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[12] Structural Study of Metastable Amyloidogenic Protein Oligomers by Photo-Induced Cross-Linking of Unmodified Proteins

By GAL BITAN

Abstract

Oligomers of amyloidogenic proteins are believed to be key effectors of cytotoxicity and cause a variety of amyloid-related diseases. Dissociation or inhibition of formation of the toxic oligomers is thus an attractive strategy for the prevention and treatment of these diseases. In order to develop reagents capable of inhibiting protein oligomerization, the structures and mechanisms of oligomer formation must be understood. However, structural studies of oligomers are difficult because of the metastable nature of the oligomers and their existence in mixtures with monomers and other assemblies. A useful method for characterization of oligomer size distributions *in vitro* is photo-induced cross-linking of unmodified proteins (PICUP) (Fancy and Kodadek, 1999). By providing ''snapshots'' of dynamic oligomer mixtures, PICUP enables quantitative analysis of the relations between primary and quaternary structures, offering insights into the molecular organization of the oligomers. This chapter discusses the photochemical mechanism; reviews the scope, usefulness, and limitations of PICUP for characterizing metastable protein assemblies; and provides detailed experimental instructions for performing PICUP experiments.

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Introduction

The Role of Protein Oligomers in Amyloidosis

Amyloidogenic proteins are characterized by their tendency to aggregate into β-sheet-rich amyloid fibrils, leading to a variety of pathologic conditions. Diseases characterized by accumulation of amyloid fibrils are termed *amyloidoses* (Buxbaum, 1996). These diseases can be systemic (Buxbaum, 2004) (e.g., light-chain amyloidosis), or affect particular tissues, such as the pancreas in type II diabetes mellitus (Marzban *et al.*, 2003). Some of the most devastating amyloidoses affect the central nervous system, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), prion diseases (e.g., ''mad cow'' disease), and amyotrophic lateral sclerosis (ALS, Lou Gehrig disease) (Trojanowski and Mattson, 2003). The amyloidogenic proteins that cause these diseases have diverse sequences, origins, and structures. Nevertheless, they all share the tendency to aggregate into amyloid fibrils. Fibrils isolated from diseased tissues or prepared from recombinant or synthetic amyloidogenic proteins (e.g., amyloid-β protein [Aβ], α-synuclein, transthyretin, islet amyloid polypeptide [IAPP]), are cytotoxic *in vitro* and *in vivo* (Gambetti and Russo, 1998). In view of these data, for many years, the prevailing paradigm, known as the ''amyloid cascade hypothesis'' (Hardy and Higgins, 1992), mandated that aggregation of amyloidogenic proteins into fibrils caused the respective amyloidoses. However, accumulating evidence from studies in humans, normal rodents, transgenic mice, cultured cells, and *in vitro* systems now suggests that soluble, oligomeric assembly intermediates of amyloidogenic proteins are the primary pathogenetic effectors in amyloidoses (Kirkitadze *et al.*, 2002; Thirumalai *et al.*, 2003; Walsh and Selkoe, 2004b). The majority of the data regarding oligomer assembly and toxicity have been obtained in studies of Aβ, the primary cause of AD (Mattson, 2004; Walsh and Selkoe, 2004a), which is often considered an archetype of amyloidogenic proteins (Lazo *et al.*, 2005). The evidence is not limited to Aβ or AD, however. Abundant data obtained for other proteins demonstrate that oligomer formation may be a common mechanism by which amyloidogenic proteins cause disease (Conway *et al.*, 2000; Demuro *et al.*, 2005; El-Agnaf *et al.*, 2001; Malisauskas *et al.*, 2005; Reixach *et al.*, 2004). In addition, protein-folding studies have shown that under suitable conditions, globular proteins that do not normally aggregate and are not associated with amyloidosis also form oligomers and fibrils similar to those formed by amyloidogenic proteins (Chiti *et al.*, 2002). Interestingly, oligomers formed by such proteins were found to be cytotoxic, whereas the counterpart fibrils were benign (Bucciantini *et al.*, 2002). Taken together, these data have supported a paradigm shift (Kirkitadze

et al., 2002) and a revision of the amyloid cascade hypothesis (Hardy, 2002; Hardy and Selkoe, 2002) that de-emphasize the role of fibrils and ascribe pathogenetic primacy to oligomeric assemblies. Thus, protein oligomers are new key targets of strategies developed to treat diseases associated with protein misfolding and aggregation.

Challenges in Biophysical Characterization of Amyloidogenic Protein Oligomers

In order for efforts toward disrupting protein oligomers to be successful, the oligomer structures and assembly processes must be understood. However, structural and biophysical characterization of oligomers of amyloidogenic proteins is difficult, because the oligomers are metastable and often exist in dynamically changing mixtures comprising monomers, oligomers of different sizes, and polymers. Classic, high-resolution structural biology methods, such as X-ray crystallography and solution-phase nuclear magnetic resonance (NMR), are not suitable for study of metastable oligomers. Therefore, a variety of lower resolution biochemical, biophysical, immunologic, and computational techniques have been employed for oligomer characterization (Bitan *et al.*, 2005; also see Chapter 11 by Mok and Howlett, and Chapter 17 by Kayed and Glabe in this volume). Each of these methods generates a limited set of data. Therefore, current views of oligomer structure and assembly are synergistic syntheses of multiple data sets obtained using a variety of strategies and techniques.

