Methods for the Synthesis of Hydrophobic Peptides and Functional Studies of NACP

by

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A.B., Chemistry
Harvard University, 1993

Submitted to the Department of Chemistry
in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Chemistry

at the

Massachusetts Institute of Technology

September 1995

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ABSTRACT

The synthesis of the transmembrane domain of β Amyloid Precursor Protein was attempted. The peptide has a tendency to aggregate when being synthesized on solid-phase, and has therefore never been synthesized. Removable N-benzyl amide protecting groups were used to attempt to prevent this aggregation. A portion of this peptide was synthesized with this method.

NACP was overexpressed in *E. coli.* and purified. Preliminary structural studies show that the 14.4 kDa protein is a random-coil, heat-stable protein. The protein elutes from a gel filtration column at 55 kDa, but is a monomer in solution. Affinity experiments and immunoprecipitation experiments were performed in order to find an NACP-binding protein. A 96 kDa protein was identified and will be analyzed for N-terminal amino acid sequencing.

Thesis Supervisor: Peter T. Lansbury, Jr.
Title: Associate Professor of Chemistry

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Acknowledgments

I would like to thank Cheon-Gyu Cho and Santosh Nandan for advice during the synthesis portion of the N-benzyl amide project; Paul Weinreb for sharing ideas and experiments on the NACP project; and Krista Evans for suffering with me during the "learning and borrowing" phases of the molecular biology work.

Thanks go to all the members of the Lansbury group for their help, and friendship, not to mention the poker games and lunches. In particular, I'd like to mention my fellow classmates, Raul Zambrano and Jim Harper; I wish Raul the best of luck in medical school, and Jim in whatever he decides to do.

Special thanks go to the entire Stubbe group and the Liu group, for their great attitude towards our invasion of their labs.

Lastly, I'd like to thank my family for their support, and Mathai, who gave me the courage to do what I thought was right.

Chapter 1

Methods for the Synthesis of Hydrophobic Peptides

Introduction

Alzheimer's disease is a neurodegenerative disease characterized by the presence of extracellular amyloid plaques, intracellular neurofibrillary tangles, and cerebrovascular amyloid deposits[1]. Extracellular amyloid plaques consists of dystrophic axons and dendrites surrounding a proteinaceous core of fibrils. The core of the amyloid plaque consists of a 40 to 43 amino acid peptide called β amyloid (Aβ)[2,3]. Aβ is a fragment of a larger protein called β Amyloid Precursor Protein (βAPP)[4]. βAPP is a cell surface glycosylated transmembrane protein; it consists of a extracellular domain (624 amino acids), a single transmembrane region (24 residues), and a cytoplasmic domain (47 residues). Aβ is derived from the 28 amino acids immediately preceding the amino terminus of the transmembrane (TM) domain of βAPP plus the first 12-15 residues of the TM domain[4] (Figure 1).

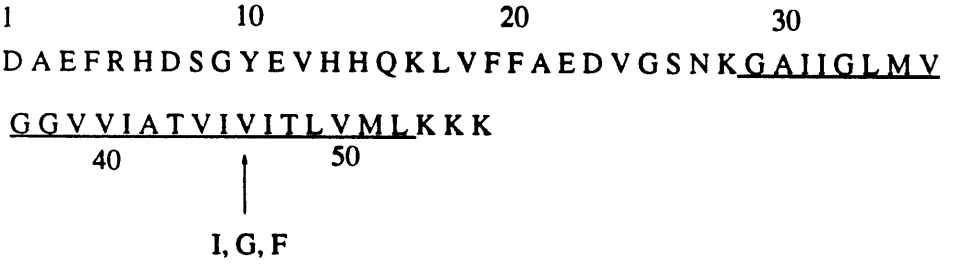


Figure 1. Sequence of βAPP containing Aβ (1 to 40-43) and transmembrane domain (underlined). Numbering refers to Aβ sequence. Arrow shows site of mutations in transmembrane domain.

Although it is unclear what role Aβ has in causing Alzheimer's disease, several mutations in the sequence of βAPP cause familial Alzheimer's disease[5]. The ability of these mutations in the sequence of βAPP to cause AD would seem to signal the importance of amyloid in the pathology of AD. One set of mutations (Figure 1) lies inside the TM domain, but outside the Aβ region. These mutations do not affect the overall rate of cleavage to generate Aβ, but may affect the site of cleavage, increasing the ratio of 1-42 to 1-40[6]. In *in vitro* experiments, β1-42 aggregates much faster than β1-40; increasing the concentration of β1-42, *in vivo*, might cause nucleation and aggregation of amyloid[7]. The mechanism of cleavage of βAPP is unknown[8].

The mutations may cause a change in the site of cleavage by changing the structure of the TM region in the lipid bilayer. One hypothesis is that the TM domain is capable of dimerizing; the ability to dimerize may be related to the production of amyloid. The mutations may change the structure of βAPP by altering the ability of the protein to dimerize. There are precedents in the literature. Another transmembrane protein, glycophorin, has previously been shown to form a dimer in lipid bilayers. Mutations L75 to V, A, or I and I76 to A or F can eliminate dimerzation of glycophorin[9]. Another example is the *neu* proto-oncogene. A single point mutation (V664 to E) in the transmembrane domain increases *neu* dimerization[10].

In order to explore the possibility of βAPP dimerization, we are attempting to synthesize the transmembrane region of βAPP along with the V46 to I, G, F mutants. By studying the behavior of the peptides in lipid bilayers or micelles, we may be able to find differences between the wild type and mutants that give clues to the mechanism of Aβ formation.

Synthesis of Transmembrane Sequences

The synthesis of transmembrane domains of proteins is not a trivial exercise. Extremely hydrophobic sequences have generally been difficult to synthesize, being prone to aggregation on the resin support[11]. Aggregation of difficult peptides on resin support has been shown to be often a result of beta-sheet formation by the peptides. The N-terminal end of the growing peptide then becomes sequestered in the aggregate, making chain extension sterically impossible. One method to prevent aggregation is to place a 2-hydroxy-4-methoxy benzyl group onto the nitrogen of every sixth amino acid in the sequence[12]. This removable protecting group is likely to be effective by removing the hydrogen involved in beta sheet formation and by sterically preventing the chains interacting.

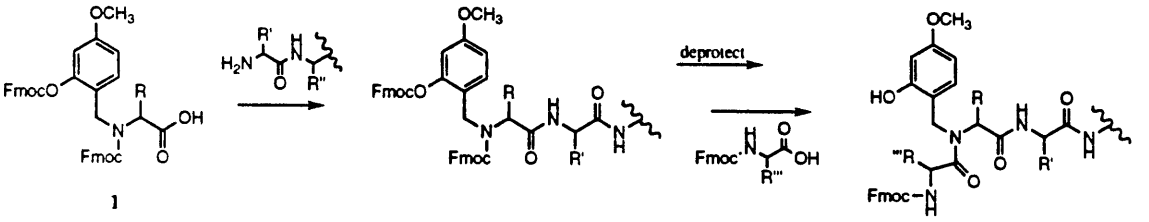
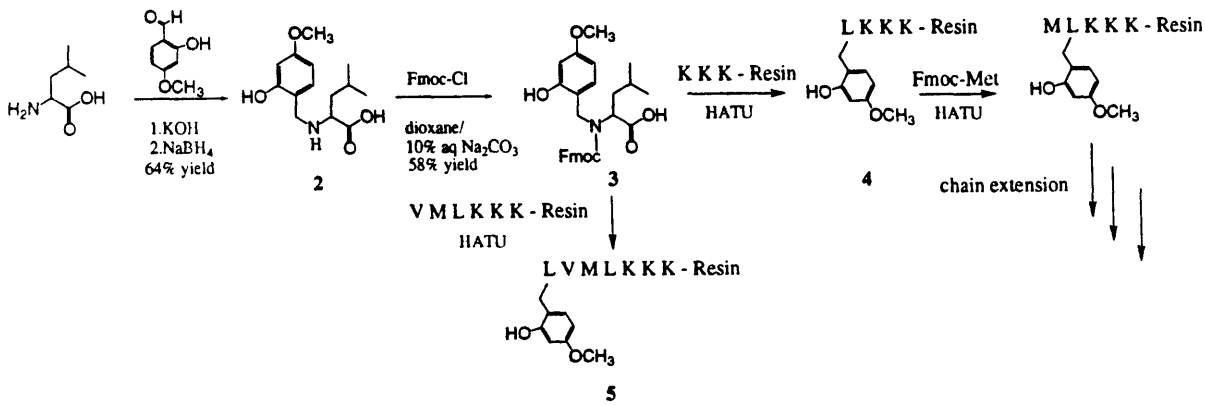


Figure 2. General usage of benzyl amide protecting groups.

