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Rapid Photochemical Cross-Linking—A New Tool for Studies of Metastable, Amyloidogenic Protein Assemblies

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ABSTRACT

Amyloidoses comprise a class of diseases characterized pathologically by the presence of deposits of fibrillar, aberrantly folded proteins, known as amyloids. Historically, these deposits were considered the key factors causing disease. However, recent evidence suggests that soluble protein oligomers, which are precursors for amyloid fibrils, are the primary toxic effectors responsible for the disease process. Understanding the mechanism by which these oligomers exert their toxicity requires knowledge of the structure, kinetics, and thermodynamics of their formation and conversion into larger assemblies. Such studies have been difficult due to the metastable nature of the oligomers. For the amyloid β-protein (Aβ), a consensus about the size and relative abundance of small oligomers has not been achieved. We describe here the application of the method Photoinduced Cross-Linking of Unmodified Proteins (PICUP) to the study of Aβ oligomerization. This approach distinguishes oligomerization patterns of amyloidogenic and nonamyloidogenic proteins, allows quantification of each component in oligomer mixtures, and provides a means of correlating primary structure modifications with assembly characteristics. PICUP thus is a powerful tool for the investigation of small, metastable protein oligomers. The method provides essential insights into the factors that control the assembly of pathogenic protein oligomers, facilitating efforts toward the development of therapeutic agents.

Introduction

Aberrant protein folding is involved in the etiology of many diseases, including neurodegenerative, metabolic, and systemic disorders.[1,2] Misfolding often leads to amyloid formation. (Misfolding is a relative term used here only to distinguish positions in conformational space differing from those associated with the native folds of the respective amyloid proteins. Outside of this context, the term misfolding has no intrinsic meaning relevant to understanding protein folding and function.) The family of diseases in which this is a prominent feature is called amyloidoses.[3] The term amyloid, which is derived from *amylum*, the Latin word for starch, was used originally because the gross appearance and histochemical staining characteristics of protein deposits were starch-like.[4] The occurrence of cerebral amyloid deposits (plaques) in patients with dementia was reported almost a century ago by Alois Alzheimer,[5] after whom the most common form of dementia is named. With the development of electron microscopy (EM), amyloids from a variety of diseases, including Alzheimer's disease (AD), were shown to contain an abundance of fibrillar material.[6] This material, rather than being starch, was proteinaceous. In 1984, Glenner and Wong reported the first protein sequence of vascular amyloid from AD,[7] leading to the cloning of its structural gene. Because the amyloid protein contained large amounts of β-sheet, it was named the amyloid β-protein (Aβ).[8,9] Amyloid fibrils also are deposited in a variety of other diseases, including Parkinson's disease, Huntington's disease, prion diseases, amyotrophic lateral sclerosis (Lou Gehrig's disease), familial amyloidotic polyneuropathy, type II diabetes, and light chain amyloidosis.[3,10] In all cases, the main component of the deposit is a protein normally expressed in the body.[11] These observations lead to the hypothesis that protein misfolding is the key etiologic factor in amyloidoses. This hypothesis is supported by the finding that fibrillar deposits in many of these diseases are surrounded by active inflammatory processes and dying cells.[12] In addition, fibrils isolated from diseased tissues, or prepared from recombinant or synthetic amyloid proteins (e.g., Aβ, α-synuclein, transthyretin), are cytotoxic in vitro and in vivo.[3] This suggests that the most important etiologic consequence of protein misfolding is amyloid fibril formation. Recently, however, studies in transgenic mice expressing human Aβ have demonstrated that neuronal dysfunction can occur in the

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Dr. Bitan received a B.Sc. cum laude in chemistry and biochemistry (1988), a M.Sc. cum laude in organic chemistry (1989), and a Ph.D. summa cum laude in organic chemistry (1996) from the Hebrew University of Jerusalem, Israel. As a graduate student with Prof. Chaim Gilon, Dr. Bitan was a Golda Meir Foundation Scholarship winner. Dr Bitan is a coinventor and a coauthor of patented methodologies for peptide cyclization—methodologies that formed the basis for the creation of the biotechnology company Peptor Inc. in 1993. As a postdoctoral fellow, Dr. Bitan worked with Profs. Michael Rosenblatt and Michael Chorev at Beth Israel Deaconess Medical Center and Harvard Medical School on the structural biology of ligand−receptor systems including integrins and G-protein-coupled receptors. In 1999, Dr. Bitan joined the group of Prof. David Teplow at the Center for Neurologic Diseases, Brigham and Women's Hospital and the Department of Neurology, Harvard Medical School. Dr. Bitan has made fundamental contributions to the study of oligomerization of the amyloid β-protein, including the discovery of the earliest assembly intermediate, the paranucleus. Dr. Bitan is currently an Instructor in Neurology at Harvard Medical School.

