Biochemistry

 Volume 35, Number 43 October 29, 1996

Accelerated Publications

NACP, A Protein Implicated in Alzheimer's Disease and Learning, Is Natively Unfolded†

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Received July 22, 1996; Revised Manuscript Received September 19, 1996⊗

ABSTRACT: The "non-Aβ component of Alzheimer's disease amyloid plaque" (NAC) is a minor peptide component of the insoluble fibrillar core of the Alzheimer's disease (AD) neuritic plaque. NAC amyloid fibrils seed the polymerization of Aβ1−40, the major AD amyloid protein. NAC is derived from a 14 kDa precursor protein, designated NACP, a member of a highly conserved family of heat-stable brain-specific acidic proteins which have been suggested to be involved in synapse formation and/or stabilization. NACP has also been suggested to play a role in AD. We present herein a conformational analysis of human NACP. NACP has a much larger Stokes radius (34 Å) but sedimented more slowly ($s_{20,w} = 1.7$S) than globular proteins of similar molecular weight, indicating that the native protein is elongated. Circular dichroism (CD) and Fourier-transform infrared spectroscopy (FTIR) indicate the absence of significant amounts of secondary structure in NACP, while CD and ultraviolet spectroscopy suggest the lack of a hydrophobic core. The conformational properties of NACP were unchanged by boiling and were independent of concentration, pH, salt, and chemical denaturants. These features indicate that NACP exists as a mixture of rapidly equilibrating extended conformers and is representative of a class of "natively unfolded" proteins, many of which potentiate protein−protein interactions.

Alzheimer's disease (AD)[1] brain is characterized by the presence of insoluble fibrillar proteinaceous deposits known as amyloid plaque (Selkoe, 1995). The primary protein constituents of AD amyloid fibrils are variants of the β amyloid protein (Aβ). However, a minor fibrillar component of amyloid plaque, the non-Aβ component of AD amyloid (NAC), has recently been identified (Uéda et al., 1993). NAC, a 35 amino acid peptide which is present at *ca.* 10% of the level of Aβ, forms amyloid fibrils which seed *in vitro* fibril formation by β1−40 (Han et al., 1995). This behavior suggests that NAC amyloid may accelerate AD amyloidogenesis by seeding *in vivo* plaque formation (Han et al., 1995).

NAC is derived from a precursor protein, NACP, which is a member of a highly conserved family of brain-specific

† This paper is dedicated to the memory of Dr. Tsunao Saitoh, whose pioneering work in this area inspired our entry into the field. This work was supported by the National Institute on Aging (AG08470) and by graduate fellowships from the U.S. Department of Defense (A.W.P.) and the National Science Foundation (K.A.C.).

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⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1996.

[1] Abbreviations: Aβ, β amyloid protein; AD, Alzheimer's disease; BSA, bovine serum albumin; CD, circular dichroism; FTIR, Fourier-transform infrared spectroscopy; GdnHCl, guanidine hydrochloride; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HPLC, high-performance liquid chromatography; LDMS, laser desorption mass spectrometry; MALDI-TOF, matrix-assisted laser desorption time-of-flight mass spectrometry; NAC, non-Aβ component of Alzheimer's disease amyloid plaque; NACP, non-Aβ component of Alzheimer's disease amyloid plaque, precursor; PNP-14, bovine phosphoneuroprotein 14; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFE, trifluoroethanol.

S0006-2960(96)01799-0 CCC: $12.00

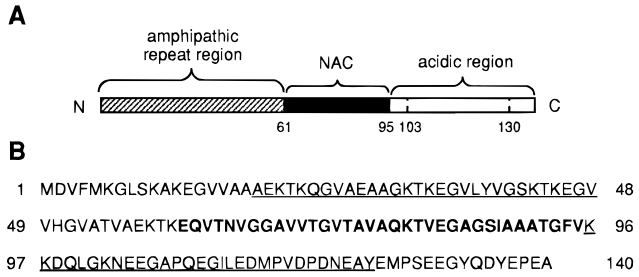

FIGURE 1: Amino acid sequence of NACP. (A) Schematic of the NACP sequence, with the three distinct regions indicated. The 103−130 sequence (delineated by dashed lines) is removed in an alternatively spliced form. Human and rat NACP share *ca*. 95% sequence homology (Iwai et al., 1995), while canary NACP (synelfin) (George et al., 1995) is *ca*. 85% homologous to both sequences. (B) NAC is indicated with bold lettering, and the other peptides used in this work (NACP19−48 and NACP96−125) are underlined.

acidic proteins found in several species. This family includes *Torpedo* synuclein (Maroteaux et al., 1988), rat synucleins 1, 2, and 3 (Maroteaux & Scheller, 1991), rat and bovine phosphoneuroprotein 14 (PNP-14) (Nakajo et al., 1990, 1993; Tobe et al., 1992; Shibayama-Imazu et al., 1993), canary synelfin (George et al., 1995), and human β-synuclein (Jakes et al., 1994). Human NACP (Figure 1) is a heat-stable 140 amino acid protein with a molecular weight of 14 460 (Uéda et al., 1993; Jakes et al., 1994). The amino terminal sequence of NACP, which is highly conserved among all members of the family, is composed almost entirely of variants of an 11 amino acid consensus motif (X**K**T**KE**G**V**XXXX; the residues in bold are completely conserved) strongly resembling that found in the amphipathic helices of the exchangeable apolipoproteins (Segrest et al., 1992; George et al., 1995). This local homology suggests a direct interaction of NACP with membranes, which is consistent with its affinity for synaptosomes (Maroteaux & Scheller, 1991; George et al., 1995). The central region, which is excised to produce the amyloid peptide NAC, is hydrophobic and is partly conserved in β synuclein/PNP14. Local homology between a sequence in this region (NACP66−73), the critical C-terminus of the Aβ protein (Aβ36−42), and a sequence in the prion protein (PrP117−124) has been noted (Han et al., 1995). The acidic C-terminal region is not conserved among family members, and the 103−130 sequence is entirely omitted in one alternatively spliced form (Uéda et al., 1994).

