

# Evidence that the Precursor Protein of Non-A $\beta$ Component of Alzheimer's Disease Amyloid (NACP) Has an Extended Structure Primarily Composed of Random-coil

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**The precursor protein of the non-A $\beta$  component of Alzheimer's disease amyloid (NACP) is a presynaptic protein of the central nervous system. The physiological function of NACP is not known, but the localization of NACP at presynaptic nerve terminals suggests that it may be involved in the neuronal function. To better understand the physiological function of NACP and its role in the pathogenesis of Alzheimer's disease (AD), the biochemical and biophysical properties of NACP were investigated. The NACP behaves abnormally on FPLC gel-filtration chromatography with the apparent molecular mass of 70 kDa, and the results from chemical cross-linking, limited proteolysis and CD experiments suggest that significant parts of NACP may be unfolded. The abnormal hydrodynamic behavior and extreme protease sensitivity of NACP could result from the extended structure which may be related to the physiological function and pathological role of this protein in the development of AD.**

The precursor protein of the non-A $\beta$  component of Alzheimer's disease amyloid (NACP) is a presynaptic protein of the central nervous system (Iwai *et al.*, 1995a) composed of 140 amino acid residues (Ueda *et al.*, 1993). NACP, also known as  $\alpha$ -synuclein in the human brain (Jakes *et al.*, 1994), is highly homologous to synuclein from Torpedo electroplaques and rat brain (Maroteaux and Scheller, 1991; Maroteaux *et al.*, 1988), and to phosphoneuroprotein 14 of bovine (Nakajo *et al.*, 1993). The primary structure of NACP is characterized by the appearance of six or seven repeated motifs, which have a consensus sequence of KTKEGV, at the amino-terminal region, and of hydrophobic residues in the middle of the protein (Jakes *et al.*, 1994; Ueda *et al.*, 1993). The non-A $\beta$  component of Alzheimer's disease amyloid (NAC) found in senile plaques of a patient's brain as the second major component is derived from the hydrophobic region (Ueda *et al.*, 1993). The carboxy-terminal region of NACP and its homologues is rich in proline and acidic residues, even though this region is more variable than the amino-terminal region.

The physiological function of NACP is not known, but NACP and its homologues found in Torpedo to human are highly homologous between different species (Jakes *et al.*, 1994). However, the localization of the NACP family at presynaptic nerve terminals suggests that it may be involved in the neuronal function (Iwai *et al.*, 1995a; Jakes *et al.*, 1994; Maroteaux *et al.*, 1988). This hypothesis is further supported by the evidence that the NACP family found in rat brain is reversibly associated with brain synaptosomal membranes (Maroteaux and Scheller, 1991). In this sense,

together with the appearance of NAC in the senile plaques, it is not surprising that the NACP may also play an important role in the pathogenesis and development of Alzheimer's disease as has been implicated by tight association of NAC with A $\beta$  (Ueda *et al.*, 1993; Yoshimoto *et al.*, 1995).

To better understand the physiological function of NACP and its role in the pathogenesis of Alzheimer's disease, the biochemical and biophysical properties of NACP were investigated. The apparent molecular mass of NACP determined by FPLC gel-filtration chromatography is about 70 kDa, while the value deduced from the amino acid sequence is 14.5 kDa, suggesting that NACP could be an oligomeric protein or could have an unusually extended structure. However, chemical cross-linking experiments failed to prove the oligomeric state of NACP. On the other hand, NACP was extremely sensitive to the proteases tested suggesting that significant parts of NACP may be unfolded. The CD spectrum of NACP also indicates that NACP is primarily composed of random coil and a few  $\beta$ -strands.

## Materials and Methods

### Materials

Recombinant DNA of NACP was a kind gift from Dr. R. Jakes (Jakes *et al.*, 1994). Isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) was purchased from

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The abbreviations used are: A $\beta$ ,  $\beta$ -amyloid; AD, Alzheimer's disease; CD, circular dichroism; NAC, non-A $\beta$  component of AD amyloid; NACP, the precursor protein of NAC.

