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Biophysical Studies of the Amyloid β-Peptide: Interactions with Metal Ions and Small Molecules

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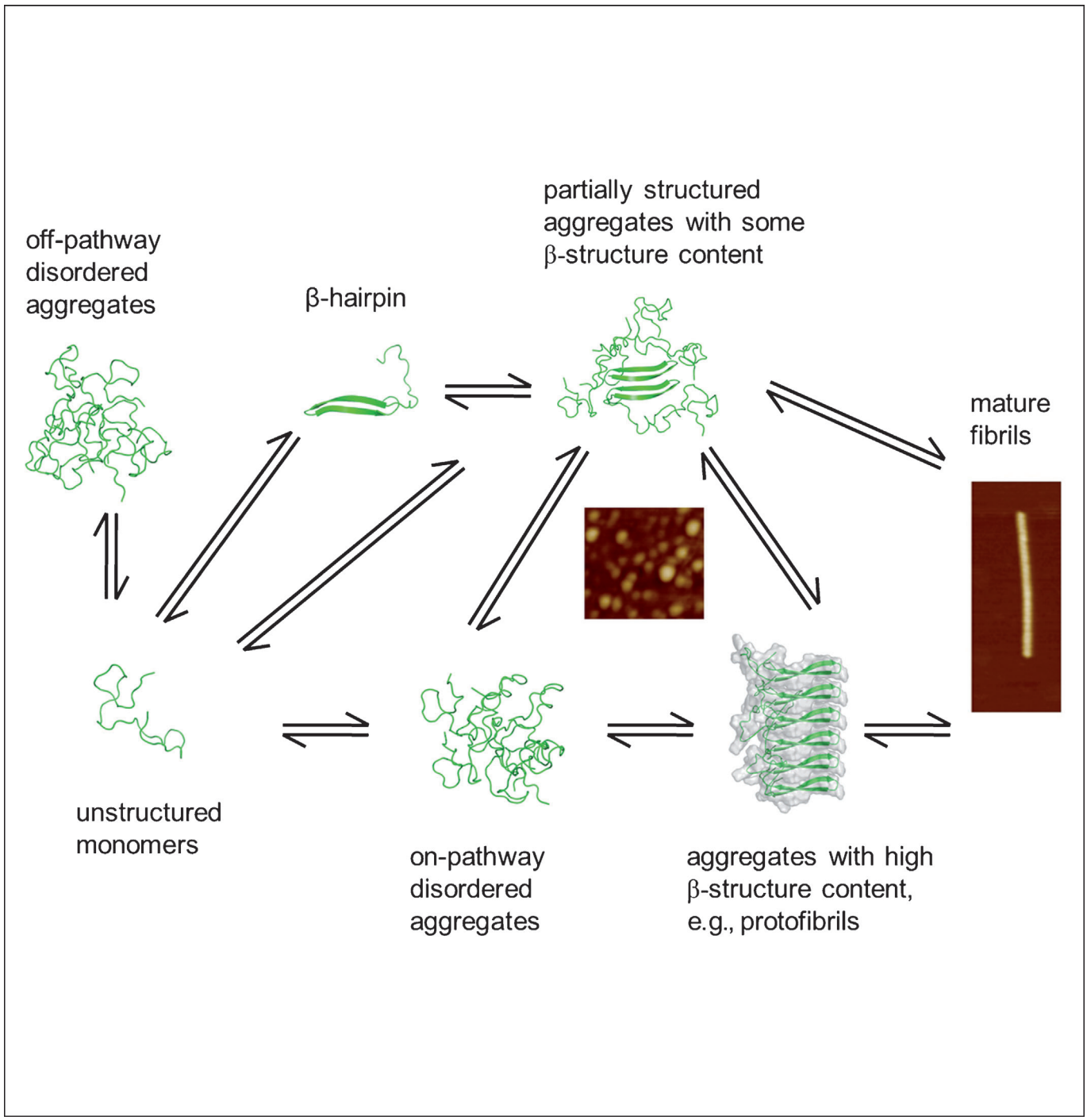
This review article is dedicated to Prof. Ivano Bertini.



Alzheimer's disease is the most common of the protein misfolding ("amyloid") diseases. The deposits in the brains of afflicted patients contain as a major fraction an aggregated insoluble form of the so-called amyloid β-peptides (Aβ peptides): fragments of the amyloid precursor protein of 39–43 residues in length. This review focuses on biophysical studies of the Aβ peptides: that is, of the aggregation pathways and intermediates observed during aggregation, of the molecular structures observed along these pathways, and of the interactions of Aβ with Cu and Zn ions and with small molecules that modify the aggregation pathways. Particular emphasis is placed on studies based on high-resolution and solid-state NMR methods. Theoretical studies relating to the interactions are also included. An emerging picture is that of Aβ peptides in aqueous solution undergoing hydrophobic collapse together with identical partners. There then follows a relatively slow process leading to more ordered secondary and tertiary (quaternary) structures in the growing aggregates. These aggregates eventually assemble into elongated fibrils visible by electron microscopy. Small molecules or metal ions that interfere with the aggregation processes give rise to a variety of aggregation products that may be studied in vitro and considered in relation to observations in cell cultures or in vivo. Although the heterogeneous nature of the processes makes detailed structural studies difficult, knowledge and understanding of the underlying physical chemistry might provide a basis for future therapeutic strategies against the disease. A final part of the review deals with the interactions that may occur between the Aβ peptides and the prion protein, where the latter is involved in other protein misfolding diseases.

1. Introduction

For almost 30 years Alzheimer's disease (AD) has been a "molecular disease", that is, since the pioneering 1984 work of Glenner and Wong,[1] who reported on the isolation and partial characterization of a new protein component, named amyloid β-protein (Aβ protein), from patients suffering from the disease. Since then a vast amount of work, including physiological, biochemical, and biophysical studies, has gone into the elucidation of the molecular processes involved in the disease, with at least partial success so far. The so-called amyloid cascade mechanism,[2,3] involving growing aggregates of the culprit Aβ peptides, is generally accepted as a basis for the disease-associated formation of insoluble senile plaques in the brain. The biochemical similarities to other neurodegenerative diseases such as Parkinson's or prion diseases have given rise to a nomenclature for these conditions as "protein misfolding" diseases.[3,4]

Understanding the properties of the Aβ peptides—especially their self-aggregating behavior—is generally considered the key to elucidating the molecular mechanisms behind AD. Much effort has been devoted to developing strategies for interfering with the peptide aggregation process. The Aβ peptides are cleavage products of the amyloid precursor protein (APP), which contains cleavage sites for different secretase enzymes (Figure 1). APP is a membrane protein with one transmembrane α-helix, and the γ-secretase cleavage site is located in the middle of this α-helix. The sites for α- and β-secretase are located outside of the cell membrane region (Figure 1). Cleavage involving β- and γ-secretases yields Aβ peptides of 39–43 residues in length, out of which the version of 40 residues in length is the most abundant. The peptide of 42 residues in length, which appears in smaller amounts, is the most amyloidogenic, that is, it has the highest propensity to self-aggregate.[5] Cleavage with α-secretase results in peptide fragments that do not self-aggregate, and hence do not contribute to the pathological processes.[6]