Dr. Teplow received undergraduate degrees in Biochemistry (1974) and Bacteriology/Immunology (1975) from the University of California, Berkeley. He did graduate work on the protein biochemistry of lymphocyte receptors at the University of Washington, receiving his Ph.D. in 1981. As a postdoctoral fellow at Caltech, Dr. Teplow worked with Profs. William Dreyer and Lee Hood on the development and application of microchemical methods to the synthesis and structural analysis of proteins and DNA. Later at Caltech, as a Research Biologist and then as a Member of the Beckman Institute, Dr. Teplow focused on the structure−function analysis of nervous system proteins, including seminal work with Dr. Stanley Prusiner on primary structure analysis of the scrapie prion. Dr. Teplow joined the Center for Neurologic Diseases, Brigham and Woman's Hospital and the Department of Neurology, Harvard Medical School, in 1991. Dr. Teplow currently is an Associate Professor of Neurology, Harvard Medical School and is Director of the Biopolymer Laboratory at Brigham and Women's Hospital. Dr. Teplow's group has made fundamental contributions to our understanding of the biochemistry, biophysics, and cell biology of the amyloid β-protein (Aβ), including the discovery of protofibrils and paranuclei, fibril assembly intermediates with potent neurotoxic activity that may be key etiologic agents in Alzheimer's disease.

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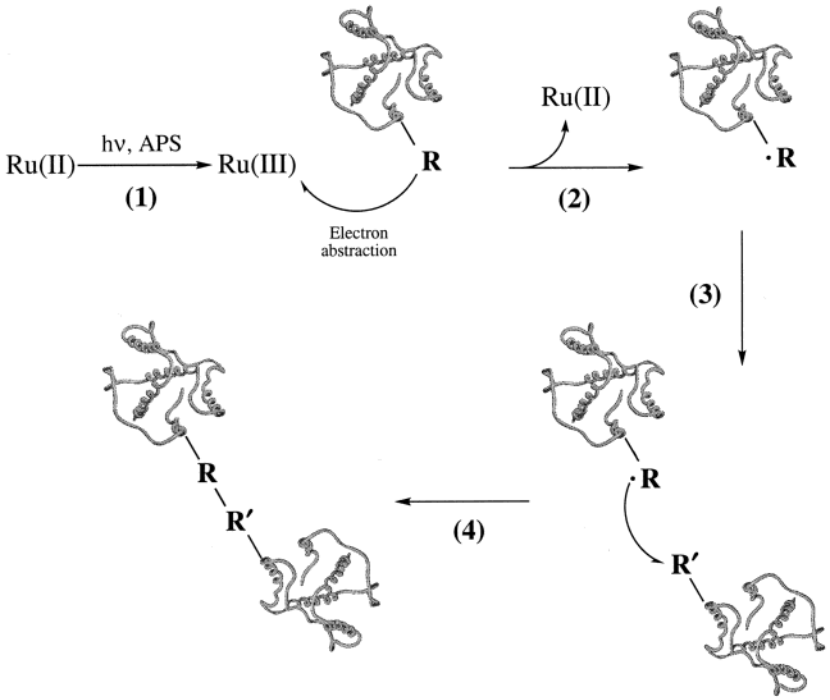
Scheme 1



absence of amyloid deposition.[13] These, and other data, have supported a paradigm shift.[14] This shift deemphasizes the role of fibrils, ascribing pathogenetic primacy to oligomeric assemblies. In agreement with this concept, soluble, oligomeric assemblies of amyloidogenic proteins were found to be potent cytotoxins.[14] Interestingly, studies of model proteins have shown that although essentially any protein may undergo aberrant folding and fibrillogenesis under suitable conditions, the fibrillar form may be benign whereas the precursor oligomers are cytotoxic.[15] Protein oligomers thus may be key targets in strategies to treat diseases associated with protein misfolding.

To test the hypothesis that oligomers of amyloidogenic proteins are critical pathogenetic agents, a clear understanding of the structural, kinetic, and thermodynamic features of their assembly is necessary. However, achieving this goal has been difficult due to the metastable nature of these oligomers and the complex equilibria associated with their formation (involving monomers, low and high order oligomers, and fibrils).

Aβ oligomerization has been the subject of very active investigation. Biochemical and biophysical methods, including size-exclusion chromatography (SEC), sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), ultracentrifugation, dynamic light scattering, fluorescence resonance energy transfer, and chemical or enzymatic cross-linking have been used to assess Aβ oligomer size and relative abundance (for a more comprehensive discussion, see ref 16 and references therein). These studies have provided important information about the early events of Aβ assembly, but a consensus on the oligomer size distribution has not been achieved. Nascent Aβ was reported to comprise a variety of species, ranging from monomer through hexamer, depending on the preparation conditions, the biochemical/biophysical method used, and the actual sequence studied. Oligomerization of other amyloidogenic proteins also has been studied, providing valuable morphologic, kinetic, and thermodynamic data and suggesting the existence of common protofibrillar structures and mechanisms of assembly.[17]

Because oligomer assembly is a dynamic process, in which many different species may exist simultaneously, an ideal method for determining the oligomer composition would provide accurate, quantitative snapshots of the different species present in the mixture at different time points. To do so, the method should be applicable within a time interval significantly smaller than the lifetimes of the species involved in the assembly process. In addition, to accurately reveal the native oligomerization state of protein populations, the method should require no prior modification of the protein. In this Account, we describe the application of a method with these characteristics, Photoinduced Cross-Linking of Unmodified Proteins (PICUP).[18] This method has allowed the study of the earliest oligomerization events in Aβ assembly and is readily applicable in studies of other peptides and proteins.

