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Review

Protein Misfolded Oligomers: Experimental Approaches, Mechanism of Formation, and Structure-Toxicity Relationships

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The conversion of proteins from their native state to misfolded oligomers is associated with, and thought to be the cause of, a number of human diseases, including Alzheimer's disease, Parkinson's disease, and systemic amyloidoses. The study of the structure, mechanism of formation, and biological activity of protein misfolded oligomers has been challenged by the metastability, transient formation, and structural heterogeneity of such species. In spite of these difficulties, in the past few years, many experimental approaches have emerged that enable the detection and the detailed molecular study of misfolded oligomers. In this review, we describe the basic and generic knowledge achieved on protein oligomers, describing the mechanisms of oligomer formation, the methodologies used thus far for their structural determination, and the structural elements responsible for their toxicity.

Introduction

Polypeptide chains have a high intrinsic propensity to self-assemble into a variety of misfolded aggregates, ranging from dimers to highly organized fibrils consisting of thousands of protein molecules (Chiti and Dobson, 2006; Dobson, 2003; Eichner and Radford, 2011; Jahn and Radford, 2008). Protein aggregation can occur starting from any of the conformational states adopted by the initially monomeric protein, including the fully unfolded state, the folded state, and any partially folded states, although the latter have the highest propensity to self-assemble (Bemporad et al., 2006; Chiti and Dobson, 2006). Protein aggregation is deleterious for any living organism as it prevents a protein from adopting its functional state and because the resulting protein oligomers are inherently toxic (Chiti and Dobson, 2006; Walsh and Selkoe, 2007). In fact, the failure of proteins to remain soluble—occurring as a consequence of mutation, aging, local increases of protein concentration, medical treatment, or other circumstances—results in pathological states that are very diverse and depend on the protein undergoing aggregation and on the tissues involved (Chiti and Dobson, 2006).

The end product of protein aggregation processes occurring in pathology is generally represented by extracellular amyloid fibrils or structurally equivalent intracellular inclusions, often associating further to form larger assemblies that are visible with optical microscopy. However, protein oligomers forming early during the process of amyloid fibril formation or, alternatively, released by mature fibrils, have acquired increasing importance over the past 10–15 years. This is first due to the fact that formation of protein oligomers is a key event of the overall process of amyloid fibril formation and has been long regarded as the rate-limiting step, responsible for the lag phase in aggregation kinetics (Morris et al., 2009; Orte et al., 2008). Second, oligomers are thought to be the pathogenic species associated with the formation of amyloid in diseases (Billings et al., 2005; Bucciantini et al., 2002; Cleary et al., 2005; Koffie et al., 2009; Lesné et al., 2006; Winner et al., 2011). As the importance of protein oligomers was increasingly realized, many reports have appeared with information on their mechanism of formation and structure, with the ultimate goal of identifying the structural determinants of their pathogenicity, the molecular events of disease onset, and the molecular targets for therapeutic intervention. In this review, we describe how oligomers form, the structure of such species, including the technological progress recently achieved to gain insight into their molecular structure and the structural elements responsible for their toxicity.

Mechanism of Formation of Amyloid Oligomers

One of the most widely accepted mechanisms proposed for the assembly of monomers into oligomers is the so-called "nucleation growth" mechanism (Jarrett and Lansbury, 1993; Lomakin et al., 1996). According to this mechanism, monomers convert into a nucleus through a thermodynamically unfavorable process taking place in the lag phase of amyloid aggregation kinetics (pathway A → C/D → H → G; red arrows in Figure 1). The nucleus can be defined as the least thermodynamically stable species in solution (i.e., the multimer of minimal size able to initiate assembly; Morris et al., 2009). Alternatively, the nucleus can also be defined as the aggregate size after which the association rate exceeds the dissociation rate for the first time (Ferrone, 1999). The nucleus could even be a monomer that acts as a template for the rapid growth of the amyloid aggregate through the association of further monomers (H → G → I in Figure 1) (Lomakin et al., 1996).

After the nucleation growth model was proposed, the concept of nucleus was investigated in further detail and other more accurate models were proposed. In the nucleated conformational conversion, native monomers initially convert into misfolded conformations, which initiate self-assembly through a