A major drawback to the use of the benzyl amide protecting groups is that it involves the coupling of a secondary amine to the next amino acid in the sequence (Figure 2). In addition, steric hindrance by the side chains of the coupling partners is also likely to be a problem. Therefore, use of the amide benzyl group should be limited to amino acids without beta branched side chains[12].

Previous work in the group has been unsuccessful at synthesizing the TM of βAPP, due to its tendency to aggregate on the resin. This work was repeated using MBHA Rink Amide resin, under standard coupling conditions (3 eq amino acid, 3 eq PyBOP, 5.3 eq DIEA, in DMF). Aggregation began by I47 (assayed by concentration of accessible amine, as determined by quantitative ninhydrin test[13]) and was complete by T43 (accessible amine reduced to 6.6% of original concentration).

The use of the benzyl amide protecting groups was attempted. As stated previously, use of the protecting groups should be limited to amino acids without beta branching. However, most of the amino acids in the C terminal end of the TM region are beta branched. There are three potential sites of benzyl amide attachment: L52, L49 and A42. Fmoc(N-2-hydroxy-4-methoxybenzyl)-leucine **3** was synthesized from leucine (Scheme I). The N-benzyl leucine **2** was synthesized by reacting leucine with 2-hydroxy-4-methoxy benzaldehyde in a reductive amination[14]. N-benzyl leucine **2** was reacted with Fmoc-Cl in dioxane/aq Na_2CO_3 to yield **3**. However, contrary to the literature[12], only **3** was formed, not the bis-Fmoc-N-benzyl derivitized amino acid **1**.

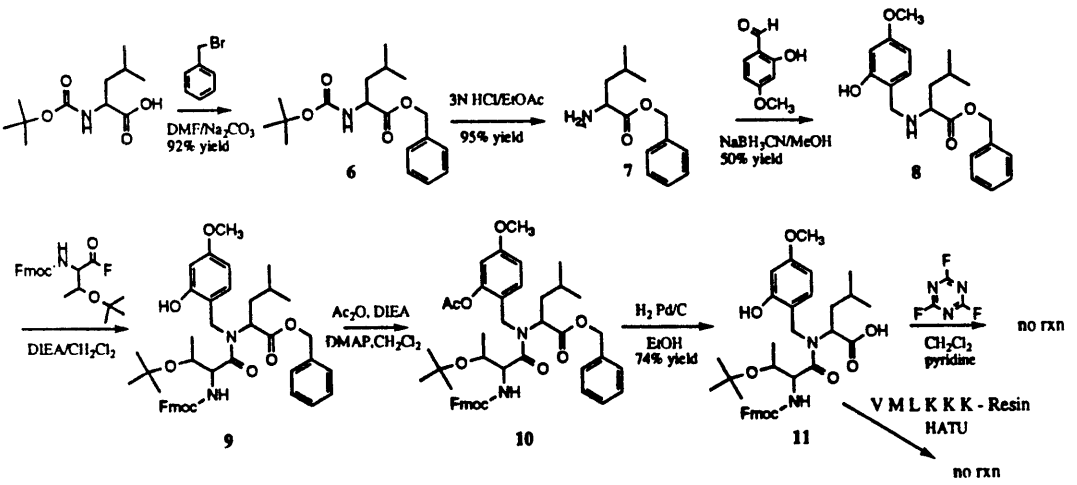


Scheme I. Synthesis of Fmoc(N-2-hydroxy-4-methoxybenzyl)leucine and its coupling to the resin.

3 was attached to KKK-Resin (3 eq, 6 eq HATU, 5.3 eq DIEA in DMF) (Scheme I). According to the literature, HATU is a faster coupling agent than PyBOP[15]. Fmoc-Met was then attached to resin-bound chain 4 (10 eq Fmoc-Met, 10 eq HATU, 5 eq DIEA in DMF) in 69% yield (as assayed by quantitative ninhydrin). Repeated couplings did not increase yield. Chain extension (by standard coupling methods) was still hindered by aggregation: after the coupling of T43, the concentration of accessible amine was reduced to 14.5% of the original concentration. Although the benzyl amide appears to have helped prevent some aggregation, significant aggregation occurs before the next benzyl amide (A42) can be used.

L49 is closer to A42 in the sequence. Unfortunately, the amino acid following L49 is a sterically hindered T. Attempts to couple Fmoc-Thr-OH or Fmoc-Thr(OtBu)-OH to resin-bound chain 5 (10 eq amino acid, 10 eq HATU, 5 eq DIEA, in DMF) failed (Scheme I).

Low coupling yields on the resin could be avoided by performing the difficult coupling of the secondary amine in solution, and attaching the resulting dipeptide to the growing peptide chain (Scheme II). Boc-leucine was treated with benzyl bromide in sodium carbonate/DMF to yield Boc-Leu-OBn 6.



Scheme II. Synthesis of Fmoc-Thr(OtBu)-(N-2-hydroxy-4-methoxy-benzyl)-leucine and its attempted coupling to the resin.

The amino acid was deprotected with 3N HCl/ethyl acetate to yield Leu-OBn **7**. Reductive amination with 2-hydroxy-4-methoxy benzaldehyde yielded N-benzyl derivitized Leu-OBn **8**. Dipeptide **9** was formed from the coupling of **8** and the acid fluoride of Fmoc-Thr(OtBu). Use of HATU to perform this coupling yielded no product. The dipeptide **9** was acylated to protect the alcohol, forming dipeptide **10**. The O-benzyl group was removed by catalytic hydrogenation (10% Pd/C) to yield dipeptide **11**.

Attempts to attach dipeptide **11** to the resin were unsuccessful. Use of HATU as the coupling agent gave no yield. Dipeptide **11** was then reacted with cyanuric fluoride (Scheme II). Very small amounts of acid fluoride were formed (seen by TLC). The dipeptide **11** is most likely too sterically hindered to become activated by the cyanuric fluoride.

The success of the coupling of **8** to the acid fluoride of Fmoc-Thr(OtBu) caused a reevaluation of the possibility of coupling Thr to the secondary amine of resin-bound peptide **5** (Scheme I). Preliminary results indicate that the coupling of the acid fluoride of Fmoc-Thr(OtBu) to resin-bound peptide **5** (10 eq acid fluoride, 5 eq DIEA, in DMF, 24 h; recoupled twice) proceeds to 90% yield (by quantitative ninhydrin). Formation of the peptide TLVMLKKK has been confirmed by PDMS (M+H = 960.1; calc = 960.31). It now remains to ascertain if the benzyl amide is capable of preventing aggregation until the next benzyl-amino acid (A42) is placed onto the resin.

abbreviations used: Boc = tert-butoxycarbonyl; Fmoc = 9-Fluorenylmethoxy-carbonyl; PyBOP = Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexa-fluorophosphate; DIEA = diethylisopropylamine; DMAP = 4-dimethylamino-pyridine; HATU = 1-Hydroxy-7-azabenzotriazole; DMF = dimethylformamide;

Rink Amide MBHA Resin = 4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin.

Experimental Section

Equipment, Materials, and Methods

All chemicals were purchased from Aldrich unless otherwise stated. Protected amino acids were purchased from Novabiochem or Advanced Chemtech. DIEA was purchased from Aldrich and distilled from ninhydrin under reduced pressure. PyBOP and Rink Amide MBHA resin were purchased from Novabiochem. HATU was obtained from Millipore. Cyanuric fluoride was purchased from Fluka.

PDMS of compounds were measured on a Bio-Ion Plasma Desorption Mass Spectrophotometer. ^1H NMR were measured on a 250 MHz Bruker instrument.

General Procedures for the Synthesis of Peptides

The Rink Amide MBHA resin was placed in a reaction vessel and swollen with CH_2Cl_2. The following procedure (standard coupling procedure) was used for the addition of each amino acid: 1) wash resin with DMF, 2) wash with 50 % piperdine/DMF, 3) shake resin in 50% piperdine/DMF (15 min), 4) wash alternately with DMF and CH_2Cl_2 (3x), 5) remove a small amount and perform Kaiser test[13] for free amine (test should be positive), 6) wash with DMF, 7) add Fmoc-amino acid (3 eq, 0.1 M in DMF) and PyBOP (3 eq, 0.1 M in DMF), shake resin for 30 s, 8) add DIEA (5.3 eq) and shake mixture for 1 h at RT, 9) wash

alternately with DMF and CH_2Cl_2 (3x), 10) remove a small amount and perform Kaiser test: if positive, repeat steps 6-9 until a negative result is obtained.