PICUP Chemistry

PICUP was developed in the Kodadek laboratory at the University of Texas as a method to study proteins that naturally form stable homo- or heterooligomers.[18] The chemistry is based on visible light excitation of a tris(2,2′-bipyridyl)dichlororuthenium(II) complex.[19] When the photoexcitation is performed in the presence of an electron acceptor, Ru(II) is oxidized to Ru(III). Ru(III) is a strong one-electron oxidizer that can abstract an electron from a nearby protein molecule, producing a protein radical and recycling back to Ru(II) (Scheme 1). Reoxidation of

Ru(II) then initiates a new cycle of radical generation. Radical formation may occur, in principle, at any site along the polypeptide chain. However, the radical would form preferentially at side chains that offer stabilization through aromatic or neighboring-group effects (e.g., Tyr, His, or Met).[20] If these side chains are not near the Ru(III) oxidizer, it may abstract an electron from less reactive side chains or react with the reaction medium. Once the protein radical is formed, it can attack a neighboring protein to form an intermolecular cross-link. PICUP, unlike traditional chemical[21] or photoaffinity[22] cross-linking methods, results in covalent bond formation directly between unmodified protein molecules, without the insertion of a spacer moiety or an unnatural photoreactive group. In addition, the PICUP reaction requires very short time periods (≤1 s) and occurs across a wide pH range.[23] The high cross-linking efficiency of PICUP (70−80%) and its ability to cross-link side chains directly make it an attractive tool for study of the early events leading to formation of metastable protein assemblies. Snapshots of dynamic protein association events occurring in solution can be taken at various time points in an assembly process by using PICUP to covalently freeze components of the mixture and then analyzing the resulting cross-linked components. For more information about the scope and limitations of the PICUP chemistry, see refs 24 and 25.

Determination of Protein Oligomer Size Distributions

Efficient protein cross-linking allows the analysis of three important states of proteins: stable monomers, stable oligomers, and unstable oligomers. Fancy and Kodadek showed that when PICUP was performed on proteins known to form stable oligomers, the main products observed in SDS−PAGE analysis were the expected oligomers.[18] For example, upon cross-linking of UvsY, a native hexamer involved in phage T4 recombination, the main product was a hexamer.[18] Similar results were obtained for glutathione *S*-transferase and a number of other enzymes, all of which form stable dimers.[24,26−29] It should be noted that even when a protein forms a stable oligomer, small amounts of lower order oligomers and monomer may still be observed following PICUP chemistry and SDS−PAGE analysis because the efficiency of cross-linking is <100% and noncross-linked oligomers can dissociate in the presence of SDS. In addition, for proteins existing in an equilibrium involving monomer and oligomer(s), diffusion-controlled cross-linking of preexisting oligmers with monomer may yield higher order artificial oligomers. However, the abundance of such artificial oligomers, produced as a cross-linking artifact, follow simple probabilistic rules. These side products are therefore readily distinguished from genuine, preexisting oligomers. The demonstrated concordance of PICUP-derived protein oligomer distributions with those determined by classical methods suggested to us that PICUP could be of significant value for elucidating features of peptide oligomerization, in particular those involving Aβ.

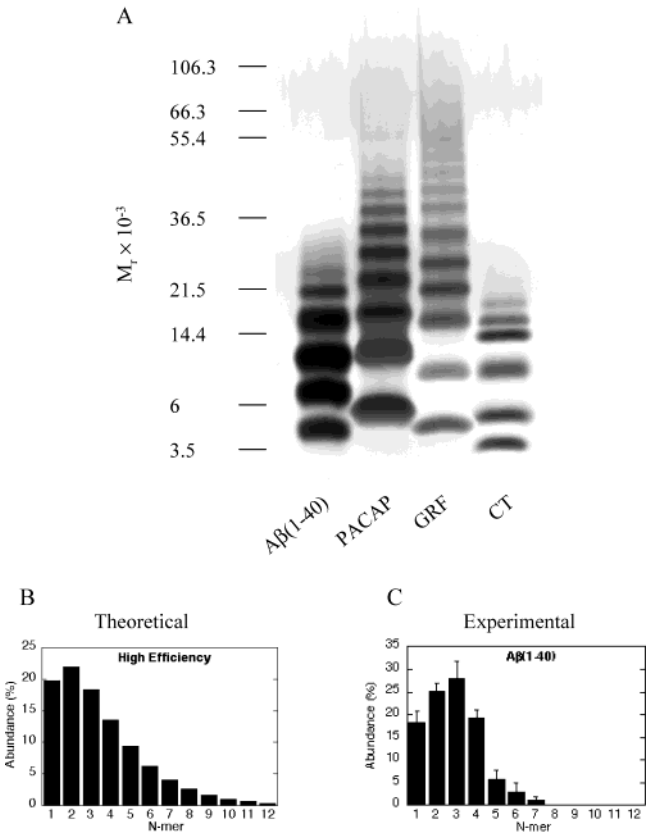


FIGURE 1. SDS−PAGE and densitometrtic analysis of PICUP products of amyloidogenic and nonamyloidogenic peptides as compared to theoretical monomeric proteins. (A) Aggregate-free Aβ-(1−40), PACAP, GRF, and CT were prepared by filtration through a 10 kDa molecular weight cutoff filter and cross-linked immediately. A silver-stained gel is shown. Positions of molecular weight standards are shown on the left. (B) A theoretical distribution of cross-linked, monomeric proteins obtained using the model described in ref 16. (C) Densitometric analysis of the cross-linking products obtained for Aβ(1−40) experimentally (Reproduced with permission from ref 16. Copyright The American Society for Biochemistry & Molecular Biology 2001).