Boehringer Mannheim (Indianapolis, IN), and glutaraldehyde, 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC), trypsin,  $\alpha$ -chymotrypsin, papain, iodoacetamide, leupeptin, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma (St. Louis, MO). Dithiobis(succinimidyl propionate) (DSP) was from Pierce (Rockford, IL). The FPLC system and column materials were from Pharmacia (Uppsala, Sweden).

#### Purification of recombinant NACP

*E. coli* BL21 (DE3) PlysS transformed with the recombinant DNA of NACP was grown in 6 liters of LB media containing 50  $\mu$ g/ml ampicillin, and induced with 0.5 mM IPTG at a late log phase for four hours. Bacteria were harvested by centrifugation, and the recombinant NACP was purified as described in Jakes *et al.* (1994) with some modification. Bacterial pellet was lysed in 200 ml of 20 mM Tris-HCl buffer (pH 7.5) with 0.1 M NaCl, 1  $\mu$ M EDTA, 1 mM DTT, 0.1 mM PMSF, 1  $\mu$ g/ml leupeptin, 0.1 mg/ml lysozyme and 1  $\mu$ g/ml DNase by the freeze and thaw method. Following a 20 min centrifugation at 8,000 rpm, the supernatant was heated in a boiling water bath for 10 min. Following a 20 min centrifugation at 12,000 rpm, the supernatant was applied to a DEAE Sepharose CL6B column equilibrated in 20 mM Tris-HCl buffer (pH 7.5) with 2 mM EDTA, and 0.1 M NaCl. NACP was eluted with a linear gradient of 0.1-0.4 M NaCl in 20 mM Tris (pH 7.5). Fractions containing NACP were identified by Coomassie blue staining of SDS-PAGE gels containing aliquots of each fraction. The fractions of NACP were pooled and concentrated using a Centriprep10 concentrator (Amicon, Beverly, MA). Final purification was achieved by FPLC gel-filtration chromatography using the Superdex 75 column equilibrated in 20 mM Tris-HCl buffer (pH 7.5) with 0.1 M NaCl and 0.02% sodium azide.

#### Chemical cross-linking of NACP

Chemical cross-linking experiments with DSP were performed based on the protocol of dePont *et al.* (1980). NACP was dialyzed against 4 liters of 20 mM HEPES buffer (pH 7.5) and the protein concentration was adjusted to 5 mg/ml. NACP was cross-linked with 1 mM DSP for 10, 30, and 60 min at room temperature, and quenched by the addition of 1 M Tris solution (pH 8.0) at a final concentration of 100 mM. Chemical cross-linking reactions with EDC were performed based on the protocol of Willing *et al.* (1989). About 5 mg/ml of NACP was cross-linked by 2.5-25 mM EDC for 30 min at room temperature and quenched with 100 mM sodium acetate (pH 5.5) and 100 mM Tris (pH 8.0). NACP was also cross-linked with 0.25% glutaraldehyde for 30 min and 60 min at room temperature, and quenched with the addition of 1 M Tris solution (pH 8.0) at a final concentration of 100 mM. The cross-linking products were analyzed by 12% SDS-PAGE under non-reduc-

ing conditions.

#### Limited proteolysis analysis

NACP was digested with papain as described by Kaufman and Strominger (1979). Briefly, 1 mg/ml of papain was incubated in 10 mM Tris-HCl buffer (pH 8.0) with 1 mM DTT and 1 mM EDTA for 5 min at 37  $^{\circ}$ C and then diluted to 0.1 mg/ml with the same buffer. Diluted papain (0.2 and 1 volume) was added to 8.8 and 8 volumes of purified NACP (1.5 mg/ml) in 20 mM HEPES (pH 7.5), respectively, and incubated at 37  $^{\circ}$ C for 1 h. The digestions were terminated by the addition of 1 volume of 200 mM iodoacetamide in 100 mM Tris (pH 8.0). Reducing sample buffer was added and the samples were boiled and analyzed by SDS-PAGE.