An important aspect of the structural characterization of protein oligomers is determination of oligomer order. Attempts to characterize the oligomer order of amyloidogenic proteins in general, and Aβ in particular, using various biophysical and biochemical methods have not yielded a consensus (Bitan *et al.*, 2001). Reasons for lack of consensus have included using methods with limited resolution (e.g., dynamic light scattering, electron microscopy, size-exclusion chromatography, ultracentrifugation) or prone to artifacts (e.g., sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE]) (Bitan *et al.*, 2005). An ideal method for determining oligomer size in a situation in which metastable oligomers exist in dynamically changing mixtures would provide accurate, quantitative "snapshots" of the distributions. Because oligomers dissociate back into monomers and associate into larger assemblies over time, the method should be applicable within intervals significantly shorter than the lifetime of the assemblies under study. In addition, in order to reveal accurately the native oligomerization state of the protein under investigation, the method should require no *pre facto* protein modifications and be applicable under physiological conditions. Photo-induced cross-linking of unmodified proteins (PICUP), a method originally developed by Fancy and Kodadek for

study of stable protein complexes (Fancy and Kodadek, 1999), has most of the characteristics of an ideal method for this task. PICUP enables cross-linking of proteins within time intervals of 1 s without *pre facto* modification of the native sequence and is applicable within wide pH and temperature ranges, including physiological values. Other cross-linking methods, such as chemical cross-linking using bifunctional linkers (Das and Fox, 1979; Kluger and Alagic, 2004) or benzophenone/arylazide-based photoaffinity labeling (Knorre and Godovikova, 1998; Kotzyba-Hibert *et al.*, 1995) require substantially longer reaction times. In addition, some chemical cross-linking reactions necessitate nonphysiological pH, and photoaffinity labeling relies on incorporation of nonnative functional groups into the protein. Therefore, PICUP is superior to these methods for studying native, metastable protein oligomers.

PICUP

PICUP Photochemistry

The photochemistry of PICUP is based on photo-oxidation of Ru^{2+} in a tris(bipyridyl)Ru(II) complex (Ru(Bpy)) to Ru^{3+} by irradiation with visible light in the presence of an electron acceptor. Ru(Bpy) is a common, commercial chemical used in a variety of photochemical reactions (Bjerrum *et al.*, 1995). In Ru(Bpy), Ru^{2+} can become excited upon absorption of photons with $\lambda_{max} = 452$ nm ($\varepsilon = 14{,}600\ M^{-1}$ [Kalyanasundaram, 1982]) (Reaction 1):(1)

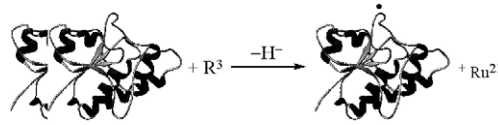
$$Ru^{2+} \xrightarrow[\lambda_{max}=452\ nm]{h\nu} Ru^{2+*} \tag{1}$$

If a suitable electron acceptor, A, is available, the Ru^{2+*} ion will donate the excited electron to the acceptor and become oxidized to Ru^{3+} (Reaction 2). A common electron acceptor in PICUP chemistry is ammonium persulfate (APS). An alternative acceptor is $Co(III)(NH_3)_5Cl^{2+}$ (Fancy *et al.*, 2000):(2)

$$Ru^{2+*} + A \longrightarrow Ru^{3+} + A^{\bullet -} \tag{2}$$

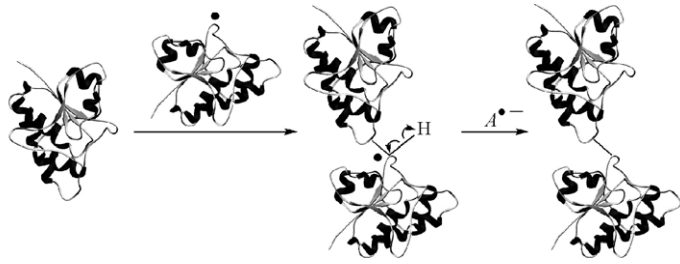
(Note that $A^{\bullet -}$ represents the oxidation state of a generic electron acceptor after Reaction 2. The actual ionization state of the reduced acceptor following reaction with Ru^{2+*} depends on its initial oxidation state. For example, following reduction, the persulfate anion ($S_2O_8^{2-}$) decomposes into $SO_4^{2-} + SO_4^{\bullet -}$, whereas $Co(III)(NH_3)_5Cl^{2+}$ is reduced to $Co(II)(NH_3)_5Cl^{+}$.)

Ru^{3+} is a strong (+1.24 V) one-electron oxidizer capable of abstracting an electron from a neighboring protein molecule, generating a protein radical (Reaction 3). As long as irradiation continues and sufficient electron acceptor is available, Ru^{2+} can be recycled into Reaction 1, get oxidized again to Ru^{3+}, and generate more protein radicals:(3)



(3)

Radicals are unstable, highly reactive species and therefore disappear rapidly through a variety of intra- and intermolecular reactions. One route a radical may utilize to relieve the high energy caused by an unpaired electron is to react with another protein monomer to form a dimeric radical, which may subsequently lose a hydrogen atom and form a stable, covalently cross-linked dimer (Reaction 4). The dimer then may react further through a similar mechanism with monomers or other dimers, leading to the formation of higher order oligomers:



(4)