NACP is expressed predominantly in brain, especially in the olfactory bulb, frontal cortex, striatum, and hippocampus, regions of the brain which are most affected by AD neurodegeneration (Iwai et al., 1995). The expression of a rat NACP homolog, PNP 14, increases linearly during the first month of life (Shibayama-Imazu et al., 1993), and the canary homolog, synelfin, is locally upregulated in the song control circuit during a critical period for song learning, suggesting a potential role of the protein in developing and maintaining synaptic contacts ("synaptic plasticity") (George et al., 1995). NACP levels are also elevated in the brains of "mild" AD patients whereas more severe cases show no difference from age-matched controls, suggesting that the protein may play a role in "a compensatory mechanism to the ongoing synaptic damage" (Iwai et al., 1996) which is characteristic of the degenerating AD brain (Terry et al., 1994). A polymorphism in the promoter region of the NACP gene appears to confer protection against risk associated with the apoE4 allele (Xia et al., 1996), although the effect of this polymorphism on NACP expression level has not been determined.

We sought to uncover clues regarding the function of NACP via structural studies of the recombinant protein. We report herein a conformational analysis of human NACP, using several independent spectroscopic and hydrodynamic techniques. NACP was shown to have the properties of a "random coil" under physiological conditions, as do a number of other proteins which may have related general functions.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. NAC was synthesized *via* a published procedure (Han et al., 1995). NACP19−48 and rat NACP96−125 were synthesized using standard Fmoc chemistry on the Rink amide MBHA resin and purified by reversed-phase HPLC. Each purified peptide eluted from a C_{18} analytical reversed-phase HPLC column (Waters) as a single sharp peak: NACP19−48, 15% CH_3CN (0.1% TFA), 2 min, followed by a linear gradient to 25% (30 min), R_v = 39 mL; rat NACP96−125, 10% CH_3CN (0.1% TFA), linear gradient to 18% (10 min), followed by linear gradient to 25% (30 min), R_v = 48 mL. Amino acid analysis: NACP19−48, Glx 5.1 (5), Ser 1.0 (1), Gly 5.2 (5), Thr 3.2 (3), Ala 4.0 (4), Tyr 1.0 (1), Val 4.0 (4), Leu 0.9 (1), Lys 6.2 (6); rat NACP96−125, Asx 3.8 (3), Glx 5.6 (7), Ser 1.9 (2), Gly 4.0 (4), Ala 1.0 (1), Pro 3.0 (3), Tyr 2.9 (2), Val 1.1 (1), Met 1.8 (2), Ile 0.9 (1), Leu 0.9 (1), Lys 4.8 (3). Laser desorption mass spectrometry (LDMS): NACP19−48, calcd for [M + H^+] 3064.5, found 3064 (±3.1); rat NACP 96−125, calcd for [M + H^+] 3329.6, found 3327 (±3.3). The peptide rat NACP96−125 differs from the analogous human sequence at the five underlined positions: KKDQMGKGE-EGYPQEGILEDMPVDPSSEAY. The sequences of NAC and NACP19−48 are identical in the human and rat proteins.

NACP Expression and Purification. A pRK172 plasmid containing the human NACP (α-synuclein) sequence (kindly supplied by R. Jakes and M. Goedert, MRC) was used to overexpress NACP in *Escherichia coli* (Jakes et al., 1994; Gast et al., 1995). NACP was purified by boiling the cell homogenate, which precipitates most cellular proteins, leaving NACP as the major component of the soluble fraction. This boiled lysate was loaded onto a size-exclusion column (2.5 × 45 cm, Sephacryl S-300 HR, Pharmacia), and NACP eluted as a protein of approximately 58 kDa relative to globular protein standards [bovine serum albumin (BSA), 68 kDa, carbonic anhydrase, 29 kDa, cytochrome *c*, 11.7 kDa]. NACP-containing fractions were loaded onto a DEAE Sepharose CL-6B column (2.5 × 15 cm, Pharmacia) and eluted with NaCl (0−1 M gradient, NACP eluted at approximately 200 mM). An alternate purification procedure, which did not require a boiling step, was also developed: Cell lysate was treated with 47% ammonium sulfate, and the pellet was resuspended and loaded on a size-exclusion column (S-300). NACP-containing fractions were pooled and loaded onto the CL-6B column and eluted with NaCl. From a 500 mL cell culture, 5−40 mg of purified NACP was obtained by these methods.

Purified NACP eluted from a C_4 analytical reversed-phase HPLC column (Vydac, Hesperia, CA) as a single sharp peak [30% CH_3CN (0.1% TFA), linear gradient to 40% (20 min), R_v = 31 mL]. The molecular weight of the recombinant

protein was determined by MALDI-TOF mass spectrometry: calcd for [M + H^+] 14 461.2, found 14 463 (±4.6 experimental error). 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). PDMS of crude trypsin proteolytic fragments: 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/or 44−58), 1930.0 (61−80), 2158.1 (59−80). The recombinant protein was immunoreactive toward a mouse monoclonal antibody to residues 129−143 of canary synelfin (EMPPEEEEYQDYEPEA, differences from the homologous human NACP sequence are underlined, see Figure 1) (George et al., 1995).

