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Review

Conformational constraints for amyloid fibrillation: the importance of being unfolded

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Abstract

Recent reports give strong support to the idea that amyloid fibril formation and the subsequent development of protein deposition diseases originate from conformational changes in corresponding amyloidogenic proteins. In this review, recent findings are surveyed to illustrate that protein fibrillogenesis requires a partially folded conformation. This amyloidogenic conformation is relatively unfolded, and shares many structural properties with the pre-molten globule state, a partially folded intermediate frequently observed in the early stages of protein folding and under some equilibrium conditions. The inherent flexibility of such an intermediate is essential in allowing the conformational rearrangements necessary to form the core cross-beta structure of the amyloid fibril.

Keywords: Fibrillation; Partially folded intermediate; Conformational stability; Natively unfolded protein; Amyloid

A number of human diseases, including the amyloidoses and many neurodegenerative diseases, originate from the deposition of stable, ordered, filamentous protein aggregates, commonly referred to as amyloid fibrils. In each of these pathological states, a specific protein or protein fragment changes from its natural soluble form into insoluble fibrils, which accumulate in a variety of organs and tissues [1–6]. Although approximately 20 different proteins are known to be involved in the amyloidoses (extracellular deposits) (see Table 1), they are unrelated in terms of sequence or structure. In addition, a number of diseases also involve the deposition of fibrillar intracellular deposits, as well as non-fibrillar deposits. Prior to fibrillation, amyloidogenic polypeptides may be rich in β-sheet, α-helix, β-helix, or contain both α-helices and β-sheets (see Table 1). They may be globular proteins with rigid 3D-structure or belong to the class of natively unfolded (or intrinsically unstructured) proteins [7–15]. Despite these differences, the fibrils from different pathologies display many common properties including a core cross-β-sheet structure in which continuous β-sheets are formed with β-strands running perpendicular to the long axis of the fibrils [16]. All fibrils have similar morphologies, and mature fibrils usually have a twisted, rope-like structure, reflecting a filamentous substructure (see Fig. 1). Typically mature fibrils consist of two to six unbranched protofilaments, 2–5 nm in diameter, associated laterally or twisted together to form fibrils with 4–13-nm diameter (e.g. see Refs. [17–19]). Amyloid fibrils have been formed in vitro from disease-associated [20–26] as well as from disease-unrelated proteins and peptides [27–46] (see Table 1). Moreover, there is an increasing belief that the ability to from fibrils is a generic property of the polypeptide chain, i.e. many proteins, perhaps all, are potentially able to form amyloid fibrils under appropriate conditions [2,38,39,47]. If so, this would dramatically extend the structural diversity of polypeptide chains with the potential to fibrillate.

To explain the molecular basis of amyloid fibril formation, it has been proposed that fibrillation can occur when the rigid native structure of a protein is *destabilized*, favoring partial unfolding and culminating in the formation

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Table 1
Amyloidogenic proteins and amyloid-based clinical disorders

Disease-associated amyloidogenic proteins			
Amyloidogenic protein	Type of structure	Disease	Tissue distribution of protein deposits
Prion protein and its fragments	N-terminal fragment (23–121) is natively unfolded; C-terminal domain (121–230) is α-helical (predominantly)	Creutzfeld–Jacob disease (CJD) Gerstmann–Straussler–Schneiker syndrome (GSS) Fatal familial insomnia (FFI) Kuru Bovine spongiform encephalopathy (BSE) and scrapie	Brain
Amyloid-β and its fragments	Natively unfolded	Alzheimer's disease (AD) Dutch hereditary cerebral hemorrhage with amyloidosis (HCHWA, also known as cerebrovascular amyloidosis) Congophilic angiopathy	Brain Brain, spinal cord
ABri	Natively unfolded	Familial British dementia	Brain
Cystatin C	α/β	Hereditary cystatin c amyloid angiopathy (HCCAA)	Brain
Huntingtin	α-Helical (but exon 1 is unfolded and forms fibrils)	Huntington Disease	Brain
Androgen receptor protein	Ligand-binding (LBD) and DNA-binding domains (DBD) are α-helical; amino-terminal domain (NTD) is natively unfolded	Spinal and bulbar muscular atrophy (SBMA)	Brain, scrotal skin, dermis, kidney, heart, and testis, spinal cord
Ataxin-1	Unknown (likely natively unfolded)	Spinocerebellar ataxia (SCA) Neuronal intranuclear inclusion disease (NIID)	Brain, spinal cord Central and peripheral nervous system
DRPLA protein (atrophin-1)	Unknown (likely natively unfolded)	Hereditary dentatorubral-pallidoluysian atrophy (DRPLA)	Brain
Serum amyloid A and its fragments	α/β	AA amyloidosis (inflammation-associated reactive systemic amyloidosis)	Bladder, stomach, thyroid, kidney, liver, spleen, gastrointestinal tract
Medin (245–294 fragment of lactadherin)	β-Sheet	Aortic medial amyloidosis	Aortic smooth muscle
Islet amyloid polypeptide (IAPP, Amylin)	Natively unfolded	Pancreatic islet amyloidosis in late-onset diabetes (type II diabetes mellitus)	Pancreas
Calcitonin	Natively unfolded	Medullary Carcinoma of the Thyroid (MCT)	Thyroid
Lysozyme	α + β	Hereditary systemic amyloidosis	Several visceral organs and tissues
Gelsolin	α/β	Hereditary systemic amyloidosis Finnish-type familial amyloidosis	Several visceral organs and tissues
Transthyretin	β-Sheet (predominantly)	Senile systemic amyloidosis (SSA) (or senile cardiac amyloidosis) Familial amyloid polyneuropathy (FAP)	Almost all organs and tissues, including heart, gland, arteries, bones, liver, digestive tract, etc. Various organs and tissues.
Apolipoprotein A1	α-Helical	Hereditary systemic amyloidosis	Eyes
β_2-Microglobulin	β-Sheet	Amyloid associated with hemodialysis (AH or Aβ2M) (athropathy in hemodialysis)	Musculoskeletal tissues (large and medium-sized joints, bones, muscles), peripheral nervous system, gastrointestinal tract, tongue, heart, urogeniteal tract
Tau protein	Probably natively unfolded	Alzheimer's disease (AD), Pick's disease, Progressive supranuclear palsy (PSP)	Brain
Immunoglobulin light chain variable domains	β-Sheet	Light chain associated amyloidosis or AL amyloidosis Light chain deposition disease or LCDD Light chain cast nephropathy Light chain cardiomyopathy	Almost all organs and tissues, including heart, kidneys, liver, spleen, gastrointestinal tract, skin, tongue, endocrine glands, peripheral nervous system, etc. Liver, spleen, bone marrow, vessel walls, parenchymal tissue, kidneys, heart, liver, skin, lungs, tongue, ovary, pancreas, etc. Kidneys Heart