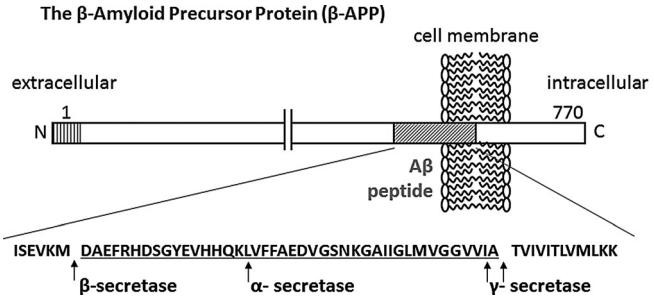


Figure 1. The β-amyloid precursor protein (β-APP) has an α-helical transmembrane segment inserted into the cell membrane. Cleavage by the β- and γ-secretases produces amyloid β-peptides of 39–43 residues in length, whereas cleavage involving α-secretase produces peptides that do not self-aggregate.

A wide range of environmental conditions and interacting molecules and ions affect the outcomes of the Aβ aggregation processes in vivo. Chemical or physical interference can either promote or counteract the appearance of certain intermediate or final aggregation states, such as the not yet fully understood toxic states. Understanding such interference mechanisms becomes particularly relevant in the search for therapeutic strategies against AD. Interference with the Aβ aggregation processes may be studied under controlled conditions in vitro, and this review focuses on the properties of certain small molecules and metal ions that have been shown to interact with

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Axel Abelein studied physics at the University of Bayreuth (Germany) and at Stockholm University (Sweden) where he received his M.Sc. in 2010. Afterwards, he started his Ph.D. studies in biophysics under the supervision of A.G. investigating the aggregation mechanisms of the Aβ peptide. His Ph.D. work deals with modulation of Aβ self-assembly by small molecules, with NMR and optical spectroscopy as the main methods.

Jens Danielsson graduated in 2007 with a Ph.D. in biophysics under the supervision of A.G. at Stockholm University. After that he moved to Professor Flemming Poulsen's lab for a postdoc position at the University of Copenhagen, where he worked on high-resolution studies of protein folding. He then moved back to Stockholm University to join Professor Mikael Oliveberg's group, where he continues to investigate protein folding physics.

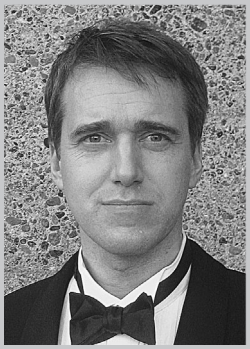
Jüri Jarvet received his Ph.D. at Stockholm University in 1999 for developing NMR relaxation methods for studies of peptide structure and dynamics under the supervision of A.G. He is a Senior Research Associate at the Estonian National Institute of Chemical Physics and Biophysics and a visiting scientist at Stockholm University Department of Biochemistry and Biophysics.

Astrid Gräslund's laboratory focuses on NMR and EPR spectroscopy of biomolecules and their interactions. Systems studied by NMR include the Aβ peptide and cell-penetrating peptides. She received her M.Sc. in applied physics at the Royal Institute of Technology in Stockholm and her Ph.D. in biophysics at Stockholm University in 1974 and is at present Professor of Biophysics at Stockholm University. She was Professor of Medical Biophysics at Umeå University from 1988 to 1993. Since 1993 she has been a member of the Royal Swedish Academy of Science (chemistry) and since 1996 secretary of the Nobel Committee for Chemistry.

Jinghui Luo is a Ph.D. candidate in biophysical structural chemistry at Leiden University, in Jan Pieter Abrahams' group. His current research interests focus on studies of amyloid protein aggregation and nanocrystallography. He expressed, purified, and crystallized a membrane protein (*E. coli* DgkA) at Trinity College, Dublin (Martin Caffrey's group) and replicases (SARS and MHV) at EMBL (Paul Tucker's group). His M.Sc. research on the interaction of the Aβ peptide and the ApoE protein was carried out in A.G.'s group.

Kajsa Löfgren Söderberg graduated from Stockholm University in 2011 with a Ph.D. in biochemistry, having worked in prion research under the supervision of A.G. She then moved to the group of associate Prof. Katrin Mani at Lund University (Sweden) as a postdoctoral research associate in biomedicine, focusing on the glycobiology of Aβ pathology, also being engaged in teaching of medical students. In 2013 she returned to Stockholm University for further prion research. Her interests involve how misfolded proteins are shuttled within and between cells.

Ann Tiiman received her Ph.D. in gene technology from Tallinn University of Technology (Estonia) in 2012, studying the metal ion interactions of the Aβ peptide in the laboratory of Prof. Peep Palumaa. She is currently a postdoctoral fellow at Stockholm University in A.G.'s group.



Sebastian K. T. S. Wärmländer is mainly an NMR spectroscopist, but also employs related spectroscopic and imaging techniques in his study of biomolecules. His research on the structure and dynamics of DNA with noncanonical bases earned him a Ph.D. in biophysics from Stockholm University in 2003. Inspired by postdoc visits to UCLA and UCSB, his research interests now include amyloidogenic proteins and environmental toxins. In addition to biophysics, he has interests in law, legal medicine, and archaeology. He is the director of an archaeological expedition in Estonia and has also been spotted excavating his Viking forefathers in Iceland.

the Aβ peptides and to change the outcomes of their aggregation. The review also covers recent structural work on the Aβ peptides. We use an overall biophysical approach, with some emphasis on NMR spectroscopy, and describe work done in vitro and involving peptides produced either synthetically or in bacteria. An important characteristic of the Aβ peptides is their ability to bind metal ions such as those of copper and zinc. NMR studies of paramagnetic ions such as Cu^{II} in interaction with biomolecules rely to a large extent on the scientific work by Ivano Bertini,[7] to whom this review is dedicated.

