Perspective

Thermodynamic and Kinetic Origins of Alzheimer's and Related Diseases: a Chemical Engineer's Perspective

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Introduction

Alzheimer's disease (AD) is an irreversible late-onset neurodegenerative disease characterized by a slow decline in thinking ability and memory, usually over a period of 5–10 years, culminating in severe dementia and death. There is no known cure. Although the exact causes of Alzheimer's disease are unknown, autopsies on the brains of its victims invariably find extracellular plaques composed of ordered aggregates, called fibrils or "amyloid", of the A-beta protein.[1] The disease was identified 100 years ago by Dr. Alois Alzheimer, who reported the appearance of abnormal clumps in the postmortem brain of his patient, Frau August D., a 55-year old woman with severe dementia.[2] Five million Americans (approximately 5–10% of 65–74 year olds, and 50% of 85 year olds) have Alzheimer's, at a cost to society of $148 billion/year. Despite the staggering projected costs of AD as the huge baby boomer generation hits their 60s, the U.S. research budget into Alzheimer's is a paltry $650 million (cf. 5.5 billion for cancer research).[3,4]

Research into Alzheimer's disease is personal for me. It started with my father, Harris J. Klein (1906–1987), a dynamic Damon Runyon type figure who served as New York City Transit Commissioner in the 1950s, and chaired the now-famous JFK birthday party at Madison Square Garden. Our family's world fell apart in 1973, when he began to exhibit bizarre behavior, bewildering himself, his family and his "public", at a time when Alzheimer's was not yet a household word. An autopsy performed after his death resulted in a posthumous diagnosis of Pick's disease, a rare amyloid disease (supposedly not hereditary) closely related to AD. My mother, Celia R. Klein (1918–2007), began showing symptoms of Alzheimer's disease when she entered her 80s. A woman before her time, she encouraged me to become a physicist (of all things) at a time when young women were discouraged from having careers, graduated with honors from college at age 63, and cared heroically for my father during the long course of his illness. She died last year at the age of 89, a shell of her former self, except for occasional flashes of humor and warmth, and impromptu sing-a-longs.

Alzheimer's is only one of the many "amyloidoses", which are also called "protein deposition diseases" or "protein conformational diseases."[5,6] Thus far, 40 different proteins have been found in amyloid deposits in various human organs. The deposits can be extracellular or intracellular, neurodegenerative (when the aggregates occur in the brain), localized (for example in the liver or kidney) or systemic. The amyloidoses are usually associated with serious clinical manifestations; most are fatal. Examples of amyloidogenic proteins (and their associated diseases) are: beta amyloid (Alzheimer's), α-synuclein (Parkinson's), prion protein (transmissible spongiform encephalopathies, including Creutzfeldt-Jakob disease and Kuru), excessive glutamine repeats in the protein huntingtin (Huntington's), tau (Alzheimer's, Pick's), IAPP or amylin (Type II diabetes), and excessive alanine repeats in the protein PABP2 (oculopharyngeal muscular dystrophy). Interestingly, a large fraction of these proteins are natively unfolded, which means that they do not fold to a native state below a certain temperature, as most proteins do. Although some of the amyloidoses (~10%) such as Huntington's are hereditary, most (~85%) including Alzheimer's and Parkinson's are sporadic, although hereditary forms do exist. A small fraction (~5%) of the diseases, particularly the spongiform encephalopathies, can be transmitted from human to human or mammal to human.[6]

The causes of amyloid formation are unknown. Particularly perplexing is the observation that many of these proteins are present in normal, as well as diseased individuals. Consider for example, Alzheimer's disease. The transmembrane protein APP (amyloid precursor protein) is present in healthy as well as diseased individuals; it is cleaved by secretases to form the 40- and 42-residue long Alzheimer's proteins, Aβ (1-40), and Aβ (1-42). In Alzheimer's these proteins aggregate to form fibrils; in healthy individuals they do not—we don't know why. Generally speaking, Aβ(1-42) is more likely to be associated with early-onset (hereditary) forms of Alzheimer's, with increased risk of getting Alzheimer's disease, with