Table 1 (*continued*)

Disease-associated amyloidogenic proteins

Amyloidogenic protein	Type of structure	Disease	Tissue distribution of protein deposits
α-Synuclein	Natively unfolded	Parkinson's disease (PD) Diffuse Lewy bodies disease (DLBD) Lewy bodies variant of Alzheimer's disease (LBVAD) Dementia with Lewy bodies (DLB) Multiple system atrophy (MSA) Hallervorden–Spatz disease	Brain
NAC (central fragment of α-synuclein)	Natively unfolded	Alzheimer's disease (AD)	Brain
Fibrinogen and its fragments	β-Sheet	Hereditary renal amyloidosis	Kidney
Atrial natriuretic factor	"Small protein"	Atrial amyloidosis	Heart
Insulin	Predominantly α-helical	Injection-localized amyloidosis	Skin, muscles

Non-disease-related amyloidogenic proteins and peptides

Protein (peptide), reference	Type of structure	Protein (peptide), reference	Type of structure
Betabellins 15D and 16D [294]	β-Sandwich	Prothymosin α [41]	Natively unfolded
Cytochrome c_{552} [133]	α-Helical	Myoglobin [38]	α-Helical
Methionine aminopeptidase [35]	α-Helical	Muscle acylphosphatase [32]	α/β
Phosphoglycerate kinase [34]	α/β	Hen egg white lysozyme [33]	α + β
Hen egg white lysozyme, β-domain [33]	β-Sheet	Acidic fibriblast growth factor [42]	β-Barrel
PI3-SH3 domain [27]	β-Barrel	OspA protein, BH^{9-10} peptide [36]	β-Turn
β-Lactoglobulin [44]	β-Sheet (predominantly)	De novo αtα peptide [37]	α-Helix-turn-α-helix
Monellin [29]	α/β	Lung surfactant protein C [40]	α-Helix
Immunoglobulin light chain LEN [87,88]	β-Sheet	α-Lactalbumin [45]	α + β
HypF, N-terminal domain, [295]	α/β	V_L domain of mouse antibody F11 [296]	β-Sheet
Human complement receptor 1, 18–34 fragment [297]	Unfolded	Apolipoprotein C-II [213–215]	Natively unfolded
Human stefin B [298]	α/β	Cold shock protein A [299]	β-Barrel
GAGA factor [300]	Natively unfolded	Protein G, B1 Ig-binding domain [79,301]	Four-stranded β-sheet with a flanking α-helix
Yeast prion Ure2p [302]	α-Helical/unfolded	Cold shock protein B, 1–22 fragment [303]	Unfolded
Herpes simplex virus glycoprotein B fragment [304]	β-Structural	De novo proteins from combinatorial library [305]	β-Structural
The fiber protein of adenovirus, 355–396 peptide from shaft [306]	Fibrillar	Soluble homopolypeptides, [262]: poly-L-lysine poly-L-glutamic acid poly-L-threonine	Unordered

of a *partially unfolded conformation* or intermediate [1–6,48–51]. The logic behind this hypothesis is as follows. Since all fibrils, independent of the original structure of the given amyloidogenic protein, have a common cross-β structure, considerable conformational rearrangements have to occur for this to happen. Such changes cannot take place in the typical tightly packed native protein conformation, due to the constraints of the tertiary structure. Thus, formation of a non-native partially unfolded conformation is required. Presumably, such a partially unfolded conformation enables specific intermolecular interactions, including electrostatic attraction, hydrogen bonding and hydrophobic contacts, which are necessary for oligomerization and fibrillation [1–6,48–51].

This model, however, cannot directly apply to intrinsically unstructured (natively unfolded) proteins, as they are devoid of secondary structure to start with. Instead, the primary step of their fibrillogenesis has been shown to be

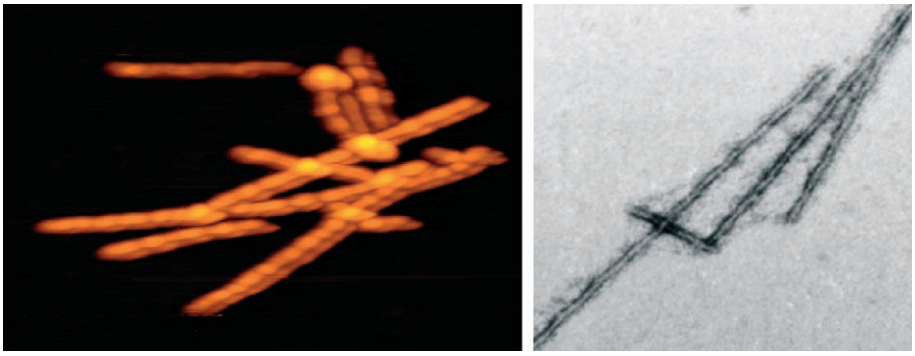

Fig. 1. Typical morphology of amyloid fibrils. Left: An atomic force microscope (AFM) of a bundle of fibrils of the immunoglobulin light chain variable domain SMA image [307]. Right: Negatively-stained electron microscopic image of SMA fibrils. The twisted structure is commonly observed in amyloid fibrils, and represents several (usually two to five) protofilaments interacting to form a rope-like structure. Typical dimensions are 7–10 nm in diameter and hundreds of nanometers in length.

the *stabilization of a partially folded conformation*, i.e. partial folding rather than unfolding occurs in such cases [24,45,52–54]. Thus, by taking the intrinsically unstructured proteins into consideration, a general hypothesis of fibrillogenesis might be formulated as follows: structural transformation of a polypeptide chain into a partially folded conformation represents an important prerequisite for protein fibrillation.