Polyacrylamide Gel Electrophoresis. SDS and non-dissociating PAGE were run using an XCell II Mini-Cell electrophoresis system (Novex, San Diego, CA). SDS-slab gel electrophoresis of NACP was carried out using 12% polyacrylamide/bis (29:1) in the presence of 2-mercaptoethanol. Non-dissociating gels were prepared and run in the absence of SDS. Gels were stained with Coomassie Blue R-250. Apparent molecular weight was determined relative to the Mark-12 wide-range molecular weight standard (Novex).

Spectroscopic Methods. Samples for circular dichroism (CD) spectroscopy were prepared by dissolving lyophilized protein or peptide in buffer (10 mM Tris, pH 7.5). NACP concentration was determined spectroscopically, using $\epsilon_{274} = 5600\ M^{-1}\ cm^{-1}$ (estimated based on the tyrosine content of the protein and consistent with quantitative amino acid analysis). All spectra were recorded at absorbances below unity. Spectra were collected on a JASCO J-500C spectropolarimeter using a 0.1 cm cell at room temperature. The percentages of secondary structural elements were estimated based on the method of Greenfield and Fasman (1969). Infrared spectra were recorded on a Perkin Elmer 1600 Series FTIR instrument using a 0.05 mm CaF_2 solution cell at a concentration of 4 mg of NACP/mL in D_2O. UV spectra were recorded on a HP8452A UV/vis spectrophotometer.

Sucrose Gradient Ultracentrifugation. For sedimentation experiments, 5−20% sucrose gradients (13 mL) in buffer (10 mM Tris, pH 7.5) were prepared in Beckman UltraClear tubes (14 × 89 mm). Onto each gradient was layered 200 μL of a 5 mg/mL solution of one of the following proteins (all obtained from Sigma) in buffer: NACP, BSA (4.6S), chicken egg ovalbumin (3.55S), bovine erythrocyte carbonic anhydrase (2.85S), chicken egg white lysozyme (2.1S), or bovine heart cytochrome *c* (1.9S). Samples were centrifuged in a Beckman L8-M ultracentrifuge equipped with a Beckman SW41Ti rotor at 40 000 rpm for 23 h at 4 °C. Fractions (0.45 mL) were collected from the bottom of each tube and proteins were localized by UV absorption (A_{280}).

RESULTS

Mild Purification Method Permitted Characterization of Native NACP. A plasmid encoding the human α-synuclein sequence (kindly provided by R. Jakes and M. Goedert, MRC) was used to overexpress NACP in *E. coli* (Jakes et al., 1994). The published purification procedure begins with the boiling of the cell homogenate, which precipitates most cellular proteins, leaving NACP as the major component of the soluble fraction (Jakes et al., 1994). In order to preserve the presumed folded structure, we developed an alternative purification which circumvented this potentially denaturing step. In lieu of boiling the cell homogenate, an ammonium sulfate precipitation step was used for the initial fractionation, followed by size-exclusion and ion-exchange chromatography. Recombinant NACP produced by this method was chemically and conformationally indistinguishable from NACP purified via heat-treatment. Recombinant NACP was characterized by several independent methods, including MALDI-TOF mass spectrometry, amino acid analysis, identification of tryptic peptides, and Western blotting with an anti-NACP monoclonal antibody (George et al., 1995).

NACP Exhibits Properties Characteristic of an Extended Protein. The exact molecular weight of the NACP used in this work was found to be [M + H^+] = 14 463 (±4.6) by MALDI-TOF mass spectrometry, consistent with the molecular weight of 14 460 predicted on the basis of the encoded amino acid sequence. On SDS−polyacrylamide gel electrophoresis (SDS−PAGE), the protein exhibits an apparent M_r of approximately 19 000 (Uéda et al., 1993), which may be attributable to abnormally low binding of SDS by the highly acidic NACP C-terminal sequence. A similar abnormal mobility has been demonstrated by other highly charged proteins (Williams et al., 1986; Yoo & Albanesi, 1990; Thomas et al., 1991; Gast et al., 1995).

In the course of developing methods for the purification of NACP, we noted that the protein eluted with an unusually high mobility from gel filtration columns. Unlike globular proteins of comparable molecular weight, NACP eluted in the void volumes of both Bio-Gel P-10 and P-100 columns. To quantify this observation, the Stokes radius (R_{ST}) was estimated by comparison of the mobility of NACP to that of a series of standard proteins on a Sephacryl S-300 column (Figure 2A). R_{ST} is sensitive to both the size and the shape of a particle and can be directly related to molecular weight when globular proteins are compared (Siegel & Monty, 1966). NACP eluted with an effective R_{ST} of 34 Å. Boiling of recombinant NACP prior to chromatography had no effect on its observed mobility. For a typical globular protein, a Stokes radius of 34 Å corresponds to a molecular mass of about 58 kDa, approximately four times higher than that of NACP (14.4 kDa). Although this behavior has not previously been noted for NACP, β-synuclein, which also has a mass of 14 kDa, was reported to have a similar Stokes radius (corresponding to $M_r \approx 57$ kDa) (Nakajo et al., 1990). There are two possible explanations for this result: either native NACP is an oligomer or it has an elongated shape relative to the globular proteins used as standards.