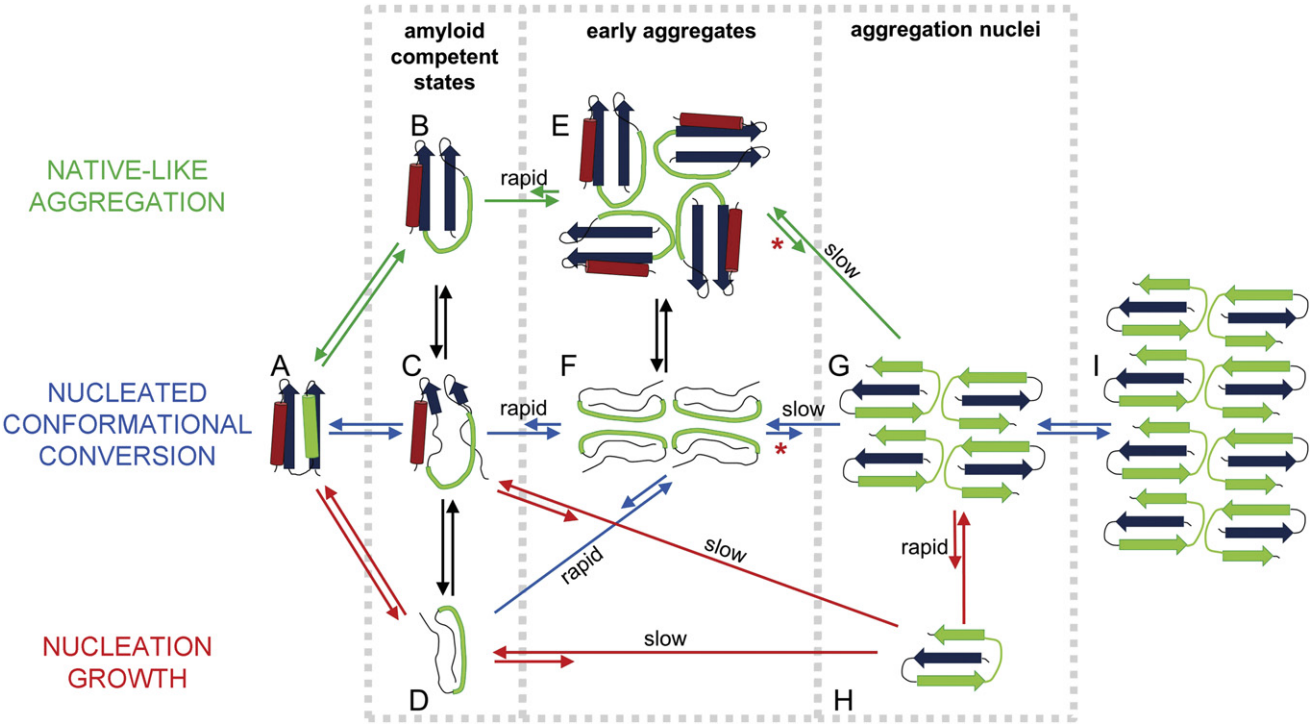

Figure 1. Mechanisms Leading to the Formation of Protein Oligomers

Proteins initially populate a native conformation (A) in which amyloid-prone segments (in green) are structured/buried and unable to initiate polymerization. However, native states can convert, under certain conditions, into aggregation-prone states, a native-like (B), a partially folded (C), and an unfolded (D) monomer. In these conformational ensembles, aggregation-prone segments become exposed to the solvent and can establish intermolecular interactions, with the resulting formation of early aggregates, which can be native-like aggregates (E) or molten aggregates of misfolded monomers (F). The early aggregates convert later into amyloid oligomers competent for fibril nucleation (G). The pathway that transits through the native-like state (A → B → E → G, indicated by green arrows) is referred to as native-like aggregation. The pathway that transits through fully or partially unfolded monomers (A → C/D → F → G, indicated by light blue arrows) is the nucleated conformational conversion. There is finally another possible pathway, in which a misfolded monomer converts into an amyloid-competent monomer (A → C/D → H, indicated by red arrows). This acts as a template for formation of the oligomer (G). This pathway is usually referred to as the nucleation growth mechanism. The red asterisks denote two steps (E → G and F → G) whose removal makes oligomer formation an off-pathway process that leads to species unable to further convert into amyloid fibrils.

template-independent mechanism, with formation of a molten oligomer lacking persistent structure (A → C/D → F; light blue arrows in Figure 1) (Serio et al., 2000). This aggregated species then undergoes a structural reorganization into an amyloid-like oligomer, which acts as a nucleus (F → G). The nucleus rapidly triggers aggregation as other molten oligomers acquire the amyloid conformation through a templating or induced-fit mechanism at the aggregate ends (Serio et al., 2000). This leads to the formation of higher order oligomers and eventually fibrils (G → I).

These two models differ for a number of aspects. While in the nucleation growth mechanism, the monomer directly interacts with the nucleus and the nucleus could be as small as a monomer, in the nucleated conformational conversion both the building block and the nucleus are described as oligomeric species. Moreover, in the first model the rate-limiting step is represented by the formation of the nucleus (C/D → H in Figure 1), whereas in the second model oligomers form rapidly, with the rate-limiting step being the conversion of the misfolded oligomer into the amyloid oligomer (F → G in Figure 1). Different reports support either model. In many cases, the nucleation growth mechanism seems to account for the observed aggregation kinetics (Bhattacharyya et al., 2005; Morris et al., 2008). Furthermore, proteins populating amyloid conformations directly at the monomer level have been described (Sandal et al., 2008), while some authors have reported (e.g., in the case of insulin) template-dependent conversion of monomers at the growing fibril end (Pease et al., 2010). However, it was recently shown that the monomers can rapidly form small globular oligomers that are kinetically competent to slowly convert to amyloid oligomers and later to amyloid fibrils following the nucleated conformational conversion. This was observed for example in ADA2h (Cerdà-Costa et al., 2007), Aβ (Lee et al., 2011; Petty and Decatur, 2005), huntingtin (Thakur et al., 2009), and IAPP (Wei et al., 2011).

In fact, it has been observed that formation of oligomers by a protein can occur through different pathways. In some cases, a certain pathway can be enhanced by changes in solution parameters such as pH and protein concentration (Bader et al., 2006; Gosal et al., 2005) or by introducing mutations (Bitan et al., 2003; Kumar and Udgaonkar, 2009). In other cases, aggregation can proceed via competing pathways occurring concomitantly in the same sample (Jain and Udgaonkar, 2011; Kayed et al., 2007; Kaylor et al., 2005). Differences in pathways imply differences in morphology (Bader et al., 2006; Gosal et al., 2005), dimensions (Bitan et al., 2003), and compactness (Kaylor et al., 2005) of the resulting oligomers. It is important to note that

alternative pathways differ for the stage at which conformational conversion from a nonamyloid to an amyloid conformation occurs (Kumar and Udgaonkar, 2009). Thus, the two models described earlier could be the limit cases of a more complicated scenario where multiple pathways are accessible and selected depending on the conditions.

A possible unifying view able to combine the two apparently competing models described earlier is that the pathway prevalently followed depends on the structural nature of both the amyloid-competent monomer and the oligomer (Figure 1). If the amyloid-competent conformational ensemble has a sufficient degree of dynamical fluctuations and hydrophobic clusters exposed to the solvent, then formation of molten oligomers lacking persistent structure is fast, while conversion of the molten oligomer into an amyloid oligomer takes place only later and is accelerated by the template effect (nucleated conformational conversion; light blue arrows in Figure 1). If the misfolded conformational ensemble has not sufficient hydrophobic clusters exposed to the solvent and/or is prone to hydrogen bonding, then the other mechanism is likely to take place (nucleation growth; red arrows in Figure 1). Consistently, it was recently shown that short hydrophobic peptides give rise prevalently to misfolded oligomers, while peptides prone to establish intermolecular hydrogen bonds with their side chains rapidly form β-rich nuclei (Bleiholder et al., 2011).

So far, we have considered that a folded protein needs to unfold to generate a partially or fully unfolded state to become competent for aggregation. However, it was recently shown that normally folded proteins retain a small but significant tendency to form amyloid without the need of reactions that cross the major energy barrier for unfolding. In these cases, the amyloid-competent state is best described as a monomeric conformation that possesses an extent of secondary and tertiary structure still comparable with that of the fully folded state yet being endowed with aberrant features that enable self-assembly (A → B in Figure 1, indicated by green arrows). Formation of this conformational ensemble, usually referred to as a native-like state, can be induced either as a consequence of a local unfolding event, as in the case of Cu,Zn-uperoxide dismutase (Banci et al., 2005), β_2-microglobulin (Eakin et al., 2006), and transthyretin (Olofsson et al., 2004; Quintas et al., 2001) or due to cooperative increase in thermal fluctuations, as it was shown in the case of lysozyme (Canet et al., 2002) and Sso AcP (Pagano et al., 2010). In either case, aggregation-prone segments that are normally buried or structured in the fully folded state become exposed to the solvent or gain flexibility, triggering the formation of native-like aggregates (B → E); these then convert directly into amyloid-like oligomers and fibrils (E → G → I).

Are Oligomers On- or Off-Pathway Species?

One long debated issue is whether protein oligomers represent on-pathway structures that must be necessarily populated along the pathway leading to fibril formation or rather off-pathway particles that are placed at a dead end of the reaction scheme and act only as a reservoir of monomers, while fibril formation proceeds directly through association of monomers to the amyloid nucleus. In the case of the immunoglobulin light chain LEN, it was shown that oligomers represent off-pathway species: Fibril formation occurs only after the amyloid-incompetent oligomer dissociates and monomers convert into a more amyloidogenic conformation (Souillac et al., 2003; Souillac et al., 2002). Other off-pathway oligomers have been reported for β_2-microglobulin (Gosal et al., 2005) and albebetin (Gosal et al., 2005; Morozova-Roche et al., 2004; Souillac et al., 2002). A recent computational study has shown that the amyloid-β peptide (Aβ) can form off-pathway oligomers; these assemblies are too curvy, are too compact, and display an amount of β-structure not enough for the conversion into larger amyloid oligomers (Yu and Zheng, 2011). Despite these reports, many proteins, such as the PI3-SH3 domain, barstar, sup35p, IAPP, and Aβ itself have been shown to form on-pathway oligomers that represent either the building block or the nucleus for fibril formation (Bader et al., 2006; Ehrnhoefer et al., 2008; Harper et al., 1997; Kumar and Udgaonkar, 2009; Serio et al., 2000; Wei et al., 2011).