The formation of amyloid-like fibrils is not the only pathological hallmark of ‘‘conformational’’ or protein deposition diseases. In several disorders (as well as in numerous in vitro experiments), protein deposits are composed of

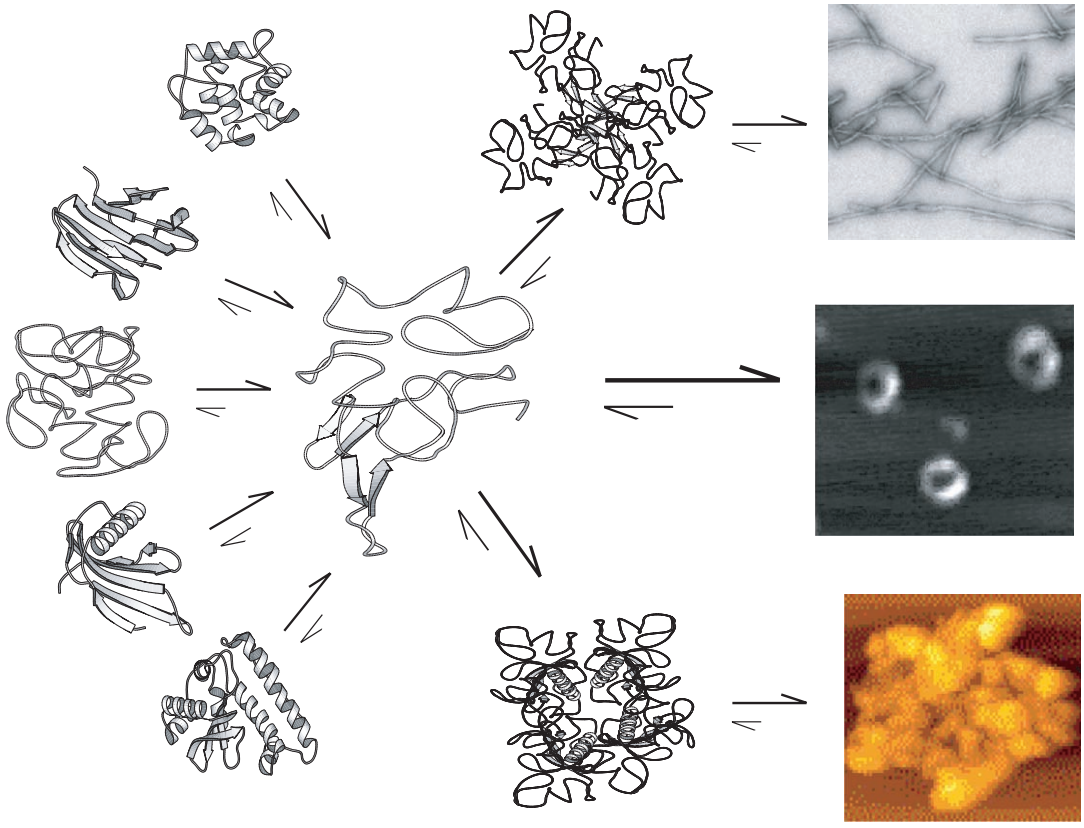

Fig. 2. General model of protein aggregation. Aggregation is initiated by a structural transformation in the protein into a partially folded conformation. Proteins with different types of structure (α-helical, β-structural, natively unfolded, α + β or α/β; single-domain or multi-domain; etc., marked as 1a–1e) transform into the partially folded conformation; 2: partially folded molecules can assemble into specific oligomers (nucleus, or protofibrils, 3a and 3b, respectively). Depending on the peculiarities of the amino acid sequence and the environmental conditions, the protein may end up as amyloid fibril, 4a, soluble oligomer (torus-shaped in this case), 4b, or amorphous aggregate, 4c.

amorphous aggregates, without local order. Similarly, soluble oligomers represent another alternative final product of the aggregation process. The choice between the three aggregation pathways, fibrillation, amorphous aggregate formation or oligomerization, is determined by the amino acid sequence and by the details of the protein environment. Fig. 2 represents a simplified model of protein aggregation and illustrates the idea that aggregation is an extremely complex process, which can be divided into three major steps. The model shows that proteins with different types of structure (α-helical, β-structural, natively unfolded, α + β or α/β; single-domain or multi-domain; etc., marked on the picture as structures 1a-1e) are equally subject to aggregation [55]. The structural transformations of these diverse soluble proteins into the "sticky" aggregation-prone precursor or intermediate (marked as 2) represent the first stage of the aggregation process. As this intermediate plays a crucial role in the process, it is presented as an enlarged image. A single conformation of the intermediate is shown for the sake of brevity only. In fact, these aggregation-prone intermediates would be structurally different for different proteins. Furthermore, the intermediate might contain different amounts/types of ordered structure even for the same protein undergoing different types of aggregation. Overall, we believe that it is likely that the precursor of soluble aggregates is the most structured, whereas amyloid fibrils are formed from the least ordered conformation (cf. Ref. [26]). It has been also pointed out that variations in the amount of ordered structure in the amyloidogenic precursor might be responsible for the formation of fibrils with distinct morphologies [56].