2. The Aβ Aggregation Process

The Aβ aggregation process in many ways resembles a polymerization process. Unstructured or weakly structured peptides are converted into intermediate metastable and finally stable "amyloid" states, in which the secondary structure is at least partially β-form. The early intermediate state(s) is/are soluble in aqueous solutions, whereas the final state(s) is/are not. The initial aggregation process follows a characteristic and approximately sigmoidal kinetic curve: after a lag phase followed by rapid elongation the sample finally reaches a steady state.[8] This kinetic trace is often derived from fluorescence of the dye thioflavin T (ThT) when bound to the amyloid material (Figure 2). Indeed, binding of ThT to the aggregated material

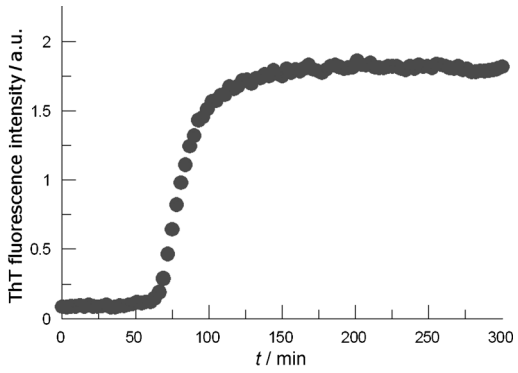


Figure 2. Illustration of typical Aβ amyloid formation process monitored by ThT fluorescence (arbitrary units, a.u.) at 450 nm [10 μM Aβ(1–40) peptide in 50 mM Tris buffer (pH 7.4) with 5 μM ThT at 37 °C with agitation]. This tripartite process typically involves a lag phase, an elongation phase, and a final steady state.

and the resulting increase in fluorescence is considered an operational definition of the term "amyloid". The ThT molecule contains two aromatic ring structures connected by a C–C bond permitting free rotation. In aqueous solution, this motion quenches the fluorescence of the dye. When the dye is bound to amyloid material, though, the free rotation is hindered, and the fluorescence increases.[9] The Aβ aggregation process has been investigated with the aid of elaborate kinetic models,[10,11] and is strongly dependent on experimental conditions such as sample concentration, pH, temperature, and agitation.

Many different techniques have been used to follow the Aβ aggregation process and the intermediate and final states. The nomenclature and description of these states vary in the literature and depend on the methods involved. In a very broad sense one can distinguish three major states: monomer (water-soluble monomeric peptide), oligomer (water-soluble or -insoluble aggregates of peptides, typically from two to less than 100 peptide molecules with a varying degree of β-structure), and fibril (water-soluble or -insoluble large peptide aggregates with a typical linear and straight appearance by microscopy and with a high degree of β-structure). The term protofibril is often used for smaller (shorter and less straight) fibrils. As pointed out in the review by Masters and Selkoe,[5] the nomenclature for the assemblies varies, with new proposed names often depending on the peptide source and method of analysis. Figure 3 gives a schematic view of various possible

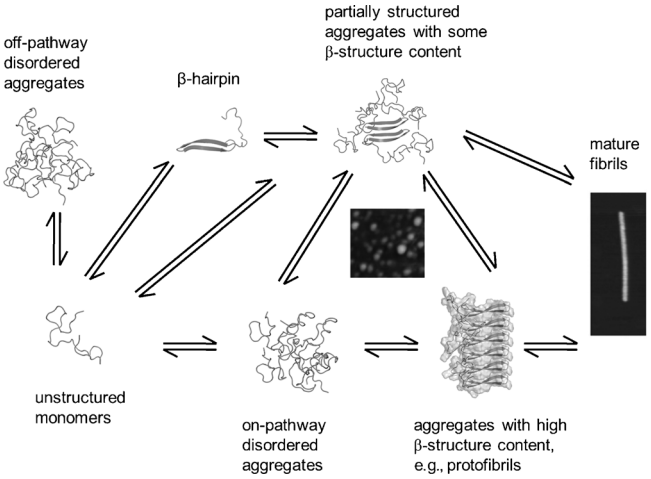


Figure 3. Schematic model for possible Aβ aggregation pathways—the mature fibrils can be formed from aggregated states of Aβ and monomers in combination. The AFM images were recorded in our laboratory and show Aβ oligomers (2–10 nm in diameter) and mature fibrils (4 nm in diameter).

states and the connections between them. The oligomeric states are now generally considered to be the most neurotoxic forms of the peptide.

3. Biophysical Methods for Studies of Aβ Chemistry and Structures

The physico-chemical studies of Aβ are numerous and cannot all be reviewed here. Many biophysical methods have been used to identify different peptide structures and their characteristics. Commonly used nonkinetic methods include computational methods such as MD simulations, as well as experimental methods such as solution-state NMR spectroscopy, CD spectroscopy, IR spectroscopy, small-angle X-ray scattering (SAXS), dynamic light scattering (DLS), solid-state NMR spectroscopy, AFM, XRD, and TEM, listed in an order going from solution-state towards solid-state studies.[12–15] Among the problems encountered when comparing results obtained by different methods are the varying sample conditions required for

application of a particular method, which affect the local properties of the peptide.

Amyloid Aβ fibrils represent the endpoints of long aggregation pathways. The molecular structures along these pathways are highly heterogeneous and diverse in vitro; the diversity in vivo should be even more pronounced. An emerging picture (see Figure 3) is that of an uncontrolled protein folding process that can occur at high peptide concentrations in aqueous solution. After a certain lag time, the unfolded peptide interacts with its identical neighbors and undergoes a hydrophobic collapse. Secondary structures are then formed, while the aggregate structure becomes increasingly compact and ordered. The final result is well-ordered fibrils consisting of stacked Aβ peptides in parallel β-sheet form.[16] Multiple fibril structures seem to exist, all based on parallel β-sheet secondary structures, and the dynamic exchange between these forms is arguably the reason why it has been all but impossible to obtain X-ray diffraction patterns for Aβ fibrils: even if one fibril form is isolated, re-equilibration re-establishes the polymorphic steady state.

One 3D structure of Aβ(1–42) fibrils has been determined by use of a combination of solid-state NMR data, amide hydrogen-exchange measurements, pairwise mutagenesis, thioflavin T binding, and high-resolution cryoelectron microscopy.[17] In this structure, β-sheet hairpins formed from residues 18–42 are stacked parallel and in-register so that the hairpin legs are perpendicular to the fibril axis. Intermolecular side-chain H-bonds, parallel to the fibril axis, are formed between the odd-numbered residues of strand β1 of the *n*th molecule and the even-numbered residues of strand β2 of the $(n-1)$th. At least two Aβ(1–42) molecules are required to achieve the repeating structure of a protofilament.

No high-resolution atomic structure of an intermediate form has so far been reported,[17,18] although biophysical studies of fibrillar Aβ oligomers show that they have the same kind of "cross-β" spine as mature amyloid fibrils (see above),[19] first shown in the yeast Sup35 protein.[20,21]

A high-resolution solution NMR structure of Aβ in a 1:1 complex with a dimeric Affibody, a protein selected to bind strongly to the Aβ monomer, shows the Aβ peptide in a β-hairpin structure similar to that proposed for fibrils.[22,23] IR spectroscopy studies indicate that the Aβ oligomers occurring early in the aggregation pathway might contain more antiparallel than parallel β-structures.[24]

4. NMR Methods for Studies of Aβ Interactions

The Aβ(1–40) peptide can be produced either by solid-phase chemical synthesis or in *E. coli* bacteria after cloning. The recombinant peptides might contain an additional N-terminal methionine, depending on the production method, but are easily labeled with ^{15}N and/or ^{13}C isotopes, to facilitate solution NMR studies. Such isotope-labeled samples are very useful for studying Aβ interactions with unlabeled molecules. Without agitation and under conditions judiciously chosen in terms of aqueous solution (low salt) and temperature (low temperature), Aβ(1–40) is relatively stable (for several days) in concentrations below 100 μM. The Aβ(1–42) peptide is an order of magnitude more prone to aggregate, making it less amenable to solution NMR, because even modern high-field spectrometers require concentrations exceeding 10 μM. Typical NMR studies are conducted with use of a ^{15}N-labeled 50 μM Aβ(1–40) sample dissolved from lyophilized material by well-established procedures (see, e.g., ref. [25]). A two-dimensional $^{1}H,^{15}N$ HSQC NMR spectrum yields one cross-peak for each amide bond along the peptide sequence, and these cross-peaks can easily be assigned to the corresponding residues.

a. High-resolution NMR studies

Many small molecules and metal ions are able to induce particular kinds of Aβ aggregates by modifying the "normal" aggregation pathways. Addition of such molecules or ions to an NMR sample in a suitable buffer solution might produce characteristic changes in the HSQC cross-peak intensities and/or chemical shifts. In favorable situations, these changes can yield information about residue-specific interactions.