Of note, by varying the solution conditions, one can modulate the nature of the oligomers, switching from β-rich toxic on-pathway oligomers to unstructured nontoxic off-pathway assemblies (Ehrnhoefer et al., 2008; Ladiwala et al., 2010). Changes from off- to on-pathway oligomers can be also obtained by mutating the peptide sequence (Jain and Udgaonkar, 2011). Furthermore, it was shown, using short polyglutamine-containing huntingtin fragments, that the same sample can contain contemporaneously on-pathway and off-pathway oligomers, with the latter acting as a reservoir of monomers that support fibril elongation (Jayaraman et al., 2011). Thus, these observations may suggest a scenario where structural features of the oligomers determine their off-pathway or on-pathway nature. For example, highly stable oligomers may populate a deep minimum in the energy landscape; consequently, their conversion into an amyloid-like conformation may be slower than their dissociation. Consistently with this idea, the structural rearrangement from off-pathway to on-pathway oligomers was shown to consist in a significant increase of disordered secondary structure, an increase in solvent accessibility, and a decrease in intrinsic stability of the soluble oligomeric species (Souillac et al., 2003).

Protein misfolded oligomers do not form only as on- or off-pathway species. They can also form as species released directly by mature fibrils. In particular, it has been recently shown that mature fibrils are not necessarily highly stable end products populating the deepest minima in the energy landscape (Eichner and Radford, 2011) and that amyloid fibrils can directly release monomers and oligomers (I → G in Figure 1). It was shown, for example, that fibrils of the Aβ peptide are destabilized by the presence of lipid vesicles and brain lipid extracts, releasing toxic oligomers (Martins et al., 2008). It is interesting that these "backward" oligomers are biophysically and biochemically similar to the "forward" oligomers (i.e., to the oligomers formed along the pathway going from monomers to fibrils) (Martins et al., 2008). Leakage of Aβ oligomers from fibrils has also been observed in vivo (Koffie et al., 2009; Lesné et al., 2006). The toxic effect observed in a mouse model expressing the Aβ peptide was shown to be proportional to the amount of oligomers released from amyloid plaques surrounding cells (Lesné et al., 2006). Solubilization of transthyretin, β2-microglobulin, and insulin fibrils by doxycycline, tetracyclins, and weakly basic pH, respectively, also led to toxic oligomers (Cardoso and Saraiva, 2006; Giorgetti et al., 2011; Heldt et al., 2011b). Hence, these observations show that amyloid fibrils can be secondarily

Table 1. Techniques Used to Study Protein Oligomers at the Molecular Level

Technique	Obtained information	Advantages	References
H/D exchange coupled to fragmentation methods and MS	Involvement of different protein segments in secondary structure formation	Noninvasive detection of multiple oligomers, good resolution (if fragmentation is obtained with top-down techniques)	(Kheterpal et al., 2006; Kheterpal and Wetzel, 2006; Pan et al., 2011)
H/D exchange coupled to both MS and NMR	Involvement of individual residues in secondary structure formation	Detection of multiple oligomers, information at the residue level, high resolution	(Carulla et al., 2009)
Solution NMR	Secondary and tertiary structures in low-molecular-weight oligomers	High resolution, information on both secondary and tertiary structures at the residue level	(Pagano et al., 2010; Yu et al., 2009)
Solid-state NMR	Secondary and tertiary structure in high-molecular-weight oligomers	Information at the residue level	(Chimon et al., 2007)
DEST solution NMR	Secondary and tertiary structures in oligomers	Information at the residue level, applicable to low- and high-molecular-weight oligomers	(Fawzi et al., 2011)
Labeling with fluorescent probes	Intermolecular interactions between labeled residues, burial of labeled residues	Information at the residue level, standard lab instrumentation required	(Campioni et al., 2010; Krishnan and Lindquist, 2005)
Scanning proline mutagenesis	Regions of the sequence involved in oligomer formation	Standard lab instrumentation required, good resolution	(Williams et al., 2005)
Small angle X-ray scattering	Simultaneous detection of size and shape	Detection of multiple oligomers	(Langkilde and Vestergaard, 2009)
Single-molecule fluorescence (TCCD or FRET)	Compactness, stability and size of oligomers	Direct monitoring of single oligomer particles, detection of multiple oligomers	(Orte et al., 2008, 2011)
Ion mobility MS	Mass and shape of oligomers	Direct monitoring of single oligomer particles, detection of multiple oligomers	(Bernstein et al., 2009; Bleiholder et al., 2011; Pease et al., 2010)
FRET sensor	Direct observation of oligomer formation in vivo	Noninvasive, applicable in vivo	(Kaminski Schierle et al., 2011)

toxic via the release of cytotoxic oligomers and that fibrils could speed up oligomer formation directly acting as a reservoir of amyloid nuclei.

Experimental Approaches to Study the Oligomer Structure

A number of biophysical and biochemical methods have been used to obtain structural information on protein oligomers. These include far-UV circular dichroism (CD), Fourier transform infrared (FTIR), and hydrogen/deuterium (H/D) exchange coupled to mass spectrometry (MS) to determine the content of β sheet and α-helical structure; ultracentrifugation, size exclusion chromatography, photo-induced crosslinking of unmodified proteins (PICUP), and dynamic light scattering to determine the oligomeric state (i.e., the amount of protein molecule in the oligomers); ANS and acrylodan binding to determine the solvent exposure of hydrophobic clusters; Thioflavine T (ThT) and Congo Red (CR) binding to determine the level of structural order; imaging techniques such as transmission electron microscopy (TEM) and atomic force microscopy (AFM) to determine the morphology and size; and so forth. Although such techniques provide valuable information on the average structure and oligomeric state of the aggregates, they do not reveal their molecular details at the residue level. This is a challenging problem as protein misfolded oligomers, similarly to amyloid fibrils, cannot be crystallized and generally have a size prohibitive for solution nuclear magnetic resonance (NMR), thus preventing the use of techniques commonly used in structural biology. In addition to the complexities encountered for fibrils, oligomers are structurally more heterogeneous and metastable than fibrils, thus raising further problems.

