All-d-Enantiomers of β-Amyloid Exhibit Similar Biological Properties to All-l-β-Amyloids*

(Received for publication, August 6, 1996, and in revised form, December 6, 1996)

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The amyloidogenic peptide β-amyloid has previously been shown to bind to neurons in the form of fibrillar clusters on the cell surface, which induces neurodegeneration and activates a program of cell death characteristic of apoptosis. To further investigate the mechanism of Aβ neurotoxicity, we synthesized the all-d- and all-l-stereoisomers of the neurotoxic truncated form of Aβ (Aβ25-35) and the full-length peptide (Aβ1-42) and compared their physical and biological properties. We report that the purified peptides exhibit nearly identical structural and assembly characteristics as assessed by high performance liquid chromatography, electron microscopy, circular dichroism, and sedimentation analysis. In addition, both enantiomers induce similar levels of toxicity in cultured hippocampal neurons. These data suggest that the neurotoxic actions of Aβ result not from stereoisomer-specific ligand-receptor interactions but rather from Aβ cellular interactions in which fibril features of the amyloidogenic peptide are a critical feature. The promiscuous nature of these β-sheet-containing fibrils suggests that the accumulation of amyloidogenic peptides in vivo as extracellular deposits represents a site of bioactive peptides with the ability to provide inappropriate signals to cells leading to cellular degeneration and disease.

Alzheimer’s disease (AD),1 vascular dementia, and hereditary cerebral hemorrhage with the Dutch type are diseases that share an invariant pathological feature, the accumulation of an amyloidogenic peptide into insoluble fibrillar extracellular deposits. In all three cases, the major component of the extracellular debris is the β-amyloid peptide (Aβ) that is derived from the proteolytic processing of the large membrane-anchored amyloid precursor protein (APP) encoded by a single gene located on chromosome 21 (1). However, the biological significance of these amyloid deposits has been extensively debated as to whether they are a causative factor of each disease or merely a metabolically inert end product lacking in biological activity. Evidence in support of a causative role for Aβ in neuropathology comes from genetic analysis of the APP gene where several autosomal dominant mutations have been linked with AD and hereditary cerebral hemorrhage with the Dutch type (2, 3). In a recent in vitro study, the β-APP717 mutation consistently caused a significant increase in the percentage of the longer and more amyloidogenic Aβ1-42 over the shorter Aβ1-40 (4). Incorporation of this same mutation into a transgenic mouse model yields Aβ deposition and neuropathology that closely parallels that observed in AD (5). Additional in vitro evidence suggesting that the Aβ peptide itself may be biologically active comes from a transgenic model overexpressing Aβ1-42, in which Aβ transgene expression was detected in a variety of peripheral tissues but histopathological changes were restricted to the brain. Moreover, the neurodegeneration was largely limited to the cerebral cortex, hippocampus and amygdala, all areas affected in AD, and was essentially undetectable in the cerebellum, which is typically not affected in AD (6). Finally, the amount of β-amyloid that accumulates in the brain appears to correlate well with the decline of brain function (7).

Insights into the inherent biological activity associated with Aβ have come from in vitro studies that show synthetic Aβ can spontaneously assemble into β-sheet-containing fibrils (8, 9), that this fibrillar-Aβ can induce neuritic dystrophy in neuronal cultures similar to that seen in the AD brain, and that the mechanism of Aβ-triggered degeneration is via programmed cell death (PCD) (10). These observations have led to the general hypothesis that the biological activity of Aβ is dependent on its transformation into a highly stable protease-resistant antiparallel β-sheet conformation and higher order quaternary assemblies (11) similar to those found in senile plaques. This is of fundamental importance because it suggests that the biological activity of Aβ depends on protein conformation and the transition into this conformation. The consequences of such a relationship between biological activity and protein conformation are critical to understanding the role of Aβ and other β-pleated sheet protein assemblies such as prion protein in disease.