For unusual amino acid couplings, PyBOP was substituted with HATU (5-10 eq), or an acid fluoride of an Fmoc-amino acid[16] (10 eq) was substituted for amino acid and coupling reagent.

N-(2-hydroxy-4-methoxybenzyl)leucine (2). Leucine (3 mmol, 303 mg) was dissolved in a 0.5 M KOH solution (3 mmol, 6 mL). To this solution was added 2-hydroxy-4-methoxy-benzaldehyde (456 mg, 3 mmol); enough ethanol was added to solubilize the benzaldehyde. The reaction was stirred for 10 min, then sodium borohydride (114 mg, 3 mmol) was added in H_2O (1 mL) over 45 minutes. The reaction was stirred for an extra 20 min, then acidified to pH 6. Product crystallized, and the solution was cooled at 4°C for 4 h, to complete crystallization. The white precipitate (504 mg, 1.9 mmol, 64% yield) was collected and used without further purification: ^1H NMR (D_2O) δ 0.3 (dd, 6H), 1.2 (m, 3H), 3.2 (s, 3H), 3.3 (t, 1H), 3.7 (s, 2H), 6.0 (d, 2H), 6.7 (d, 1H).

Fmoc(N-2-hydroxy-4-methoxybenzyl)leucine (3). Dioxane (16 mL) was added to a solution of **2** (300 mg, 1.1 mmol) in 10% sodium carbonate (30 mL). Fmoc-Cl (710 mg, 2.7 mmol) was added in small amounts over 1 h. As each portion was added, the Fmoc-Cl gradually dissolved, then a gel-like precipitate formed. After stirring overnight, H_2O was added, and the product was acidified to pH 4 and extracted with ethyl acetate (3x). The organic layers were combined, dried over magnesium sulfate, and concentrated by rotary evaporation. The compound was purified by silica column chromatography (5% methanol/ chloroform). Yield = 58% (310 mg, 0.64 mmol): ^1H NMR ($CDCl_3$) δ 0.9 (d, 6H),

1.5 (t, 2H), 1.7 (m, 1H), 3.7 (s, 3H), 3.9 (s, 2H), 3.4 (t, 1H), 4.5 (d, 2H), 6.3 (dd, 1H), 6.5 (d, 1H), 6.8 (d, 1H); PDMS $[M+H]^+$ = 489.0 (calc = 487.5)

N-(t-Butoxycarbonyl)-O-benzyl-leucine (6). N-*t*Butoxycarbonylleucine (3.7 g, 15 mmol) was suspended in DMF. Sodium bicarbonate (3.78 g, 45 mmol) and benzyl bromide (2.7 mL, 22.5 mmol) were added, and the reaction was stirred overnight. H_2O was added, and the product was extracted into ether (3x). The organic layers were combined, washed with 1N HCl (3x), and dried over magnesium sulfate. The solution was concentrated by rotary evaporation and chromatographed on silica gel (2.5% to 10% ethyl acetate/hexane). Yield of the clear oil was 92% (4.42 g, 13.8 mmol): ^1H NMR ($CDCl_3$) δ 0.9 (dd, 6H), 1.4 (s, 9H), 1.7 (m, 3H), 4.3 (t, 1H), 5.1 (d, 2H), 7.3 (s, 5H).

O-benzylleucine (7). Into 3N HCl/ethyl acetate (20 mL) was dissolved **6** (4.42 g, 13.8 mmol). The reaction was stirred for 2 h, after which the reaction was brought to pH 10 with 3M NaOH. The product was extracted with ethyl acetate (3x). The solution was dried over magnesium sulfate, and concentrated by rotary evaporation to a clear oil (2.9 g, 13.1 mmol, 95% yield). The product was pure by NMR, and used without further purification: ^1H NMR ($CDCl_3$) δ 0.9 (dd, 6H), 1.7 (m, 3H), 4.0 (t, 1H), 5.1 (d, 2H), 7.3 (m, 5H).

N-(2-hydroxy-4-methoxy)-O-benzylleucine (8). Sodium cyanoborohydride (6.24 mg, 10 mmol) and **7** (1.46 g, 6.6 mmol) were added to a solution of N-hydroxy-4-methoxy-benzaldehyde (1 g, 6.6 mmol) in anhydrous methanol. The pH was adjusted to 7 with glacial acetic acid. Hot molecular sieves were added, and the reaction was purged with argon. The reaction was stirred under argon for 48 h, during which the orange solution formed a precipitate. The

reaction was quenched with a solution of saturated sodium bicarbonate, and extracted with ether (3x). The organic layers were combined, dried over magnesium sulfate, and concentrated. The residue was chromatographed twice on silica gel (2.5% ethyl acetate/chloroform), to give a 50% yield (1.2 g, 3.3 mmol): ^{1}H NMR ($CDCl_3$) δ 0.9 (dd, 6H), 1.5 (t, 1H), 1.75 (m, 1H), 3.4 (t, 1H), 3.75 (s, 3H), 3.8 (dd, 2H, J=75 Hz), 5.2 (d, 2H), 6.3 (dd, 1H), 6.5 (d, 1H), 6.8 (d, 1H), 7.4 (s, 5H).

N-Fmoc-(O-t-butyl)-threonine-N-(2-hydroxy-4-methoxy-benzaldehyde)-O-benzyl-leucine (9). N-Fmoc-(O-t-butyl)-threonine (250 mg, 630 μmol) was dissolved in methylene chloride. Cyanuric fluoride (54 μl, 630 μmol) and pyridine (76 μl, 945 μmol) were added to the solution; the solution was stirred for 3 h. Ice water was added, and the organic layer was retained. The organic layer was reduced by rotary evaporation. The acid fluoride was redissolved in methylene chloride, and added to 120 mg of **8** (336 μmol) in methylene chloride. A 10% solution of sodium bicarbonate (1.6 mL) was quickly added. The reaction was stirred overnight. The aqueous and organic layer were separated, and the organic layer was reduced by rotary evaporation. The reaction was chromatographed on silica gel (5% ethyl acetate/methylene chloride), but was not completely purified. The product (90 mg) was too difficult to purify, and was used without further purification: PDMS $[M+H]^+$ = 737.5 (calc = 736.9).

N-Fmoc-(O-t-butyl)-threonine-N-(2-O-acyl-4-methoxy-benzaldehyde)-O-benzyl-leucine (10). The impure dipeptide **9** (90 mg) was dissolved in methylene chloride and treated with acetic anhydride (60μl, 0.6 mmol), DIEA (102 μl, 0.6 mmol), and a catalytic amount of DMAP. After 30 min, the reaction was washed with saturated sodium bicarbonate (1x) and with 1 N HCl (1x). The organic layer

was dried over magnesium sulfate, concentrated, and chromatographed on silica gel (5% ethyl acetate/methylene chloride). 55 mg (71 μmol, 21% total yield from **8**) was isolated: ^{1}H NMR ($CDCl_3$) δ 0.9 (dd, 6H, J = 25 Hz), 1.0 (d, 3H), 1.2 (s, 9H), 1.7 (m, 2H), 2.0 (m, 1H), 2.2 (s, 3H), 3.7 (s, 3H), 4.0-4.4 (m, 5H), 4.7 (m, 1H), 5.1-5.4 (m, 3H), 6.5 (m, 3H), 7.3 (s, 5H), 7.3-7.8 (m, 9H); PDMS $[M+H]^+$ = 780.1 (calc = 778.9).

N-Fmoc-(O-t-butyl)-threonine-N-(2-O-acyl-4-methoxy-benzaldehyde)-leucine (11). Dipeptide **10** (55 mg, 71 μmol) was dissolved in ethanol containing a few drops of ethyl acetate and 1 drop of glacial acetic acid. A catalytic amount of Pd/C was added and the reaction was purged with H_2. After 3 h under H_2, the Pd/C was filtered and rinsed with ethyl acetate. The filtrate was reduced with the addition of cyclohexane to yield pure white crystals (36 mg, 52 μmol, 74% yield): ^{1}H NMR ($CDCl_3$) δ 0.73 (dd, 6H, J = 18, 42 Hz), 1.09 (s, 3H), 1.16 (s, 9H), 1.5 (m, 2H), 1.8 (m, 1H), 2.25 (s, 3H), 3.6 (s, 3H), 3.8 (d, 1H), 3.9 (t, 1H), 4.2 (t, 2H), 4.4-4.6 (m, 5H), 6.7 (m, 2H), 6.9 (d, 1H), 7.3 (m, 5H), 7.7 (dd, 4H, J = 8.8, 42 Hz).