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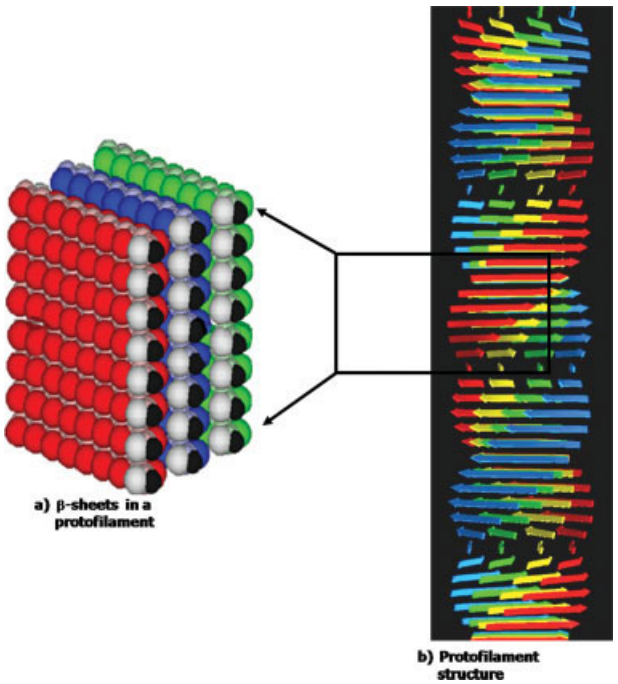

Figure 1. (a) Generic protofilament containing three β-sheets (red, blue and green), with eight peptides per sheet.

Hydrogen bonds between the backbone atoms (shown in gray) hold the peptides together in the sheet; hydrophobic interactions between the side chains (red, green and blue spheres) cause the sheets to stack on top of one another, and (b) another view of a protofilament showing the axial twist,[11] the latter is reproduced with permission.

enhanced neurotoxicity, and with faster formation of fibrils *in vitro*.[7–9]

Alzheimer's disease is also characterized by the presence within neurons of fibrillary tangles, meshes of the microtubule-associated protein, tau. For the past twenty years a controversy has swirled over the question of which of the two proteins, beta amyloid or tau, is the cause of Alzheimer's disease. Current thinking favors the Aβ link, because amyloid plaques appear earlier in the disease process than tau tangles, and because mutations in the amyloid precursor protein APP are linked to hereditary forms of Alzheimer's disease.[10] In this article, we focus primarily on amyloid.

Fibril Structure

Despite a lack of similarity in the sequence, structure and function of the 40 different proteins associated with the 40 different amyloidoses, the fibrils share many basic structural features in common.[6,11,12] All amyloid fibrils are ordered, insoluble structures 70–120 Å in dia., and can be as long as 1 μm. Most are made of up two or more smaller fibrillar structures, called protofilaments, which are themselves long ribbons containing two to four layers of "crossed" beta-sheets propagating along the fibril axis. A beta sheet is a planar array of beta-strands (peptides in elongated conformations) held together by hydrogen bonds between the backbone NH and C=O group, with intrastrand distances of 4.7 to 4.8 Å. In a cross-beta structure, the beta sheets stack approximately 10 Å apart (to shield their hydrophobic side chains from contact with solvent), and extend in the direction of the fibril axis as shown in Figure 1a. There is an axial twist as shown in Figure 1b. Fibril structure can be measured using transmission electron microscopy (TEM), atomic force microscopy (AFM), X-ray diffraction, and solid-state NMR (ssNMR).

Recent advances in solid-state NMR have led to exciting advances in our understanding of the structure of the fibrils formed by the Alzheimer's peptide β-amyloid.[13–15] By combining ssNMR results with computational energy minimization, the Tycko group has proposed the structure shown in Figure 2 for the Aβ(1-40) protofilament.[16,17] Each A-β monomer loops back on itself due to a salt-bridge between Asp23 and Lys28, forming a U-shaped "β-strand (red)-loop (green)-β-strand (blue)" structure. The C-terminal of this structure (blue), then interacts with exposed hydrophobic residues on the C-terminal (blue) of a second U-shaped structure to form a protofilament. Residues 1–9 (green) appear to flop around off to the side.