The potential for a particular functional group in a protein to react with Ru^{3+}, donate an electron, and form a radical or to react with another protein radical depends on a number of parameters, including the capability of the group to stabilize an unpaired electron, the proximity of the group to the Ru^{3+} ion or to a radical on a neighboring protein, and the structure of the protein. Stabilization of a radical can occur via mechanisms like resonance, hyperconjugation, neighboring group effect, or a combination of the three. Based on electronic considerations, the probability for the side chains of the amino acids Trp and Tyr to be sites of radical formation and/or reaction is highest, whereas that of the side chain of Ala is lowest among the 20 natural amino acids. A radical also can form, in principle, on the protein backbone. However, this is unlikely, because steric interference hinders backbone atoms from being in close proximity to the Ru^{3+} ion or to a neighboring protein radical. For the same reason, the probability of radical formation/reaction on the α-carbon (C_α) of Gly is low. The surrounding environment of each functional group strongly influences the potential for radical formation on, or reaction with, this group. Tyr is highly prone to form a radical upon reaction with Ru^{3+} (Reaction 3) (Fancy, 2000; Fancy *et al.*, 2000). The human amyloidogenic peptides, Aβ(1–40) (40 residues), calcitonin (CT, 32 residues), and IAPP (37 residues) each contain a single Tyr residue (residue 10 in Aβ, residue 12 in CT, and residue 37 in IAPP). Because Trp is not present in these peptides, Tyr likely is the

most reactive residue in each of these peptides in PICUP chemistry. When subjected to PICUP, ≈80% of Aβ and ≈75% of CT monomers react to form cross-linked oligomers (Bitan *et al.*, 2001), whereas only ≈30% of IAPP monomers form oligomers (G. Bitan, unpublished results), demonstrating the strong influence of the environment of the Tyr residue in each peptide on its reactivity in PICUP chemistry. The difference in reactivity is not merely an effect of the C-terminal position of Tyr^{37} in IAPP, because when Tyr^{10} in Aβ is repositioned at the C-terminus, as in the analogue [Phe^{10},Tyr^{40}]Aβ(1–40), ≈80% of the monomer reacts to form oligomers, similar to wild-type (WT) Aβ(1–40) (S. K. Maji and D. B. Teplow, personal communication). When neither Trp nor Tyr is present in a peptide, the overall cross-linking efficiency is substantially lower than even in the presence of a single Tyr. For example, when Tyr^{10} in Aβ is substituted by Phe as in [Phe^{10}]Aβ(1–40) or [Phe^{10}]Aβ(1–42), the cross-linking yield (monomer conversion into oligomers) decreases from ≈80% for both WT Aβ(1–40) and Aβ(1–42), to 51% and 33%, respectively (S. K. Maji and D.B. Teplow, personal communication). Similarly, when residues 1–10 of Aβ are deleted, as in Aβ(11–40) and Aβ(11–42), the cross-linking yields of the N-terminally truncated peptides are 43% and 38%, respectively (Bitan *et al.*, 2003c). Notably, Aβ alloforms lacking Tyr always form abundant dimers and, in some cases, trimers and tetramers as well, indicating that amino acid residues other than Tyr and Trp are reactive in PICUP chemistry. It will be important and interesting to determine the reactivity of each of the 20 natural amino acids in PICUP chemistry, both in forming a radical (Reaction 3) and in reacting with one (Reaction 4) in different protein conformations. Such data will enable making predictions about the feasibility and usability of PICUP for particular protein systems. Until such data become available, reaction conditions must be optimized empirically for each experimental system.

Optimizing the Experimental System

For optimization of an experimental system, it is important to consider the factors that determine the result of a PICUP experiment, which include the reactivity of the protein under study, the steady state concentration of Ru^{3+} ions, $[Ru^{3+}]^{\ddagger}$, and the protein/Ru(Bpy) ratio (the Ru(Bpy)/APS ratio should be kept at 1:20). $[Ru^{3+}]^{\ddagger}$ is a function of the initial concentration of Ru^{2+}, the characteristics of the irradiation system, and the time of irradiation. Practically, for optimization of cross-linking yield, it is convenient to maintain constant protein and Ru(Bpy) concentrations and modify the irradiation time systematically. Using this protocol, we found that for 60 μM Ru(Bpy) and a Ru(Bpy)/Aβ(1–40) concentration ratio of 2:1, efficient cross-linking occurred with 0.5–8 s of illumination using a 150-W incandescent lamp positioned 10 cm from the reaction vessel (Bitan *et al.*, 2001). Within this

time range, irradiation time had only a moderate effect on the observed oligomer size distribution of Aβ(1–40). At shorter irradiation times, formation of trimer and tetramer decreased substantially. At higher irradiation times, extensive radical reactions caused protein degradation, and ''fading away'' of entire lanes (Bitan *et al.*, 2001). A similar effect was observed using excess (fivefold) Ru(Bpy) and 1-s irradiation (G. Bitan, unpublished results).

For studies of the relation between protein concentration and oligomerization state, once an optimal irradiation period has been determined, it is important to maintain a constant protein/Ru(Bpy) ratio. For example, under the experimental conditions described above, we determined that the oligomer size distributions of Aβ(1–40) and Aβ(1–42) at 30 and 300 μM were essentially unchanged, whereas a shift in abundance toward smaller oligomers was observed when Aβ(1–40) was diluted below 10 μM or when Aβ(1–42) was diluted below 3 μM (G. Bitan and D.B. Teplow, unpublished results). The distributions observed for dilute (<3 μM) Aβ(1–42) were similar to those observed by other investigators who used nanomolar concentrations of Aβ(1–42) (Crouch *et al.*, 2005; LeVine, 2004).