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Chapter 2

Structural and Functional Studies of NACP

Introduction

The major constituent of amyloid plaques in Alzheimer's disease is the Aβ protein. NAC (non-Aβ component of AD amyloid), a peptide of at least 35 amino acids in length, has also been determined to be an intrinsic component of amyloid. The core of amyloid plaques may consist of up to 10% of NAC[1]. Immunohistochemical studies have shown that other proteins, such as α1-antichymotrypsin[2], apolipoprotein E[3], and many others, have been found to be associated with the core of the amyloid plaque. NAC, however, is unique in that it has been copurified from the SDS-insoluble fraction of amyloid plaques.

NAC is a fragment of NACP (NAC-precursor) which is a 140 amino acid protein, with an expected molecular weight of 14,460. NACP purified from human brain tissue has a molecular mass of 14,681, indicating that native NACP may have a post-translational modification[4]. The amino-terminal amino acid of NACP is unamenable to Edman degradation, and is therefore possibly modified. A myristoylation (MW 211) may be indicated. There are no known signal sequences, glycosylation sites, or phosphorylation motifs in the NACP sequence. However, there is evidence that a highly homologous protein, bovine phosphoneuroprotein 14 (PNP14), is phosphorylated[5].

NACP, also called α synuclein, is highly homologous to *Torpedo* synuclein[6], rat synuclein 1,2, and 3[7], bovine phosphoneuroprotein 14 (PNP-14)[5], canary synelfin[8], and human β synuclein[4]. There are two major isoforms in this family,

differing in their C-termini. NACP, canary synelfin, and rat synuclein 1 share the same acidic C-terminus; Human β synuclein and PNP-14 do not contain the NAC sequence (Figure 1).

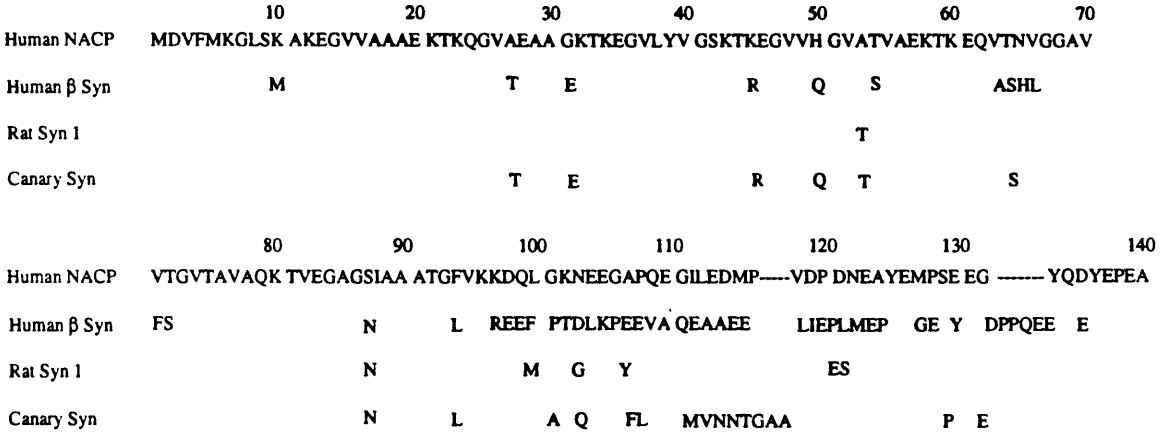

Figure 1. Sequence of the various homologous forms of NACP; NAC is underlined. The full sequence for NACP is shown; only different amino acids were shown for β synuclein, rat synuclein 1, and canary synelfin.

All forms of synuclein share a very similar N-terminus, containing a KTKEGV motif, which occurs 5-6 times, within an 11 amino acid periodicity. It has been suggested that this stretch of the protein forms an amphipathic helix capable of associating with lipids[8].

The synucleins are primarily expressed in nervous tissue, and are found in relatively small amounts in the other organs of the body [1,6,9]. Immunohistochemistry studies show that rat synuclein expression is highest in the pyramidal cell layer of the hippocampus[7], which is a major site of AD caused neurodegeneration: the process of amyloid deposition begins along the projections of the pyramidal neurons within the hippocampus and parahippocampal structures[10]. NACP has been found to be most enriched in the telencephalon, which includes the hippocampus and cerebral cortex[11].

NACP is an intracellular protein, localized to the presynaptic terminal[11]. Although there has been speculation that NACP is a synaptic vesicle associated protein[11], NACP exists mostly as a soluble protein, and it is unclear if NACP is associated with lipid membranes[8].

We are interested in the relationship between NACP and Alzheimer's disease. NAC *in vitro* forms ordered fibrils, which can be classified as amyloid. [12] Similar to the *in vitro* aggregation of β1-40, the aggregation of NAC was found to be nucleation dependent and could be seeded with preformed NAC fibrils. NAC amyloid fibrils were able to seed the aggregation of β1-40; fibrils made of β1-40 were also able to seed the aggregation of NAC.

It is possible that, *in vivo*, accumulated NAC aggregates seed the aggregation of β1-40, causing the neurodegeneration associated with Alzheimer's disease. Alternatively, β1-40 may seed the aggregation of NAC, and the loss of NACP may be the cause of the disease.

The function of NACP may well be related to memory and learning: the mRNA of NACP is upregulated in canaries during a critical period of song learning[8]. Other than its immunohistochemical location, and involvement in canary learning, little else is known about NACP. This lab is currently involved with studying NACP and peptides from NACP, with the hope that these studies will lead to insight about its relationship to neurological diseases. We describe, here, research on the structure and function of NACP.

Purification and Properties of NACP

A pRK172 plasmid containing the NACP expression sequence[4] was obtained from Michel Goedert. The plasmid was transformed into BL21(DE3) competent cells, and overexpressed. The bacteria were lysed in a french press, and centrifuged.

The supernatant was treated with streptomycin sulfate to precipitate the DNA, and then boiled at 100°C for 5 min. Previous studies[4,13] had indicated that NACP stayed soluble after heat treatment, while most other proteins denatured and precipitated. Final purification was performed by Paul Weinreb and was achieved by gel filtration.

Consistent with previous observations[4,6], recombinant NACP migrates as a 19 kDa protein in SDS-polyacrylamide gels. This behavior is attributed to the highly acidic nature of the C-terminus[4], which affects binding of SDS to the protein.

The CD of NACP (Paul Weinreb, unpublished results) indicated that this preparation of NACP lacked secondary structure. The heat treatment, however, may have denatured the protein, and it was thus necessary to purify the protein without having first boiled it.

NACP was overexpressed in bacteria, which were lysed and treated with streptomycin sulfate. Ammonium sulfate was added to 46.6% saturation. The pellet was redissolved in lysis buffer and loaded onto an S-300 size-exclusion column. Although the protein is 14.4 kDa, it eluted at approximately 55 kDa. The final purification step was a CL-6B DEAE Sepharose column, in which the protein eluted at approximately 200 mM NaCl. The fractions were dialyzed against distilled water and the protein was quick frozen in dry ice-acetone and lyophilized.

We were interested in the possibility that synuclein could be eluting from the size exclusion column as an oligomer. Nakajo, *et al.* found that PNP-14 eluted at 57 kDa by gel filtration; they interpreted this to mean that PNP-14 exists as a tetramer in saline solution at neutral pH. In our hands, purified boiled NACP, however, also eluted at 55 kDa when run on the S-300.

Ferguson plots of boiled and unboiled NACP showed that the protein is likely to be a monomer, not an oligomer (Figure 2). In the electrophoresis of proteins in a native gel, there is a linear relationship between K_r (the slope of a plot of $\log_{10}$

relative mobility vs gel concentration for any particular protein) and the molecular mass of native proteins[14]. By constructing a plot of K_r vs molecular weight for a series of known proteins, the molecular weight of an unknown protein can be determined from a series of native gels.