The cross-beta protofilament structure is also found in proteins other than those associated with amyloid diseases, which suggests that the forces that stabilize protofilaments are common to all proteins—hydrophobic interactions and backbone hydrogen bonding—and not associated with specific interactions between side chains.[18,19] The side chain interactions, and, hence, the specific sequence along the peptide chain do, however, influence the way that the sheets are arranged within the protofilaments (as described earlier for the Aβ protein), and the way that the protofilaments stack to form fibrils.[6] The

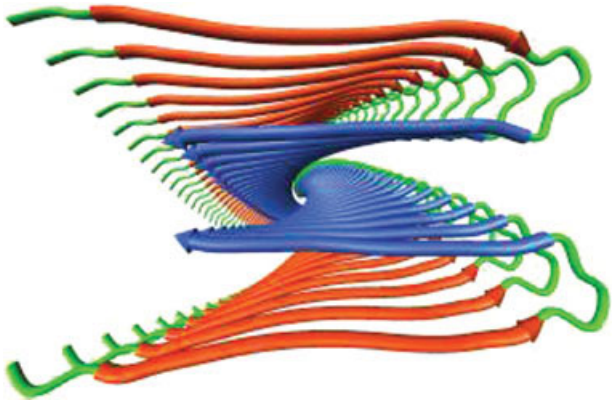

Figure 2. Cartoon taken from the Tycko group's research website (http://spin.niddk.nih.gov/tycko/rob_cv_2004_files/image004.jpg.) showing a cross-section of their proposed quaternary structure for the Aβ fibril.[16]

Each peptide has an unstructured N terminus (green), and two beta strands (red and blue) separated by a loop (green). The residues along the strands hydrogen bond to their neighboring peptides (red to red and blue to blue), creating a beta sheet folded back on itself. The two folded sheets are stacked together at their C terminus to form the fibril. Reproduced with permission.

regularity of amyloid structures is being explored by numerous groups seeking to use peptides as the building blocks in supramolecular functional self-assembling nanostructures, with potential applications in nanoelectronics, templating, cell culture, biofiltration, biosensing, etc.[20]

The study of fibril structure is important for diagnostic as well as treatment purposes. Currently, absolute diagnosis of Alzheimer's is only possible post mortem. However, knowledge of the structure of Aβ fibrils could lead to new techniques that can detect and image these structures in the brain of living patients. A major breakthrough occurred in 2004 when Klunk et al. successfully imaged amyloid deposits in the brains of Alzheimer's patients by applying positron emission tomography (PET) using a new tracer, Pittsburgh compound B.[21]

Molecular-level computer simulations

Computer simulations offer an additional avenue for gaining a better understanding of the molecular-level events associated with amyloid formation.[22] Atomistic simulations based on force-fields such as CHARMM and Amber are being used to assess the stability of different types of fibril structure.[23] The idea here is to start with the structure of interest as an initial conformation, and then simulate for many nanoseconds to see if the structure changes or melts. For example, Zheng et al.[24] recently applied all-atom molecular dynamics simulation to Aβ (17–42) in an attempt to learn which of the two possible sheet-sheet interfaces (C-terminal- C-terminal or N-terminal-N-terminal) in the β-strand-turn-β-strand U-shaped motif is more stable, and how Alzheimer's mutations influence the stability of the protofilament structures. They concluded that the C-terminal-C-terminal interface is more stable due to shape complementarity and strong hydrophobic contacts, in agreement with the Tycko group's proposed structure.[16,17] While atomistic simulations offer insights on the stability of postulated amyloid fibril structures, they do not tell us much about the assembly process due to their inability to access time scales greater than tens of nanoseconds. An alternative approach for the study of aggregate assembly is the class of models known as low-resolution models.