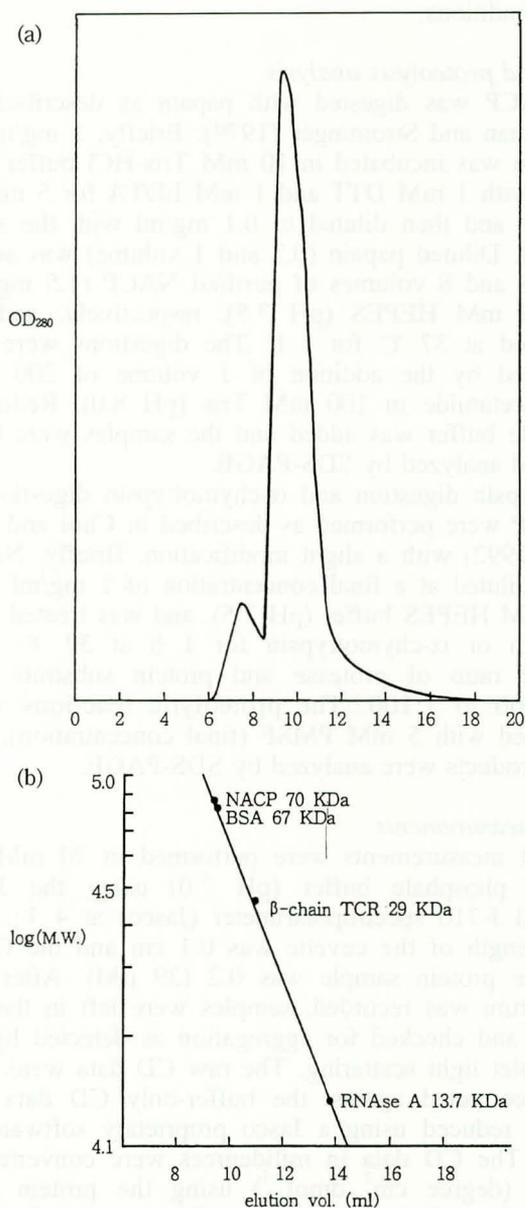
Trypsin digestion and  $\alpha$ -chymotrypsin digestion of NACP were performed as described in Choi and Zalkin (1992) with a slight modification. Briefly, NACP was diluted to a final concentration of 1 mg/ml with 20 mM HEPES buffer (pH 7.5), and was treated with trypsin or  $\alpha$ -chymotrypsin for 1 h at 37  $^{\circ}$ C. The molar ratio of protease and protein substrate was 1:1,000 to 1:100. The proteolytic reactions were stopped with 5 mM PMSF (final concentration), and the products were analyzed by SDS-PAGE.

#### CD measurements

CD measurements were performed in 20 mM sodium phosphate buffer (pH 7.0) using the Jasco model J-710 spectropolarimeter (Jasco) at 4  $^{\circ}$ C. The pathlength of the cuvette was 0.1 cm and the OD<sub>280</sub> of the protein sample was 0.2 (39  $\mu$ M). After CD spectrum was recorded, samples were left in the cuvette and checked for aggregation as detected by ultraviolet light scattering. The raw CD data were base line corrected against the buffer-only CD data and noise reduced using a Jasco proprietary software, J-700. The CD data in millidegrees were converted to theta (degree cm<sup>2</sup> dmol<sup>-1</sup>) using the protein concentration of the sample and the number of amino acid residues, and the  $\alpha$ ,  $\beta$  contents of NACP were calculated using the CDANAL program by Provencher and Gloeckner (1981) with the calculated extinction coefficient of NACP.