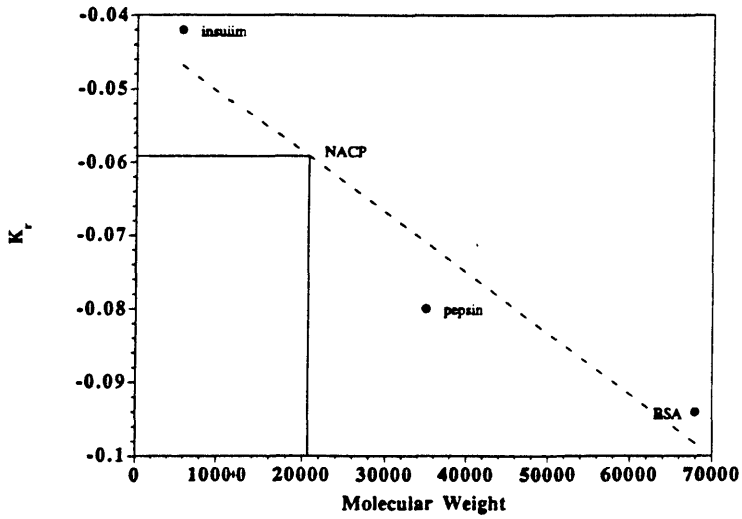

Figure 2. Ferguson Plot (K_r vs MW) of insulin, BSA, and pepsin. NACP (K_r = -.061) plots to an approximate MW of 20,000.

The accuracy of Ferguson plots are highly dependent upon the similarity in shape and hydrodynamic radius between the protein of interest, and protein standards. We, however, needed only a rough approximation of the size of NACP, and therefore chose readily available proteins as our standards.

CD's of the boiled and unboiled NACP were identical (Figure 3). Recombinant NACP is random coil. Although we were initially surprised that NACP was a heat stable random coil protein, there are many other proteins which have these same characteristics (tau[15], chromogranin A[16], several phosphatase inhibitors[17,18], and others). None are enzymes; this is consistent with the amino acid sequence of NACP: a protein containing such an extensive amino acid repeat would most likely be a structural protein or binding protein, not an enzyme.

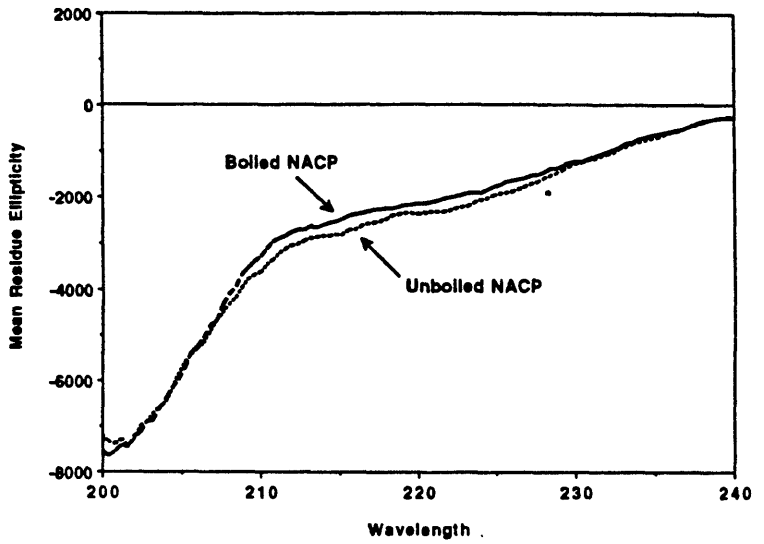

Figure 3. CD of boiled vs. unboiled NACP (courtesy of Paul Weinreb).

Affinity Chromatography

We are interested in understanding the relationship between Alzheimer's disease, amyloid, and NACP. We believe that a significant step would be taken if the function of NACP were known. Towards this end, we have attempted to isolate binding proteins for NACP via affinity chromatography.

Affinity chromatography is a fairly standard technique used to isolate binding proteins for a particular ligand[19]. In general, a ligand is affixed to a solid support (usually Sepharose 4B), and a mixture is passed over the beads. A binding protein for the ligand will be selectively retained on the column; the protein can be desorbed from the column after unbound material has been washed away. The ligand should have a K_d for the binding protein in the range of 10^{-4} to 10^{-8} in free solution[19]; a limitation of this technique is that a low affinity binding protein to NACP will not be identified.

Currently, many affinity columns are generated through the use of GST-fusion proteins, or biotin-strepavidin systems. Covalent attachment of the ligand to the column, however, is the traditional way of making a column, and there have been many successes[20].

Proteins are usually covalently attached to Sepharose 4B by primary amino groups or thiol groups. If neither is available, a protein may be attached by carboxyl groups. NACP contains no cysteines; it, however, has many lysines. Cyanogen bromide (CNBr) activated Sepharose 4B is a commercially available product (Pharmacia Biotech) for convenient attachment of primary amino groups to Sepharose (Figure 4). There is, however, the possibility of ligand leakage, via nucleophilic attack on the isourea group. Multipoint attachment of the ligand to the Sepharose is therefore advisable. NACP has many lysines by which multipoint attachment may be achieved.

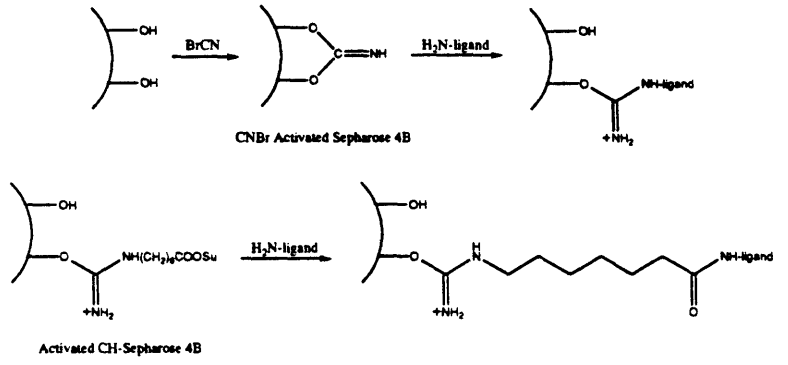

Figure 4. Structure of CNBr activated Sepharose 4B and Activated CH-Sepharose 4B, and their attachment to ligands.

Multipoint attachment, however, may cause problems if the binding region of NACP is blocked from interacting with the binding protein. It may, therefore, be useful to work with beads containing a low concentration of NACP.

Steric hindrance can also be a problem when a protein is held too closely to the surface of the bead for its binding site to be accessible. It is common to employ

spacer arms; a drawback to the use of spacer arms is that they can become sites of non-specific binding, increasing the background of an affinity experiment. A commercially available resin from Pharmacia Biotech is CH-Sepharose 4B (Figure 4).

A major drawback to the covalent attachment of NACP to Sepharose is the location of the lysines in the protein sequence; most of the lysines are clustered in the N-terminal repeat region. If the binding site is in the region, binding proteins will most likely not be isolated.

There are two general methods for performing the chromatography: column or batch[19]. Unlike the column method, the batch method can be done on a very small scale. It is a useful way of beginning affinity experiments.

Recombinant NACP (purified by boiling) was fixed to CNBr-activated Sepharose 4B. The substitution level was approximately 18 μg NACP/mg beads. Control beads were generated in the same fashion with lysozyme as the ligand. Lysozyme was chosen because the protein is similar in molecular weight to NACP, and is very inexpensive. In addition, NACP was attached to activated CH-Sepharose 4B at a substitution level of 20 μg NACP/mg beads. Lastly, beads with a low substitution of NACP was prepared, at a level of 4 μg NACP/mg beads. Controls (beads substituted with lysozyme) were generated for both sets of beads. Substitution levels of the beads were estimated by examining the concentration of uncoupled protein in the coupling washes.

Rat brain cytosol (from Liu lab) was incubated with the high substitution-CNBr beads, after which they were isolated via centrifugation. The beads were washed in one of two ways: 1) with lysis buffer; or 2) with lysis buffer, and then with buffers of increasing salt concentration. The beads were then boiled in 1 x SDS gel loading buffer and run on polyacrylamide gel. Candidate proteins were difficult to identify due to the high background of the gels. Repeated washings to reduce the background of the gels did not help.

Even if a candidate protein is identified, it is necessary to show that the protein can be released from the beads by washing with the ligand (NACP). Competition experiments were performed on the beads, yet no candidate proteins were identified, again due to high background.

The same set of experiments was performed with the other sets of beads. Although high background was not a problem with these beads, no candidate proteins were seen on these gels.

There is the possibility that the binding protein of NACP is membrane associated. Bovine brain membrane fractions were obtained from the Liu lab and experiments were performed with all three sets of beads. No proteins were identified.