The formation of different oligomers (protofibrils, 3a; or protoaggregates, 3b) represents the second stage of the aggregation process, which is usually considered as a nucleation step [55], in which formation of the nucleus is a kinetically disfavored event, and leads to the lag period preceding significant formation of aggregates. However, once a critical nucleus has been generated, the conditions change in favor of a rapid increase in size [55]. As a result, any available aggregation-prone conformation quickly becomes entrapped in the fibrils, 4a; soluble oligomer, 4b; or amorphous aggregate, 4c.

Several recent investigations lend support to the idea that fibrillar proteins (as in senile plaques in AD or Lewy bodies in PD) are not necessarily the toxic entity, but rather the formation of some "protofibrillar" structures is responsible for the toxicity [57–61]. However, these issues (multiple pathways of aggregation and cytotoxicity of protein aggregates) are outside the primary scope of this review, which is devoted to the idea that the appearance of the partially folded conformation is a critical early stage of fibrillogenesis and precedes the appearance of any aggregated material. Furthermore, in this paper we show that the amyloidogenic conformation is significantly unfolded and shares many structural properties with the pre-molten globule state.

1. Fibrillogenesis of globular proteins: the requirement for partial unfolding

Data have been reported indicating that the first critical step in protein fibrillogenesis is the partial unfolding of the protein [1–6,48–51]. It is difficult to trap and characterize such partially folded species under physiological conditions because they are only transiently populated on the fibrillation pathway. However, the reality of partial unfolding as an important prerequisite of fibrillation follows from several indirect lines of evidence. Due to structural fluctuations (conformational breathing), the structure of a globular protein under physiological conditions represents a mixture of tightly folded and multiple partially unfolded conformations, with the former greatly predominating [62,63]. Most mutations associated with accelerated fibrillation and protein deposition diseases have been shown to *destabilize* the native structure, *increasing* the steady-state concentration of partially folded conformers [1,2,6,43,64–69]. A variety of compounds have been shown to significantly affect the rate of fibrillation in vitro, through a variety of mechanisms. For example, DNA-induced destabilization of the prion protein (in the presence of 0.12 μM nucleic acid the T_m drops from 70 to 63 °C) leads to its enhanced polymerization to amyloid fibrils [70]. Conversely, it has been shown that the amyloidogenicity of a protein can be significantly *reduced* by *stabilization of the native structure* [25], for example, via specific binding of ligands [25,71–78].

It has been shown for several proteins that destabilization of the native globular structure (e.g. low or high pH, high temperatures, low to moderate concentrations of strong denaturants, organic solvents, etc.) may significantly accelerate the rate of fibril formation. This is well illustrated in recent studies on the fibrillogenesis of bovine β-lactoglobulin [44]. It has been shown that this protein will form fibrils in urea solutions; the process is denaturant concentration-dependent, showing the highest efficiency in the vicinity of 5 M urea, which corresponds roughly to the half-transition point. Significantly, it was shown that a molten globule-like intermediate did not accumulate during the urea-induced unfolding of β-lactoglobulin [44]. Thus, it has been concluded that amyloid fibril formation by bovine β-lactoglobulin is promoted under conditions where significant accumulation of relatively unfolded protein occurs, but is inhibited under conditions where higher denaturant concentrations further destabilize intermolecular interactions [44]. Generally speaking, it has been concluded that amyloid formation in vitro can be achieved by destabilizing the native state of the protein under conditions in which non-covalent interactions still remain favorable [27–29,32,33,35,44,79].

The following are examples of proteins that form amyloidogenic intermediates and for which there is evidence for relatively unfolded amyloidogenic intermediates as critical species in fibrillation.

1.1. Transthyretin (TTR)

TTR, also known as prealbumin, is a homotetramer composed of 127 amino acid subunits. TTR is found in human plasma and cerebral spinal fluid, with the plasma form being the amyloidogenic precursor. Wild-type TTR amyloidogenesis may cause senile systemic amyloidosis, characterized by deposition and pathology in the heart after age 60 [80]. Early-onset amyloid formation (as early as the second decade), due to one of more than 80 single-site TTR variants, leads to a number of diseases collectively termed familial amyloid polyneuropathy (FAP, see Table 1) characterized by neuropathy and/or organ dysfunction [81]. TTR can be converted into amyloid in vitro by acid-mediated dissociation of the tetramer into monomers. The pH required for disassembly also results in tertiary structural changes within the monomeric subunits, finally leading to the enhanced fibrillation [82]. Recently, in a quest to understand the relationship between the tertiary structural changes and amyloidogenicity, a monomeric mutant has been designed, which has native-like structure and stability, but was non-amyloidogenic, unless partially unfolded [83]. Ligands that bind to the native tetramer and stabilize it have been shown to minimize fibril formation [71–78,84]. In addition, it has been shown that anions, including chloride, are able to bind to the TTR tetramer [85], dramatically stabilizing its quaternary structure. Furthermore, a buffer containing 1.5 M Cl^- was shown to be capable of saturating the Cl^- interaction sites in the tetrameric TTR, rendering the protein non-amyloidogenic (pH 4.4) and not denaturable by urea [85].