#### Results

NACP was purified to a homogeneity by taking advantage of the heat stability of the protein (Jakes *et al.*, 1994; Nakajo *et al.*, 1990) and by using conventional column chromatography techniques. Purified NACP was very soluble and stable for at least six months at 4  $^{\circ}$ C. Purified NACP appeared as a 19 kDa band on the SDS gel (Fig. 2a, lane 2) as had been reported in Ueda *et al.* (1993) and Jakes *et al.* (1994), while the molecular weight deduced from the amino acid sequence was 14,460 (Ueda *et al.*, 1993) and the molecular mass of recombinant NACP de-



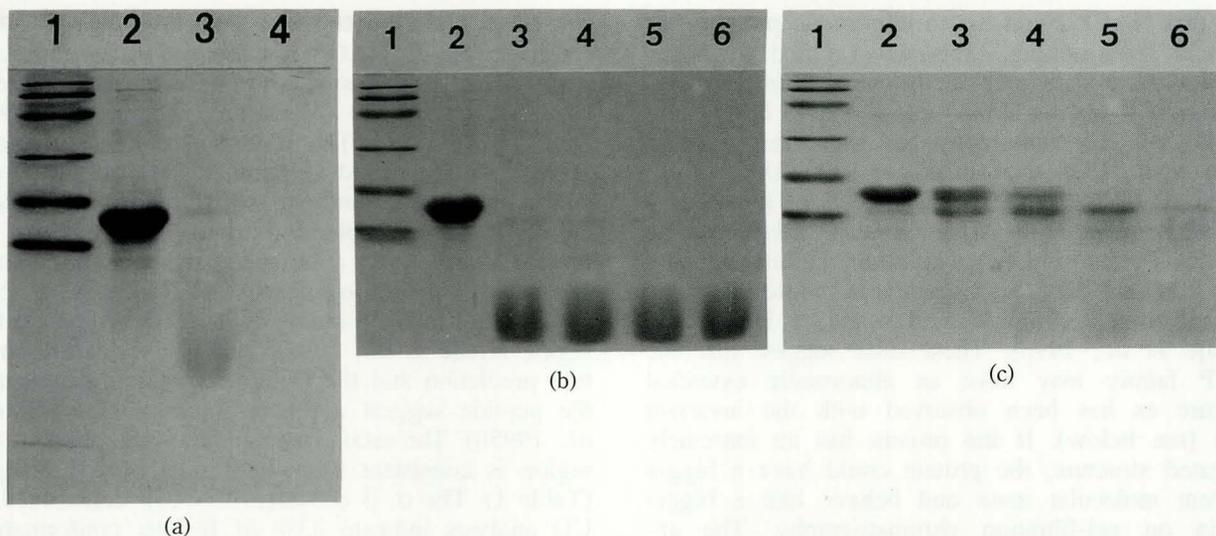
**Figure 1.** a) A typical gel-filtration FPLC profile of NACP. Superdex 75 column equilibrated in 20 mM Tris buffer (pH 7.5) with 0.1 M NaCl was used. NACP was eluted as a broad peak at the position of the 70 kDa protein. The elution profile was unaffected by different buffer solutions and a similar behavior was observed with the Superdex 200 column. b) Determination of the apparent molecular mass of NACP by FPLC gel-filtration chromatography. BSA (67 kDa),  $\beta$ -chain T cell receptor (TCR, 29 kDa) and RNase A (13.7 kDa) were run on the same column and used as size markers. The apparent molecular mass of NACP is about 70 kDa.

terminated by mass spectrometry was 14,462 (Jakes *et al.*, 1994). Earlier studies have shown that the 19 kDa band represents the NACP by Western blotting and the recombinant and native NACP show the same gel mobilities (Jakes *et al.*, 1994). NACP also behaved abnormally on FPLC gel-filtration chro-

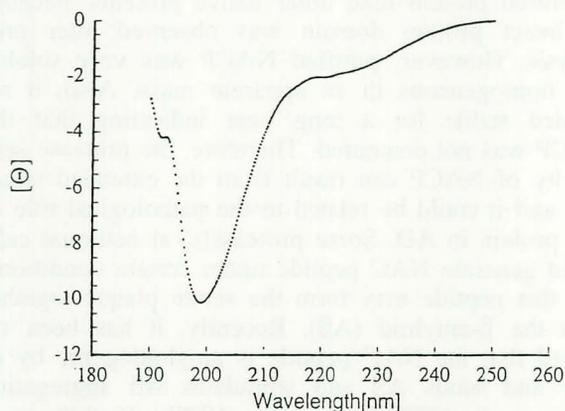
matography. Figure 1a shows a typical FPLC gel-filtration chromatography profile of NACP. The apparent molecular mass of NACP determined by FPLC gel-filtration chromatography was about 70 kDa (Fig. 1b). This result suggests that the NACP could be an oligomeric protein or could have an unusually extended structure, or could have both properties. To address the question about the oligomeric state of NACP, chemical cross-linking experiments were performed. However, significant intermolecular cross-linking of NACP was not observed by chemical cross-linking reactions with DSP, EDC or glutaraldehyde (data not shown). These negative results aroused a suspicion about the oligomeric state of NACP, although the possibility can not be ruled out at this stage because other type of cross-linkers might be able to cross-link the NACP.