High background difficulties can possibly be avoided by performing these experiments in a column, and repeatedly passing large amounts of homogenate through the column. The column may become saturated with proteins of interest, thereby reducing the high background of these experiments. It is also possible that these experiments are inherently flawed: NACP is attached to the Sepharose through the lysines contained in the repeat region of the N-terminus. If binding occurs there, these experiments will not identify any ligands.

The C-terminus of NACP contains many carboxylic acid groups, which can be used for attachment to the Sepharose. Unfortunately, all commercial preparations of such beads are based upon amide bond formation, between an amino group on the resin and an activated carboxylic acid group. NACP has many lysines; it would be very difficult to prevent oligomers of NACP from forming during the activation procedure.

Affinity chromatography with recombinant NACP may not be an appropriate method for discovering binding proteins. Native NACP may have a post-

translational modification which is critical for ligand binding. For this reason, immunoprecipitation from rat brain homogenate may be a better technique.

Immunoprecipitation

An alternative technique to affinity chromatography for finding a protein's ligand is immunoprecipitation[21]. In this technique, an antibody to NACP would be allowed to bind to NACP in rat brain homogenate. If the antibody is polyclonal, a large number of antibody molecules may bind to the protein; the antibody-NACP combination may precipitate and can be collected by centrifugation. The use of a mixture of monoclonal antibodies, specific for different epitopes, can also cause immunoprecipitation. It is hoped that any protein bound to NACP would also be precipitated in the process, thus isolating any ligands of NACP. Only tight binding ligands will likely be isolated.

The monoclonal antibody H3C (mouse IgG1) was provided by David Clayton in the form of reconstituted mouse ascites[8]. It is an antibody to the canary C terminal sequence YEMPPEEEYQDYEPEA. The rat C terminal sequence only differs by two amino acids (YEMPSEEGYQDYEPEA), and is recognized by the H3C antibody (Figure 6). Because the antibody is specific to the C-terminus of NACP, we can investigate the possibility that the N-terminus is involved in ligand binding; this was not possible with the affinity chromatography experiments.

This antibody was thus used as the primary antibody in all immunoprecipitation experiments. Monoclonal antibodies are usually not desirable for immunoprecipitation because only one antibody molecule can bind to each NACP molecule. Isolation of the antibody-NACP complex is achieved through one of two methods:

1) an excess of polyclonal antibody, specific to the primary antibody, is added; or 2) Protein A-Sepharose or Protein G-Sepharose is added.

Protein A and Protein G are cell wall proteins of specific bacteria which bind to the constant region (F_C) of antibodies[21]. The two proteins have different affinities for antibodies, depending upon their species and subclass. Protein G has a higher affinity for mouse IgG1, and was the reasonable choice for precipitating agent. Because Protein G contains a second binding site which will bind to albumin, GammaBind Plus Sepharose (Pharmacia Biotech) was used in all experiments. Gammabind Plus Sepharose has been engineered to not bind to albumin. Albumin binding is undesirable, because the background of any experiment would be heightened.

The cytosolic protein of rat frontal cortex consists of as much as 0.5% - 1% of NACP[11]. Ten stripped whole rat brains were homogenized and separated into cytosolic and membrane fractions. The cytosolic total protein concentration of prepared rat brain cytosol was 6.8 mg/mL as assayed by BCA. Estimating that the rat frontal cortex consists of 25% of the total rat brain mass, it was estimated that between 100 µg and 200 µg of H3C per mL rat brain cytosol was necessary for efficient immunoprecipitation. Through analyzing Western blots, it was estimated that the concentration of H3C ascites was approximately 100 mg/mL, but could range between 40-200 mg/mL. Through these calculations, the quantity of antibody required per mL of rat brain cytosol was estimated.

3 µl of H3C was used per mL of rat brain cytosol in order to form the 1° antibody - NACP - ligand complex. 50 µl of Protein G beads were added (beads have an antibody capacity of 18 mg/ml) to isolate the complex. The beads were boiled in 1 X SDS loading buffer to generate sample G1. In order to control for nonspecific binding, two controls were run. Rat brain cytosol was incubated with Protein G beads (control 1 beads, C1); this controls for nonspecific binding to the Protein G

beads. To control for nonspecific binding to the constant regions of IgG1 and to control for proteins contained in mouse ascites, rat brain cytosol was incubated with mouse monoclonal α-Neurofilament 200 (Sigma, ascites), before isolation with Protein G beads (control 2 beads, C2). α-Neurofilament 200 was chosen as a control antibody, because it is an mouse IgG1 against a common brain protein.

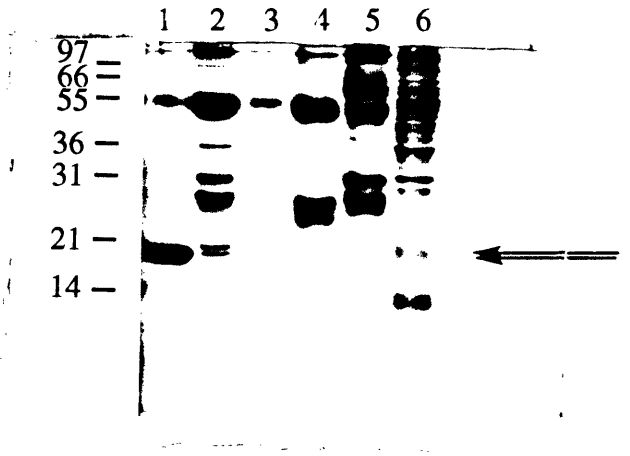

Figure 5. 12 % polyacrylamide gel. Lanes: 1, recombinant NACP; 2, experimental G1; 3, control C1; 4, control C2; 5, antibody H3C; 6, rat brain cytosol. Comparison of lane 2 with 3-6 show that a doublet band (arrows) at MW 19 and 20 kDa is selectively immunoprecipitated in G1.

Comparison of lane 2 (G1) against lanes 3, and 4 (C1, C2), and 5 (H3C) show that a doublet 19 and 20 kDa protein is specifically precipitated when the H3C antibody is used (Figure 5). The bottom band runs at the same level as recombinant NACP; Western blotting with H3C as the 1° antibody shows that the doublet band is specifically recognized by H3C (Figure 6, lane 2). Iwai, *et al.* also noted that their antibodies to NACP recognized a doublet band in both human and rat homogenates[11]. They speculate that the higher molecular weight is NACP with a post-translational modification. However, post-translationally modified human NACP

has previously been observed to comigrate with recombinant NACP[4]. It is unlikely that the higher molecular weight band is either rat synuclein 2 or 3, because they do not contain the proper epitope. The high molecular weight band may be an as-yet-undiscovered alternatively spliced form of rat synuclein 1.

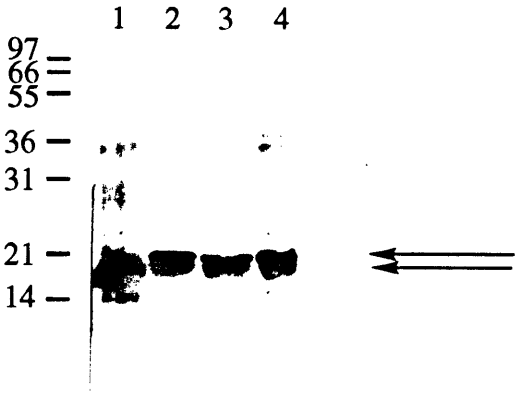

Figure 6. Western blot analysis, detected with anti-NACP monoclonal antibody H3C. Lanes: 1, recombinant NACP; 2, experimental G1; 3, experimental IP1; 4, rat brain cytosol. Arrows indicate doublet band of NACP.

Examination of a 7% acrylamide gel (Figure 7) shows that, in lane 2 (G1) there is a band at approximately 96 kDa which does not exist in any control lanes (3, 4, and 5). By Western blotting, this 96 kDa protein does not react with the H3C antibody (Figure 6). Interestingly, Nakajo, *et al.* perform an immunoprecipitation with polyclonal α-PNP-14, and coprecipitated two proteins along with PNP-14: an 82 kDa protein, and a 96 kDa protein[5]. The 96 kDa protein may therefore be an NACP binding protein.