Low-resolution or minimalist models rely on a coarse-grained representation of protein geometry and energetics.[22] Typically, they account for the motion of groups of atoms along the protein and ignore the motion of the solvent atoms in order to enhance computational efficiency. The absence of solvent atoms in low-resolution models means that effective potentials, or potentials of mean force, must be used to describe the interactions between residues. The most common type of low-resolution model is the lattice model, which represents a protein as a linear chain of beads (residues) confined to a lattice. The group of Blanch/Prausnitz has employed lattice protein models to examine the competition between protein folding and aggregation on a fundamental level.[25] By comparing the folding of proteins in isolation with folding in the presence of additional proteins, they were able to show that the free energy landscape of a protein loses its normal funnel-shape (with the native state at the bottom) as the environment becomes more crowded, portending the tendency for the protein to misfold to non-native states.[26] Such misfolded proteins in turn expose their normally-buried hydrophobic residues to similarly-exposed hydrophobic residues on other misfolded proteins, leading to aggregation. In related work, the Blanch/Prausnitz group showed that mutations that have little effect on the folding of a protein in isolation can significantly alter the tendency of a protein to aggregate under crowded conditions.[27]

Intermediate-resolution protein models represent protein energetics and geometry at a resolution between those adopted in atomistic and low-resolution protein models.[22] Our group has developed an implicit-solvent intermediate-resolution protein model, called PRIME, and applied it to a relatively simple protein, polyalanine, which forms fibrils in test tubes.[28] In PRIME the level of molecular detail in the protein representation and interaction potential is reduced just to the point at which the key physical features governing protein folding and aggregation remain and the other features are neglected. Each amino acid residue is composed of four spheres: a 3-sphere backbone comprised of united atom NH, $C_\alpha H$, and CO, and a single-sphere side chain (CH_3- for alanine). Ideal backbone bond angles, C_α-C_α distances and residue L-isomerization are maintained. The force field includes: steric interactions, hydrogen bonding between the backbone NH and CO, and hydrophobic interactions between the alanine side chains. By combining this model with the very-fast discontinuous molecular dynamics technique, we were able to simulate the spontaneous formation of a protofilament in a system of ninety-six 16-residue polyalanine peptides, KA14K—the first time that anyone had been able to simulate spontaneous formation of fibrils.[29] Figure 3 contains a series of snapshots taken from a simulation of the formation of a protofilament. The thermodynamics, kinetics, and structure of the formed fibrils were in very good agreement with experimental observations.

A snapshot from a simulation of a 96-peptide protofilament is presented in Figure 4. Five to six sheets are stacked on top of each other; the fibril axis is into the page. The peptides are more or less in register, which means that there is little staggering of the peptides within a sheet. The figure on the cover shows a detail of this protofilament viewed from the side. Four beta sheets are depicted—colored pink, blue, green and purple. The alanine side chains (shown in red) oscillate from one side of the zig-zag sheet to the other, occupying the pockets created on the adjacent sheets. This structure is consistent with X-ray diffraction measurements.[29] The structure of the protofilament formed spontaneously in our simulations is the same as the basic protofilament structure shown in Figure 1. The take home message here is that hydrogen bonding between backbone NH and CO, and hydrophobic interactions between side chains are the key driving forces for fibril formation.

Mechanism and Kinetics of Fibril Formation and Growth

Fibril formation is believed to follow a nucleation and growth mechanism,[6,30,31] which means that there is a time lag before fibrils first appear. During this time, the peptides associate one by one or in groups in a series of energetically unfavorable steps. Once a critical size and shape is reached, the so-called "nucleus", the process rapidly proceeds downhill in free energy. Protofilaments form as the nucleus associates