In order to understand the degenerative processes induced by Aβ, it is essential to define the characteristics of Aβ salient to its function as a neurotoxic stimulus. In a previous study, we used a series of synthetic Aβ peptides with progressively truncated C-termini to demonstrate that the length of this hydrophobic region is a crucial determinant of peptide ability to both aggregate and induce neurotoxicity in vitro (12). We have also synthesized a series of truncated Aβ peptides to examine the effects of N-terminal heterogeneity, which occurs in vivo on the assembly and biological activity of Aβ. The N-terminal truncated isoforms produced enhanced aggregation into neurotoxic β-sheet fibrils, which suggests that these truncated peptides may initiate the pathological neurodegeneration in AD by acting as a nucleation site for Aβ deposition (13). Thus far, we have observed that assembled, bioactive Aβ peptides exhibit β-sheet structure and that amino acid substitutions that dis-
rupt Aβ assembly also prevent β-sheet structure and abolish toxicity (14). Further analysis of peptides will be useful in elucidating the specific requirements for both the assembly and bioactivity of Aβ.

Since numerous ligand-target interactions are stereospecific, one means to both examine the nature of the Aβ-cellular interactions and assess the validity of several proposed mechanisms of Aβ-induced cell death is to determine whether Aβ bioactivity exhibits stereospecificity. Similar issues of ligand stereospecificity have been investigated in several recent studies by comparing the binding and or activities of d- and l-enantiomers of small peptide ligands (15, 16). In the current study, we have utilized a comparable paradigm, synthesizing the all-d- and all-l-amino acid stereoisomers of the truncated biologically active Aβ25–35 and the full-length Aβ1–42 peptide, and compared their physical and biological properties to determine whether the interaction of Aβ with biologically relevant cells is stereospecific.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Aβ1–42, Aβ25–35 (GSNKGAIGILM), and scrambled sequence Aβ25–35 (12) were synthesized from either all-d- or all-l-amino acids using solid phase Fmoc (N-(9-fluorenylmethoxycarbonyl) amino acid chemistry and purified by reverse phase HPLC, as described previously (9). The purified peptides were then routinely analyzed by electrospray mass spectroscopy (9). The all-d-Aβ25–35 and -Aβ1–42 enantiomers gave mass values of 1060.4 and 4513.9, respectively. HPLC analysis of the all-l-enantiomers produced identical elution times to the all-l-Aβ peptides, and mixtures of all-l-Aβ25–42 and all-l-Aβ25–35 produced a single peak by HPLC. Peptides were solubilized in sterile doubly deionized water as 2.5 mM stock solutions and allowed to aggregate at room temperature for at least 1 h before using. Aliquots of the peptide stocks were then diluted with an equal volume of 2× Dulbecco’s modified Eagle’s medium (DMEM) and then with DMEM supplemented with 0.7% (v/v) fetal bovine serum (FBS) to yield a final concentration of 0.7 µg/ml (14). Sedimentation analysis was then used to control for lot to lot variability in the biological activity of the peptides. Comparison of the all-d- and all-l- forms of Aβ25–35 by electrospray mass spectroscopy gave essentially identical mass values, and HPLC analysis showed that the two enantiomers had similar elution times. Mixed samples of both enantiomers eluted as a single peak from the HPLC (Fig. 1A). All-d-Aβ25–35 was allowed to assemble in parallel with the all-l-Aβ25–35, and then both peptides were subjected to a series of commonly used assays to monitor the properties of fibrillar Aβ. Both enantiomers rapidly produced visible aggregates in aqueous solution (Fig. 2, A-D), and analysis of negatively stained specimens by electron microscopy showed similar fibrillar structures (Fig. 1, B and C). Sedimentation analysis was then used to compare the extent of peptide assembly for both enantiomers. Three different lots of the all-l-Aβ25–35 and two lots of the all-d-Aβ25–35 were used, and both forms gave similar results (data not shown). The above results show that both peptides have similar physical properties and can only be distinguished by CD analysis where mirror image spectra are generated (data not shown).