1.2. Immunoglobulin light chains

Light chain, or AL, amyloidosis is a pathological condition arising from systemic extracellular deposition of monoclonal immunoglobulin light chain variable domains in the form of insoluble amyloid fibrils, especially in the kidneys and heart (Ref. [86], see Table 1). Structural and fibrillation properties of one of the amyloidogenic light chain variable domains, SMA, have been analyzed under a variety of conditions [26]. The results of biophysical analysis revealed that a decrease in pH resulted in the accumulation of two partially folded intermediates. A relatively native-like intermediate, designated I_N, was observed between pH 4 and 6, and was characterized by little loss of secondary structure, but significant changes in tertiary structure, and enhanced binding of the hydrophobic dye 1-anilinonaphthalene-8-sulfonic acid (ANS). At pH below 3, a relatively unfolded, but compact, intermediate, I_U, with decreased tertiary and secondary structure was observed. The I_U intermediate readily forms amyloid fibrils, whereas I_N preferentially leads to amorphous aggregates [26]. Comparable data have been recently reported for another light chain variable domain, LEN [87,88]. In fact, a general correlation between reduced thermodynamic stability and increased amyloidogenicity has been reported for number of other immunoglobulin light chains [89–94]. For example, the thermodynamic stabilities and hydrogen-deuterium (H-D) exchange rates of two other human monoclonal light chains, an amyloid-associated protein, BIF, and a homologous non-pathological light chain, GAL, have been compared [90]. It has been shown that an amyloid-associated protein readily formed fibrils in vitro and had a lower free energy of unfolding than a homologous non-pathological protein, which did not form fibrils in vitro. H–D exchange was much faster for the pathological protein, suggesting it had a greater fraction of partially unfolded molecules [90]. The thermodynamic stabilizer sucrose was shown to completely inhibit fibril formation by the pathological protein, shifting the values for its physical parameters to those measured for the nonpathological protein in buffer alone. In contrast, urea sufficiently destabilized the nonpathological protein such that its physical properties were equivalent to those of the pathological protein in buffer. Furthermore, the nonpathological protein was shown to become amyloidogenic when destabilized by urea [90]. These observations indicate that fibril formation by light chains is predominantly controlled by thermodynamic stability. Based on these observations, it has been suggested that a rational strategy to inhibit amyloidosis is to design high affinity ligands that specifically increase the stability of the native protein [90].

1.3. Insulin

Insulin is a small α-helix-containing protein hormone that is crucial for the control of glucose metabolism and in diabetes treatment. Amyloid deposits comprised of fibrillar insulin have been observed both in patients with diabetes [95,96] and in normal aging [97], as well as after continuous subcutaneous insulin infusion and after repeated insulin injections [98]. Insulin is composed of two polypeptide chains, the A-chain (21 residues) and the B-chain (30 residues), linked together by two disulfide bonds [99,100]. In solution, insulin exists as an equilibrium mixture of monomers, dimers, tetramers, hexamers, and possibly higher associated states, depending on concentration, pH, metal ions, ionic strength, and solvent composition [98,101]. In 20% acetic acid, insulin is monomeric and has a native-like conformation [25,102]. Exposure to elevated temperatures, low pH, organic solvents and the presence of hydrophobic surfaces (e.g. the air–water interfaces formed on agitation) leads to destabilization and fibrillation [25,103–105].

The fibrillation of insulin is accelerated in the presence of denaturants such as urea, whereas stabilizers, such as trimethylamine *N*-oxide (TMAO) and sucrose, decrease the fibrillation rate [25]. Based on these observations, it has been proposed that the early stages of insulin fibril formation involve the dissociation of native associated states (hexamer, tetramer, and dimer) to give native monomer, which is in equilibrium with the fibrillation-competent

partially folded intermediate. Once formed, the intermediate, which must have a strong propensity to oligomerize, will inevitably lead to fibrils above some critical threshold concentration. The amyloidogenic partially folded intermediate was assumed to be relatively unfolded [25]. This conclusion has been confirmed via a systematic investigation of the effect of Gdm·Cl-induced structural perturbations on the mechanism of fibrillation of insulin [106].

1.4. Prion protein

The central event in the pathogenesis of prion diseases is a major conformational change of the prion protein (PrP) from an alpha-helical (PrP^C) to a beta-sheet-rich isoform (PrP^{Sc}). The mature PrP^C species consists of an N-terminal region of about 100 amino acids, which is unstructured in the isolated molecule in solution, and a C-terminal segment, also approximately 100 amino acids in length. The C-terminal domain is folded into a largely α-helical conformation (three α-helices and a short antiparallel β-sheet) and stabilized by a single disulfide bond linking helices 2 and 3 [107]. Although unstructured in the isolated molecule, the N-terminal region contains tight binding sites for Cu^{2+} ions and therefore may acquire structure following copper binding [108–110]. The structured C-domain folds and unfolds reversibly in response to denaturants, and recent work on the folding kinetics of mouse PrP^C demonstrates that there are no populated intermediates in the folding reaction and that the protein displays unusually rapid rates of folding and unfolding [111]. This shows that no partially unfolded forms or intermediates have a population greater than the unfolded state. These findings have been confirmed by hydrogen/deuterium exchange measurements on the human protein, which show that the conformation of human PrP^C is not abnormally plastic and the stable core of PrP^C has extensive contributions from all three α-helices, which show protection factors equal to the equilibrium constant for the major unfolding transition. Moreover, a residual, hyper-stable region was retained upon unfolding [112]. Based on these data, it has been assumed that the most likely route for the conversion of PrP^C to PrP^{Sc} is through a highly unfolded state that retains, at most, only this small nucleus of structure, rather than through a highly organized folding intermediate [112].