To distinguish between these two possibilities, alternative methods for molecular weight determination were employed. Polyacrylamide gel electrophoresis in the absence of SDS separates on the basis of both size and charge (Hames, 1990). Multiple acrylamide concentrations can be used to control for the effect of charge and to estimate "native" molecular weight (Ferguson, 1964). Analysis of NACP by this method (Figure 2B) led to the estimation of a molecular weight of 20 kDa (±3 kDa) for NACP, indicating that NACP is not an oligomer. Addition of saturating concentrations of urea to samples prior to native gel electrophoresis had no effect on the mobility of NACP.

The sedimentation coefficient, a measure of the velocity of a particle in a centrifugal field, also provides information

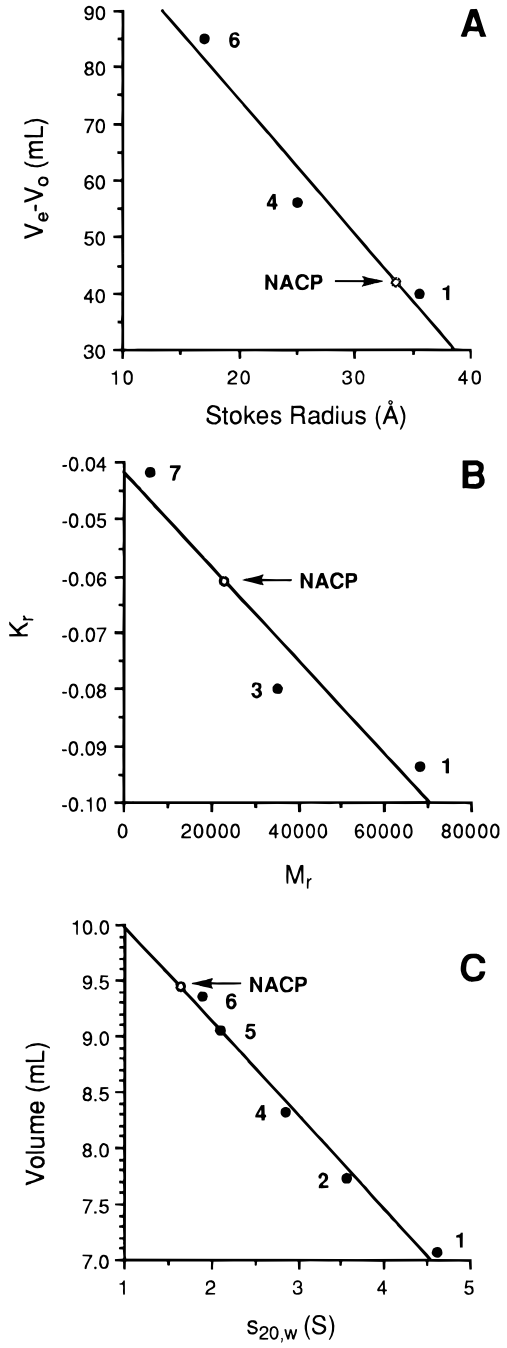

FIGURE 2: Hydrodynamic properties of NACP. (A) Determination of the Stokes radius of NACP by gel filtration chromatography on a Sephacryl S-300 column. NACP (indicated with an arrow) eluted with a Stokes radius of 34 Å, corresponding to a molecular weight of *ca.* 58 kDa for a globular protein. (B) Molecular weight determination using non-dissociating PAGE. NACP and standard proteins were run on 6%, 10%, and 14% non-dissociating polyacrylamide gels. For each protein, K_r was calculated as the slope of a plot of log R_f vs % acrylamide (Ferguson plot). A plot of K_r vs MW was used to estimate an approximate M_r of 20 kDa for NACP. (C) Determination of the sedimentation coefficient of NACP by sucrose density gradient ultracentrifugation. NACP (indicated with an arrow) sedimented at 1.7S. The protein standards used were as follows: **1**, BSA (68 kDa); **2**, ovalbumin (43 kDa); **3**, pepsin (35 kDa); **4**, carbonic anhydrase (29 kDa); **5**, lysozyme (14 kDa); **6**, cytochrome *c* (11.7 kDa); and **7**, insulin (5.7 kDa).

about the size and shape of a particle (Martin & Ames, 1961). For example, if one compares two particles of the same general shape (i.e., two globular proteins), the one with the higher mass will sediment more quickly. On the other hand, if one compares particles of identical mass but very different shapes (i.e., an elongated fibrous protein with a globular protein), a more compact particle will sediment more quickly than an extended one. To determine the sedimentation coefficient ($s_{20,w}$) for NACP, sucrose gradient ultracentrifugation experiments were performed (Figure 2C). The mobility of NACP on a 5−20% sucrose gradient was determined, and $s_{20,w}$ was determined by comparison to standard proteins (Martin & Ames, 1961). By this method, $s_{20,w}$ for NACP was found to be 1.7S, a value which corresponds to a globular protein of less than 10 kDa. This result indicates that native NACP is not an oligomer. The observation that NACP sediments more slowly than globular standards of comparable molecular weight is consistent with it having an elongated solution structure.