Addition of certain ions or small molecules can sometimes cause the NMR signals from the amide protons to become almost uniformly reduced in intensity. If this happens immediately after addition of the compound, the sample will typically be stable for some time. The situation resembles that in which Aβ is stored without added effectors and allowed to aggregate slowly on its own. In cases in which monomeric Aβ undergoes chemical exchange with an NMR-invisible species it can be possible to apply more advanced NMR methods involving relaxation studies to investigate the properties of the "lost" component in the sample, also known as "the dark state".[26–31] Such studies can provide information on induced secondary structure in the non-monomeric state, on the sizes of the aggregates formed, on the exchange kinetics on intermediate or relatively slow NMR timescales (typically ms to μs), and on the proportions of the peptide populations involved.

Addition of paramagnetic ions such as Cu^{II} will inevitably produce paramagnetic broadening and therefore cause loss of specific NMR signals from nuclei in the vicinity of the binding site. Similar broadening of specific NMR signals can arise from chemical exchange between different conformations or aggregation states, induced either by diamagnetic (e.g., Zn^{II}) or paramagnetic (e.g., Cu^{II}) metal ions, or by ligands such as small molecules.

b. Solid-state NMR studies

Solid-state NMR is one of the few methods that can give detailed information on amyloid structure and metal binding on a molecular level.[15] The structures of Aβ(1–40), Aβ(1–42), and shorter Aβ fragments have been studied by use of specific labeling with ^{13}C and ^{15}N, with an emphasis on fibrillar structures. Wild-type Aβ fibrils have been shown by a variety of techniques to contain cross-β structures, with β-strands perpendicular to the fibril axis and in which the β-sheets have an in-register parallel supramolecular organization. Multiple-quan-

tum solid-state NMR data indicate a parallel organization of β-sheets in Alzheimer's β-amyloid fibrils.[16] NMR dipolar recoupling methods for the measurement of interpeptide distances have established that the central core of Aβ(10–35) consists of a parallel β-sheet structure in which identical residues in adjacent chains are in-register.[32]

Recent studies indicate that fibrils formed in vitro by the Aβ(1–40) Iowa mutant (D23N), which is associated with early onset of neurodegeneration, can contain either parallel or antiparallel β-sheets,[33] and both forms were found to be cytotoxic to neuronal cell cultures.

Ivano Bertini and his co-workers recently published a new structural model of well-ordered fibril samples of Aβ(1–40), consisting of a β1-turn-β2 motif involving residues 13–39, a structured N-terminal segment, and inter-protofilament pairing involving the β2-strands.[34] The structural constraints were obtained by ^{13}C–^{13}C 2D dipolar-assisted rotational resonance (DARR, with <100 ms mixing time) and ^{15}N–^{13}C 2D proton-assisted insensitive nuclei cross-polarization (PAIN-CP) measurements. The sequential assignment and the analysis were achieved from NCA, NCO, NCACX (3D), NCOCX (3D), and CANCO (3D) experiments.

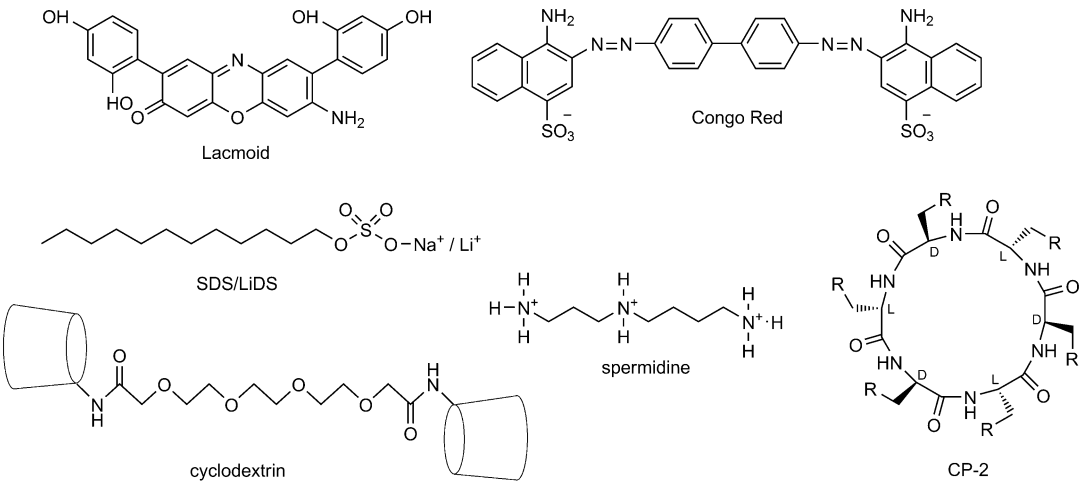
Different symmetric organization of protofilaments has been found within distinct morphologies—termed "twisted pairs" and "striated ribbons"—of wild-type Aβ(1–40) fibrils.[35,36] For the Arctic mutant (E22G) of the Aβ peptide, very broad variation was found in the morphologies of aggregation intermediates, in the polymorphism of amyloid fibrils, and in the aggregation kinetics.[37] The new structure of the wild-type Aβ(1–40) interprotofilament interface found by Bertini's group[34] differs from those in previous reports, which demonstrates the importance of the method and conditions for fibril preparation, as well as the polymorphism of the Aβ fibril structure.

Solid-state ^{13}C NMR has also been used to investigate Cu^{II} binding to Aβ(1–40) fibrils, with use of paramagnetic signal quenching in 1D and 2D NMR spectra to unravel the molecular details of the binding.[38] Primary Cu^{II} binding sites were found at N^{ε} in H13 and H14, and also at the carboxylic acid groups in V40 and in the side chains of the glutamic acid residues (E3, E11, and/or E22). Not all possible binding sites were isotope-labeled in the study, so other additional binding sites, such as H6, might exist. ^{13}C chemical shift analysis demonstrated no major structural changes upon Cu^{II} binding in the hydrophobic core regions (residues 18–25 and 30–36). Although production of reactive oxygen species (ROS) through M35 oxidation in the presence of Cu^{II} has long been suspected as a mechanism contributing to the neuronal cell damage observed in AD, the signal of $^{13}C^{\varepsilon}H_3$-S in M35 showed little change after Cu^{II} binding; this indicates that Cu^{II} binding alone does not induce M35 oxidation (see also ref. [39]).