In our initial control experiments, we applied PICUP to natively monomeric peptides and proteins. Cross-linking the monomeric peptides pituitary adenylate cyclase-activating polypeptide (PACAP) and growth hormone-releasing factor (GRF), followed by SDS−PAGE analysis, yielded a ladder of oligomers displaying an exponential decrease in abundance with increasing oligomer order (Figure 1A).[16] To evaluate these results, we developed a simple mathematical model that simulated the cross-linking reaction. The model assumed that the substrate molecules were monomeric spheres and the only interaction among them was random elastic collision.[16] An example of a theoretical oligomer distribution generated using this model under high cross-linking efficiency conditions is shown in Figure 1B. The experimental distributions observed for PACAP,[16] GRF,[16] and lysozyme [24] were very similar to this theoretical distribution, a result expected for diffusion-controlled collisions and cross-linking among activated monomers. To demonstrate that

these results were an accurate reflection of the monomeric state of the peptides and were not sequence-specific effects, we studied the protein transthyretin (TTR). TTR is a tetramer under physiologic conditions but can be stabilized in a monomeric form under appropriate conditions.[30] Cross-linking of TTR, preincubated in monomer-stabilizing conditions, yielded an oligomer ladder expected for a monomeric protein, similar to the theoretical distribution in Figure 1B. In contrast, under physiologic conditions, cross-linking of TTR yielded monomer, dimer, and tetramer.[16] These data confirmed that PICUP was capable of distinguishing between monomeric and oligomeric states of the same protein and that the oligomer distributions obtained accurately reflected the assembly state of each protein, independent of its unique amino acid sequence.

To evaluate the utility of PICUP for the study of amyloidogenic proteins, the technique was applied to aggregate-free preparations of the amyloidogenic peptides Aβ(1−40) and calcitonin (CT). The resulting oligomer distributions clearly differed from those produced by monomeric peptides (Figure 1A) and from the theoretical distribution calculated for monomeric peptides (cf. Figure 1B,C). Aβ(1−40) yielded an oligomer size distribution ranging from monomer through heptamer. The distribution was characterized by similar amounts (17−24% of the total protein content, measured by densitometry) of monomer, dimer, trimer, and tetramer (Figure 1C).[16] These data were consistent with the preexistence of monomer through tetramer in the solution, a state which would lead to very efficient cross-linking. As discussed previously, larger oligomers may form by a diffusion-dependent reaction of preexisting oligomers with free monomer. Consistent with this interpretation, the pentamer through heptamer were formed in small amounts and displayed a sharp exponential decrease in abundance with increasing oligomer order, demonstrating that they were produced artificially and did not exist prior to the cross-linking reaction. The results demonstrated that preexisting, metastable oligomers could be "frozen" by cross-linking and then analyzed and quantified. Cross-linking of CT also produced a limited oligomer size distribution, characterized by predominant monomer, dimer, and tetramer bands (Figure 1A).[16] Similar oligomer size distributions, distinct from those of monomeric or stable oligomeric proteins, also were observed for the amyloidogenic peptides calcitonin gene-related peptide (CGRP) and islet amyloid polypeptide (IAPP, amylin) (G. Bitan, unpublished results). Thus, PICUP revealed that peptides with amyloidogenic propensity form mixtures of kinetically unstable oligomers before fibrils assemble. These quasi-equilibrium mixtures are difficult to analyze by other means but can be detected and quantified using PICUP.

PICUP as a Tool for Structural Studies

Small changes in protein primary structure are known to have dramatic effects on protein assembly and activity. Examples relevant to protein misfolding are familial forms of amyloidosis that are caused by mutations resulting in single amino acid substitutions in the respective amyloid protein(s).[31] These mutations can affect the thermodynamics and the kinetics of fibril assembly relative to the wild-type (WT) protein or alter the stability of intermediate assemblies. An important question is which of these effects is relevant clinically. If oligomeric assemblies rather than fibrils are the key etiologic factors in amyloidosis, as is now suggested,[15] small structural modifications may result in the formation or stabilization of particular oligomers. However, as discussed previously, investigation of the relationship between the primary structure of amyloidogenic proteins and their quaternary structure at the oligomer level has been difficult. PICUP offers an opportunity to study primary−quaternary structure relationships involved in early assembly of amyloidogenic proteins by comparing oligomer size distributions of WT and structurally altered alloforms. We have applied this approach to study primary−quaternary structure relationships of Aβ (Figure 2).

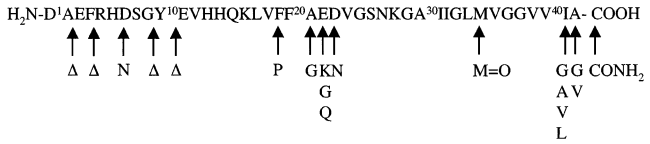


FIGURE 2. Schematic representation of structural modification introduced in the Aβ sequence and studied using PICUP. Substitutions are indicated using a one letter code. Deletion N-terminally to the arrow point is indicated by Δ.