Assuming that NACP is a monomer, as suggested by non-dissociating gel electrophoresis and sedimentation analysis, the Stokes radius can be used to calculate the frictional ratio (f/f_o) of NACP, a measure of the departure of the molecule from spherical shape (Siegel & Monty, 1966).[2] Typical globular proteins have frictional ratios close to 1 [e.g., 1.09 for cytochrome *c* and 1.35 for BSA (Siegel & Monty, 1966)] whereas, for extended structures [e.g., fibrinogen, $f/f_o = 2.34$ (Siegel & Monty, 1966)] and denatured proteins [e.g., BSA in 6 M GdnHCl, $f/f_o = 2.6$ (Horiike et al., 1983)], the ratios are significantly higher. The frictional ratio of NACP was calculated to be 2.09, consistent with an extended conformation. None of these experiments, which measure hydrodynamic properties, can distinguish between a single, ordered, extended structure, such as that found in a fibrous protein, and a family of rapidly interconverting unstructured conformations, such as that found in a denatured protein.

CD, UV, and FTIR Spectroscopy Indicate NACP Has a "Random Coil" Conformation. The CD spectrum of NACP in buffer (10 mM Tris, pH 7.5) is shown in Figure 3A. The maximum negative ellipticity at *ca.* 200 nm is characteristic of random coil or denatured proteins (Greenfield & Fasman, 1969). The CD spectra of NACP before and after boiling were indistinguishable, indicating that heat-treatment has no effect on the conformation. The more convenient published purification procedure (Jakes et al., 1994) was therefore used throughout. Analysis of the CD spectrum using the method of Greenfield and Fasman (1969) indicates 68% random coil and less than 2% helical content (the remainder is calculated to be β-sheet, although it is difficult to quantify β-sheet structure by CD). The CD spectrum is independent of concentration over a range of 10−600 μM, strongly suggesting that NACP is monomeric over this concentration range. Altering the pH (from 4.5 to 10) also had no effect on the spectrum, nor did the addition of NaCl (2 M final concentration), $MgCl_2$ (2 M), $CaCl_2$ (10 mM), or ATP (0.1 mM), indicating that the conformation of NACP is not stabilized by ionic interactions. The CD spectrum was devoid of any significant ellipticity between 250 and 400 nm at concentrations up to 100 μM, indicating the absence

[2] The frictional ratio (f/f_o) was calculated according to the following equation (Siegel & Monty, 1966):

$$(f/f_o) = \alpha/\left(\frac{3\upsilon M}{4\pi N}\right)^{1/3}$$

where M = molecular weight, α = Stokes radius, υ = partial specific volume (0.721 mL/g for NACP, calculated from the amino acid sequence), and N = Avogadro's number.