Limited protease digestion experiments were carried out to investigate whether NACP contains any unfolded parts and to check whether the NAC peptide found in AD patient's plaques could be generated by *in vitro* protease digestion. The limited proteolysis patterns of NACP are shown in Figure 2. NACP was extremely sensitive to papain digestion. In contrast to the immunoglobulin (Kaufman and Strominger, 1979) and a class II MHC molecule HLA-DR1 (Gorga *et al.*, 1987), NACP was completely digested in 30 min with 1-10  $\mu$ g/ml papain treatment (Fig. 2a). NACP was also completely digested by  $\alpha$ -chymotrypsin (Fig. 2b). It is noteworthy that the molar ratio of protease and protein substrate was about 1:1,000, and most protein domains are pretty resistant under this condition (Choi and Zalkin, 1992; Park *et al.*, 1995; Kim *et al.*, unpublished results). NACP was also very sensitive to trypsin digestion. NACP was truncated to a 15 kDa fragment by a 1 h incubation with 1.0  $\mu$ g/ml trypsin (Fig. 2c, lane 3), but it was completely digested with 10.0  $\mu$ g/ml trypsin treatment (Fig. 2c, lane 6). Even though the NACP was very sensitive to the proteases tested, the proteolytic fragments were soluble. The senile plaque composed of NAC peptide, or any precipitation, was not observed under an optical microscope in any of the reaction conditions. This result suggests that the NAC peptide may be generated by other proteases, if any, or may be aggregated with the aid of other factor(s) *in vivo*.

The results of FPLC gel-filtration chromatography, chemical cross-linking experiments and protease digestion experiments suggest a possibility that the NACP has an extended structure with significant parts that are unfolded in solution. To obtain the secondary structural information of NACP, circular dichroism (CD) measurements were performed with the Jasco J-710 spectropolarimeter. The CD spectrum of NACP was unchanged by introducing a boiling step during the preparation and was independent of concentration, pH, and NaCl concentration. The CD spectrum of NACP (Fig. 3) indicates that the NACP al-



**Figure 2.** a) Papain digestion analysis of NACP. Proteolytic fragments were applied to a 18% SDS-PAGE gel and detected with Coomassie blue R-250. Lane 1, size marker proteins (97, 66, 45, 31, 21.5, and 14.5 kDa from the top); lane 2, NACP control; lane 3, NACP treated with 1.5  $\mu\text{g/ml}$  papain for 30 min at 37  $^{\circ}\text{C}$ ; lane 4, NACP treated with 10  $\mu\text{g/ml}$  papain. b)  $\alpha$ -Chymotrypsin digestion analysis of NACP. Proteolyzed samples were analyzed by a 20% SDS-PAGE gel. Lane 1, size marker proteins (same as Fig. 2a; lane 2, NACP control; lane 3, NACP treated with 1 mg/ml  $\alpha$ -chymotrypsin for 1 h at 37  $^{\circ}\text{C}$ ; lane 4, NACP treated with 2  $\mu\text{g/ml}$   $\alpha$ -chymotrypsin; lane 5, NACP treated with 5 mg/ml  $\alpha$ -chymotrypsin; lane 6, NACP treated with 10  $\mu\text{g/ml}$   $\alpha$ -chymotrypsin. c) Trypsin digestion analysis of NACP. Proteolyzed samples were analyzed by a 20% SDS gel. Lane 1, size marker proteins (same as Fig. 2a); lane 2, NACP control; lane 3, NACP treated with 1  $\mu\text{g/ml}$  trypsin for 1 h at 37  $^{\circ}\text{C}$ ; lane 4, NACP treated with 2  $\mu\text{g/ml}$  trypsin; lane 5, NACP treated with 5  $\mu\text{g/ml}$  trypsin; lane 6, NACP treated with 10  $\mu\text{g/ml}$  trypsin.