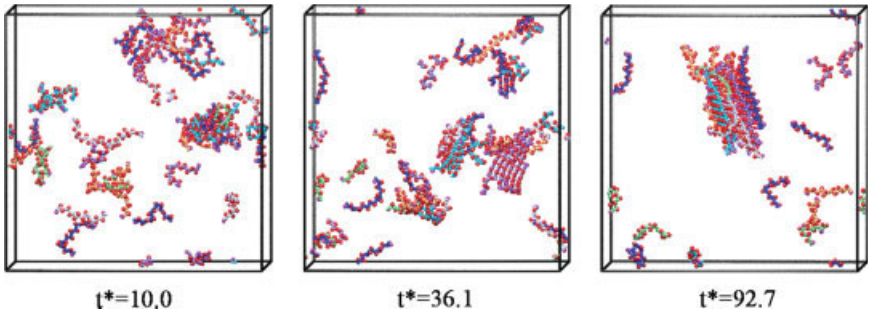

Figure 3. Discontinuous molecular dynamics simulation[29] of a system of 48 polyalanine peptides at various reduced times t*.
Hydrophobic side chains are red; the backbone atoms on each beta sheet have a unique color to make it easier to distinguish the various sheets in the resulting fibril. United atoms are not shown full size for ease of viewing. Reproduced with permission.

with monomers (single proteins) or oligomers (small groups of proteins), and elongate either through monomer addition at the tip or end-to-end association of short protofilaments. There is some debate as to how the protofilaments join together to from fibrils. As is the case in other nucleated phenomenon, e.g., crystallization, the addition of a preformed fibrillar seed to a system of proteins eliminates the lag time, presumably because it provides a ready-made nucleus.

The events preceding the formation of a protofilament have received considerable attention lately, because they are thought to be likely candidates for intervention in the fibril formation process. *In vitro* studies have shown that before forming protofilaments, the Alzheimer's peptide Aβ forms "protofibrils", metastable nonfibrillar structures 2–5 nm in dia. containing $\sim$ 20 molecules that are rich in beta sheet structure.[19,32,33] These can be spherical beads, linear or curly chains of beads or annular structures formed from circular chains of beads. Recent studies of even earlier events in the fibril formation process have shown that unstructured oligomers containing 2–6 peptides form even before protofibrils; such oligomers have been observed in the brains of AD victims. As will be discussed later, both protofibrils and early oligomers are being investigated as the potential toxic species in AD.

Murphy is one of the pioneers of the application of mathematical modeling to the kinetics of protein aggregation. She and Pallitto postulated a multistep pathway for Aβ fibril formation and growth, derived the appropriate differential and algebraic equations, and fit the model parameters to their own experimental data on aggregate mass and size as a function of time and concentration.[31] Roberts and coworkers have developed an irreversible model of non-native protein aggregation kinetics that combines the classic Lumry-Eyring model with nucleation/polymerization ideas.[34] A number of possible aggregation pathways were considered; the model was solved for several limiting cases.

Identity of the Toxic Species

The "amyloid cascade hypothesis", the idea that protein aggregation triggers the disease process, has evolved over the years particular as applied to Alzheimer's disease.[7,8,35] Originally, the beta amyloid plaques (the mature fibrils) were thought to be at the root of the dementia and memory loss that characterize Alzheimer's disease. However, the lack of correlation between the extent of plaque formation in postmortem brains and the severity of Alzheimer's symptoms, the presence of multiple Aβ species (ranging in size from monomers to mature fibrils) in the brains of AD victims, and the appearance of plaques in the brains of symptom-free 70-year olds has caused the paradigm to shift in recent years. In the last five years there has been mounting evidence that the precursors to fibril formation—intermediates such as early oligomers or protofibrils that appear along the fibril formation pathway—are most toxic to cells. This is supported by observations that preparations enriched in protofibrils are toxic to cultured neurons, interfere with long-term potentiation in the hippocampus (a cellular mechanism for learning and memory), and