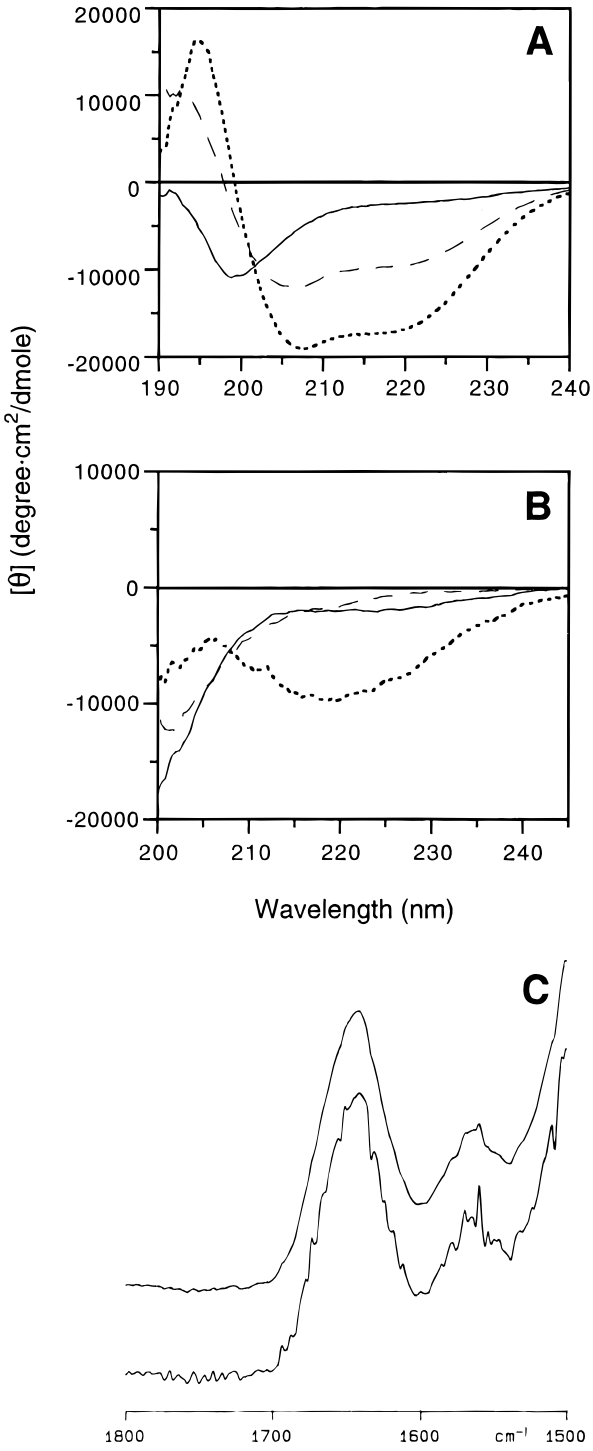

FIGURE 3: Spectroscopic analysis of NACP. (A) CD spectra of recombinant human NACP under various conditions. Percent secondary structures (Greenfield & Fasman, 1969): NACP (20 μM, solid line), 2% α, 30% β, 68% rc; NACP with 10% (v/v) HFIP (20 μM, dotted line), 52% α, 1% β, 47% rc; NACP with 10 mM SDS (20 μM, dashed line), 27% α, 13% β, 60% rc (α = α-helix, β = β-sheet, rc = random coil). (B) CD spectra of NACP-based peptides (note difference in low-wavelength cutoff as compared to panel A). Percent secondary structures (Greenfield & Fasman, 1969): NACP19−48 (38 μM, solid line), 5% α, 23% β, 72% rc; NAC (11 μM, dotted line), 6% α, 60% β, 34% rc; and NACP96−125 (49 μM, dashed line), 6% α, 17% β, 77% rc. (C) FTIR spectrum of NACP. The lower curve is the band-width-deconvoluted spectrum. The most intense amide I absorption band is centered at 1642 cm^{-1}, consistent with a random coil conformation. The smaller amide I bands at 1612, 1619, 1667, 1673, 1688, and 1694 cm^{-1} may represent minor amounts of ordered structure. The band at 1580 cm^{-1} derives from the amide II absorption.

of oriented aromatic residues, such as those typically found in the hydrophobic cores of globular proteins.

Helical conformations of NACP could be stabilized by the addition of either hexafluoro-2-propanol (HFIP) (Figure 3A) or trifluoroethanol (TFE) (data not shown). The maximal helical content was reached in 10% HFIP or 50% TFE, leading to 52% and 39% helix, respectively. The addition of SDS micelles (10 mM) also led to the induction of helical structure (27%, Figure 3A), suggesting an interaction of NACP with micelles. This interaction may involve the proposed amphipathic helix motif at the N-terminus of the protein (George et al., 1995).

The CD spectra of synthetic peptides corresponding to portions of the NACP sequence were also measured (Figure 3B). In aqueous buffer (pH 7.5), NACP19−48, which contains the 11 amino acid amphipathic helix motif, exhibited random coil structure comparable to NACP. As with the full-length protein, a helical conformation could be induced by the addition of HFIP [20% (v/v) gives 64% α] or SDS (15 mM gives 23% α) (data not shown). A random coil CD spectrum was also measured in aqueous buffer for rat NACP96−125, a model for the acidic C-terminal region. Even in the presence of 50% TFE, this peptide exhibited little tendency toward secondary structure formation (6% α), possibly due to its high charge density. In contrast to the spectra of the other two peptides, the CD spectrum of a saturated solution (11 μM) of the NAC peptide (NACP61−95) in aqueous buffer indicated a significant degree of β-sheet structure (*ca.* 35% β, Figure 3B). Similar CD spectra have been measured for aqueous solutions of Aβ amyloid peptides (Barrow & Zagorski, 1991).

FTIR spectroscopy confirmed that native NACP is unstructured. The position of the amide I infrared absorbtion band of proteins is indicative of the constituent secondary structure (Krimm & Bandekar, 1982; Bowler et al., 1994). The FTIR spectrum of NACP (Figure 3C) showed a broad amide I absorbtion band centered at 1642 cm^{-1}. This band is characteristic of a denatured or "random coil" protein, and the breadth of the peak is probably due to the existence of small amounts of defined secondary structures with nanosecond lifetimes. The bandwidth-deconvoluted spectrum revealed minor peaks at 1612, 1619, 1688, and 1694 cm^{-1}, indicating small amounts of antiparallel β-sheet, and at 1673 and 1667 cm^{-1}, which may arise from β-turn structure. These minor bands suggest that an ordered conformer is populated to a slight extent or, more likely, that many conformers contain a small amount of secondary structure.

The ultraviolet absorption of aromatic residues in proteins depends on the environment of the chromophore (Wetlaufer, 1962). Upon protein denaturation, the aromatic amino acids which are buried within the hydrophobic core of the molecule become solvent-exposed, leading to a shift in λ_{max} to a lower wavelength. NACP contains four tyrosine residues (at positions 39, 125, 133, and 136), three of which are in the acidic C-terminus of the protein. The absorption spectrum of NACP in the aromatic region was unchanged in the presence of 6 M guanidine hydrochloride (data not shown), indicating that the tyrosine residues are completely solvent-exposed in the native state. This result was consistent with the CD spectrum of NACP, which showed no significant ellipticity in the near-UV region; both results are inconsistent with the existence of a hydrophobic core.

Table 1: Representative "Natively Unfolded" Proteins

protein[a]	heat stable?	CD indicates random coil?	p*I*[b]	molecular weight (kDa) calcd[c]	SDS−PAGE	gel filtration	reference
NACP	YES	YES	6.1	14	19	58	this work
β-synuclein	YES	NA	5.5	14	19	56	Nakajo et al. (1990)
tubulin/actin binders							
tau	YES	YES	10.4	∼50	∼50	600	Schweers et al. (1994)
MAP2	YES	YES	5.0	199	300	NA	Hernandez et al. (1986)
Op18/stathmin	YES	NA	6.0	17	18	45	Belmont and Mitchison (1996)
Ca^{2+}/calmodulin binders							
chromogranin A	YES	YES	5.0	48	75	300	Yoo and Albanesi (1990)
chromogranin B	YES	YES	<5	71	110	200	Yoo (1995)
caldesmon	YES	YES	5.7	93	140	>500	Lynch et al. (1987)
phosphatase/kinase inhibitors							
DARPP-32	YES	YES	4.7	22	32	59	Hemmings et al. (1984)
PP1 inhibitor	YES	NA	<5	18.7	26	NA	Nimmo and Cohen (1978)
PKI	YES	YES	5.3	7.8	16.9	22	Thomas et al. (1991)
other							
prothymosin α	YES	YES	3.6	11.9	20.6	59	Gast et al. (1995)

[a] Abbreviations: MAP2, microtubule-associated protein 2; Op18, oncoprotein 18; DARPP-32, dopamine and adenosine 3′:5′-monophosphate-regulated phosphoprotein; PP1, protein phosphatase 1; PKI, CAMP-dependent protein kinase inhibitor. [b] Some p*I*'s estimated from amino acid composition; others determined experimentally. [c] Calculated from amino acid sequence data or determined by mass spectrometry.