1.5. β_2-Microglobulin

This is a small, non-glycosylated protein with two antiparallel pleated sheets of β-strands linked together by a disulfide bridge; i.e. an immunoglobulin fold [113]. β_2-Microglobulin is part of the major histocompatibility complex class 1 antigen [114], and has a normal plasma, concentration in adults of 1.1–2.7 mg/l. The daily production of β_2-microglobulin is in the range of 150–200 mg, of which 97% is excreted in the kidneys. The concentration of β_2-microglobulin, therefore, is significantly increased in patients with renal failure. Aggregation and fibrillation of β_2-microglobulin are hallmarks of dialysis-related amyloidosis. This type of amyloidosis does not seem to require a mutated, processed, or chemically modified precursor protein [115]. β_2-Microglobulin can form amyloid fibrils in vitro. Fibrillogenesis of β_2-microglobulin was shown to be critically dependent on the pH and the ionic strength of the solution, with low pH and high ionic strength favoring fibril formation. Formation of a partially folded conformation has been detected under these same conditions [116–119]. Circular dichroism studies show that titration of one or more residues with a pK_a of 4.7 destabilizes native β_2-microglobulin and generates a partially unfolded species. On average, these molecules retain some secondary structure and are able to bind ANS, show line broadening in one-dimensional ^{1}H NMR spectra, and are weakly protected from hydrogen exchange. These data are consistent with a model for β_2-microglobulin fibrillogenesis in vitro involving the association of partially unfolded molecules into ordered fibrillar assemblies [118,119]. Recently, it has been shown that β_2-microglobulin is able to form fibrils with distinct morphologies under the acidic conditions in vitro [56]: (a) short, curved fibrils (< 600 nm in length, which formed rapidly with a maximum rate at pH 3.5); (b) long straight fibrils (~ 1 μm) were produced at pH ≤ 3.0 [56]. β_2-Microglobulin at pH 1.5 was shown to be considerably unfolded [56]. In order to investigate the role of different partially folded states in generating fibrils of each type, and to analyze the effect of protein stability on fibril formation, 13 variants of β_2-microglobulin containing point mutations in different regions of the native protein have been created and their structure, stability and fibrillation propensities have been investigated as a function of pH [56]. It has been shown that while destabilization of the native state is important in the generation of amyloid fibrils, population of specific denatured states is a prerequisite for amyloid formation from this protein. Furthermore, it has been established that the formation of fibrils with different morphologies in vitro correlates with the relative population of different precursor states [56]. These observations confirm the idea that variations in the structure of the amyloidogenic precursor might be responsible for the variety of fibrillar morphologies.

1.6. Serum amyloid A (SAA) protein

SAA is a family of closely related apolipoproteins associated with high-density lipoprotein. The protein is very well conserved throughout evolution, indicating an important biological function [120]. Reactive systemic (AA, secondary) amyloidosis occurs in chronic inflammatory diseases, and most patients present with nephropathy. The amyloid fibrils are derived from the circulating acute-phase reactant SAA protein. The serum concentra-

tion of SAA can increase from the healthy reference range of under 10 mg/l to more than 1000 mg/l during active inflammation, and these levels can persist indefinitely [121]. Structure and propensity to aggregate have being compared for several SAA isoforms. It has been shown that mouse SAA2 has about one-half of the α-helix content of the SAA1 analogue. Importantly, decreased helical content correlated with increased propensity to aggregate for the SAA2 form compared with SAA1 [122].

1.7. Human lysozyme

The enzyme lysozyme is involved in an amyloid-related human disorder [123] in which the disease is associated with single point mutations in the lysozyme gene, and fibrils are deposited widely in tissues (Table 1, Ref. [123]). Wild-type human lysozyme and its two amyloidogenic variants have been found to form a partially folded state at low pH [124]. This state was characterized by extensive disruption of tertiary interactions and partial loss of secondary structure. Incubation of these proteins at pH 2.0 resulted in the formation of large numbers of fibrils over several days of incubation [124]. The amyloidogenic mutant proteins were significantly less stable than the wild-type protein, leading to higher populations of the partially unfolded intermediate and thus greater propensity to form fibrils.

1.8. α-Lactalbumin

α-Lactalbumin, α-LA, is a small acidic protein with a Ca^{2+}-binding site and a similar three-dimensional fold to lysozyme. It is very attractive for studies of partially folded conformations since at acidic pH, or moderate Gdm·Cl concentrations, or elevated temperatures (apo form), it adopts the classic molten globule conformation [125]. α-LA is comprised of a large α-helix domain and a small β-sheet domain connected by a calcium-binding loop and four disulfide bridges [125]. α-LA forms amyloid fibrils in vitro at low pH. *S*-carboxymethyl-α-lactalbumin, a disordered form of the protein with three of the disulfide bridges reduced, was even more susceptible to fibrillation. *S*-carboxymethyl-α-lactalbumin exhibits the properties of a pre-molten globule and its fibrillation is orders of magnitude faster than when starting with the molten globule conformation [45]. Other partially folded conformations induced in α-LA at neutral pH, either by removal of Ca^{2+} or by binding of Zn^{2+} to the Ca^{2+}-protein, did not fibrillate, although Zn^{2+}-loaded α-LA precipitated out of solution as amorphous aggregates (i.e. structure-less deposits). Based on these data, it was concluded that the transformation from native state to a substantially unfolded conformation is required for successful fibril formation, whereas less unfolded species may form amorphous aggregates [45].

1.9. Tetramerization domain of human tumor suppressor protein p53

p53 is a nuclear protein believed to play an important role, through mutation and overexpression, in the progression of human malignant tumors [126,127]. One example is a p53 mutant (R337H) associated with adrenocortical carcinoma (ACC) in children [128]. It has been established that the substitution of R337 by histidine results in pH-dependent instability of the p53 tetramerization domain (p53tet-R337H) [129]. In the native protein residue R337 forms a stable salt bridge with residue D352 of an adjacent p53 subunit. It has been shown that substitution of this residue by histidine weakens this salt bridge in a pH-dependent manner [129]. It has been reported recently that the tetramerization domains of wild-type p53 (p53tet-wt) and the mutant, R337H (p53tet-R337H), can be converted from the soluble native state to amyloid-like fibrils at pH 4.0 through incubation at elevated temperatures [130]. The mutant, p53tet-R337H, had a significantly higher propensity to form amyloid-like fibrils then the wild-type. The far-UV CD and FTIR spectra revealed that the conformation preceding the appearance of amyloid fibrils was substantially unfolded [130].

