as determined by the BCA protein concentration assay (Pierce).

**Immunoprecipitation of NACP**

Rat brain cytosolic extract (1.5 mL) was prescreened with 200 µL of a 1:1 slurry of Protein G-Sepharose (Gammabind Plus, Pharmacia), incubated for 1 h at 4°C with 4 mL of a mouse monoclonal antibody to the C-terminus of canary synelfin
(residues 129-143) (kindly provided by D. Clayton, University of Illinois),\textsuperscript{7} followed by incubation with 200 μL bead slurry with gentle agitation for 1-2 h at 4°C. Beads were washed 3x with lysis buffer and boiled in SDS-PAGE sample loading buffer.

**ATPase Activity Staining of VCP**

VCP was eluted from the immunoprecipitate using 0.5 M NaCl. This sample was run on a 6% non-dissociating polyacrylamide gel, which was incubated with ATPase buffer (24 mM Tris, 192 mM glycine, pH 8.3; 2mM ATP, 2 mM MgCl\textsubscript{2}; 0.05% Pb(OAc)\textsubscript{2}•3H\textsubscript{2}O) for 18 h at room temperature. The gel was washed thoroughly with distilled H\textsubscript{2}O and immersed in a solution of 0.5% sodium sulfide. ATPase activity was manifested as a dark brown precipitate in the gel. After prolonged incubation in this solution, the gel became soft and unmanageable.

**In Vitro Binding Experiments**

A 50% slurry of glutathione-Sepharose 4B (50 μL) (Pharmacia) was washed 3x with binding buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM DTT, 1 mM EDTA) and incubated with crude GST-VCP cell lysate (50 μL) with gentle agitation at 4°C for 1 h. Beads were washed thoroughly with buffer, until no significant A\textsubscript{280} was observed in the wash. Recombinant NACP (50 μL of a 200 μM solution in binding buffer) was added, the suspension was agitated at 4°C for 2-3 h, the beads were washed 3 - 5 x with buffer and boiled in SDS gel loading buffer. As a control experiment, NACP (200 μM) was incubated with glutathione-Sepharose 4B. A small amount of non-specific binding of NACP to the beads in the control experiment could not be avoided, even upon addition of 50-100 mM NaCl or 0.05% Tween-20 to the binding buffer. Samples were loaded on 12% SDS-PAGE and silver-stained.
Peptide Competition Experiments

50 μL of a 1:1 slurry of GST-VCP immobilized on glutathione Sepharose 4B was incubated at 4°C overnight with 25 μL of NACP (~40 μM) and 25 μL of either 1) NACP19-48 (1.8 mM); 2) NACP111-125 (2 mM); 3) NACP96-125 (2 mM); 4) NACP61-95 (NAC, 60 μM); or 5) buffer (control). Peptides NACP19-48 and NAC were N-terminally biotinylated. The beads were washed 3 x 1mL with binding buffer, and boiled in 20 μL of 2x SDS gel loading buffer. 20 μL of each sample was loaded onto a 12% SDS gel and the gel was silver-stained. No competition, as evidenced by a decrease in the intensity of the bound NACP band, was observed.
References for Chapter V

22. Saitoh, T., personal communication.
27. Ferguson, K.A. *Metabolism* 1964, 13, 985.
Appendix

$^1$H NMR Spectra (500 MHz, buffered D₂O)

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Mass Spectra

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<tr>
<td>Immunoprecipitated NACP (MALDI-TOFMS)</td>
<td>175</td>
</tr>
</tbody>
</table>
Ac-Gly-Sar-CONH₂
Ac-Ala-Gly-Sar-Ala-CNH$_2$
Ac-Ala-Ala-Gly-Sar-Ala-Ala-CONH₂
Ac-Ala-Gly-Sar-Val-CONH$_2$
Ac-Val-Gly-Sar-Ala-CNH₂
Ac-Ile-Gly-Sar-Ile-CONH₂
Ac-Phe-Gly-Sar-Phe-CONH₂
Ac-Lys-Gly-Sar-Glu-CONH$_2$
Ac-Val-Gly-Sar-Val-Val-CNH₂
Ac-Val-Gly-Sar-Val-Val-Ile-CONH₂
Ac-Met-Val-Gly-Sar-Val-CONH₂
Ac-Met-Val-Gly-Sar-Val-Val-CONH$_2$
Ac-Glu-Val-Gly-Sar-Val-Glu-CONH₂
Ac-Leu-Glu-Val-Gly-Sar-Val-Glu-CONH$_2$
Ac-Glu-Val-Gly-Sar-Val-Glu-Ile-CONH₂
Ac-Leu-Glu-Val-Gly-Sar-Val-Glu-Ile-CONH₂
NAC Peptide (PDMS)
Calculated (M+H⁺) = 3262.7
Recombinant NACP (MALDI-TOFMS)
Calculated (M+H⁺) = 14461
(M+2H⁺) = 7231
Immunoprecipitated NACP (MALDI-TOFMS)
Lower $M_r$ Band of Immunoreactive Doublet