The choice of detection method for protein oligomers following PICUP depends on the starting protein preparation and the protein concentration. In the examples mentioned in the previous paragraph, SDS-PAGE and silver staining were used in our laboratory, whereas LeVine (2004) and Crouch *et al.* (2005) used Western blot analysis for visualization of Aβ(1–42) oligomers. The results were qualitatively similar. Immunodetection must be used for biological samples in which the protein of interest exists in a mixture with other proteins (e.g., in cell extracts or conditioned cell culture media). Caution must be exercised when Western blot analysis is used for detection of PICUP products, because antigenic epitopes may be modified by radical reactions and such modifications may affect certain products more than others. This would complicate data interpretation, because it would be difficult to distinguish between a situation in which certain oligomers form with a low yield because of inherent instability and low detection of stable oligomers because of modification of antigenic epitopes. This potential problem may be overcome by using several antibodies recognizing different epitopes of the same protein.

PICUP products may be analyzed without fractionation using a variety of morphological and spectroscopic methods (e.g., Bitan *et al.*, 2003a). Fractionation using size exclusion chromatography (SEC) provides lower resolution than SDS-PAGE but enables further analysis of isolated oligomers individually, without the need to remove SDS from the isolated fractions (Bitan *et al.*, 2003a). Analyzing cross-linking products using mass spectrometry (MS) would offer advantages relative to SDS-PAGE, because oligomers can be assigned unambiguously based on their mass rather than their electrophoretic mobility, which does not always correlate

directly to mass (Bitan *et al.*, 2005). However, detection of oligomers by MS following PICUP has been difficult. We have attempted to analyze Aβ40 that had been subjected to PICUP using both matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) techniques. Crude PICUP reaction mixtures yielded no signal in either technique. Fractionation of the mixtures by high-performance liquid chromatography (HPLC) or SEC interfaced with an ESI source produced predominantly monomer signals. Detection of Aβ oligomers (dimer through hexamer) by MALDI time of flight (TOF) was enabled eventually following purification of the oligomers by SEC, using ammonium acetate as the mobile phase, and lyophilization of this volatile buffer (G. Bitan, D. Teplow, R. Loo, and J. Loo, unpublished results).

The type of protein preparation dictates not only the choice of method for analysis of the PICUP products but also the reaction conditions and the way the data are interpreted. When pure proteins are studied, the Ru(Bpy)/protein stoichiometry should be maintained at ~2:1. As mentioned above, lower ratios will decrease the cross-linking yield and may lead to misrepresentation of higher order oligomers, whereas higher stoichiometric ratios increase formation of artifactual, diffusion-controlled cross-linking products and may promote protein degradation. When the protein preparation is more complex, (e.g., cell culture medium or cell extract), other reactive molecules, including proteins and carbohydrates, compete for reaction with Ru^{3+}. Therefore, substantially larger (10–100-fold) amounts of cross-linking reagents are required. In these preparations, in addition to cross-linking of oligomers, if they exist, cross-linking of the protein of interest to other proteins (or nonproteinaceous molecules) also may be observed. This provides an opportunity to study interactions of a protein of interest with its binding partners but may complicate interpretation of the data (Lin and Kodadek, 2005). For example, it may be difficult to distinguish between a cross-linked dimer and a cross-linked complex of two different proteins of similar size.

For experiments using biological samples, it should be noted that APS and $Co(III)(NH_3)_5Cl^{2+}$ are not cell-permeable. Therefore, cross-linking of intracellular proteins using these reagents is not feasible unless the cells are permeabilized artificially.

Scope and Limitations of PICUP

PICUP was originally developed for studies of stable protein assemblies. Proof of concept was given using UvsY, a native protein hexamer involved in phage T4 recombination (Beernink and Morrical, 1998). When UvsY was cross-linked using PICUP, the main product was a hexamer (Fancy and Kodadek, 1999). Similar results were obtained for the enzymes