In the past 5 years, however, a number of approaches have been introduced for a deep molecular characterization of the oligomers (Table 1). H/D exchange followed by peptide fragmentation and MS has proven a valuable technique, exploiting the principle that backbone amide hydrogen atoms are protected to H/D exchange if they are engaged in stable β sheet or α-helical contacts. Oligomers are subjected to H/D exchange upon incubation in deuterated water (D_2O), fragmented with limited proteolysis or top-down techniques (e.g., electron capture dissociation) and then analyzed with MS (Kheterpal et al., 2006; Kheterpal and Wetzel, 2006; Pan et al., 2011). The determination of the extent of protection to deuterium incorporation in the fragments detected in the mass spectra has revealed the extent to which the fragments are engaged in secondary structure formation in the intact oligomers, often with a resolution down to

individual residues (Kheterpal et al., 2006; Pan et al., 2011). MS methods coupled to top-down techniques have the advantage of being noninvasive and photographing the solution of interest (no purification, chemical modification, or treatment of the oligomers is required), of detecting and structurally determining a multiplicity of coexisting species rather than their average structural properties, and of obtaining a spatial resolution down to individual residues (Pan et al., 2011).

In another study, H/D exchange and MS methods were coupled to NMR (Carulla et al., 2009). The PI3-SH3 protein domain was incubated at low pH in the presence of D_2O, under conditions favoring its rapid unfolding and subsequent aggregation. After a variable time interval (Δt_{agg}), the sample was transferred into H_2O for a fixed time and the H/D exchange was then quenched by freeze drying and dissolution in DMSO, which is a denaturing and disaggregating solvent preventing H/D exchange. Samples left to aggregate for variable Δt_{agg} time values were then analyzed with both electrospray ionization-MS (ESI-MS) and NMR. This approach allowed the concomitant detection of various oligomeric species during aggregation to follow their kinetics of appearance and disappearance and to determine their structure in terms of amide protection to H/D exchange at the residue level.

Other H/D independent studies aimed at determining the degree of structural flexibility and solvent exposure of side chains in the aggregates. Twenty mutants of the HypF-N protein containing a single cysteine residue at various positions were labeled with pyrene and allowed to aggregate under two different conditions; the fluorescence spectra of the resulting samples were acquired, revealing in each case whether the pyrene moiety was buried inside the oligomers and in contact with another pyrene moiety or rather flexible and solvent exposed (Campioni et al., 2010). This study allowed the identification of the regions of the sequence that were most structured and buried in the interior of the protein oligomers, revealing significant differences between the two oligomer populations that could explain differences in their cytotoxicity (discussed later).

Low-molecular-weight oligomers have also been studied with conventional solution NMR (Pagano et al., 2010; Yu et al., 2009), whereas large aggregates have been successfully studied with solid state NMR using approaches normally applied to amyloid fibrils (Chimon et al., 2007). A novel, very interesting solution NMR technique, named dark-state exchange saturation transfer (DEST), has been very recently presented to probe the structure of $A\beta_{40}$ and $A\beta_{42}$ protofibrils and circumvent the problems of limiting solution NMR to small oligomers (Fawzi et al., 2011). In all such cases, secondary and/or tertiary information of the molecules forming the oligomers have been obtained for individual residues.

Detailed molecular structure of protein oligomers was also obtained for $A\beta_{40}$ protofibrils using scanning proline mutagenesis (Williams et al., 2005). This approach consisted in the systematic substitution of all peptide residues with proline and in the subsequent evaluation of the equilibrium between soluble and aggregated peptide for all mutants having a single substitution. Mutants that produced the largest changes in such equilibrium relative to the wild-type peptide were meant to indicate the involvement of the mutated residues in the protofibrillar structure. Another interesting method is small angle X-ray scattering. This technique gives information about shape and dimensions of oligomers ranging from 1 to about 100 nm (Langkilde and Vestergaard, 2009) and has been used in different systems such as insulin (Vestergaard et al., 2007), α-synuclein (Giehm et al., 2011), and the immunoglobulin light chain LEN (Souillac et al., 2002).

Many of the approaches described here to study protein oligomers were inspired by previous studies successfully applied to stable amyloid-like fibrils. The complexities arising from the structural heterogeneity and metastability of the oligomers has been circumvented with various strategies; for example, by using compounds stabilizing the oligomers (Williams et al., 2005), protocols based on the disaggregation of fibrils at high pH (Heldt et al., 2011a), proteins forming stable oligomers (Campioni et al., 2010) or by using MS methods that allow the concomitant detection of various species in a sample (Carulla et al., 2009; Pan et al., 2011). The study performed on the PI3-SH3 domain provides a nice example of an approach to effectively circumvent such problems, as the oligomeric species could be studied in spite of their transient appearance and coexistence with other species (Carulla et al., 2009).

The problem of structural heterogeneity is also being overcome by a number of emerging cutting-edge methodologies that allow the oligomers to be studied at the single molecule level. In two-color coincident detection (TCCD), two subpopulations of a protein sample are labeled with two different probes. By inducing the coaggregation of the resulting subpopulations, it is possible to observe the formation of individual oligomers through the coincident detection of the two fluorescent probes (Orte et al., 2008). Intensity of the observed two fluorescence signals provided information about oligomer size distribution. The same approach was extended to perform single-molecule fluorescence resonance energy transfer (FRET) studies (i.e., monitoring the energy transferred from a donor probe to an acceptor probe through measurements of the fluorescence emitted by the latter); the observed fluorescence arises from FRET events within individual oligomers and provided information about the distance between the two labeled positions (Orte et al., 2011). Finally, in ion mobility MS, the oligomers were separated in two steps: (1) according to their mobility in a chamber containing a carrier buffer gas that opposes their ion motion and (2) according to their mass-to-charge ratio. This technique gave direct information about mass, size, and shape for individual oligomer species, detecting concomitantly the presence of different oligomer subpopulations, and was applied to Aβ (Bernstein et al., 2009; Kłoniecki et al., 2011), β_2-microglobulin (Smith et al., 2010), and insulin (Pease et al., 2010), as well as to a set of synthetic peptides (Bleiholder et al., 2011).

It is interesting that a noninvasive FRET sensor has been recently set up to monitor in vivo the formation of oligomers and their interconversion (Kaminski Schierle et al., 2011). In this approach, changes in fluorescence lifetime reflect changes in the oligomer status and can be related to differences in toxicity, providing a new tool to study noninvasively oligomer-related toxicity in vivo.

Structure of the Oligomers

The existence of parallel pathways in protein aggregation, and the metastability of the various species accumulating during

the process, creates a multiplicity of oligomers, often with very diverse characteristics. This complexity has led to the utilization of different criteria for their classification; for example, on the grounds of their size, β sheet content (or other purely structural characteristics), stability to SDS solubilization or other treatments, productive role in amyloid fibril formation, reactivity to conformation-specific antibodies, and so forth. Even by focusing on a single system such as Aβ, a full description of the various oligomers described in the literature would require a review or book chapter per se. Therefore, in this section, we try to describe the key structural features shared by protein oligomers from different peptides/proteins and how such structures change as aggregation proceeds.