DISCUSSION

NACP Is a "Natively Unfolded" Protein. The results presented herein provide evidence that, in solution, NACP exists as a mixture of rapidly equilibrating monomeric conformers which, on average, contain little secondary structure and no hydrophobic core. This conclusion is based on spectroscopic and hydrodynamic probes of protein structure, as well as the behavior of the protein in the presence of additives and cosolvents. The microtubule-associated protein tau, which has similar properties, has been termed "natively denatured" by Mandelkow and co-workers (Schweers et al., 1994). The use of the word denatured is confusing, since it is operationally defined: we prefer the alternative designation "natively unfolded".

A typical globular protein folds into a single, minimum energy structure stabilized by packing of a hydrophobic core. When partially unfolded *in vitro*, or incompletely folded *in vivo*, the exposed hydrophobic patches tend to associate intermolecularly, leading to aggregation. NACP, in contrast, is apparently energetically minimized as an extended, non-compact mixture of disordered conformers. It is interesting to speculate as to why these unfolded conformers do not aggregate *in vitro* and are not recognized and degraded in the cytoplasm. A cursory inspection of the NACP amino acid composition does not indicate anything unusual, that is, the ratio of hydrophobic residues to total residues seems to be normal. The unusual properties of NACP may derive from the concentration of hydrophobic residues in the central (NAC) region of the sequence and/or the the role of the anionic C-terminal domain in preventing aggregation. It may be significant that NACP is over 100-fold more soluble than the NAC peptide (Han et al., 1995). Thus proteolysis may play an important role in AD pathogenesis.

Conformational Properties Define a Family of "Natively Unfolded" Proteins. While the unusual conformational properties of NACP do not directly suggest a function, examination of proteins with similar properties may provide clues. Table 1 lists several such natively unfolded proteins, which share one or more of the following diagnostic properties: (1) stability to heat denaturation; (2) a highly charged (often acidic) amino acid sequence; (3) "random coil" structure as demonstrated by CD; (4) an abnormally high Stokes radius; and (5) abnormal SDS binding leading to unusual mobility on SDS−PAGE. The charged regions of these proteins may interfere with SDS binding (affecting SDS−PAGE calibration), and may strongly disfavor intramolecular interactions, rendering them "unfoldable" and, by definition, resistant to thermal denaturation.

Several natively unfolded proteins bind and regulate the assembly of cytoskeletal proteins such as tubulin and actin [e.g., MAP-2 (Hernandez et al., 1986) and tau (Schweers et al., 1994)]; some bind Ca^{2+} and/or calmodulin [e.g., chromogranin A (Yoo & Albanesi, 1990) and caldesmon (Lynch et al., 1987)], and still others are phosphatase inhibitors [e.g., DARPP-32 (Hemmings et al., 1984)]. In each case, their disordered nature may be critical to facilitate protein−protein interactions. Tau, for example, binds to and stabilizes microtubules and is only ordered in the context of this complex. Unstructured domains can increase association rates for macromolecules by allowing rapid formation of nonspecific "encounter complexes" (Pontius, 1993), similar to those between some DNA-binding proteins and DNA (Earnshaw, 1987).

Natively Unfolded Conformation Suggests a Functional Role of NACP. The conformational similarity of NACP to the proteins in Table 1 suggests that it too may function to facilitate protein−protein interactions. The NACP C-terminus may bind a cationic target, while the N-terminus may be involved in weak interactions with a membrane cytoplasmic surface. Thus, NACP may serve to carry a cationic target protein to the inner surface of the neuronal membrane or to the outer surface of a vesicle [members of the NACP family have affinity for synaptosomes (Maroteaux & Scheller, 1991; George et al., 1995)]. How such a multicomponent structure could relate to synaptic plasticity, learning, and Alzheimer's disease will be the subject of future investigations.

ACKNOWLEDGMENT

We are grateful to Prof. Tsunao Saitoh for sharing unpublished results with us. We thank D. Clayton (University of Illinois) for NACP antibodies and M. Goedert and R. Jakes (MRC, Cambridge) for the NACP expression vector. Mass spectral data were provided by A. Rhomberg of the MIT Mass Spectrometry Facility, which is supported by NIH (RR00317 to K. Biemann). Amino acid analysis was performed at the MIT Biopolymers Laboratory.