Aβ is produced through endoproteolytic cleavage of its precursor, the amyloid β-protein precursor (AβPP). The predominant alloforms found in the brain are 40 (Aβ(1−40)) or 42 (Aβ(1−42)) amino acids in length. Aβ(1−40) is the most abundant form, yet several lines of evidence have shown that Aβ(1−42), not Aβ(1−40), is linked most strongly to the etiology of AD. For example, mutations increasing the Aβ(1−42)/Aβ(1−40) concentration ratio cause early-onset, familial forms of AD and treatments that reduce Aβ(1−42) levels correlate with decreased risk for AD.[32] Aβ(1−42) also displays enhanced neurotoxicity relative to Aβ(1−40). In particular, oligomers of Aβ(1−42) recently have been shown to be substantially more neurotoxic than those of Aβ(1−40).[33,34] The mechanistic basis for these toxicity differences is not known.

Using PICUP, we found that the oligomer size distributions of Aβ(1−40) and Aβ(1−42) differed. Unlike the equilibrium mixture of monomer, dimer, trimer, and tetramer formed by Aβ(1−40), Aβ(1−42) preferentially formed pentamer/hexamer units that self-associated into larger assemblies, including dodecamers and octadecamers (Figure 3A).[35] Bar graphs representing the densitometric analyses of these data and the PICUP results described hereafter are available in the original publications. The data suggested that pentamer/hexamer units were building blocks of the higher order oligomers. The term paranuclei thus was introduced to describe the pentamer/hexamer units. Paranuclei are the earliest assemblies observed for Aβ(1−42). The differences in oligomerization between Aβ(1−40) and Aβ(1−42) offer a plausible expla-

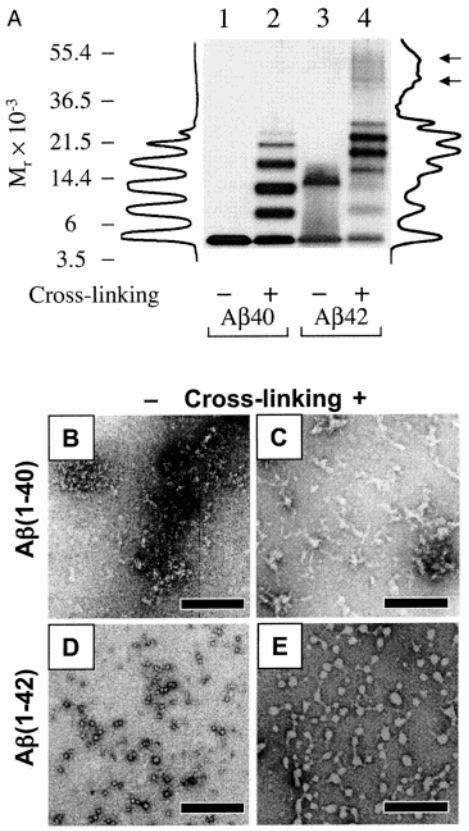


FIGURE 3. Aβ(1−40) and Aβ(1−42) display distinct oligomer distributions and morphologies. Aβ(1−40) and Aβ(1−42) were cross-linked and analyzed by SDS−PAGE/silver staining or by EM. (A) Noncross-linked (lanes 1 and 3) or cross-linked (lanes 2 and 4) Aβ(1−40) and Aβ(1−42). Densitometric intensity profiles of lanes 2 (cross-linked Aβ(1−40) and 4 (cross-linked Aβ(1−42) are shown on the left and right sides of the gel, respectively. Arrows indicate intensity maxima in lane 4 corresponding to Aβ(1−42) dodecamer and presumptive octadecamer. Positions of molecular weight standards are shown on the left. Aβ(1−40) (B and C) or Aβ(1−42) (D and E) were analyzed by electron microscopy (EM). Aliquots of noncross-linked (B and D) or cross-linked (C and E) peptide were spotted on glow-discharged, carbon-coated grids and stained with uranyl acetate prior to examination by EM. Scale bars are 100 nm. (Reproduced with permission from ref 35. Copyright National Academy of Sciences 2003).