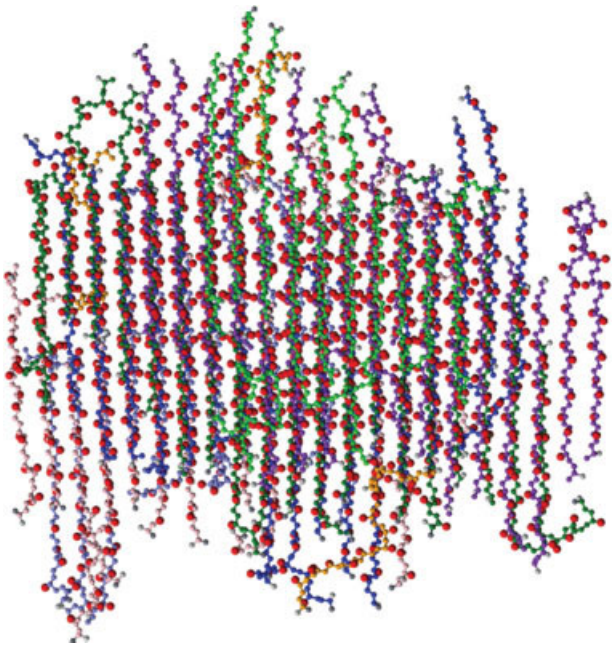

Figure 4. Final structure of protofilament formed in a discontinuous molecular dynamics simulation of a system of 96 polyalanine peptides.[29]
Five to six sheets are stacked on top of each other; the fibril axis is into the page. The color scheme is as indicated in Figure 3. Reproduced with permission.

adversely impact cognition and synaptic plasticity. In fact, it has been suggested that fibril formation might be nature's way of sequestering bad actors, preventing them from damaging cells. If this were indeed the case, then blocking the growth of mature fibrils or dissociating them could accelerate the disease process by allowing the toxic species to buildup. Despite the controversy over the nature of the toxic species, several drugs are in the pipeline aimed at disrupting the formation of fibrils.

We don't yet know how Aβ protofibrils and/or early oligomers damage cells. Most likely it is a combination of effects.[6] Since Aβ protofibrils and/or early oligomers are "misfolded", they have significant numbers of exposed hydrophobic residues on their surfaces. This makes them susceptible to inappropriate interactions with cell membranes, proteins, and other parts of cell machinery, disrupting cell functioning including regulator mechanisms and clearance. The aforementioned reasoning is consistent with the fact that AD and the other amyloid diseases are late onset; the cell's ability to function in the face of disruption diminishes with age as its clearance mechanisms degrade, leading to an imbalance between Aβ production and clearance. Another possibility is that annular protofibrils insert themselves or assemble within cell membranes, forming channels that disrupt membrane permeability to calcium.[36]

Therapeutic Agents for the Treatment of Amyloidoses

Even though there is an ongoing debate on the identity of the toxic species, significant work toward developing therapeutic agents that either address the major symptoms of Alzheimer's disease (severe dementia and diminished cognitive function) or target the plaques (amyloid fibrils) is underway. Four drugs designed to reduce the symptoms of Alzheimer's disease are currently in use today. Three of these, donepezil (Aricept), rivastigmine (Exelon) and galantamine (Razadyne) are cholinesterase inhibitors, which means that they slow the action of an enzyme that degrades acetylcholine, a neurotransmitter vital to transmission of nerve signals across synapses. These are prescribed for mild to moderate AD. The fourth, memantine(Namenda), lowers levels of another neurotransmitter, glutamate, in the brain, and is prescribed for mild to severe AD. These four drugs produce marginal improvement of cognitive function in AD victims but do not slow the progression of the disease.[37]

Many potential strategies and drugs for treatment and prevention of AD are now being investigated. Examination of the NIH website, ClinicalTrials.gov, shows that they include familiar drugs: anti-inflammatory agents like celebrex and ibuprofen; cholesterol lowering statin drugs like Lipitor and Zocor; nutritional supplements like DHA (an omega 3 fatty acid); Avandia (a diabetes drug); vitamin E; resveratrol (a component of red wine); hormones like estrogen; ginkgo biloba, and the steroid prednisone.