**Figure 3.** A CD spectrum of NACP collected in 20 mM sodium phosphate buffer (pH 7.0) at 4  $^{\circ}\text{C}$  on a Jasco spectropolarimeter. A similar CD spectrum was obtained with the protein in 20 mM Tris (pH 7.5)/0.1 M NaCl. The raw CD data were base line corrected against the buffer-only CD data and noise reduced using a Jasco proprietary software, J-700. The CD spectrum is represented in units of mean residue ellipticity,  $[\theta]$  (degree $\text{Cm}^2\text{dmol}^{-1}$ ). The vertical axis is in a scale of  $10^{-3}$ .

most lacks secondary structural elements, i.e.  $\alpha$ -helix or  $\beta$ -strand. The 208 nm and 222 nm absorption band characteristics of  $\alpha$ -helical peptide, and the 215 nm band of  $\beta$ -stranded peptide are almost absent in this spectrum, while Figure 3 shows a strong absorption band at 199 nm, the characteristic of a random-coiled

**Table 1.** Secondary structural elements of NACP determined by CD analysis. The  $\alpha$ ,  $\beta$  contents of NACP were calculated using the CDANAL program by Provencher and Gloeckner (1981)

Secondary structural elements	$\alpha$ -helix	$\beta$ -sheet	$\beta$ -turn	random coil
Percentage	0.0	33	3.0	64

polypeptide. The absence of a strong peak around 190-195nm also indicates that NACP has little content of conventional secondary structural elements. The  $\alpha$ ,  $\beta$  content of NACP calculated by the CDANAL program based on the CD spectrum (Table 1) is generally consistent with the qualitative interpretation. Table 1 shows that NACP lacks  $\alpha$ -helix and is composed of 64% random coil and 33%  $\beta$ -sheet conformation. The  $\alpha$ ,  $\beta$  contents of NACP are remarkably low. In view of these results, it is likely that the NACP, at least in this particular preparation, has an abnormally extended structure with significant parts unfolded and is primarily composed of random-coil and a few  $\beta$ -strands.

## Discussion

The apparent molecular mass of NACP determined by FPLC gel-filtration chromatography is about 70 kDa (Fig. 1), while the value deduced from the amino acid sequence is 14.5 kDa. This discrepancy sug-

gests that NACP could be an oligomeric protein, but chemical cross-linking experiments failed to prove the oligomeric state of NACP. The abnormal behavior of NACP and its homologues on SDS PAGE and gel-filtration chromatography has also been reported (Jakes *et al.*, 1994; Nakajo *et al.*, 1990; Ueda *et al.*, 1993). The apparent molecular mass of PNP14 (NACP homologue in bovine brain) determined by SDS PAGE and FPLC gel-filtration chromatography is 19 kDa and 57 kDa, respectively, while the value deduced from the amino acid sequence is 14 kDa (Nakajo *et al.*, 1993). These data suggest that the NACP family may have an abnormally extended structure as has been observed with the invariant chain (see below). If the protein has an extremely elongated structure, the protein could have a bigger apparent molecular mass and behave like a bigger protein on gel-filtration chromatography. The abnormal heat stability of NACP (Jakes *et al.*, 1994; Nakajo *et al.*, 1990) is also interesting in the context of its three dimensional structure.