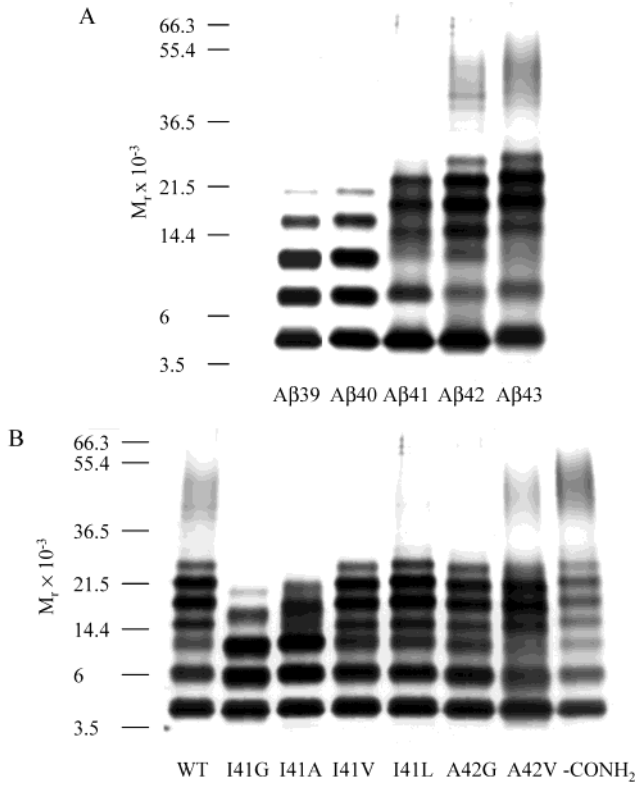


FIGURE 4. Dependence of Aβ oligomerization on C-terminal length and structure. (A) Aβ(1−39), Aβ(1−40), Aβ(1−41), Aβ(1−42), and Aβ(1−43) were cross-linked individually and analyzed by SDS−PAGE. Positions of molecular weight standards are shown on the left. (B) PICUP was applied to Aβ(1−42) analogues containing the modifications Ile-41 → Gly, Ile-41 → Ala, Ile-41 → Val, Ile-41 → Leu, Ala-42 → Gly, Ala-42 → Val, or Aβ42-carboxamide. WT Aβ(1−42) was used as control. Positions of molecular weight standards are shown on the left. (Reproduced with permission from refs 35 and 36. Copyright National Academy of Sciences 2003 and Copyright American Society for Biochemistry & Molecular Biology 2003).

nation for the differences in neurotoxicity observed for the two alloforms.

Following the discovery of paranuclei, complementary biophysical methods were used to further characterize them. For example, the morphologies of the paranuclei were assessed by EM and were consistent with the PICUP data. Thus, whereas Aβ(1−40) showed mainly amorphous structures that became slightly larger upon cross-linking (Figure 2B,C), Aβ(1−42) formed predominantly spheroids, ∼5 nm in diameter, which appeared either individually or associated into small groups (Figure 3D).[35] Following cross-linking, chains of spheroidal structures were apparent, most of which were connected to each other by narrow (1−2 nm) threads (Figure 3E). The differences between the early oligomerization of Aβ(1−40) and Aβ-(1−42) did not result from substantial conformational differences, as both alloforms yielded similar circular dichroism (CD) spectra, characterized mainly by irregular secondary structures.[35] Insight into the mechanisms controlling the distinct oligomerization behavior of Aβ(1−40) and Aβ(1−42) was obtained by examination of the PICUP-derived oligomer size distributions of Aβ analogues ending at positions 39−43. The distribution of Aβ(1−39) was essentially identical to that of Aβ(1−40), but the distributions obtained for Aβ(1−41), Aβ(1−42), and Aβ(1−43) were distinct (Figure 3A).[35] Paranucleus formation did not occur in the absence of Ile-41. Elongation of the Aβ(1−41) peptide by the addition of Ala-42, or of Ala-42 and Thr-43, facilitated paranucleus self-association. Ile-41 thus was necessary and sufficient for formation of paranuclei.

To gain additional mechanistic insight into the roles of amino acids 41 and 42 in paranucleus formation and self-association, the side chains of these residues were substituted systematically by smaller or larger residues. In addition, the C-terminal carboxyl group was changed to a carboxamide (Figure 2). PICUP proved to be a useful method for elucidation of the effects of single residue and subresidue structural modifications on Aβ assembly. Substitution of Ile-41 by Gly, eliminating both the side