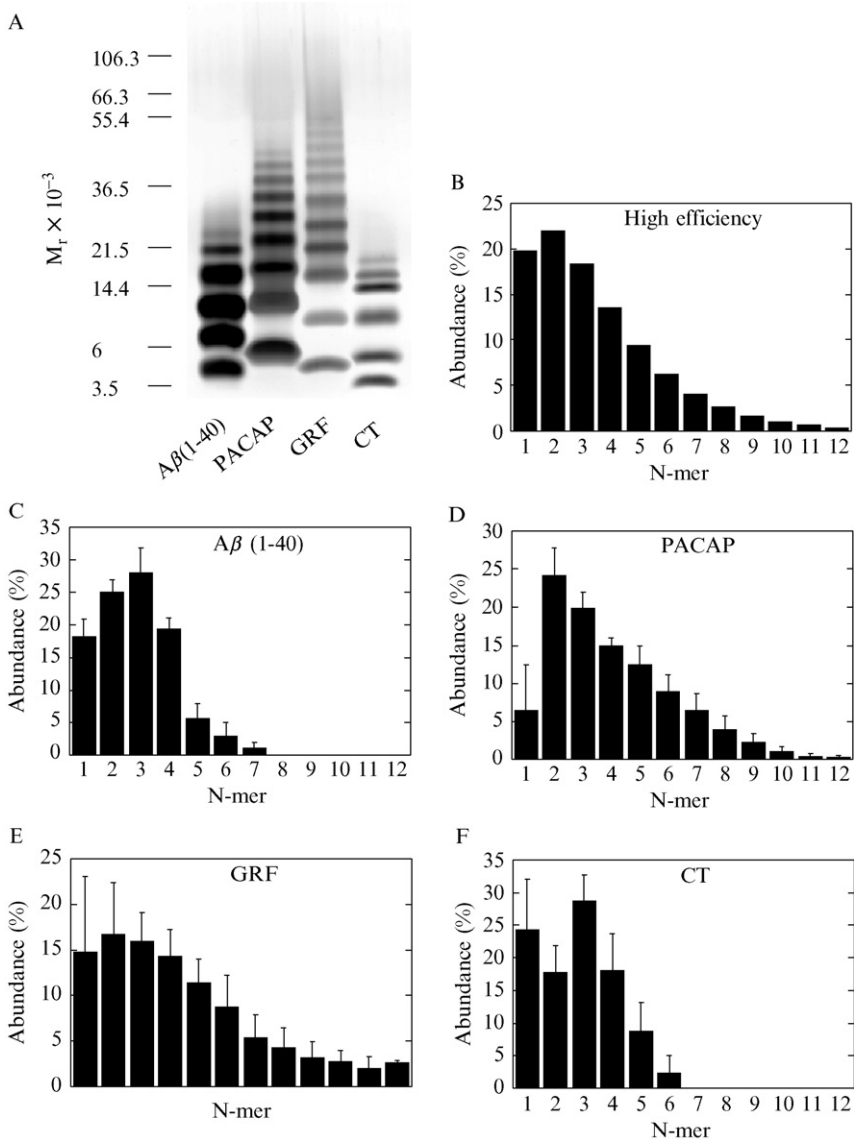
glutathione *S*-transferase (Fancy *et al.*, 2000), glyoxylate aminotransferase (Lumb and Danpure, 2000), muscle acylphosphatase (Paoli *et al.*, 2001), hormone-sensitive lipase (Shen *et al.*, 2000), the prokaryotic RNA-editing enzyme tadA (Wolf *et al.*, 2002), and the yeast transcription factor Pho4 (Fancy *et al.*, 2000), all of which form stable dimers. Other studies found the predicted oligomerization patterns for the yeast mating-type proteins SMTA-1 and SMTa-1, which form homo- and heterodimers (Jacobsen *et al.*, 2002), and for Cowpea mosaic virus subunit, which is a stable pentamer (Meunier *et al.*, 2004). PICUP also has been applied successfully to characterization of protein-ligand interactions, including mapping the interaction of signal recognition particle (SRP) with various signal sequences (Cleverley and Gierasch, 2002), binding of the transcription factor ETS-1 to stromelysin-1 promoter (Baillat *et al.*, 2002), and affinity labeling of G-protein-coupled receptors for bioactive peptide hormones, including bradykinin, angiotensin, vasopressin, and oxytocin, using agonists and antagonists derived from the native hormones (Duroux-Richard *et al.*, 2005). The latter study demonstrated the usefulness of PICUP not only for cross-linking of proteins in buffers or cell-extracts but for studies of membrane-bound proteins. Additional uses of PICUP included ''fishing out'' specific interactions in mixtures of peptides and proteins (Lin and Kodadek, 2005) and modulation of cell adhesion to glass (Luebke *et al.*, 2004).

The studies listed above demonstrate the usefulness of PICUP in stabilizing protein oligomers for analysis using denaturing methods (e.g., SDS-PAGE). In addition, important features of the method itself were gleaned. In all cases, in addition to the predicted stable oligomer(s), monomers and, where appropriate, lower order oligomers, were observed following PICUP and SDS-PAGE analysis. These products reflect the fact that the cross-linking efficiency is $<100\%$ and non-cross-linked oligomers can dissociate in the presence of SDS. An opposite effect also was observed in certain cases—diffusion-controlled cross-linking of pre-existing oligomers with monomer yielded artifactual, higher order oligomers.

An important question for studies of oligomer size distributions of metastable protein oligomers is whether artifactual oligomers formed by diffusion-controlled cross-linking can be distinguished from bona fide pre-existing oligomers. To answer this question, we applied PICUP to two amyloidogenic peptides, Aβ(1–40) and CT, and two peptides of similar size, growth hormone-releasing factor (GRF) and pituitary adenylate cyclase-activating polypeptide (PACAP), which have not been reported to oligomerize or form amyloid under physiological conditions. In all cases, oligomers were observed following cross-linking (Bitan *et al.*, 2001). To distinguish pre-existing oligomers from those formed by diffusion-controlled cross-linking of monomers, the observed distributions were compared with theoretical distributions produced using a mathematical

model, which assumes no association among molecules except for random, diffusion-controlled elastic collision (see Bitan *et al.*, 2001 for details).