REFERENCES

Bachmair, A., Finley, D., & Varshavsky, A. (1986) *Science 234*, 179.
Barrow, C. J., & Zagorski, M. G. (1991) *Science 253*, 179−182.
Belmont, L. D., & Mitchison, T. J. (1996) *Cell 84*, 623−631.
Bowler, B. E., Dong, A., & Caughey, W. S. (1994) *Biochemistry 33*, 2402−2408.
Earnshaw, W. C. (1987) *J. Cell Biol. 105*, 1479−1482.
Ferguson, K. A. (1964) *Metabolism 13*, 985.
Gast, K., Damaschun, H., Eckert, K., Schulze-Forster, K., Maurer, H. R., Müller-Frohne, M., Zirwer, D., Czarnecki, J., & Damaschun, G. (1995) *Biochemistry 34*, 13211−13218.
George, J. M., Jin, H., Woods, W. S., & Clayton, D. F. (1995) *Neuron 15*, 361.
Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108−4116.
Hames, B. D. (1990) in *Gel Electrophoresis of Proteins* (Hames, B. D., & Rickwood, D., Eds.) Oxford University Press, New York.
Han, H., Weinreb, P. H., & Lansbury, P. T. (1995) *Chem. Biol. 2*, 163−169.
Hemmings, H. C., Jr., Nairn, A. C., Aswad, D. W., & Greengard, P. (1984) *J. Neurosci. 4*, 99−110.
Hernandez, M. A., Avila, J., & Andreu, J. M. (1986) *Eur. J. Biochem. 154*, 41−48.
Horiike, K., Tojo, H., Yamano, T., Nozaki, M. (1983) *J. Biochem. 93*, 99−106.
Iwai, A., Masliah, E., Sundsmo, M. P., DeTeresa, R., Mallory, M., Salmon, D. P., & Saitoh, T. (1996) *Brain Res. 720*, 230−234.
Iwai, A., Masliah, E., Yoshimoto, M., Ge, N., Flanagan, L., Rohan de Silva, H. A., Kittel, A., & Saitoh, T. (1995) *Neuron 14*, 467−475.
Jakes, R., Spillantini, M. G., & Goedert, M. (1994) *FEBS Lett. 345*, 27−32.
Krimm, S., & Bandekar, J. (1982) *Adv. Protein Chem. 38*, 181−364.
Lynch, W. P., Riseman, V. M., & Bretscher, A. (1987) *J. Biol. Chem. 262*, 7429−7437.
Maroteaux, L., & Scheller, R. H. (1991) *Mol. Brain Res. 11*, 335−343.
Maroteaux, L., Campanelli, J. T., & Scheller, R. H. (1988) *J. Neurosci. 8*, 2804−2815.
Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372−1379.
Nakajo, S., Omata, K., Aiuchi, T., Shibayama, T., Okahashi, I., Ochiai, H., Nakai, Y., Nakaya, K., & Nakamura, Y. (1990) *J. Neurochem. 55*, 2031−2038.
Nakajo, S., Tsukada, K., Omata, K., Nakamura, Y., & Nakaya, K. (1993) *Eur. J. Biochem. 217*, 1057−1063.
Nimmo, G. A., & Cohen, P. (1978) *Eur. J. Biochem. 87*, 341−351.
Pontius, B. W. (1993) *Trends Biochem. Sci. 18*, 181−186.
Schweers, O., Schönbrunn-Hanebeck, E., Marx, A., & Mandelkow, E. (1994) *J. Biol. Chem. 269*, 24290−24297.
Segrest, J., Jones, M., DeLoof, H., Brouillette, C., Venkatachalapathi, Y., & Anantharamalah, G. (1992) *J. Lipid Res. 33*, 141−166.
Selkoe, D. J. (1995) *J. NIH Res. 7*, 57.
Shibayama-Imazu, T., Okahashi, I., Omata, K., Nakajo, S., Ochiai, H., Nakai, Y., Hama, T., Nakamura, Y., & Nakaya, K. (1993) *Brain Res. 622*, 17−25.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346−362.
Terry, R. D., Masliah, E., Hansen, L. A. (1994) in *Alzheimer Disease* (Terry, R. D., Katzman, R., & Bick, K. L., Eds.) pp 179−196, Raven Press, NY.
Thomas, J., Van Patten, S. M., Howard, P., Day, K. H., Mitchell, R. D., Sosnick, T., Trewhalla, J., Walsh, D. A., & Maurer, R. A. (1991) *J. Biol. Chem. 266*, 10906−10911.
Tobe, T., Nakajo, S., Tanaka, A., Mitoya, A., Omata, K., Nakaya, K., Tomita, M., & Nakamura, Y. (1992) *J. Neurochem. 59*, 1624−1629.
Uéda, K., Fukushima, H., Masliah, E., Xia, Y., Iwai, A., Yoshimoto, M., Otero, D. A. C., Kondo, J., Ihara, Y., & Saitoh, T. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11282−11286.
Uéda, K., Saitoh, T., & Mori, H. (1994) *Biochem. Biophys. Res. Commun. 205*, 1366−1372.
Wetlaufer, D. B. (1962) *Adv. Protein Chem. 17*, 303.
Williams, K. R., Hemmings, H. C., Jr., LoPresti, M. J., Konigsberg, W. H., & Greengard, P. (1986) *J. Biol. Chem. 261*, 1890−1903.
Xia, Y., de Silva, H. A. R., Rosi, B. L., Yamaoka, L. H., Rimmler, J. B., Pericak-Vance, M. A., Roses, A. D., Chen, X., Masliah, E., DeTeresa, R., Iwai, A., Sundsmo, M., Thomas, R. G., Hofstetter, C. R., Gregory, E., Hansen, L. A., Katzman, R., Thal, L. J., Saitoh, T. (1996) *Ann. Neurol. 40*, 50−58.
Yoo, S. H. (1995) *J. Biol. Chem. 270*, 12578−12583.
Yoo, S. H., & Albanesi, J. P. (1990) *J. Biol. Chem. 265*, 14414−14421.

BI961799N