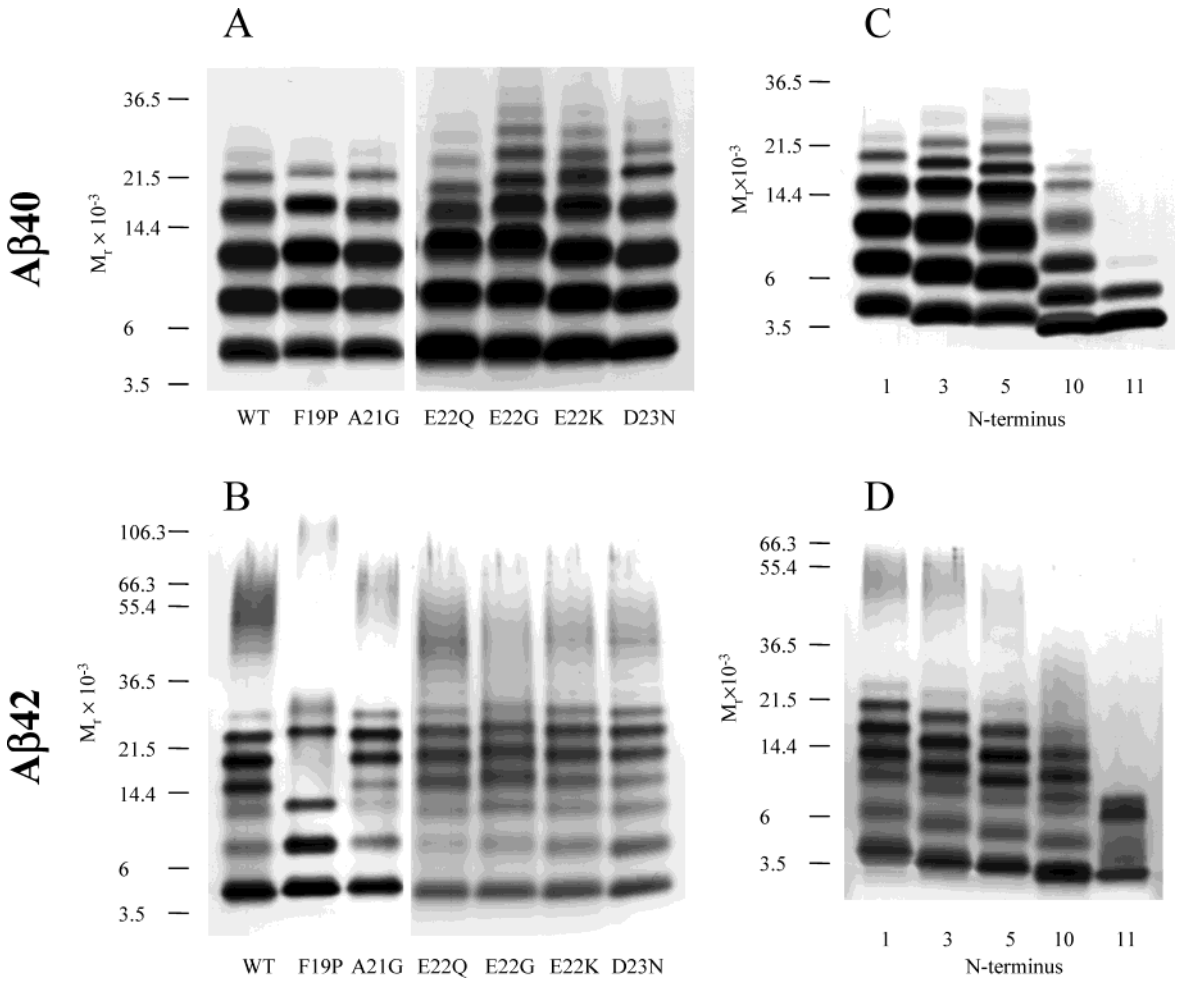


FIGURE 5. Effects of central region and N-terminus structure on early Aβ assembly. Aβ(1−40) (A) and Aβ(1−42) (B), each containing the substitutions Phe-19 → Pro, Ala-21 → Gly, Glu-22 → Gln, Glu-22 → Gly, Glu-22 → Lys, or Asp-23 → Asn and of Aβ(x-40) (C) and Aβ(x-42) (D), in which $x = 1, 3, 5, 10$, or 11, were cross-linked, analyzed by SDS−PAGE, and visualized by silver staining. WT Aβ(1−40) and Aβ(1−42) were used as controls. Positions of molecular weight standards are shown on the left of each panel. (Reproduced with permission from ref 36. Copyright American Society for Biochemistry & Molecular Biology 2003).

chain and the stereocenter of the C_α group, yielded a distribution that was qualitatively similar to that of Aβ-(1−40) (Figure 4B).[36] When Ile-41 was substituted by Ala, the oligomer size distribution was a composite of the distributions characteristic of Aβ(1−40) and Aβ(1−42) (Figure 4B), whereas substitution of Ile-41 by Val or Leu yielded distributions that were similar to WT Aβ(1−42) in the amount of paranuclei formed but displayed substantially fewer high order oligomers.[36] These data indicated that a minimal alkyl side-chain in position 41, such as the methyl group of Ala, was essential for paranucleus formation. Moreover, whereas the propyl and *iso*-butyl side chains of Val and Leu, respectively, supported paranucleus formation, the *sec*-butyl side chain of Ile-41 was important for paranucleus self-association (Figure 4B).[36] When the C-terminal carboxyl group was changed to a carboxamide, a large increase in the abundance of oligomers between 30 and 60 kDa was observed (Figure 4B), revealing that the charged C-terminal carboxyl moderates paranucleus self-association.[36]

In addition to structure−activity studies based on theoretical considerations, familial forms of AD (FAD) and cerebral amyloid angiopathy (CAA; a related, vascular amyloidosis) reveal sites in the Aβ molecule linked to disease. A hot spot for diseases caused by mutations in the Aβ region of the AβPP gene is the segment encoding residues 20−22 (for a review of the structural modifications discussed hereafter and the relevant references, see ref 37). The mechanism by which amino acid changes in these sites cause disease is not completely understood. One hypothesis is that the disease-associated amino acid substitutions alter Aβ assembly.[17] We thus used PICUP to examine the effects of FAD/CAA-linked substitutions in the central region of Aβ (Figure 2). Consistent with our finding that Aβ(1−40) and Aβ(1−42) oligomerize through distinct mechanisms, identical amino acid substitutions in the 20−22 region had different effects on peptide oligomerization (Figure 5). Substitution of Glu-22 by Gln (Dutch mutation), Gly (Arctic mutation), or Lys (Italian mutation), or substitution of Asp-23 by Asn (Iowa mutation) led to formation of Aβ(1−40) oligomers of higher order than those observed for WT Aβ(1−40) (Figure 5A).[36] The same substitutions had essentially no effect on the oligomer size distributions of Aβ(1−42) analogues (Figure