1.10. Cytochrome c_{552}

The *c*-type cytochromes are electron-transfer proteins that contain a heme prosthetic group which is covalently attached through thioether linkages to two cysteine residues that are in a CXXCH motif in the protein [131]. The wild-type form of cytochrome c_{552} from *Hydogenobacter thermophilus* adopts the helical class 1 cytochrome fold containing four α-helices but no β-sheet structure [132]. The release of the heme group from cytochrome c_{552} leads to substantial destabilization and structural perturbations in this protein [133]. In particular, a threefold decrease in α-helical content with a concomitant increase in random coil structure has been observed [133]. This *apo* form of cytochrome c_{552} was shown to be sufficiently unstable that it was readily converted into amyloid fibrils. Here the fibrils appear to form from the partially folded *apo* state which has a CD spectrum characteristic of a mainly random coil conformation with approximately 14% α-helical secondary structure (compared to 43% α-helix in the *holo* state) [133].

1.11. Acidic fibroblast growth factor

Acidic fibroblast growth factor from newt (*Notophthalmus viridescens*), nFGF-1, is a ~ 15-kDa, all β-sheet protein devoid of disulfide bonds. The protein lacks helical segments, and the secondary structural elements include 12 β-strands arranged into a β-barrel architecture [134]. Thermally induced amyloid formation in this β-barrel protein has been analyzed [42]. Maximum fibril formation was

observed at 65 °C. Using a variety of biophysical techniques, including multidimensional NMR, fibril formation was shown to occur from a relatively unfolded intermediate that accumulated in the thermal unfolding pathway of nFGF-1 [42]. Similarly, partial unfolding induced in nFGF-1 by low concentrations of 2,2,2-trifluoroethanol and detected by intrinsic fluorescence, far-UV CD, ANS binding, multidimensional NMR, and Fourier transformed infrared spectroscopy was shown to be accompanied by a dramatic increase in the propensity of this protein to fibrillate [135].

1.12. SH3 domains

The SH3 domain is a well-characterized small protein module (60–85 residues) with a simple fold found in many proteins. The structure of PI3-SH3 domain (84 residues) of the p85α subunit of bovine phosphatidylinositol 3-kinase is a β-barrel composed of two perpendicular, antiparallel β-sheets of three and two strands, respectively [136]. At acidic pH, this SH3 domain was shown to slowly form a gel, comprised of typical amyloid fibrils [27]. The soluble form of PI3-SH3 at acid pH, from which fibrils were generated, had been characterized by several biophysical techniques including CD (far- and near-UV), ANS fluorescence, and ^{1}H NMR [27]. The low-pH form of PI3-SH3 possessed a far-UV CD spectrum typical of the pre-molten globule state. The hydrodynamic radius of the PI3-SH3 acid state was ~ 23% higher than that of the native protein and was ~ 20% lower than that of the unfolded protein [27]. These hydrodynamic dimensions are consistent with the definition of the pre-molten globule state [10,11,137].

1.13. Monellin

Monellin is a plant protein isolated from the fruit of the tropical plant *Dioscoreophyllum cumminsii*. It is a sweet-tasting protein composed of two subunits, one being 45 residues (A chain) and the other 50 residues (B chain). X-ray and NMR methods indicate the structure of monellin has a five-strand anti-parallel β-sheet and a 15-residue α-helix (Ref. [138], Table 1). Monellin undergoes irreversible thermal unfolding at pH 2.5 and 85 °C, leading to an unfolded-like conformation [29]. Incubation under these conditions for 3 h leads to amyloid-like fibrils of ~ 10-nm width, which were shown to bind Congo red [29].

1.14. Phosphoglycerate kinase

Yeast PGK is a single-chain α/β protein of 415 amino acids and no disulfide bonds. The enzyme comprises two domains of about equal size connected by a flexible hinge-region. Under physiological conditions, 35% and 13% of its amino acids are in α-helix and β-sheet conformations, respectively (Ref. [139], Table 1). At acidic pH, PGK is unfolded, although partial refolding can be induced by the addition of anions. Interestingly, it has been shown that if the anion concentrations exceed critical limits, the protein tends to aggregate and anion-induced refolding gives rise to the formation of amyloid fibrils [34].

1.15. Acylphosphatase (AcP)

AcP is a small globular enzyme of 98 residues. It catalyzes the hydrolysis of acyl phosphates such as 1,3-bisphosphoglycerate, acetyl phosphate, carbamoyl phosphate, succinyl phosphate, and the β-aspartyl phosphate formed during the activity of membrane pumps [140,141]. AcP has been purified and sequenced from several vertebrates as two isoenzymes with ~ 50% amino acid sequence identity (see Ref. [142], and references therein), which are known as the muscle and erythrocyte AcP. The structure of muscle AcP has been determined by NMR [143–147], whereas the erythrocyte isozyme structure has been solved by X-ray crystallography [148]. Both AcP isozymes are single-domain proteins with no disulfide bonds, consisting of two α-helices packed against an antiparallel five-stranded β-sheet. There is clear evidence that AcP unfolds in a highly cooperative and reversible manner and this little persistent residual structure is maintained in the denatured protein [149]. AcP is a relatively unstable protein with a $\Delta G(H_2O)$ of 22 ± 1 kJ mol^{-1} at pH 7 and 25 °C. The midpoints of both thermal and chemical denaturation curves are also relatively low [150]. AcP can be converted into amyloid fibrils by addition of trifluoroethanol, TFE [32]. The propensity to form fibrils has been investigated for a series of mutants of AcP by monitoring the range of TFE concentrations that result in aggregation [151]: the tendency to aggregate correlates inversely with the conformational stability of the native states of mutants. Notably, the most strongly destabilized AcP variant, F94L mutant, forms amyloid fibrils in aqueous solution in the absence of TFE. Based on these results it has been concluded that the fibrillation takes place from an ensemble of denatured conformations under conditions in which some noncovalent interactions are still favored.