Figure 1 shows SDS-PAGE analysis of the four cross-linked peptides (Fig. 1A), a modeled distribution obtained under high-efficiency conditions (Fig. 1B), and densitometric analysis of each lane (Fig. 1C–F). In the



absence of pre-existing oligomers and under high-efficiency cross-linking conditions, ≈80% of monomer is converted to oligomers ranging from dimer through dodecamer (Fig. 1B). The experimental oligomer size distributions obtained for PACAP (Fig. 1D) and GRF (Fig. 1E) were similar to this theoretical distribution, with the exception of a higher cross-linking efficiency observed for PACAP, leading to consumption of ≈95% of the monomer. Both distributions were characterized by formation of a ''ladder'' of oligomers extending up to a dodecamer and by an exponential decline in oligomer abundance. These distributions were distinct from those observed for Aβ(1–40) (Fig. 1C) or CT (Fig. 1F). Importantly, for both Aβ(1–40) and CT, the cross-linking efficiency was similar to that of the model and of GRF, consuming ≈75–80% of the monomer. As discussed above, cross-linking efficiency depends on the local environment of each reactive group. Therefore, when oligomer size distributions of different peptides are compared, it is important that the cross-linking yield is similar for all peptides. Aβ(1–40) and CT yielded oligomer size distributions that did not extend beyond hexamer (CT) or heptamer (Aβ(1–40)). In addition, the abundance of monomer through tetramer for Aβ(1–40) and of monomer through trimer for CT diverged from an exponential pattern. These differences indicated that the solutions of Aβ(1–40) and CT contained species other than peptide monomers and suggested that these species were pre-existing oligomers. In both cases, the abundance of higher oligomers (pentamer through heptamer for Aβ(1–40) and tetramer through hexamer for CT) declined exponentially, demonstrating that diffusion-controlled cross-linking of pre-existing oligomers to monomers is an inevitable side reaction. A conservative interpretation of these data is that oligomers whose abundance diverges from an exponential decline pattern are bona fide pre-existing oligomers, whereas oligomers whose abundance declines exponentially likely are generated by diffusion-controlled cross-linking.

FIG. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometric analysis of photo-induced cross-linking of unmodified proteins (PICUP) products of amyloidogenic and nonamyloidogenic peptides. (A) Low-molecular-weight (LMW) preparations of amyloidogenic (amyloid β protein (Aβ)(1–40) and calcitonin [CT]) and nonamyloidogenic (pituitary adenylate cyclase-activating polypeptide [PACAP] and growth hormone releasing factor [GRF]) peptides were prepared by filtration through a 10-kDa molecular-weight cutoff filter (Bitan and Teplow, 2005) and cross-linked immediately. A silver-stained gel is shown. Positions of molecular weight standards are shown on the left. (B) Theoretical distribution of monomers in the absence of preassociation under high-efficiency cross-linking conditions. (C–F) densitometric analysis of the gel bands in panel A. Reproduced with permission from Bitan *et al.* (2001).

PICUP as a Tool for Structural Studies

PICUP enables quantitative study of metastable, quaternary protein structures. Thus, by studying the effect of amino acid sequence modifications on the quaternary structure, the relation between primary and quaternary structures of metastable protein oligomers can be delineated. These relations can have a great impact on protein bioactivity. For example, certain amyloidoses are caused by mutations, resulting in single amino acid substitutions in the respective amyloid protein (Buxbaum and Tagoe, 2000). Studying the effect of such substitutions on protein oligomerization may be crucial to understanding disease mechanism.

We have applied PICUP to the study of primary-quaternary structure relations of Aβ (Bitan and Teplow, 2004). The predominant Aβ alloforms in the brain are Aβ(1–40) and Aβ(1–42). Aβ(1–40) is ≈10 times more abundant than Aβ(1–42). Nevertheless, genetic, pathologic, and biochemical evidence demonstrates that Aβ(1–42) is linked most strongly to the etiology of AD (Selkoe, 2001). Oligomers of Aβ(1–42) have been shown to be more neurotoxic than those of Aβ(1–40) (Dahlgren *et al.*, 2002; Hoshi *et al.*, 2003), but the mechanistic basis for these toxicity differences is not known. Using PICUP, we found that Aβ(1–40) and Aβ(1–42) form distinct oligomer size distributions. Aβ(1–40) forms a roughly equimolar, quasi-equilibrium mixture of monomer, dimer, trimer, and tetramer, whereas Aβ(1–42) preferentially forms pentamer/hexamer units (Fig. 2), which self-associate into larger assemblies, including dodecamers and octadecamers, and hence were termed *paranuclei* (Bitan *et al.*, 2003a). Consistent with the PICUP data, distinct particle size distributions of Aβ(1–40) and Aβ(1–42) were observed by dynamic light scattering (Bitan *et al.*, 2003a). Morphological studies showed that Aβ(1–40) oligomers were amorphous, whereas Aβ(1–42) paranuclei appeared as spheroids ≈5 nm in diameter (Bitan *et al.*, 2003a). These differences in oligomer size distribution and morphology between Aβ(1–40) and Aβ(1–42) offer a plausible explanation for the differences in neurotoxicity observed for the two alloforms.