There are a number of AD drugs in clinical trials (see ClinicalTrials.gov) designed to reduce Aβ amyloid.[8,9,35] These are based on two broad strategies: (1) partially inhibiting action of the secretases that cleave APP to form Aβ, or (2) preventing formation or enhancing clearance of Aβ protofibrils or early oligomers through passive or active vaccination against Aβ.[7,35] Passive vaccination is based on the principle that subjects exposed to an active agent, like Aβ42 will develop their own immune response to that agent by producing antibodies. In active vaccination the subjects are injected directly with laboratory- generated antibodies. Based on very promising results of antiamyloid immunotherapy in animal models, the Elan company conducted a Phase II trial in 2001 of the safety and efficacy of AN-1792, a drug based on active vaccination with Aβ 42 and a T- cell helper adjuvant. This study was halted after the second injection in January 2002 because 6% of the participants developed meningoencephalitis. Follow-on evaluation of the participants, however, has lent support to the immunological approach by showing that participants with the highest antibody levels experienced the least cognitive decline. Based on lessons learned from AN-1793, Elan and Wyeth have teamed up to test passive vaccination (a presumably safer approach). Their new drug is Bapineuzumad, a humanized monoclonal antibody, now in phase III trials. Another antiamyloid drug in Phase III clinical trials is Flurizan, a small molecule drug developed by Myriad Genetics that shifts the site of the APP cleavage by γ-secretase from Aβ 42 to shorter peptides. Alzhemed, a once considered promising small molecule drug that inhibits all Aβ aggregation by binding Aβ monomers, failed in recent Phase II trials to demonstrate efficacy (Ref. 37, ClinicalTrials.gov,www.alzforum.org).

Further development of inhibitors to prevent Aβ aggregation in Alzheimer's disease is also being explored. The Good group has found that a novel small heat shock protein, HSP20, inhibits Aβ aggregation and toxicity.[38] The Murphy group has developed inhibitors that are hybrid Aβ peptides. These combine a recognition element, a short sequence of hydrophobic residues from the Aβ peptide that binds to other Aβ peptides, with a disrupting sequence, for example, a sequence of charged or branched amino acids, that get in the way of Aβ aggregation.[39] Interestingly, the most effective hybrids were found to be those that increased solvent surface tension. Necula et al. have examined 29 small-molecule inhibitors of Aβ aggregation, and divided them into three classes according to whether they: (1) inhibit oligomerization, but not fibrillization, (2) inhibit both oligomerization and fibrillization, or (3) inhibit fibrillization, but not oligomerization.[40] An altogether different tack is being taken by Bodner et al. in investigations of therapeutic approaches for Huntington's and Parkinson's diseases. Based on the suggestion that fibrillization is nature's way of sequestering the more harmful early oligomers early oligomers, they have developed a drug that promotes the formation of "inclusions", (large intracellular protein aggregates), and have found that it lessens cellular pathology.[41]

Conclusions

There are still many questions to be answered in the pursuit of treatments or cures for amyloid diseases. What is the structure of amyloid fibrils? What reaction pathways do proteins follow during aggregation? Why do the proteins aggregate more quickly in some people than in others? Do the pathways depend on protein sequence or change with environmental conditions? Which species or steps in the aggregation process are toxic, what are the toxicity mechanisms, and how can we get around these mechanisms? It seems obvious to me that

chemical engineers, with their grounding in thermodynamics and chemical reaction kinetics, can play a unique role in the search for a cure for Alzheimer's and other amyloid diseases.

In the meantime what should each of us do to protect ourselves against AD and related diseases? I wish I knew. I do know that hasty efforts to jump on any specific treatment bandwagon are not a good idea. In my efforts to avoid the fate of my father and mother, I threw out my aluminum pots and pans years ago, based on a now-disproved connection between aluminum and AD. Later I took estrogen hormones because epidemiological studies showed that postmenopausal women on estrogen replacement therapy (ERT) got AD later than average. What we didn't know then but know now is that ERT is associated with increased risk for breast cancer! So—be cautious.

I am heartened these days by the great progress being made in our understanding of how to treat and/or prevent AD. It may be the optimist in me, but I believe that viable treatments will be available within this decade.

Acknowledgments

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