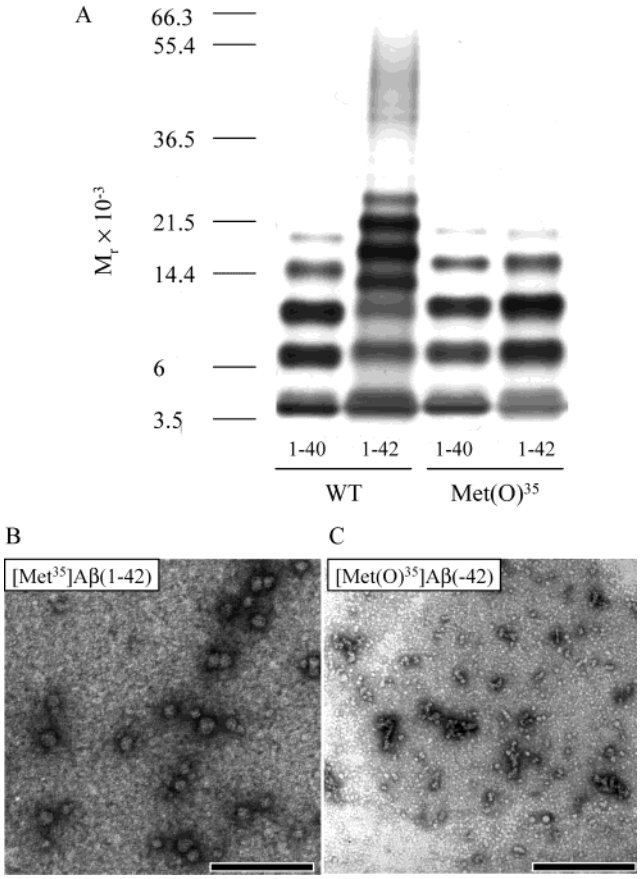


FIGURE 6. Effects of Met-35 oxidation on Aβ oligomerization. (A) PICUP was applied to WT and oxidized Aβ(1−40) and Aβ(1−42). Following cross-linking, the products were analyzed by SDS−PAGE and silver staining. Positions of molecular weight standards are shown on the left. Aβ(1−42) peptides containing Met-35 (B) or Met-(O)-35 (C) were spotted on glow-discharged, carbon-coated grids. The samples then were stained with uranyl formate and examined by EM. Scale bars are 100 nm.

5B). Similarly, truncation of the N-terminal 2 or 4 residues of Aβ(1−40) yielded distributions characterized by formation of higher order oligomers (Figure 5C), whereas equivalent truncations of Aβ(1−42) did not affect the oligomer size distributions (Figure 5D).[36] In contrast, substitution of Ala-21 by Gly (Flemish mutation), or of Phe-19 by Pro (a modification known to abolish fibril formation), altered the oligomer size distribution of Aβ-(1−42) (Figure 5B) but had little effect on the distribution of Aβ(1−40) oligomers (Figure 5A) (a full discussion of the effects of the structural modifications described here is given in ref 36).

One mechanism by which Aβ injures or kills neurons is oxidative stress.[38] It has been postulated that a particularly important oxidation event, associated with Aβ-induced oxidative damage, is the conversion of Met-35 to the corresponding sulfoxide.[39] Solution NMR studies of reduced and oxidized Aβ(1−40) and Aβ(1−42) have shown little conformational difference among the four peptides,[40] suggesting that the effect of Met-35 oxidation on the biological activity of Aβ is exerted in the assembled state rather than in the monomeric state. Recently, oxidation has been demonstrated to attenuate formation of small[41] and large Aβ oligomers.[42] We examined the effect of Met-35 oxidation on the oligomer size distribution of Aβ(1−40) and Aβ(1−42) using PICUP (Figure 6). Met-35 oxidation had little effect on oligomerization of Aβ(1−40), but it abolished the formation of Aβ(1−42) paranuclei.[43] In fact, the oligomer size distribution of [Met(O)-35]Aβ-(1−42) was indistinguishable from that of reduced or oxidized Aβ(1−40) (Figure 6A). Consistent with the PICUP data, the spherical structures commonly observed in WT Aβ(1−42) preparations (Figure 6B) were not detected for oxidized Aβ(1−42), whose morphology was amorphous (Figure 6C) and resembled that of Aβ(1−40) (Figure 3C).[43] Met-35 oxidation thus causes profound changes in Aβ-(1−42) oligomerization, suggesting that specific oxidation of Aβ(1−42) may be a plausible direction for treatment of AD.

Conclusions

Increasing clinical and experimental evidence suggests that oligomers, rather than higher order polymers (fibrils), are the primary pathologic effectors in several amyloidoses.[14,44] It thus is very important to understand how this oligomerization occurs. However, studying oligomerization using classical biophysical approaches has been difficult because oligomers have short lifetimes and exist as dynamic mixtures, including monomers and higher order assemblies. The application of the PICUP approach to this problem has provided the means to significantly advance our understanding of protein oligomerization. PICUP can provide snapshots of metastable oligomer mixtures, both during their assembly into higher order structures and during their dissociation into monomers. Coupled with electrophoresis or other analytical methods (e.g., chromatography, mass spectrometry), each component in the population may be quantified. It also becomes possible to stabilize and isolate specific oligomers to determine whether a given oligomer is cytotoxic or amyloidogenic, providing valuable information for directing future efforts to treat amyloid-related diseases. The qualitative and quantitative insights into dynamic oligomer distributions provided by PICUP may also be useful for diagnostics and for the identification of oligomerization inhibitors. For example, it may be possible to develop diagnostic systems to test for the presence of oligomers in biological fluids (e.g., plasma, CSF, urine) and to correlate disease severity with oligomer size distributions of pathologic proteins. We have only begun to explore the potential of this useful cross-linking method.

Space limitations restrict the ability to discuss and reference all of the contributions made by the many fine laboratories studying diseases involving aberrant protein assembly and activity. We acknowledge these efforts here. This Account was supported by Grants AG14366, AG18921, and NS38328 from the National Institutes of Health (D.B.T.), by the Foundation for Neurologic Diseases (D.B.T.), and by Grant 1042312909A1 from The Massachusetts Alzheimer's Disease Research Center (G.B.).

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