The biological properties of the two enantiomers were then tested by applying the enantiomers to primary cultures of rat hippocampal and cortical neurons that have previously been used to assay the neurotoxic activity of Aβ (12, 18). The all-d-Aβ25–35 produced visible aggregates in the tissue culture wells and appeared to bind to the surface of neurons equally as well as the all-l-Aβ25–35 (Fig. 2, A-D). Noticeable neuronal degeneration was apparent at 12 h, and extensive cell death was observed at 24 h for both enantiomers. In order to compare the levels of neurotoxicity between the two enantiomers, a dose response curve was generated. As can be seen in Fig. 2E, the all-d-Aβ25–35 produced similar toxicity to the all-l-Aβ25–35 over the entire range of concentrations tested. The specificity of the neurotoxicity was determined by analyzing peptides with scrambled sequences of both the all-l- and the all-d enantiomers. Neither scrambled sequence produced detectable neurotoxicity over the entire range of concentrations utilized for the dose response curve (data not shown).

During the comparison of computer-generated models of Aβ25–35 in antiparallel β-sheet conformation, we discovered, that with perfect alignment of the individual strands of peptide, that a pseudo-axis of symmetry was generated do to the planar nature of the β-sheet such that the distribution of the surface groups produced topochemically similar enantiomers. Other cases of topochemically similar peptides that bind to stereoselective receptors and posses similar activities have been reported (20, 21). Based on the high level of bioactivity associated with the all-d-Aβ25–35, we next determined whether the all-d-enantiomer of the full-length Aβ1–42 peptide would also bind to cells and produce similar neurotoxicity to the all-l-Aβ1–42. Although the β-sheet-containing fibrils of the
$\text{A}$β$_{1-42}$ are predicted to form planar sheets, the longer length of the $\text{A}$β$_{1-42}$ peptide would reduce the probability of formation of topochemically similar enantiomers since the surface topography of $\text{A}$β$_{1-42}$ would be far more complex than with $\text{A}$β$_{25-35}$.

Highly purified all-D-$\text{A}$β$_{1-42}$ was subjected to CD, and the spectra were compared with the all-L-$\text{A}$β$_{1-42}$ (Fig. 3A). The all-D-enantiomer produced the expected mirror image spectra, indicating similar secondary structure for the enantiomers. The peptides were then examined by electron microscopy, and while the filamentous structures were different from those observed with $\text{A}$β$_{25-35}$, the $\text{A}$β$_{1-42}$ enantiomers produced fibrils that were indistinguishable from each other (Fig. 3, B and C). The ability to bind certain dyes, such as Congo red and thioflavine T, is a characteristic property of amyloidogenic peptides (22–24) and can be used to measure the amount of peptide in $\beta$-sheet-containing fibrils (25). Analysis of assembled peptides of both enantiomers indicates that the all-D-$\text{A}$β$_{1-42}$ binds thioflavine with intensity equal to that of the all-L-$\text{A}$β$_{1-42}$ (data not shown).

**Fig. 1.** Comparison of the physical properties of the all-l- and all-d-$\text{A}$β$_{25-35}$ enantiomers. A racemic mixture of all-l- and all-d-$\text{A}$β$_{25-35}$ produces a single peak upon HPLC analysis using a C4 analytical column (A). Negatively stained samples of the all-l-$\text{A}$β$_{25-35}$ (B) and all-d-$\text{A}$β$_{25-35}$ (C) were examined by electron microscopy. Magnification for both samples was $\times 40,000$.

**Fig. 2.** Comparison of the cellular association and neurotoxic activities of the all-l- and all-d-enantiomers of $\text{A}$β$_{25-35}$. Primary cultures of rat neurons were treated with pre-assembled forms of the enantiomers of $\text{A}$β$_{25-35}$. Numerous visible fibrillar aggregates (arrows) of the all-l-$\text{A}$β$_{25-35}$ (A and B) and all-d-$\text{A}$β$_{25-35}$ (C and D) can be seen associated with the neurites at 6 h post-treatment with 6 µM $\text{A}$β$_{25-35}$. At higher magnification (B and D), the fuzzy appearing fibrillar aggregates can been seen decorating the swollen degenerating neurites. Dose response curves were generated to compare the relative neurotoxic activities of the two $\text{A}$β$_{25-35}$ enantiomers (E). The all-l- and all-d-$\text{A}$β$_{25-35}$ produced similar dose response curves. Scrambled sequences of all-l- and all-d-peptides were not neurotoxic even at 50 µM (data not shown).
the two $\alpha$-enantiomers is shown in the dose response curves in Fig. 4E. Both enantiomers produce noticeable neurotoxic activity at 5 $\mu$M, and at 25 $\mu$M, approximately half of the neurons are dead within 24 h.