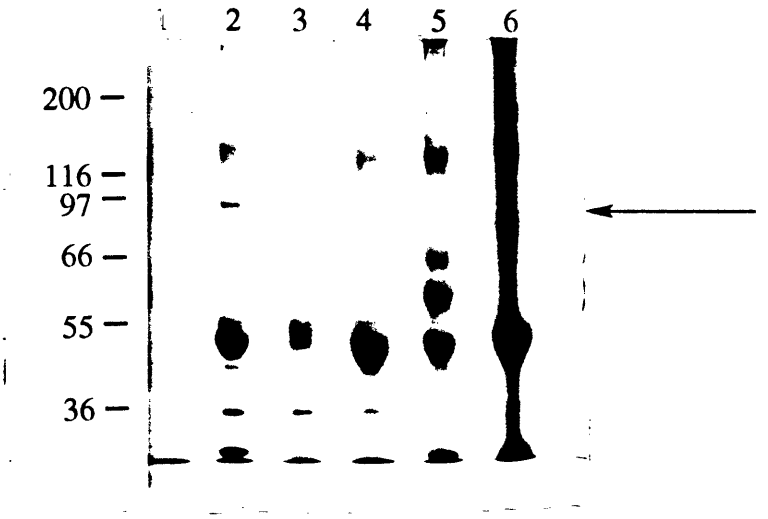

Figure 7. A 7% polyacrylamide gel. Lanes: 1, recombinant NACP; 2, experimental G1; 3, control C1; 4, control C2; 5, antibody H3C; 6, experimental IP1. Comparison of lanes 3-5 with lane 2 show that a 96 kDa protein (arrow) was selectively immunoprecipitated in G1.

If a different method than Protein G was used to isolate the complex, and the 96 kDa protein was again isolated, the 96 kDa protein would be a viable candidate ligand. Isolating the 96 kDa protein in two different ways would reduce the likelihood that the protein is non-specifically binding to the Protein G beads. Immunoprecipitation, therefore, using an excess of goat α-mouse IgG to precipitate the H3C-NACP-ligand complex was attempted. Rat brain cytosol was incubated with H3C, after which, an excess of goat α-mouse IgG was added (estimated to be 15 fold excess). White precipitate formed, which was pelletted and isolated. The pellet was dissolved in 1 X SDS gel loading buffer, to generate sample IP1. Attempts to achieve good resolution on the gels was not successful. The high concentration of IgG relative to all other proteins caused streaking (Figure 5, lane 6), making it impossible to ascertain the presence or absence of a 96 kDa band. Western blotting, however, indicated that NACP was precipitated (Figure 6, lane 3).

In order to identify the 96 kDa protein, G1 was electrophoresed on a 6% acrylamide gel. The separated proteins were blotted onto PVDF, and the membrane was stained with Commassie Blue R-250. The 96 kDa protein was submitted for N-terminal amino acid sequencing.

Future Experiments

If a sequence is obtained from the immunoblot, then the protein may be identified from a search of the protein sequence data banks. Whether the protein is identified, or is an unknown protein, we must prove that the immunoprecipitation of the 96 kDa protein is not simply an artifact. Probably the best way to do this is to make an antibody to the 96 kDa protein, and immunoprecipitate the 96 kDa protein out of rat brain cytosol. If NACP is co-precipitated with the 96 kDa protein, then we can put more confidence into the designation of the protein as an NACP-binding protein. Polyclonal antibodies can be made easily: the protein can be purified by SDS-polyacrylamide gel electrophoresis and injected into rabbits.

Another experiment which must be performed is the identification of the post-translational modification of NACP. Small amounts of NACP can be purified from rat brain cytosol by performing an immunoprecipitation, and isolating the doublet NACP from the rat cytosol. The NACP can be purified in the immuno-globulins by separating the mixture on SDS-polyacrylamide gels and blotting onto PVDF. The NACP doublet can be isolated, and eluted separately from the membrane. The quantities obtained from a membrane should be adequate for mass spectrometry. In addition a tryptic digest can be performed, and the site of the post-translational modification can be identified from the molecular weight of the fragments.

Experimental Section

Equipment, Materials, and Methods

All chemicals and materials were purchased from Sigma unless otherwise noted. Rat brains were obtained from Pelfreez. BL21(DE3) competent cells were purchased from Novagen. Monoclonal α-NACP H3C was obtained from David Clayton. The plasmid containing the NACP expression vector was obtained from Michel Goedert. All beads for affinity chromatography, gel filtration, ion-exchange, and immunoprecipitation were purchased from Pharmacia Biotech.

Gel Electrophoresis

Polyacrylamide gels were poured and run according to the method of Laemmli[22]. Native gels were poured and run according to established methods[23]. All gels were visualized by Commassie Blue R-250. All gels were run in a Novex mini-cell system.

Western Blotting

Proteins were electrophoresed on SDS-polyacrylamide gels then blotted onto PVDF in a TE 70 SemiPhor Semi-Dry Transfer Unit. The proteins were transferred according to established procedure[24]. The proteins were transferred at 100 mA for 45 min. Prestained molecular weight markers were always run in the gel to check the efficiency of transfer. After the transfer, the membrane was incubated with blocking buffer (1% BSA in 50 mM Tris, 150 mM NaCl, pH 7.5) for 15 min. The membrane was then incubated with primary antibody (H3C, 1:100,000 dilution) in blocking buffer for 2 h. The membrane was washed with washing buffer (50 mM Tris, 150

mM NaCl, pH 7.5) for 1 x 1 min, 3 x 5 min, and then incubated with secondary antibody (goat anti-mouse IgG conjugated to alkaline phosphatase, Sigma A-1682, 1:4000) in blocking buffer for 1 hour. The membrane was again washed with washing buffer (1 x 1 min, 3 x 5 min). The membrane was developed with a solution of BCIP/NBT (Sigma Fast BCIP/NBT, B-5655). Staining for total protein on membranes was done with Commassie Blue R-250.

Expression of NACP

A pRK172 plasmid containing the NACP expression sequence[4]. was dissolved in TE and added to 25 µl of competent BL21(DE3) cells. The cells were stored on ice for 30 min, heat shocked at 42°C for 30 s, then let sit at RT for 2 min. 500 µl of Luria-Bertani Broth (LB) containing 0.2% glucose was prewarmed to 37°C and added to the cells. After incubation at 37°C for 1 h, 200 µl of the bacteria were plated onto ampicillin positive agar plates[25]. The bacteria was grown for 17 h at 37°C. Control plate (competent cells without the plasmid) did not grow colonies. Two colonies were chosen. Each colony was used to inoculate a stab culture[25], which was grown for 36 h and stored at -80°C. All cultures were henceforth grown from stab culture A.

10 mL of LB, containing 100 µg/mL of ampicillin, was inoculated from stab culture A, and shaken at 37°C for 12 h. 2 x 500 mL of LB (with 50 µg/mL ampicillin) were each inoculated with 5 mL of the culture. The 500 mL cultures were grown at 37°C until absorption equaled 0.6-1.0 at 600 nm (blanked against LB). The cultures were induced with isopropyl-1-thio-β-galactoside (IPTG) to a final concentration of 0.5 mM, and shaken for 2 h at 37°C. The cultures were centrifuged at 11,000 x g for 30 min. The cells were frozen in dry ice-acetone and stored at -20°C.

Purification of NACP from *E. coli*

The frozen cells were thawed at 4°C, and resuspended in working buffer (WB, 50 mM Tris-HCl, 0.1 mM DTT, 0.1 mM PMSF, pH 7.4). The cells were lysed in a french press at 16,000 psi. The lysate was centrifuged at 14,000 x g for 30 min. The supernatant was removed, and streptomycin sulfate was added (0.2 volumes of 5% streptomycin sulfate in working buffer). After stirring on ice for 15 min, the lysate was centrifuged at 24,000 x g for 30 min. The supernatant was removed and heated in a boiling water bath for 10 min. The boiled lysate was centrifuged at 24,000 x g for 30 min. The supernatant was loaded onto a 25 g of Biogel P-10 and eluted with WB. The protein eluted in the void volume, but was acceptably pure to use.

Purification of NACP from *E. coli* Without Boiling Step

The frozen cells were thawed, lysed, and treated with streptomycin sulfate as above. Ammonium sulfate was added to 46.6% saturation and stirred 1 h at 4°C. The suspension was centrifuged at 10,000 x g for 10 min. The pellet was resuspended in WB and loaded onto a gel filtration column (S-300). NACP was eluted with working buffer; V_e/V_o approximately equaled 1.5. All fractions containing NACP (assayed by gel electrophoresis) were combined; the concentration of the WB was increased to 100 mM Tris, and 50 mM NaCl. The fractions were loaded onto 40 mL of CL-6B DEAE Sepharose gel and eluted with a NaCl gradient (50 mM to 312 mM NaCl, over 300 mL). NACP eluted at approximately 200 mM NaCl. All fractions containing NACP were combined and dialyzed against distilled H_2O (24 h, 2 changes of H_2O). The fractions were frozen in dry ice/acetone, lyophilized, and stored at -20°C.