5. Small-Molecule Interactions with the Aβ Peptides

Inhibition of amyloid formation, including that resulting from Aβ aggregation, is a topic with many facets, most of them with a practical angle for potential therapies for amyloid diseases.[40] Investigating how various interactors modulate Aβ aggregation might help us to develop a therapeutic drug, but also allows us to understand the Aβ self-assembly mechanism better in itself. Several kinds of small molecules have been shown to interact with the Aβ peptides and thereby to interfere with their aggregation processes. Different classes of compounds that affect oligomerization or fibrillation, distinguished by their specific antibody reactions or induction of turbidity/EM results, respectively, have been described. Certain compounds inhibit both endpoints.[41]

Here we concentrate on a few interacting compounds and their potential mechanisms for modulating the Aβ aggregation processes. These compounds are: monomeric and dimeric cyclodextrins, the dyes lacmoid and Congo red, the detergents SDS/LiDS, positively charged polyamines, the cyclic peptide CP-2, and a small Affibody protein (Scheme 1). SDS/LiDS and the cyclic CP-2 peptide belong to a class of Aβ-interacting small molecules that have detergent-like properties and can form supramolecular structures on their own.[42] CP-2 and the Affibody were selected from libraries by use of their affinities for Aβ as a criterion for selection.



Scheme 1. Some of the reviewed molecules, all of which interact with the Aβ peptides and modulate their aggregation in different ways.

Experimental studies relating to the effects of these compounds on Aβ aggregation can in principle deal with a) interactions with the monomeric form of Aβ, b) interference with the initial aggregation process, or c) interference resulting in effects observed during the later aggregation stages when fibrils are formed. In addition to various biochemical and immunological methods, spectroscopic techniques such as CD, IR, NMR, and ThT fluorescence can be used to observe the effects at stages (a) and (b), whereas microscopic or other solid state methods are used for stage (c). CD spectroscopy, which provides information on the secondary structure of the peptide bonds, is a very powerful technique for monitoring the structural conversion of Aβ peptides from random coil to β-structure, because each form has its characteristic CD spectrum. IR spectroscopy, however, is required to distinguish between parallel and antiparallel β-sheets.

With this range of techniques available for the experimentalist, the challenge is often to find proper connections between the various aggregation stages, because the different methods often require divergent experimental conditions (peptide concentrations, solvents etc.), and this makes it difficult to draw general conclusions and to compare results obtained by different techniques.

a. Cyclodextrins

β-Cyclodextrin is a cyclic oligosaccharide composed of seven glucose units. The compound is nontoxic and often used as an addition in drugs for human consumption.[43] The β-cyclodextrin monomer has been found to interact with Aβ(1–40) at the side chains of the aromatic residues Y10, F19, and/or F20, with a dissociation constant (K_D) of 3.9 ± 2.0 mM. A dimer of β-cyclodextrin containing a flexible linker was constructed in an attempt to improve the binding affinity to the Aβ peptide, thereby hopefully also improving the ability to disrupt the oligomer formation and/or aggregation process of Aβ. NMR translational diffusion experiments revealed a moderately increased affinity of the β-cyclodextrin dimer (apparent K_D of 1.1 ± 0.5 mM) for Aβ(1–40) relative to that of the β-cyclodextrin monomer.[44,45]

In general, the cyclodextrin interactions modulate the Aβ aggregation. Kinetic aggregation experiments based on ThT fluorescence indicate that the β-cyclodextrin dimer at 0.05–5 mM decreases the aggregation lag time of 8 μM Aβ(1–40), whereas a concentration of 10 mM increases the lag time. The β-cyclodextrin monomer only weakly affects the aggregation lag time, although it reduces the overall ThT fluorescence amplitude. The aggregation pathway is modulated by the β-cyclodextrin monomer, as evidenced by ThT binding kinetics for the aggregating peptide and by TEM analysis. The β-cyclodextrin dimer also has a significant influence on the structural outcome of the Aβ aggregation process; however, this is only partly reflected in its effects on the amyloid formation kinetics. The influence of both weakly interacting cyclodextrin variants can obviously direct the peptide aggregation process toward different amyloid morphologies, with potential intermediates that might be very different, both in structures and in neurotoxic activities.

b. Lacmoid and Congo Red

Lacmoid and Congo Red are representatives of a class of Aβ-interacting dye compounds that are intrinsically inclined to form colloidal aggregates.[46] When added to solutions of Aβ peptides in proportions at which about half the initial NMR signal is lost, these dyes rapidly induce dynamic coaggregates with a formation rate of the order of 10 s^{-1}. The presence of these coaggregates might counteract the "normal" aggregation reactions.[25] Although the kinetics for peptide exchange between monomeric and coaggregated forms are similar (about 1000 s^{-1}) for both compounds, as determined from NMR relaxation studies, the tendencies to induce secondary structures in Aβ are quite different: lacmoid induces very little secondary structure in the coaggregates, whereas β-structure is promoted in the Congo Red coaggregates. In both cases there is exchange of Aβ peptide between coaggregate and solution on the μs to ms timescale, and at any given time point only a few percent of the total Aβ amount are located in the coaggregates.[27]

c. SDS and LiDS

The surfactants SDS and LiDS can be used as model systems to investigate Aβ–lipid interactions; these are of interest because lipids have been found within and around amyloid plaques in vivo.[47,48] SDS and LiDS form micelles in aqueous solution above a critical micelle concentration (CMC) of about 8 mM. At high detergent concentrations, Aβ monomers bind to the micelle surface, and segments with α-helical secondary structure are induced.[49,50] At sub-micellar intermediate detergent concentrations, coaggregates are formed between the detergent and the Aβ peptide, whereupon CD spectroscopy shows that β-structure is induced in the peptide.[51] The situation is similar to that observed for the interaction between Aβ and Congo Red, including the display by the peptide of chemical exchange between the monomeric and coaggregated states on the μs to ms timescale. NMR relaxation dispersion data can provide information on several characteristics of the LiDS–Aβ coaggregates, including their sizes. The estimated average size is in good agreement with parallel SAXS results, thus providing a fairly good description of the properties of the coaggregates.[28]

d. Polyamines

Positively charged small molecules are a class of compounds that share certain properties with metal ions. Polyamines are multivalent cationic alkylamines that naturally exist in the body, both intra- and extracellularly. Polyamines such as spermine, spermidine, and putrescine bind to the N-terminal parts of the Aβ peptides, just like metal ions, but also to the negatively charged central Aβ residues (such as E21 and D22).[52] The polyamines promote Aβ aggregation, although they also alter the aggregation pathways, and their competition with metal ions for the N-terminal binding site might be of great biological relevance.[52] The multivalency of the polyamines to-

gether with multiple Aβ binding sites immediately suggests that one polyamine might bind multiple Aβ peptide moieties, which would readily explain how polyamines facilitate Aβ cross-linking. Although polyamine levels are altered in brains of AD patients,[53] the role of polyamines in AD pathology remains unclear.