**DISCUSSION**

This study was designed to probe the stereospecificity of the interaction between $\alpha$- and the plasma membrane of cultured neurons that in vitro leads to programmed cell death (10, 27, 28). According to classic receptor pharmacology, a $\delta$-stereoisomer of an amino acid or peptide would not be predicted to exhibit bioactivity comparable with the native $\lambda$-peptide. For example, glutamate receptors readily discriminate $\lambda$-versus $\delta$-antagonistic agents (29). We have analyzed both the physical and biological properties of the all-$\delta$-enantiomers of $\alpha_{25-35}$ and $\alpha_{1-42}$, and have compared them with their corresponding all-$\lambda$-enantiomers. With the exception of the CD spectropolarimetry study, which produced mirror image spectrums, both all-$\delta$-enantiomers exhibited essentially identical physical and biological properties to their all-$\lambda$-enantiomers.

A number of studies have been done utilizing $\delta$-enantiomers of various ligands to investigate the stereospecific requirements for binding to their respective receptor proteins. In the cases of three peptide hormones, bradykinin (30), oxytocin (31), and angiotensin (32), that must interact with chiral receptors on the plasma membrane, the all-$\delta$-forms of the ligands were inactive. However, different results were obtained in the case of a synthetic $\beta$-endorphin analog that contained 18 $\delta$-amino acid residues in the C-terminal portion of the peptide but 5 $\lambda$-residues within the actual binding site. The all-$\delta$-containing region was designed to form a left-handed amphiphilic helical segment that was topochimically similar to the native right-handed amphiphilic helix. The $\delta/\lambda$ chimeric peptide retained equal ability to bind and to activate the opiate receptor (21). In some cases, the all-$\delta$-peptide enantiomers can still resemble...
the parent compound, both in the overall spatial arrangements and with respect to the electronic nature of the functional groups. In the case of the antibiotic enniatin B, the topochemically similar enantio-enniatin B possessed similar antimicrobial activity (20). In two recent studies for example, the all-D-peptide analogs were found to bind with similar affinities to their respective receptors. In the first one, two all-D-aminophilic helical peptides were shown to interact with calmodulin in a sterically malleable fashion (16, 33), and the second example reported that the laminin segment containing the IKVAV amino acid sequence, which is responsible for cell attachment and tumor-promoting activities, was retained in the all-D-peptide. Peptide analogs with either alternating L-D-substitutions or randomized IKVAV sequence were inactive, indicating that the sequence and conformational status of the domain contribute to the biological activity but that no stereospecific requirement exists (15).

Although bilayer lipids and membranes are also chiral and contain numerous asymmetric centers, the partitioning of chiral channel-forming antibiotic peptides into membranes does not require a specific chirality. The all-D-analogs of cecropin, magainin II amide, and melittin were equally effective when tested on achiral synthetic planar bilayers and as antibiotics against bacteria containing chiral membranes (34). The reports that Aβ̂$_{1-40}$ forms giant multivalent cation channels when incorporated into synthetic bilayers (35, 36) and our findings that the neurotoxic activity associated with both Aβ̂$_{25-35}$ and Aβ̂$_{1-42}$ are not chirally dependent are consistent with the results obtained with the channel-forming antibiotic peptides. Unfortunately, after many years of extensive study on Aβ, there is no definitive evidence that Aβ forms channels in neurons. Another more likely possibility is that Aβ acts as a membrane perturbant, which may alter the microenvironment between the bilayer and membrane-bound enzymes or receptors.