Ferguson plots

Native gels were poured of 6%, 10% and 14% acrylamide concentration. BSA (68 kDa), pepsin (35 kDa), insulin (5.7 kDa), and NACP (14.5 kDa) were dissolved in 1X native gel loading buffer and run on each gel.

Preparation of Affinity Beads of High Protein Substitution

250 mg of cyanogen bromide (CNBr) activated Sepharose 4B were washed, over a fritted funnel, with 50 mL of 1 mM HCl. The gel was washed with 1.25 mL of coupling buffer (100 mM $NaHCO_3$, 500 mM NaCl, pH 8.3), then immediately added to a solution of 0.42 µmol of protein (NACP or lysozyme) in 2 mL of coupling buffer. The gel was rotated slowly at 4°C for 21 h. The beads were filtered and washed with 2 mL of coupling buffer. Approximate concentration of uncoupled protein in the filtrate was assayed by UV. The gel was resuspended in 3 mL of blocking buffer (100 mM $NaHCO_3$, 500 mM NaCl, 200 mM glycine, pH 8.0) and rotated at room temperature for 2 h. The beads were washed alternately, 4 or 5 times, with coupling buffer and acetate buffer (100 mM NaOAc, pH 4). The beads were washed with PBS (50 mM potassium phosphate, 500 mM NaCl, pH 7.2) and stored at 4°C in 1 mL PBS (total volume approximately 1.8 mL).

Preparation of CH-Sepharose 4B Based Affinity Beads

100 mg of activated CH-Sepharose 4B were washed with 10 mL of 1 mM cold HCl over a fritted funnel. The beads were washed with 200 µl of coupling buffer (100 mM $NaHCO_3$, 500 mM NaCl, pH 8.0), then added to 0.16 µmol of protein (NACP or lysozyme) in 200 µl of coupling buffer. The beads were rotated for 1 h at

RT, then filtered and washed with coupling buffer. Approximate concentration of uncoupled protein in the filtrate was assayed by UV. The beads were blocked with Tris-HCl buffer (100 mM Tris, 500 mM NaCl, pH 8.0) and rotated for 1 h at RT. The beads were filtered and washed alternately, 5 times, with Tris-HCl buffer then acetate buffer (100 mM, pH 4). The resin was stored in 400 µl of Tris buffer (20 mM Tris, 100 mM NaCl, 2 mM DTT, pH 6.3).

Preparation of Affinity Beads of Low Protein Substitution

50 mg of CNBr activated Sepharose 4B was washed with 10 mL of 1 mM HCl, followed by 1 mL of coupling buffer (100 mM $NaHCO_3$, 500 mM NaCl, pH 8.3). The beads were added to 0.5 mL of coupling buffer and rotated. After 5 h, 0.024 µmol of protein (NACP or lysozyme) in 200 µl of coupling buffer was added to the beads. After 16 h, the beads were filtered and washed with coupling buffer. Approximate concentration of uncoupled protein in the filtrate was assayed by UV. The gel was resuspended in blocking buffer (100 mM $NaHCO_3$, 500 mM NaCl, 200 mM glycine, pH 8.0) and rotated at room temperature for 2 h. The resin was stored in 200 µl of Tris buffer (20 mM Tris, 100 mM NaCl, 2 mM DTT, pH 6.3).

Affinity Experiments with Rat Brain Cytosol

100 µl of bead slurry (experimental or control) was placed into 1.5 mL microfuge tubes and washed 4 times with Tris buffer (20 mM Tris, 100 mM NaCl, 2 mM DTT, pH 6.3) by centrifuging and removing the supernatant. 800 µl of rat brain cytosolic extract (1 mg/mL) was added and the tubes were rotated at 4°C for 4 h. The tubes were centrifuged and supernatant removed (control supernatant (CS) and experimental supernatant (ES)). Beads were washed in one of three different ways:

1) washed once with Tris buffer; 2) washed successively with Tris buffer of increasing salt concentration (100 mM, 200 mM, 300 mM); or 3) washed once with Tris buffer, then twice with a 5 mg/mL solution of lysozyme or NACP. Beads (control beads (CB) and experimental beads (EB)) were boiled in 100 µl of 1X SDS gel loading buffer.

Preparation of Membrane Fraction for Affinity Experiments

1 mL of bovine brain membrane fraction (10-12 mg/mL total protein, gift of Liu lab) was washed with 6 mL of buffer (25 mM Tris, 50 mM KCl , 5 mM MgCl, 1 mM EDTA, pH 7.4) and centrifuged at 108,000 x g in a T-100 mini ultracentrifuge for 1 h at 4°C. The pelleted membrane was resuspended in 6 mL of buffer containing 0.32% Triton X100 and 1 mM PMSF. The suspension was homogenized with 10 strokes of a Dounce homogenizer, after which, it was rotated at 4°C for 4 h. The suspension was centrifuged at 108,000 x g for 1 h at 4°C. The concentration of the supernatant was 0.8 mg/mL total protein assayed by BSA. The supernatant was used for affinity chromatography experiments.

Affinity Experiments with Bovine Brain Membrane Fractions

150 µl of bead slurry (experimental or control) was placed into 1.5 mL microfuge tubes and washed 4 x with solubilization buffer (25 mM Tris, 50 mM KCl , 5 mM MgCl, 1 mM EDTA, 0.32% Triton X100 , 1 mM PMSF, pH 7.4). 800 µl of bovine brain membrane extract was added and the tubes were rotated at 4°C for 4 h. The tubes were centrifuged and supernatant removed (control supernatant (CS) and experimental supernatant (ES)). Beads were washed as described above. Beads were boiled in 50 µl of 1X SDS gel loading buffer.

Preparation of Rat Brain Cytosol and Membrane Fractions

Twenty grams of stripped rat brains (10 brains) were thawed at 4°C in 80 mL of homogenization buffer (50 mM Tris-HCl, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM DTT, pH 7.4). Each brain was diced with a clean razor blade in a petri dish lined with parafilm. The rat brains were homogenized with a Polytron, twice for 30 s on setting 5. The suspension was centrifuged at 800 x g for 15 min. The supernatant was centrifuged at 100,000 x g for 1 h. The clear supernatant was removed with a pipette. 52 mL of supernatant was recovered. The concentration of rat brain cytosol was 6.8 mg/mL as determined by BCA. Glycerol was added to the supernatant to a final concentration of 5%. The supernatant was aliquotted in 1 mL fractions. The membrane fraction was resuspended in homogenization buffer (18 mL). Glycerol was added to a final concentration of 5% and aliquotted into 1 mL fractions. All fractions were frozen in dry ice/acetone, and stored at -80°C.

Immunoprecipitation with Protein G Beads

Monoclonal antibody (H3C, mouse IgG1) to NACP was obtained from David Clayton[8]. Rat brain cytosol was thawed at 4°C. 1.35 µl of H3C was added to the 500 µl of cytosol, and it was incubated at 4°C for 2 h. 50 µl of a 1:1 slurry of GammaBind Plus Sepharose was washed twice with homogenization buffer (50 mM Tris-HCl, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM DTT, pH 7.4); the beads were then added to the rat brain cytosol, and incubated, with rotation, for 1 h. The beads were centrifuged and washed 3 times with 150 µl of homogenization buffer. The beads were boiled in 50 µl of 1x SDS gel loading buffer. Two sets of controls were also generated: instead of H3C, either 13.9 µl of monoclonal α-NF200 (Sigma, IgG1, N-0142) or nothing was added.

Immunoprecipitation With Goat anti Mouse IgG

250 µl of rat brain cytosol was treated with 6.75 µl of a 1:10 dilution of H3C. After incubating for 4 h, 1 mL of a 1 mg/mL solution of a goat α-mouse IgG (Sigma, M-8642) was added. The solution was incubated overnight, after which a white precipitate could be seen in the tube. The suspension was centrifuged in a microfuge, and the supernatant was removed. The pellet was washed twice with 1 mL of homogenization buffer (50 mM Tris-HCl, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM DTT, pH 7.4). The pellet was dissolved in 50 µl of 1 x SDS gel loading buffer.

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