e. Affibody

The $Z_{Aβ3}$ affibody is an interesting dimeric molecule that binds to the Aβ peptides with nanomolar affinity,[22,23] thereby—in vivo—preventing Aβ aggregation, dissociating preformed oligomers, and facilitating Aβ degradation.[54] Because the $Z_{Aβ3}$ dimer forms 1:1 complexes with Aβ, the affibody completely inhibits Aβ aggregation at stoichiometric levels, an almost unique property (so far known to be shared only with the BRICHOS protein).[55] The $Z_{Aβ3}$ affibody was selected by phage display and was found to lock Aβ efficiently in a hairpin conformation in which the two legs form an antiparallel β-structure. This conformation appears similar to that found in Aβ fibrils, so this makes the $Z_{Aβ3}$/Aβ complex a promising model system for studying Aβ in a biologically relevant monomeric yet non-aggregating conformation. Modified versions of $Z_{Aβ3}$ such as the nonfluorescent $Z_{Aβ3}$(12–58)Y18L have, for example, been developed to allow study of metal binding to Aβ without concern for ongoing aggregation.[23,56]

f. The cyclic peptide CP-2

Another Aβ-binding molecule selected from a library is CP-2, a nontoxic cyclic D,L-α-peptide.[57] CP-2 self-assembles, and in assembled form it stabilizes small Aβ oligomers, thereby preventing Aβ from forming larger fibrils. There is mounting evidence that the toxic species in AD are oligomers consisting of Aβ peptides in antiparallel β-sheet conformation. CP-2, however, stabilizes low-molecular-weight oligomers dominated by Aβ peptides in a possibly parallel β-structure, which appears to be less cytotoxic. Interestingly, CP-2 assemblies are also capable of dissolving already formed Aβ fibrils, and this makes CP-2 and similar cyclic peptides potentially interesting for clinical use.[57]

Taken together, the above examples illustrate the versatility of the Aβ peptide interactions. A common denominator for the interactors is hydrophobicity and/or charge, although the outcome of the aggregation process depends also on the environmental conditions. The final result in terms of whether amyloids are formed, and of whether neurotoxicity is affected, seems to depend critically on the physical chemistry—but also on more delicate and variable properties of the affected biological system.

6. Quantitative Aspects of Aβ Metal Binding

The biologically active ions of Cu and Zn are important regulators of synaptic function.[58] When released into the synaptic cleft they are weakly coordinated to their ligands and thus easily exchangeable. Cu ion concentrations in the cleft are estimated to be from 15 μM up to 100–250 μM,[59,60] and Zn ion concentrations are around 100–300 μM.[61]

The AD brain is characterized by metal dys-homeostasis,[62] and the amyloid plaques contain relatively high levels of Cu, Zn, and Fe ions.[63] The Fe ions are bound to ferritin,[64] whereas Cu and Zn ions are coordinated by the Aβ peptides.[65] In this work only the Aβ interactions with Zn and Cu ions are reviewed, mainly with consideration of two different aspects: metal ion binding to the monomeric peptide and the effect of metal ions on the Aβ aggregation processes.

Metal ion binding to the Aβ peptide is characterized by a dissociation constant (K_D), and accurate measurements of such K_D values are important for understanding of possible in vivo interactions. The Aβ complexes with Cu and Zn ions have been intensively studied by different methods, including intrinsic Aβ Y10 fluorescence, calorimetry, potentiometry, and NMR spectroscopy. The side chain of the Y10 residue in Aβ is the only intrinsically fluorescent moiety in the peptide, and its fluorescence can be affected (typically quenched) by interactions with other molecules or ions, particularly paramagnetic metal ions such as Cu^{II}. However, the reported affinities of Aβ towards Cu^{II} ions vary considerably—from aM to μM[66]—with recently reported values falling in the 0.01–1 nM range.[67–70] A secondary Cu^{II} binding site has been observed, although it is probably not biologically relevant, due to its low affinity.[71,72]

There are various possible explanations for the variability in the reported K_D values. Firstly, only buffer-independent conditional K_D values should be compared: a major source of K_D variation might be the use of different buffers, with different metal ion affinities, together with differences in the models used to account for buffer effects.[73–75] Secondly, the measurements might be influenced by Aβ aggregation; higher affinity values have often been obtained with, for example, calorimetry and potentiometry measurements. These techniques use relatively high peptide concentrations, which facilitate aggregation. Higher affinities might thus reflect Cu^{II} binding to oligomeric or dimeric complexes, whereas lower affinities might represent binding to the Aβ monomers.[73,74] One study found the Cu^{II} affinities for Aβ monomers to be around ten times lower than the affinities for Aβ aggregates, and that the affinities for the aggregates were high enough to sequester copper from human serum albumin.[76]

Because Zn^{II} is not a redox-active or paramagnetic metal ion, less attention has been paid to it. The variation in reported dissociation constants for Zn^{II}/Aβ complexes is smaller than for Cu^{II}/Aβ complexes, with approximate K_D values around 1–60 μM (without buffer correction).[74] From NMR and fluorescence studies a K_D value of about 1 μM at neutral pH was evaluated, this is similar to that estimated for Cu^{II} under similar conditions.[72] The variation could in part be explained by the different aggregation states of the Aβ peptides, because it has been shown that the Zn^{II}/Aβ complexes transform into higher-affinity complexes during longer incubation times.[66,77] It is thus possible that the weaker affinities correspond to monomeric complexes, whereas the stronger affinities correspond to aggregated complexes. The intermediate values would then

originate from measurements on mixtures of the two types of complexes.[66,73]

In conclusion, despite the ongoing debate about true affinity values, it is clear that the Aβ affinity towards Cu^{II} and Zn^{II} is in a biologically relevant range. It is thus highly possible that Aβ–metal ion interactions in certain brain areas and/or types of neuronal cells affect the behavior and the neuropathological effects of the Aβ peptides in vivo.