When aggregation is initiated by fully or largely unfolded monomers in the nucleated conformational conversion, the initial oligomers exhibit a large variety of conformations, with monomers still adopting a disordered structure. For example, using PICUP and CD spectroscopy, it was found that the early aggregates formed by $A\beta_{40}$ and $A\beta_{42}$ are dimers-tetramers and pentamers-hexamers, respectively, with a poor level of structure; these acquire β sheet structure later on in the process at the level of protofibrils (Bitan et al., 2003). The appearance of early unstructured aggregates of $A\beta_{40}$ with unstable β sheet structure was also detected in other two more recent studies, performed independently with H/D exchange (coupled to MS) and FlAsH labeling, respectively, with larger oligomers containing stable β sheet structure forming later (Lee et al., 2011; Qi et al., 2008). Furthermore, with tryptophan fluorescence, it was shown that the initial aggregates formed by α-synuclein display unfolded monomers that expose hydrophobic residues to the solvent (Dusa et al., 2006). Characterization of the aggregation pathway of the pH-unfolded PI3-SH3 domain by pulse-labeling H/D exchange coupled to MS and NMR showed that species detected early in the aggregation process are disordered on the basis of the low degree of amide hydrogen exchange protection (Carulla et al., 2009). Very recently, it has been shown, by means of ion mobility MS, that a set of peptides form initially unfolded assemblies (Bleiholder et al., 2011). Molten globule-like self-associating oligomers have been found to be populated prior to fibril formation also for IAPP (Wei et al., 2011).

If aggregation starts from native-like states, the early aggregates formed at the beginning of the process display monomers populating native-like states (Banci et al., 2005; Olofsson et al., 2004; Pagano et al., 2010). In the case of the model protein Sso AcP, aggregation is not just initiated by a native-like state; the native-like structure persists when the protein forms the initial aggregates (Pagano et al., 2010). In a mutant form of superoxide dismutase type-1, native dimers interact to form larger oligomers stabilized by transient interactions between electrostatic loops from different dimers; these oligomers retain monomers in a native-like conformation (Banci et al., 2005). Transthyretin assembles into aggregates in which monomers exhibit six β strands in a native-like conformation while two edge strands are misfolded (Olofsson et al., 2004). Early oligomers from insulin have been shown to be rich in α-helical structure (Bouchard et al., 2000; Vestergaard et al., 2007).

It therefore emerges that initial aggregates are far from the amyloid structure. In fact, regardless of the aggregation pathway followed by a protein, the initial aggregates display the same conformational features observed in the aggregation-competent monomers. The aggregates do not bind amyloid specific dyes, nor do they exhibit a significant content of stable β sheet structure (Lee et al., 2011; Plakoutsi et al., 2005).

As aggregation proceeds, oligomers undergo a structural rearrangement into species stabilized by β sheet structure, able to bind ThT and CR. In addition, oligomers undergo an increase in dimensions, compactness, stability, and order, still retaining a nonfibrillar morphology. It was proposed that fibril elongation of the NM region of the prion Sup35p is initiated following formation of ordered nuclei by conformational rearrangements of less structured, molten, oligomeric intermediates (Serio et al., 2000). During aggregation of human muscle acylphosphatase (mAcP), oligomers increase their dimensions and disaggregation induced by dilution into nonamyloidogenic conditions becomes slower as aggregation proceeds, consistently with an increase in oligomer stability (Calamai et al., 2005). The study on the pH-unfolded PI3-SH3 domain mentioned earlier also indicated the late appearance of oligomeric intermediates with highly ordered β sheet structure (Carulla et al., 2009). Single-molecule studies on the same protein provided direct evidence that the stable cross-β structure of the late aggregates emerges via internal reorganization of disordered oligomers formed during the lag phase of the self-assembly reaction (Orte et al., 2008). FRET studies carried out on α-synuclein oligomers showed a decrease in the distance between Tyr39 and Trp125 from the early oligomers to the late oligomers, suggesting an increase in compactness (Kaylor et al., 2005). During aggregation, the NNQQNY peptide undergoes a transition, beginning near the octamer, from a natively unstructured assembly to a highly ordered β sheet assembly (Bleiholder et al., 2011). Finally, as mentioned earlier, an increase of order and β sheet structure content was also observed for $A\beta_{40}$ and $A\beta_{42}$ oligomers by different authors and using different methodologies (Bitan et al., 2003; Lee et al., 2011; Qi et al., 2008; Sandberg et al., 2010).

Such a structural conversion into β sheet containing amyloid-like aggregates was also observed for native-like aggregates containing significant levels of α-helical structure. For example, the native-like aggregates formed by Sso AcP convert, with no need of disaggregation, into amyloid-like protofibrils with a higher level of order, as deduced with ThT fluorescence, FTIR, and far-UV CD (Plakoutsi et al., 2005). Similarly, α-helical native-like oligomers of insulin were also shown to convert into β sheet containing protofibrils, suggesting again a similar process (Bouchard et al., 2000). The direct conversion from α-helical to β sheet oligomers without the need of disaggregation and reassociation was also observed in many molecular dynamics simulations; for example, for a 17-residue peptide designed to form a coiled coil trimer (Strodel et al., 2008). In cases where native-like aggregation involves all-β proteins, such a structural conversion from early to amyloid-like aggregates has not been yet reported, maybe because of inherent difficulties for the detection of such a transition where β sheet structure remains the dominant secondary structure type. Alternatively, the early oligomers may possess already the structural characteristics to act as nuclei.

In addition to the observation of a transition from unstructured (or native-like) oligomers into amyloid oligomers with β sheet content, the β-structure that forms in early aggregates, when

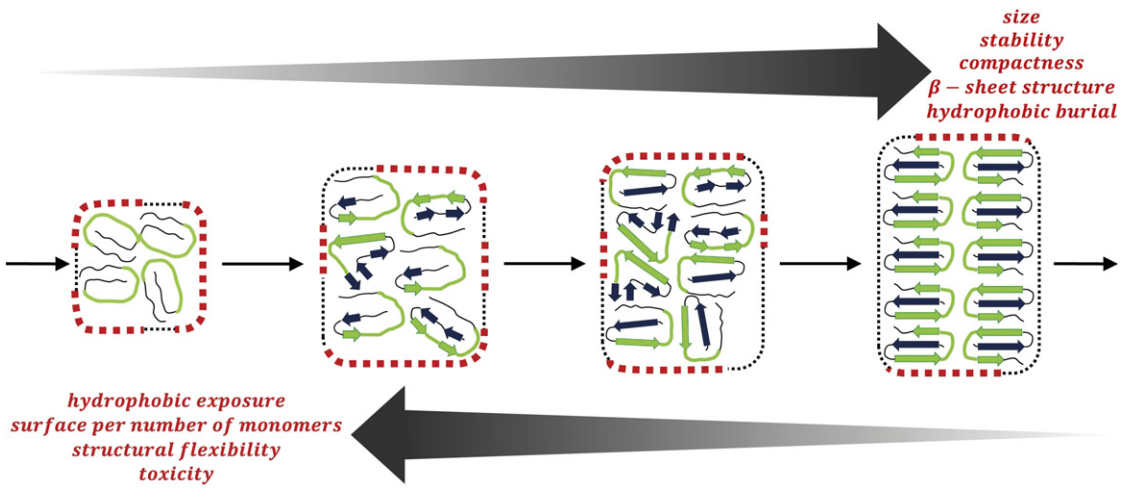

Figure 2. A Schematic Representation of the Structural Rearrangements Occurring during Oligomer Formation
For simplicity, only aggregation starting from fully or largely unfolded monomers is considered (reaction F → G in Figure 1). Amyloidogenic/hydrophobic segments are in green. The oligomer surface is drawn as a thin black and a thick red dotted line when amyloidogenic/hydrophobic segments are buried and exposed to the solvent, respectively. While aggregation proceeds (left to right), a set of structural rearrangements takes place: The top and bottom arrows show the parameters that increase and decrease, respectively. Binding of monomers to early oligomers is isotropic, whereas late oligomers can bind to monomers only at the edges. This leads to growth of thin filaments, which eventually originate amyloid fibrils.