Insight into the mechanism(s) controlling the distinct oligomerization behavior of Aβ(1–40) and Aβ(1–42) was obtained by examination of PICUP-derived oligomer size distributions of Aβ analogues ending in positions 39–43. With the exception of Aβ(1–41), these alloforms are found in Aβ samples from cultured cells (Wang *et al.*, 1996) and AD patients (Mori *et al.*, 1992; Wiltfang *et al.*, 2002). The oligomer size 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 and demonstrated that paranucleus formation did not occur in the absence of Ile-41 (Fig. 2) (Bitan *et al.*, 2003a). Subsequent studies demonstrated that the side

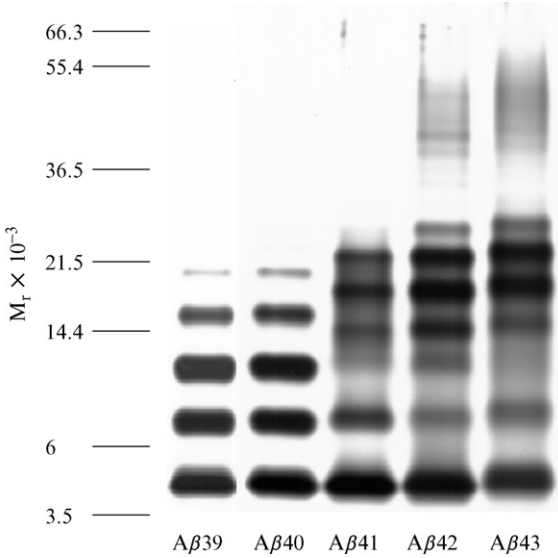


FIG. 2. C-terminal length-dependence of amyloid β protein (Aβ) oligomer size distribution. Low-molecular-weight Aβ(1–39), Aβ(1–40), Aβ(1–41), Aβ(1–42), and Aβ(1–43) were cross-linked individually and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Positions of molecular weight standards are shown on the left. Reproduced with permission from Bitan *et al.* (2003a).

chain in position 41 and the C-terminal carboxylate group of Aβ(1–42) are critical modulators of paranucleus assembly (Bitan *et al.*, 2003c). Study of clinically relevant alloforms containing substitutions in the midregion of Aβ and of N-terminally truncated Aβ analogues, which are found in plaques from AD patients, demonstrated that Aβ(1–40) oligomerization is largely affected by charge alterations at the N-terminus and in positions 22 and 23, whereas oligomer formation by Aβ(1–42) is controlled primarily by hydrophobic interactions and is highly sensitive to conformational changes at the central hydrophobic region (Bitan *et al.*, 2003c). Further study showed that oxidation of Met-35, a modification often found in Aβ extracted from AD brain (Nordstedt *et al.*, 1994), abolishes formation of Aβ(1–42) paranuclei but has no effect on early oligomerization of Aβ(1–40) (Bitan *et al.*, 2003b). Thus, structural data obtained using PICUP demonstrated that modification of as little as one atom can induce dramatic effects on Aβ assembly and provided important insights into the mechanism by which Aβ assembles into neurotoxic oligomers relevant to AD pathogenesis.

Experimental Protocol

Materials

1. Light source. Both 150-W Xe lamps and 150-W incandescent lamps have been used successfully (Bitan *et al.*, 2001; Fancy *et al.*, 2000) (Notes 1 and 2).
2. Reaction apparatus allowing controlled exposure and positioning of samples a fixed distance from the light source. We and others have used a 35-mm single lens reflex (SLR) camera body to control exposure time (Bitan *et al.*, 2001; Fancy and Kodadek, 1999). In our setting, a bellows attached to the camera in place of a lens provides a convenient means to place the sample and control its distance from the light source (Fig. 3). The data described above were obtained with the light source at a distance of 10 cm from the sample.
3. Clear, thin-walled plastic tubes (Note 3).
4. Tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate (Ru(Bpy), Sigma), 1 m*M*, in 10 m*M* sodium phosphate, pH 7.4 (Notes 4–6).

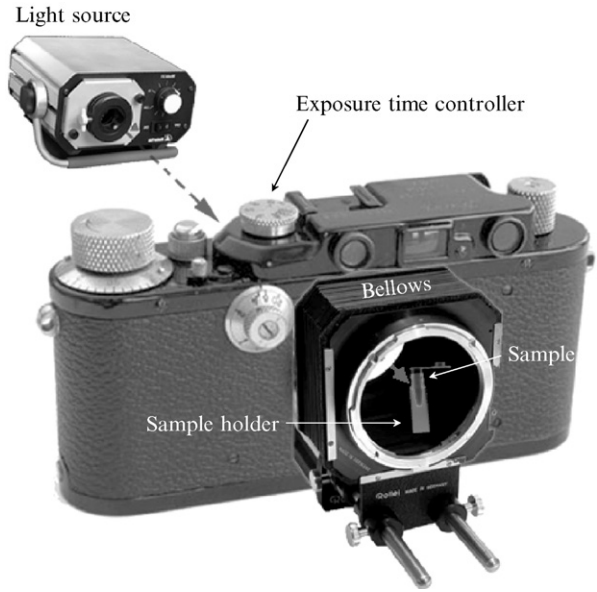


FIG. 3. Schematic cross-linking system. The reaction mixture is prepared in a polymerase chain reaction (PCR) tube (see Note 3) immediately before irradiation. A glass vial is used to hold the PCR tube within the dark chamber (bellows). The sample is illuminated through the open back of a camera body using the camera shutter mechanism to control the illumination time.

5. APS (Sigma), 20 m*M*, in 10 m*M* sodium phosphate, pH 7.4 (Notes 5 and 6).
6. Low-molecular-weight Aβ (Note 7).
7. Quenching reagent: 5% (v/v) β-mercaptoethanol (β-ME; Sigma) in 2× Sample Buffer (Invitrogen), or 1 *M* dithiothreitol (DTT; Fisher) in water (Note 8).