A second important aspect of Aβ/metal ion interactions is the effect on the Aβ aggregation process. This effect is currently less well understood, because both enhancement[78] and inhibition[79,80] of fibrillation has been observed. It has been reported, however, that at least trace amounts (<1 μM) of metal ions are required for Aβ aggregation to initiate.[81] Cu^{II} and Zn^{II} both appear to promote Aβ aggregation,[79,82–84] possibly by coordinating multiple Aβ peptides.[85] The metal-induced aggregates are amorphous rather than standard linear fibrils,[79,84] but might transform into spherical aggregates and/or "normal" elongated amyloid fibrils during longer incubation times (cf. Figure 3).[79,82–84,86] A disturbance in the metal homeostasis in the brain might thus lead to formation of metal-induced Aβ aggregates, which can then transform into amyloid fibrils. In this scenario, metal ions could be initiators of amyloid accumulation in sporadic AD.[74,87]

7. Metal Binding Sites on Aβ

NMR studies of Zn^{II} ion binding to Aβ generally indicate that H6, H13, and H14 are involved in coordinating the metal ion.[72,88,89] The identity of the fourth ligand is more problematic: D1,[72,90] Y10,[91] and E11[88,90] have all been proposed. Other studies have demonstrated that the histidine residues coordinating Zn^{II} can originate from different peptide molecules,[90,92] thereby promoting peptide aggregation.

The histidine residues also coordinate the Cu^{II} ion in its 1:1 complexation with Aβ,[68,92,93] although Cu^{II} forms at least two different types of complexes with Aβ depending on the pH value: the complex I type at low pH, and complex II type at high pH, with the midpoint around physiological pH.[68,94–96] All studies at low pH point towards a 3-nitrogen/1-oxygen coordination in the complex I type, probably with the O atom from a carboxylate side chain or a carbonyl group, one N from the N terminus, and two N atoms from the histidine residues (the three histidine residues in Aβ might alternate).[95–98]

The complex II type is less clear. NMR and EPR experiments on human[96,99] and murine[100–102] Aβ suggest that Cu^{II} might be coordinated mainly by the D1 amine nitrogen, the A2 carbonyl oxygen and amide nitrogen, and one of the three Aβ histidine residues. An alternative binding model involves the three histidine residues of Aβ together with the A2 carbonyl oxygen.[95]

The effect of simultaneous binding of Cu and Zn ions to the Aβ peptide has been somewhat neglected. At neutral pH values Cu^{II} and Zn^{II} ions compete for similar/overlapping binding sites on the Aβ peptide.[103] At lower pH values, Zn^{II} seems to lack residue-specific ligands whereas Cu^{II} retains its specificity. When Zn and Cu ions compete for the same binding site the redox-silent Zn^{II} binding might be expected to protect from harmful ROS production,[103,104] although a recent study did not observe such a protective effect.[105]

8. Theoretical Modeling of Aβ Interactions with Metal Ions and Small Molecules

In this section we review theoretical studies on the interaction of Aβ with metal ions, with small molecules that modulate Aβ aggregation, and with certain proteins. Quantum mechanics (QM) and molecular dynamics (MD) studies of monomeric Aβ peptide interaction with Cu^{II} and Zn^{II} are summarized. Through theoretical studies utilizing both docking and MD simulations, we also discuss the aggregation-inhibiting/-promoting mechanisms of the reviewed molecules.

a. Metal ion interactions

The conformational transition of the Aβ peptide from a random structure monomer to ordered fibrils passes through an oligomeric state (Figure 3). Ab initio model calculations indicate that Cu^{II} promotes the deprotonation of Aβ(1–42) at pH 7.0, binds H13, H14, and the carbonyl group in the H13–H14 peptide bond, and facilitates β-sheet structure formation.[106] The β-turn structure involving the Aβ N- and C-terminal regions observed in the Cu^{II}-free system is disrupted by the Cu^{II} binding, as shown by MD simulations of Cu^{II}/Aβ(1–42) systems in which the ab initio structures were used as templates for the copper binding site.[107] 3D models of Cu^{II}/Aβ(1–16) complexes with the three histidine residues in the coordination sphere have recently been presented.[108] Results based on homology modeling (HM) techniques combined with QM-based approaches illustrate that the final stabilities of the Cu^{II}/Aβ(1–16) complexes are caused by an energy balance between the metal coordination site and Aβ(1–16) folding in the complexes.[108]

To provide better understanding of the redox chemistry of Cu/Aβ complexes, density functional theory approximations have been used to model the structure of the Cu/Aβ complexes at physiological pH.[109] These calculations gave rise to a model that suggested a profound reorganization of the ligand environment to the Cu ion in the $+1$ and $+2$ states.[109] The impact of metal ions on Aβ fibrillation has also been explored by MD simulations. The β-hairpin conformation of Aβ is distorted in the presence of Cu^{II}, which might potentially destabilize the aggregation of Aβ into fibrils.[110] However, Miller et al.[111] have reported that oligomeric Zn^{II}/Aβ(1–42) can simultaneously coordinate two peptides, significantly decreasing the solvation energy for large Zn^{II}/Aβ(1–42) oligomers and enhancing their aggregation tendency. Aβ(1–42) aggregation is thus promoted in the presence of Zn^{II}, although the aggregates formed are of a less uniform type.

b. Small-molecule interactions

Many studies, both experimental and theoretical, focus on understanding of the activities of small molecules that interact

with Aβ and inhibit its aggregation. The scientific questions are multifaceted. From a basic point of view, the mechanisms of interaction and aggregation inhibition should be explained. In addition, there is the added interest of finding new therapeutic strategies against AD.

Molecular docking can provide information on the virtual binding site of a small-molecule aggregation inhibitor, both on Aβ monomers and on fibrils. A multi-site ligand docking method, for example, was developed to study spirostenol inhibition of Aβ-induced neurotoxicity.[112] One lead (maximum neurotoxicity inhibition) candidate—SP233—was found with a nearly equal docking affinity for two Aβ sites. In another case, in silico docking suggested that the Aβ binding site for stilbene derivatives was located at the polar N-terminal residues.[113] However, the Aβ monomer structure used for docking was solved in the presence of SDS, which is a nonphysiological condition. Experimentally, monomeric Aβ in aqueous solution adopts a random coil structure and is by necessity heterogeneous. Selecting reasonable starting structures, an important parameter in theoretical calculations, is therefore challenging in the case of monomeric Aβ.

For modeling of Aβ fibrils, an aggregate of peptides stacked as parallel β-hairpins involving residues 18–36 of Aβ(1–42) has previously been solved by solid-state NMR spectroscopy,[17] and this structure has proven useful for theoretical calculations. The Aβ N terminus, which is very flexible, is usually omitted from structural models. Congo Red and myricetin have been docked into this fibril hairpin structure and, despite having quite different molecular structures, were found to be located in the same binding pocket.[114] MD calculations combined with 3D-QSAR (quantitative structure–activity relationship) models have also identified reasonable binding sites on Aβ fibrils for 21 flavone derivatives.[115]

All current docking studies involving the Aβ peptide have certain limitations, however. The binding site of the receptor is assumed to be rigid, even though the system is known to be highly flexible, and the environment does not involve explicit or implicit solvent. Thus, even though several different binding sites for a variety of small molecules on monomeric or fibrillar Aβ have been proposed from docking simulations, the predictions still need to be confirmed by experiments and/or MD simulations.