present, tends to be variable and unstable. Aβ(16–22) peptide molecules rearrange, for example, through a realignment of β strands, from a less regular β sheet structure into a β sheet structure more stable in register (Petty and Decatur, 2005). While fibrils and large spherical amyloid intermediates (diameter, 15–35 nm) contain stable parallel β sheets, as determined with solid-state NMR (Chimon et al., 2007), solution NMR data indicate that small globulomers by Aβ (38–48 kDa) have a mixed parallel and antiparallel β sheet structure (Yu et al., 2009). In another study, it was found that initial low-molecular-weight oligomers of $A\beta_{40}$ have a less extended and more unstable β sheet structure, judging from H/D exchange data, than larger intermediate oligomers forming later (Qi et al., 2008). Consistently with these data, the oligomeric interfaces of oligomers formed by a set of model peptides display a variety of sheet-to-sheet pairing angles (Liu et al., 2011). Such internal conversions from variable to regular sheets produce oligomers more productive in terms of fibril formation, as fibrils have a stable and a regular β sheet structure (Carulla et al., 2009; Cerdà-Costa et al., 2007; Orte et al., 2008). This was found directly in a paper where oligomers exhibiting aligned strands were found to be capable of forming thermostable, long, rigid, and twisted fibrils, whereas oligomers without this strand alignment aggregate to form thin, flexible, and smooth protofibrils (Petty et al., 2005).

Thus, the general picture emerging from all such studies is that, as aggregation proceeds through a nucleated conformational conversion process, oligomers undergo a continuous rearrangement of structure (Figure 2). This reorganization involves an increase in size, stability compactness, regularity of the β sheet structure, and hydrophobic burial. It also implies a decrease in dynamical fluctuations, exposure of hydrophobic clusters, and oligomer surface per number of monomers. We show in a following section that this trend is also related with a decrease in oligomer toxicity. The conversion may occur through a multiplicity of oligomeric states or just as a two-state process, not necessarily through four oligomeric states, as depicted in Figure 2. In addition, each oligomeric state may be considered as an ensemble of oligomers with distributions of size, structure, and so forth.

The Oligomers Are Polymorphic in Size and Structure

The aforementioned existence of parallel competing pathways and the presence of multiple species even for the same pathway, imply that oligomers exist as a number of species with different morphological and structural properties. This phenomenon is usually referred to as oligomer polymorphism (Kodali and Wetzel, 2007; Stefani, 2010). Oligomers exhibit polymorphism in terms of their size (Mastrangelo et al., 2006), shape (Pountney et al., 2005), compactness (Kaylor et al., 2005), stability (Calamai et al., 2005; Souillac et al., 2003), and secondary and tertiary structure content (Bleiholder et al., 2011; Ehrnhoefer et al., 2008; Ladiwala et al., 2010; Lee et al., 2011; Serio et al., 2000). The spectrum of oligomer polymorphism extends even further if we consider that small oligomers containing both Aβ and α-synuclein molecules form in patients with mixed Alzheimer's disease and Parkinson's disease and in transgenic mice coexpressing both proteins (Tsigelny et al., 2008).

Different types of oligomers can coexist in solution at the same time (Goldsbury et al., 2005; Gosal et al., 2005; Jain and Udgaonkar, 2011; Mastrangelo et al., 2006; Relini et al., 2010) and even in vivo (Winner et al., 2011). The predominance of some particular species can be determined by a number of factors. First, mutations can alter the pathway by which oligomers form and, consequently, the morphology/structure of the oligomers. For example, in the case of Sso AcP, a set of point mutations leads to protein variants that aggregate following a pathway different from that of the wild-type protein, leading to ThT-binding β-structured oligomers and native-like aggregates, respectively (Soldi et al., 2008). Addition of two residues at the C terminus of the Aβ peptide is sufficient to change the

population of oligomers in equilibrium with the monomer, with a distribution of monomers to tetramers typical for $A\beta_{40}$ shifting to a mixture of pentamers and hexamers for $A\beta_{42}$ (Bitan et al., 2003). In the presence of membrane extracts, different mutants of $A\beta_{40}$ having single amino acid substitutions also formed different distributions of polymorphic aggregates (Pifer et al., 2011). A similar mutation dependence of oligomer formation was also observed for α-synuclein (Ono et al., 2011).

A second determinant of oligomer polymorphism is the solution conditions under which aggregation is initiated. In the case of HypF-N, it was shown that oligomers formed under two different solution conditions, differing in terms of pH and cosolvent composition, exhibit different toxicities (Campioni et al., 2010), and this can be attributed to differences in compactness and solvent exposure of hydrophobic clusters (discussed later). Two different products of lipid peroxidation have recently been shown to be able to induce α-synuclein oligomers that differ in terms of morphology, dimensions, compactness, and stability (Näsström et al., 2011). Finally, the type of seeding can affect the final fibril morphology/structure, and, consequently, it is expected to alter the properties of the oligomers that accumulate prior to fibril formation (Paravastu et al., 2009; Yamaguchi et al., 2005).

Oligomer polymorphism is not just a relevant phenomenon for a full description of the structure and mechanism of formation of oligomers and fibrils but also has many implications in biology. It was indeed found that expression of different mutants of Aβ in *Drosophila* qualitatively led to different pathologies (Iijima et al., 2008) and that different oligomers of α-synuclein and Aβ caused toxicity in cell cultures through different mechanisms (Danzer et al., 2007; Deshpande et al., 2006). On top of that, it is well established that polymorphism is a central theme to explain the propagation of prion strain infectivity (Jones and Surewicz, 2005).

Oligomers In Vitro and In Vivo

An important question is whether the large number of protein oligomers formed in vitro and described in the literature are relevant for amyloid fibril formation processes in vivo and their associated diseases. Using approaches aimed at detecting oligomers directly in vivo, a number of studies have revealed the presence of such species in human patients suffering from protein deposition diseases or related animal models. This has been observed, for example, for Aβ (Lesné et al., 2006; Shankar et al., 2008), tau (Lasagna-Reeves et al., 2012; Patterson et al., 2011), huntingtin (Nekooki-Machida et al., 2009), and α-synuclein (Tsigelny et al., 2008). Such oligomers, when isolated from the living specimen and administered to normal rats, have been shown to cause cognitive impairment in the animals (Lesné et al., 2006; Shankar et al., 2008). Models for oligomerization in vivo are now also being proposed (Larson and Lesné, 2012).