The abnormal hydrodynamic properties of the invariant chain (Ii), which binds and stabilizes class II MHC molecules in the endoplasmic reticulum and lysosomal compartments of antigen presenting cells, have been observed by FPLC gel-filtration chromatography and light scattering experiments. Cresswell and coworkers reported that native Ii (amino acids 1-216) trimers have an unusually large Stokes radius for their predicted Mr of approximately 90 kDa for each chain, arguing that the structure is extended (Cresswell, 1996; Marks *et al.*, 1990; Roche *et al.*, 1991). Recombinant Ii trimers (amino acids 72-216) made soluble by removing the transmembrane and cytoplasmic domains, also have similar hydrodynamic properties to the native molecule (Park *et al.*, 1995). The apparent molecular mass estimated by light scattering and FPLC gel-filtration chromatography for the soluble Ii is 100 kDa, approximately twice its actual molecular mass. Further characterization of the soluble Ii by proteolysis, CD and NMR analysis revealed that a core fragment (amino acids 118-193) retains the trimeric structure of the intact molecule and the other parts of Ii are unfolded (Jasanoff *et al.*, 1995; Park *et al.*, 1995). This suggests that the abnormal hydrodynamic properties of Ii result from the presence of the unfolded domain, and the abnormal hydrodynamic property could be an indicator for unfolded proteins.

The abnormal structural features of NACP can be inferred from the primary structure of NACP. First, the amino acid sequence of NACP has 6 to 8 repeating motifs at the amino terminal region and the number of repeating motifs are variable between species. The consensus sequence of this repeating motif, KTKEGV, also appears one time in Rho and Ras protein, where it forms a loop exposed to solvent around the guanine nucleotide base (Pai *et al.*, 1990). Second, the carboxy-terminal region of NACP is not

conserved well and contains many proline and acidic residues. Third, NACP has no sequence homology with other proteins studied so far, although there is some local similarity (Maroteaux and Scheller, 1991; Ueda *et al.*, 1994). The abnormal sequence elements at the N-terminal and C-terminal parts of NACP can contribute to the abnormal structure of this protein. However, the hydrophobic region at the center of the protein could have a compact structure with some secondary structural elements. The NAC peptide, consisting of at least 35 amino acids, is derived from this region (Ueda *et al.*, 1993), and the secondary structure prediction and the Congo red staining pattern of the peptide suggest a  $\beta$ -sheet conformation (Iwai *et al.*, 1995b). The secondary structure prediction of this region is consistent with the results of CD analysis (Table 1). The  $\alpha$ ,  $\beta$  contents of NACP determined by CD analysis indicate 33% of  $\beta$ -sheet conformation. Taken together, the  $\beta$ -sheet region can be assigned to the central hydrophobic region of the NACP.

NACP was extremely sensitive to the various proteases tested, and was also sensitive to the contaminated protease(s) incorporated during the preparation. The NACP prepared without the boiling step was degraded during storage at 4 °C (data not shown), while the NACP prepared with the boiling step remained intact. The limited proteolysis pattern of NACP shown in Figure 2 resembles rather that of denatured protein than other native proteins, because no intact protein domain was observed after proteolysis. However, purified NACP was very soluble and homogeneous in an apparent mass. Also, it remained stable for a long time indicating that the NACP was not denatured. Therefore, the protease sensitivity of NACP can result from the extended structure and it could be related to the pathological role of this protein in AD. Some protease(s) at neuronal cells could generate NAC peptide under certain conditions, and this peptide may form the senile plaque together with the  $\beta$ -amyloid (A $\beta$ ). Recently, it has been reported that the NAC peptide is amyloidogenic by itself, and binds A $\beta$  and stimulates A $\beta$  aggregation (Han *et al.*, 1995; Iwai *et al.*, 1995b; Yoshimoto *et al.*, 1995).

The abnormal structural features of NACP presented in this paper must be related to the physiological function of NACP, and the extreme protease sensitivity of this protein probably resulting from the abnormally extended structure may be related to the pathological role of this protein in the development of AD. However, our understanding of molecular neurobiology of NACP is currently incomplete. Further study will clarify the mystery of the structure and the function of this protein.

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#### Note added in proof

During the preparation of this manuscript, Lansbury's group reported that the NACP is natively unfolded [Weinreb *et al.*, (1996) *Biochemistry* **35**, 13709-13715].

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