Information from MD simulations of Aβ monomers in complexation with small aggregation-modifying molecules could represent the initial interaction modes between Aβ and these ligands. Using docking followed by MD simulations, we recently found that positively charged polyamines bind to the Aβ N terminus: three types of polyamines were shown to interact in different ways with the negatively charged and polar Aβ residues, in agreement with experimental results.[52] Another MD study found that lipids stabilize an α-helical Aβ conformation, thus suggesting that stabilization of an α-helical Aβ conformation might inhibit peptide aggregation.[116]

MD simulations using a fibril model for Aβ revealed that hydrophobic residues in a β-sheet structure bury the hydrophobic parts of the Congo Red and ThT molecules.[117] For the ThT molecule, two binding sites were discovered: at the β-sheet grooves and at the β-sheet end or center.[117] MD simulations also indicated that the rotation between the two ThT rings, which is free in aqueous solution, is significantly torsionally restricted after binding to Aβ fibrils,[118,119] which explains the higher fluorescence of the dye when bound to amyloid fibrils. Another study using an Aβ fibril model found that molecules based on all-D amino acids are highly efficient inhibitors of fibril formation, a result found to agree with experimental data.[120] In the study, side-chain rotamers of all amino acids were built onto a fixed protein backbone template, and the lowest-energy set of side-chain rotamers was identified by energy minimization.

The effects of macromolecules on Aβ aggregation can also be investigated by MD simulations. The β-sheet breaker peptides KLVFF and LPFFD were thus found to interfere with the intermolecular hydrogen bonds in the Aβ fibril.[120] In silico, Aβ(1–40) retards the aggregation of Aβ(1–42) and makes the D23K28 salt bridge and 18–33 fragment of Aβ(1–42) more flexible, which is in agreement with experimental data.[121] Similarly, the salt bridges of apolipoprotein E4 can be interrupted by binding to the Aβ(1–40) peptide,[122] and this might be related to the suggested role of the apolipoprotein E4 allele as a risk factor in AD.

Although the number of theoretical studies of Aβ–ligand interactions is rapidly increasing, the techniques for accurate prediction of the complexes still remain challenging (as discussed above). Several new methods that can be used for simulations of Aβ–ligand systems have been developed, including discrete MD (DMD), coarse-grained MD (CGMD), and replica-exchange MD (REMD). It is noteworthy that a combination of DMD and CGMD is computationally more efficient and gives greatly improved sampling simulations, relative to the traditional all-atom MD simulations.[123,124] We conclude that theoretical methods, despite their importance for studying aggregation-inhibition/-promotion mechanisms in Aβ systems, are arguably most successful when combined with experimental work, in which the theoretical results can help to shed light on the interactions that govern the aggregation processes in these complex systems.

9. Aβ Interactions with the Prion Protein

The Aβ peptides naturally interact with a number of common human proteins: lysozyme, for example.[125] One very interesting interaction is that between Aβ and prion proteins, especially given that Alzheimer's disease and prion pathologies appear to be closely intertwined. The Aβ peptides have been shown to exhibit a prion-like self-aggregating capacity because murine β-amyloidosis can be induced by inoculations of purified or synthetic Aβ specimens (most recently reviewed in ref. [126]). The prion protein (PrP^C) is a GPI-anchored membrane protein ubiquitously expressed throughout the brain and much of the body. Although PrP^C-knockout mice are immune to Aβ-induced neuropathology,[127] overexpression of PrP^C can confer the same protection;[128] this indicates both neurotoxic and neuroprotective functions of PrP^C in AD—and possibly also in other protein misfolding disorders.[129] Interest-

ingly, the levels of PrP^C found in cerebrospinal fluid (CSF) are lower in AD patients.[130]

Numerous studies have been conducted to outline the role of PrP^C in AD pathology (see ref. [131] for a recent review). Although the results have not been unambiguous, a certain consensus has emerged with regard to some important observations, such as the finding that memory deficits in AD transgenic mice appear to require the presence of PrP^C. PrP^C is a cell-surface receptor considered to mediate Aβ oligomer toxicity, and the presence of Aβ oligomers increases the localization of PrP^C at the cell surface.[132] Both Aβ and PrP^C are also concentrated at synaptic terminals, and through the interaction with PrP^C, Aβ might give rise to neuronal events converging with the neurotoxicity triggered by prions; this might partly explain some of the similarities between these fatal progressive dementias.

PrP^C is essential for oligomeric-Aβ-induced neuronal death,[133] with PrP^C seemingly binding, and trapping, Aβ in an oligomeric form and also disassembling fibrils.[134] The unstructured N-terminal domain of PrP^C appears to bind Aβ oligomers and thereby trigger neurotoxic effects and synaptic impairments,[135,136] outcomes possibly mediated through cross-linking of PrP^C.[137] The Aβ-induced toxicity seems to require an interaction between PrP^C and the transmembrane low density lipoprotein-receptor-like protein-1 (LRP-1), as well as association to membrane rafts.[138]

PrP^C has several binding sites for Cu^{II} and other divalent metal ions in its flexible N-terminal region, and many of the PrP^C–protein interactions depend on its copper-binding capacity. The potential role of Cu^{II} or other metal ions in PrP^C-mediated Aβ toxicity is unclear (as reviewed in ref. [139]). Although EDTA does not affect Aβ binding to PrP^C,[140] it remains to be investigated whether or not copper influences the functional aspects of this interaction. The strong affinity of Aβ for Cu^{II} could possibly disrupt synaptic functions of Cu^{II} binding to PrP^C when Aβ oligomers interact with PrP^C.[141] In addition, Aβ inhibition of ionic conductance in murine neurons is PrP^C-dependent,[142] and interaction of PrP^C with metal ions such as Cu^{II}, Zn^{II}, Mn^{II}, and Fe^{II} can induce misfolding into pathogenic prions.[139] Whereas soluble PrP^C binds soluble Aβ and inhibits Aβ fibrillization and toxicity,[143] the PrP^C specimen clinically interacting with Aβ(1–42) oligomers appear to be mainly insoluble.[144] Because PrP is often found in histological samples of Aβ plaques, and because prion misfolding enhances the aggregation of Aβ,[145] it is possible that an aggregation-prone subpopulation of PrP^C proteins could facilitate Aβ-oligomerization. In this respect, supporting roles of divalent metal ions are highly likely.

10. Future Perspectives

Despite the large amount of research devoted to the question of protein misfolding and amyloid formation, and the coupling of these phenomena to disease, it seems that even certain quite fundamental questions remain unanswered. In this review we have tried to present some aspects about which there is consensus among scientists, but would also like to point out some questions that will require new types of answers, from biophysics and other sciences:

1) What are the details of the molecular mechanism(s) of Aβ self-assembly?
2) What is/are the structure(s) of the neurotoxic Aβ species, and what mechanism(s) give(s) rise to the neurotoxicity in vivo?
3) What are the roles of the redox-active and -inactive metal ions in the neurotoxic processes involving Aβ?
4) How can the formation of neurotoxic species be inhibited? Will it be possible to develop small-molecule drugs to promote this inhibition?
5) How do the aggregation mechanisms studied in vitro relate to the aggregation processes that occur in vivo?

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