In addition, conformation-specific antibodies raised against particular types of exogenous Aβ or tau oligomers and showing no specificity for monomeric or fibrillar protein have been used to reveal the presence of the same types of oligomers in Alzheimer's disease patients, indicating the existence of such in vitro-formed oligomers in human patients as well (Hillen et al., 2010; Kayed et al., 2007, 2003; Lacor et al., 2004; Lasagna-Reeves et al., 2012, 2011; Noguchi et al., 2009; Patterson et al., 2011). Thus, although our understanding of oligomer formation mechanisms in vivo remains limited, the existence of protein oligomers with structural characteristics similar to those found and characterized in vitro suggests that we can capitalize on our current knowledge obtained in vitro.

The Structural Determinants of Oligomer-Induced Cytotoxicity

The progressive elucidation of oligomer structure is starting to reveal the structural determinants of oligomer toxicity; i.e., the structural elements that are responsible for the ability of protein oligomers to interact with the cells and cause their dysfunction. An important source of information is represented by the large body of studies on the Aβ peptides. Various oligomers have been described for both the $A\beta_{40}$ and $A\beta_{42}$, which have been attributed different names, such as Aβ-derived diffusible ligands (ADDLs), protofibrils, prefibrillar oligomers, fibrillar oligomers, annular protofibrils, amylospheroids, globulomers, spherical amyloid intermediates, and so forth. The toxicity of many of such oligomeric species has been measured on cultured cells using the MTT reduction assay, providing an opportunity to compare their toxicities.

Figure 3A reports the MTT reduction values measured by various authors for their studied oligomers versus the size of the oligomers, expressed as mean molecular weight. The MTT reduction values reported in the figures were all measured at a peptide monomer concentration of 2.0–2.7 μM and are thus comparable between different oligomeric species. The data refer to both $A\beta_{40}$ and $A\beta_{42}$ as it was shown that the same type of oligomers formed by the two species cause similar decreases of MTT reduction following their addition to the cells (Kayed et al., 2003; Kayed et al., 2009). A clear trend is present in the plot, with toxicity decreasing with the size of the oligomers until a well-defined plateau. It is interesting that the plateau obtained with the best fitting procedure was not found as 100% but as 83% ± 5%, which is similar to the values reported by different authors for amyloid fibrils (molecular weight > 2,000 kDa) formed by both $A\beta_{40}$ and $A\beta_{42}$ (Chafekar et al., 2008; Kayed et al., 2003; Walsh et al., 1999). The analysis therefore indicates that oligomer size is an important determinant of oligomer toxicity. In agreement with this analysis, it was found that three classes of small aromatic molecules can inhibit $A\beta_{42}$ oligomer toxicity by converting the small oligomers into large aggregates, fibrils, and monomers, respectively (Ladiwala et al., 2011).

Hydrophobic exposure on the aggregate surface seems to be another important determinant of oligomer-mediated toxicity. A pioneristic study that used reconstructed model membranes rather than cells reported a correlation between hydrophobic exposure in $A\beta_{40}$ aggregates and membrane fluidity, measured with bis-ANS fluorescence and membrane fluorescence anisotropy, respectively (Kremer et al., 2000). Later on, a correlation between hydrophobicity of homopolymeric amino acid stretches and cytotoxicity of their aggregates has been observed (Oma et al., 2005). In a more recent study, two types of spherical oligomers formed by the HypF-N protein using different protocols and shown to have indistinguishable size and morphology, as detected with AFM, were found to have very different toxicities, with one species found to be nontoxic altogether (Campioni et al., 2010). With site-directed pyrene labeling, it was found