Method

General Instructions. The method described here is applicable to samples volume of 20–120 μl. For volumes larger than 120 μl, the cross-linking efficiency declines with increasing sample volume. Using a ratio of 2:40:1 for Ru(Bpy), APS, and LMW Aβ, respectively, the cross-linking yield is relatively insensitive to changes in protein concentration between 10–50 μM. Lower or higher concentrations may require empirical adjustment of the Ru(Bpy)/protein ratio. The Ru(Bpy)/APS ratio should be kept at 1:20. Longer irradiation may be necessary for highly diluted samples for the same cross-linking yield to be obtained. If larger amounts of cross-linked protein are desired, several samples can be pooled together following cross-linking and quenching of each sample.

Specific Steps

1. Prepare the peptide or protein sample as appropriate. Here, LMW Aβ was isolated according to published protocols (Note 7).
2. Transfer an 18-μl aliquot to a polymerase chain reaction (PCR) tube (Note 3).
3. Add 1 μl Ru(Bpy) and 1 μl APS, and mix by drawing up and expelling solution several times from a pipette tip (Notes 9–11).
4. Place in the illumination chamber (bellows), and irradiate for 1 s (Note 12).
5. Quench immediately by mixing with either 10 μl β-ME in Sample Buffer or 1 μl DTT (Note 8).
6. Cross-linked samples may be stored in a -20° freezer for 7–10 days prior to analysis. Longer storage of samples may result in decreased resolution on a gel.

Notes

1. Other lower intensity sources of light can be used (Fancy and Kodadek, 1999). Irradiation time must be adjusted empirically to maximize cross-linking efficiency. Care should be taken, because long irradiation may induce protein degradation.

2. Filtering (IR) radiation by using distilled water to prevent sample overheating has been used by some researchers (Fancy *et al.*, 2000). We have found this to be unnecessary for short ($\leq$8 s) irradiation times.

3. We have used clear, 0.2-ml PCR tubes (Eppendorf) for sample preparation and a flat-bottom, 1.8-ml glass vial (Kimble Chromatography) as a sample holder (Fig. 3). Others have used larger (1–2 ml) sample tubes. We find that the tube size is not a critical parameter for successful cross-linking as long as the sample can be placed reproducibly at a fixed distance and angle relative to the light source. In our setting, this distance is 10 cm directly in front of the light source. Reproducibility is of critical importance. The absolute distance and angle of the sample from the light source are of lesser importance, with the understanding that cross-linking yield decreases as a function of the distance.

4. Palladium (II) porphyrins also have been used as photoactivators in PICUP chemistry (Fancy *et al.*, 2000; Kim *et al.*, 1999).

5. Buffers other than sodium phosphate can be used, but the efficiency of the cross-linking reaction in different buffers must be determined empirically. The cross-linking yield of Aβ(1–40) in different solvents is $H_2O \approx NaCl > Na_2HPO_4 > NaHCO_3 > NaBO_4$ (10 m*M* of each buffer or salt was used, buffer pH was 7.4, H_2O and NaCl solution pH was 3.3) (G. Bitan, unpublished results).

6. Dissolution of Ru(Bpy) requires vortexing for $\sim$1 min until the solution is transparent to the eye. The Ru(Bpy) solution is light sensitive and must be protected from ambient light. A simple method is to use aluminum foil to wrap the tube containing the Ru(Bpy) solution. The APS and Ru(Bpy) reagent solutions can be used for up to 48 h following preparation.

7. The method described here uses low-molecular-weight (LMW) Aβ, an aggregate-free preparation described elsewhere (Bitan and Teplow, 2005; Fezoui *et al.*, 2000; Walsh *et al.*, 1997). However, the method is readily applied to the analysis of other peptides and proteins, with appropriate optimization of reaction conditions. The most important factors to consider are the reagent stoichiometry, irradiation time, and sample preparation procedure (see the section on optimizing the experimental system). The former two issues require empirical optimization. The latter issue largely determines how the experimental data are to be interpreted. For amyloidogenic proteins in particular, determination of native oligomerization states requires using aggregate-free starting preparations.

8. The choice of a quenching reagent depends upon the purpose of the cross-linking experiment. Samples analyzed using PAGE are quenched with the appropriate sample buffer containing 5% β-ME. Samples analyzed by chromatography or other methods may be quenched with

1 *M* DTT. Lower concentrations of DTT (as low as 200 m*M*) also can be used if preferred.

9. Do not vortex samples containing amyloidogenic proteins, because vortexing may promote their aggregation.

10. In order to prevent cross-linking induced by ambient light, the procedure may be performed in a dark room. However, the efficiency of ambient light-induced cross-linking is low. In our experience, a nonirradiated mixture of Aβ(1–40) and cross-linking reagents yields a very faint dimer band following exposure to ambient light for the same time that normally is required to cross-link such a sample.

11. Proteins also can be cross-linked in biological fluids, such as cultured cell media or cell extracts. Because Ru^{3+} is a nonselective oxidizer, it will react with susceptible components of biological solutions. Therefore, cross-linking of these types of samples requires higher concentrations of reagents, up to 100 m*M* Ru(Bpy) and 2 *M* APS. Upon addition of reagents at these high concentrations to the sample, some precipitate may form. This precipitate does not appear to interfere with cross-linking and can be removed by centrifugation or dissolved upon addition of sample buffer after the cross-linking process is complete.

12. Irradiation time should be kept to a minimum and should be optimized empirically (see the section on optimizing the experimental system).

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