We currently favor a mechanism dependent on the interaction of Aβ with membrane receptor proteins on the surface of neurons, and other cell types such as astrocytes, because assembled fibrillar forms of the amyloidogenic peptides are required for activity (12, 14, 37). It is possible, for example, that Aβ acts as a ligand to cross-link receptors at the cell surface and activates cell death pathways via activation-induced cell death similar to Fas (38). Consistent with a mechanism involving membrane receptors, we have shown that the lectin ConA, which forms clusters of membrane glycoproteins on the cell surface, also causes neurodegeneration and apoptotic death in cultured neurons similar to that observed with Aβ while succinyl ConA, which binds but does not cross-link, is inactive (39). Recently, Burdick, et al. (40) have shown that a substantial portion of the Aβ that binds to cells can be removed by treatment with trypsin and several receptors that appear to bind Aβ peptides have been identified (41–46). In the case of the receptor for advanced glycation end products (RAGE), some evidence has been presented that it may be directly involved in Aβ-induced neurotoxicity (45). Experiments with all-D-Aβ and these putative Aβ receptors are in progress and should provide information on the specificity of these receptors for Aβ. Thus, we suggest that extracellular macromolecular assemblies such as Aβ can serve as stimuli or agonists that trigger a particular sequence of cellular reactions in neurons that initiate an apoptotic program of cell death. These PCD agonists are characterized in part by β-sheet fibrillar structure but, in addition, have the common ability to access critical signal transduction and downstream mechanisms that drive PCD.

Other amyloidogenic proteins, which do not share sequence homology with Aβ (e.g., prion and amylin), do form structurally similar extracellular deposits and have been found to have similar neurotoxic activity in vitro (47, 48). One possible mechanism that could explain the common biological activity seen with different amyloidogenic peptides is β-sheet augmentation, whereby a peptide forms a “peptide-surface association” (49) either by inserting itself into a β-sheet-containing domain (50), which has been proposed for other diseases involving protein conformational changes (51–54), or by adding to the edge of an anti-parallel β-strand, as has been implicated in regulating protein associations governing signal transduction pathways and assembly interactions in certain viral capsids (49). The β-sheet augmentation mechanism provides much greater flexibility than classical domain-domain association because the peptide is not constrained by a rigidly folded domain. The specificity in this model is dependent on the ability of the peptide to augment an appropriate β-strand on a protein. Consistent with this model, Aβ has shown a pronounced ability to bind to other proteins, such as α-1-antichymotrypsin, and transthyretin which are rich in β-sheet. Finally, the cell surface contains numerous proteins with Ig superfamly homology with extensive β-sheet content, which include receptors (55) and cell adhesion molecules (i.e., NCAM and N-cadherin) (56), and in several reports, the cell surface appears to be able to actually nucleate Aβ assembly (40, 57, 58), which is also consistent with the model (49).

The fact that the all-D analogs of Aβ retain bioactivity may present new avenues for therapeutic intervention by allowing the d-enantiomers of inhibitory peptides to be utilized. An approach similar to this has recently been used to identify an all-D-amino acid opioid peptide with analgesic activity capable of crossing the blood brain barrier using a synthetic combinatorial library made up of D-amino acid hexapeptides (59). In addition, the identification of D-peptide ligands through mirror image phage display using genetically encoded libraries (60) offers the promise of rapidly screening for D-peptide ligands that can block assembly and or neurotoxicity of the Aβ peptide.

All-D-ligands are generally resistant to proteolysis and D-amino acid proteins are reported to have low immunogenicity, thus making them useful for pharmacological applications (61). The results obtained in this study suggest that the neurotoxic activity of Aβ is independent of the classical stereoisomer-specific ligand-receptor interaction. Rather, Aβ-induced neurotoxicity is dependent on the primary sequence of the peptide that regulates both the ability of the peptide to assemble into active conformations and bind to cellular surfaces. While mechanisms dependent on the perturbation of cellular membranes or the formation of calcium ion channels cannot be excluded, the requirement for higher order protein assemblies by amyloidogenic peptides for biological activity does not readily support these mechanisms. Further investigation of the mechanism of Aβ-induced toxicity will likely benefit attempts to understand the neurodegeneration that occurs in AD and perhaps other amyloid-related disorders.

Acknowledgments—We thank Virany Kreng for excellent technical assistance and Dr. Charles Glabe for synthesis and characterization of the amyloid peptides.

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