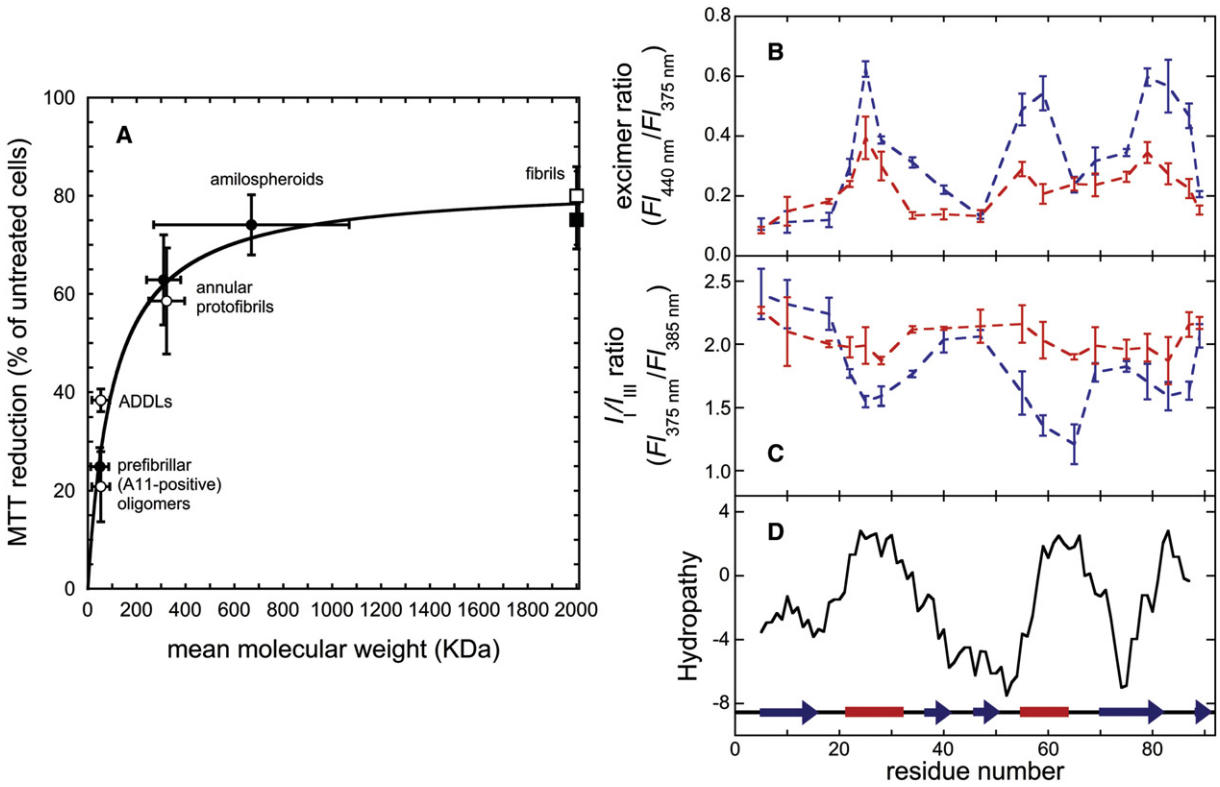

Figure 3. Structural Determinants of Oligomer-Induced Toxicity

(A) Toxicity versus size of $A\beta_{40}$ and $A\beta_{42}$ aggregates. Toxicity is measured by determining MTT reduction by cultured cells following their exposure to oligomers added to the extracellular medium. Aggregate toxicity was expressed as percentage of MTT reduction relative to untreated cells, where 0% and 100% values are two extremes of full cell death and full viability, respectively. Values and error bars are from the original papers: prefibrillar oligomers (Kayed et al., 2003), ADDLs (Lambert et al., 2001), annular protofibrils (Kayed et al., 2009), and amylospheroids (Hoshi et al., 2003). All data were obtained at a peptide concentration in the range of 2.0-2.7 μM. Aggregate size was expressed as mean molecular weight of the reported distributions in the original papers, and error bars refer to the width of the distributions, not SD or SEM: prefibrillar oligomers (Kayed et al., 2007), ADDLs (Gong et al., 2002), annular protofibrils (Kayed et al., 2009), and amylospheroids (Hoshi et al., 2003). Only data for which both molecular weight and MTT reduction values at ca. 2.0–2.7 μM Aβ are reported. Data for both $A\beta_{40}$ (filled circles) and $A\beta_{42}$ (empty circles) are presented, as the same type of oligomers or fibrils formed by the two species cause similar decreases of MTT reduction (Kayed et al., 2003; Kayed et al., 2009). All data points were fitted to a hyperbolic function of the form $y = a * x/(b + x)$. MTT reduction induced by Aβ fibrils (filled and empty squares for $A\beta_{40}$ and $A\beta_{42}$, respectively) are not taken into account in the fitting procedure and are shown for comparison (Kayed et al., 2003). It is implicit that their molecular weight is often >2000 kDa.

(B–D) Structural differences between toxic and nontoxic aggregates of HypF-N. (B) Excimer ratio of pyrene (related to the degree of structure formation) versus number of labeled residue for toxic (red lines) and nontoxic (blue lines) oligomers of HypF-N. (C) I_I/I_{III} ratio of pyrene (a correlate of the degree of solvent exposure) versus number of labeled residue for toxic (red lines) and nontoxic (blue lines) oligomers. (D) Hydropathy profile of HypF-N sequence. The three panels show that in nontoxic aggregates, unlike the toxic aggregates, the three hydrophobic regions of the sequence are structured and buried inside the oligomers.

that the three most hydrophobic regions of the protein sequence are structured and buried in the nontoxic oligomers, whereas in the toxic oligomers the same regions are more solvent exposed and flexible (Figures 3B–3D).

Other studies have appeared very recently on the importance of hydrophobic exposure for protein aggregate toxicity. Striking correlations have been found for a number of peptides/proteins between the toxicity of various forms of aggregates formed in vitro and added extracellularly to cell cultures, measured with propidium iodide incorporation, and their solvent exposure determined with ANS binding (Bolognesi et al., 2010). Another study has demonstrated that highly amyloidogenic proteins expressed intracellularly in human embryonic kidney 293T cells have levels of toxicity, measured with the MTT test, that increase with the exposure of hydrophobic clusters on the aggregate surface measured with ANS binding (Olzscha et al., 2011). In the latter study, the toxicity of the intracellularly expressed proteins was attributed to the ability of their aggregates to interact with a number of multifunctional cellular proteins and alter their function (Olzscha et al., 2011), whereas in the HypF-N study, toxicity was attributed to the ability of the extracellularly added oligomers to interact with the cell membrane and cause an uptake of calcium (Campioni et al., 2010; Zampagni et al., 2011). In addition, many other mechanisms of toxicity have been proposed. Hydrophobic exposure on oligomeric surface thus seems an important determinant of toxicity, independent of the mechanism by which oligomers cause cell dysfunction.

Another proposed determinant of oligomer toxicity is the shape of the aggregates. In particular, it has been proposed that monomers may associate to form pore-like oligomers that bind membranes or, alternatively, that monomers directly self-assemble into pores at the membrane interface (Giehm et al., 2011; Lashuel et al., 2002a, 2002b; Last et al., 2011). The proposed consequence of pore formation is that normally

intracellular or extracellular components can freely circulate between the two environments, leading to cellular dysfunction. Ion-channel oligomers have been observed by TEM for a number of systems, including Aβ, IAPP, α-synuclein, ABri, and ADan (Lashuel et al., 2002a; Last et al., 2011; Quintas et al., 2001). It should be emphasized, however, that alternative mechanisms of oligomer-mediated toxicity have been proposed for all systems found so far to form annular oligomers. For α-synuclein, for example, it has recently been proposed that spherical oligomers growing at the membrane edge captures lipids from the membrane, eventually resulting in membrane disruption (Reynolds et al., 2011). Furthermore, the correlation between size and toxicity (Figure 3A) includes annular protofibrils (Kayed et al., 2009), and these oligomers do not significantly deviate from the observed trend. It is therefore evident that a role for this specific morphology—and, more generally, for oligomer shape—in oligomer-mediated toxicity remains elusive.

Although the results reviewed here indicate that protein oligomer toxicity is determined by a well-defined set of parameters such as size and hydrophobic exposure of the oligomers, it is important to emphasize that toxicity does not reside in one or limited number of oligomeric forms of a given protein. Different oligomers from the same protein have been shown to affect cell viability to some degree. Moreover, the observation that protein oligomers formed by different proteins share similar levels of toxicity is itself a strong suggestion that toxicity is a shared property of protein misfolded oligomers rather than a characteristic of a specific structural pattern.

Conclusions

Although the increasing awareness of the existence of a myriad of different metastable oligomers seemed to make their characterization an impossible task, in the past few years considerable efforts expended by different researchers has led to the emergence of innovative methodologies that are starting to unveil the multiple structures of protein misfolded oligomers and the structural features underlying their toxicity. Oligomer particles can now be detected at the single molecule level and can be observed not only in the test tube but even in vivo. The general emerging picture is that oligomers cannot be described as a finite number of protein structures, each of them identified by a defined set of parameters. Oligomers are best described as a number of conformational ensembles, each containing an indefinite number of different assemblies that vary, almost without solution of continuity, in secondary and tertiary structure, compactness, shape, size, number of monomers, and so forth. All these parameters contribute to a complex energy landscape and determine, directly or indirectly, the toxicity of the oligomers through relationships that are slowly being revealed.

Of course, much remains to be determined. Key points that need to be addressed in future research include (1) developing new and more accurate methodologies for oligomer structure determination; (2) identifying additional molecular determinants of oligomer toxicity and the mechanisms of action of the resulting aggregates so that new molecular targets for therapeutic intervention can be identified; (3) understanding the reasons why, where, and when functional proteins convert into such aggregates, again with the aim of interfering therapeutically with their formation; and (4) developing new methodologies to detect reliably and routinely toxic oligomers in vivo in a noninvasive way and before disease onset.

It is hoped that such studies will make it possible to fully describe the "protein aggregation side" of the protein (mis-)folding landscape, to link our in vitro knowledge with systems in vivo and to reach a complete understanding of the relationships between structure and toxicity of protein misfolded oligomers.

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