1.16. Methionine aminopeptidase (MAP)

MAP from the hyperthermophile, *Pyrococcus furiosus*, is a monomeric protein of 295 residues. MAP formed amyloid-like fibrils when incubated under conditions leading to unfolding (3.37 M Gdm·Cl at pH 3.31) [35].

1.17. Myoglobin

Myoglobin is a classical all-helix protein. However, under destabilizing conditions, where it is in a partially folded intermediate conformation, it will form amyloid fibrils [152].

2. Fibrillogenesis of natively unfolded proteins: the requirement for partial folding

Certain proteins seem to require a high degree of structural disorder in their native states to fulfill their function [7,9,11,13,15,153,154]. The structural plasticity of natively unfolded (or intrinsically unstructured) proteins has been suggested to favor their interaction with ligands [11,154]. These proteins represent an intriguing aspect of molecular evolution in which the subtle boundary between risky self-aggregation and sophisticated function is easily crossed [55,155]. We now consider details of the fibrillogenesis of such intrinsically unstructured proteins. Data presented in Table 1 show that a significant portion of known amyloidogenic proteins belong to this class. It is reasonable to assume that such proteins are well suited for amyloidogenesis, as they lack significant secondary and tertiary structure and significant specific intra-chain interactions. In the absence of such conformational constraints, they would be expected to be substantially more conformationally motile, and thus able to polymerize more readily than tightly packed globular proteins. Substantial evidence suggests that the earliest stage of fibrillation of these proteins is their partial refolding. The following section considers illustrative examples of fibril formation of natively unfolded proteins.

2.1. Amylin

Pancreatic islet β-cells produce, in addition to insulin, a peptide called amylin or islet amyloid polypeptide, IAAP [156]. Dysfunction of amylin due to mutation and/or amyloid fibril formation has been associated with the development of non-insulin-dependent diabetes mellitus (NIDDM) [157–159]. Amylin is an unstructured peptide hormone of 37 amino acid residues (see Table 1). Human amylin and its 8–37 fragment were shown to form fibrils under physiological conditions. The process of polymerization was relatively fast (lag-times were 100 and 50 min for full-length amylin and its 8–37 fragment, respectively) and resulted in the appearance of typical amyloid fibrils [20]. Interestingly, both peptides showed formation of a partially folded (pre-molten globule-like) intermediate early in the fibrillation process. It takes ~ 90 min for full-length amylin to form such an intermediate, whereas this period was almost half as long for the truncated peptide, showing excellent agreement with the fibrillation lag-times [20].

2.2. Amyloid β-protein

Alzheimer's disease, AD, is the most prevalent age-dependent dementia. AD is characterized pathologically by the accumulation of extracellular amyloid deposits in the cerebral cortex and vasculature and of intracellular neurofibrillary tangles from the protein tau. Amyloid deposits contain the amyloid β-protein (Aβ), which is a 40–42-residue peptide produced by endoproteolytic cleavage of the amyloid β-protein precursor (APP). Many lines of evidence support the crucial role of Aβ in AD. Fibrillation of Aβ is associated with the development of the cascade of neuropathogenetic events, ending with the appearance of cognitive and behavioral features typical of AD. Aβ appears to be unfolded at the beginning of the fibrillation under physiological conditions (see Table 1). NMR studies have shown that monomers of Aβ(1–40) or Aβ(1–42) possess no α-helical or β-sheet structure [160], i.e. they exist predominately as random extended chains. Partial refolding to the pre-molten globule-like conformation has been detected at the earliest stages of Aβ fibrillation [140].

2.3. α-Synuclein

α-Synuclein is a small (14 kDa), soluble, intracellular, highly conserved protein that is abundant in various regions of the brain. Structurally, purified α-synuclein is a typical natively unfolded protein (Refs. [24,161], Table 1). Several observations indicate that this presynaptic protein is involved in the pathogenesis of Parkinson's disease, PD. Two different missense mutations in the α-synuclein gene, corresponding to A53T and A30P, have been identified in two kindreds with autosomal-dominantly inherited, early-onset PD. Furthermore, the production of WT or A53T α-synuclein in transgenic mice [162–164], or WT, A30P and A53T in transgenic flies leads to the motor deficits and neuronal inclusions reminiscent of PD [165]. Interestingly, the peptide derived from the central hydrophobic region of α-synuclein is a significant constituent of the amyloid plaques in AD. This 35-amino-acid peptide, known as NAC, was shown to amount to about 10% of the amyloid plaque. In fact, recent studies suggest that a very significant fraction of AD patients also have α-synuclein pathology, e.g. Lewy bodies [166]. These observations indicate that α-synuclein is a key player in the pathogenesis of several neurodegenerative disorders [167].

The fibrillogenesis of α-synuclein has been studied extensively. In particular, accumulated data strongly suggest that the formation of a partially folded intermediate (possessing the major characteristics of the pre-molten globule) represents the critical first step of α-synuclein fibrillogenesis. This partially folded intermediate can be stabilized by numerous factors, including high temperatures [24], low pH [24], the presence of several common pesticides and herbicides [168–170], or metal ions [170,171], or at moderate concentrations of trimethylamine-*N*-oxide [172], or other organic solvents [173]. Under all these conditions, α-synuclein was shown to undergo significantly enhanced fibrillation. In contrast, fibril formation was considerably slowed or inhibited under conditions favoring formation of more folded conformations [172,173], or by stabilization of the fully unfolded form, e.g. by oxidation of its methionines

[174] or by stabilization of off-pathway oligomers via nitration of tyrosines [175]. Importantly, all the conditions favoring the aggregation-prone partially folded conformation accelerated both nucleation and elongation stages of fibril assembly. This means that the pre-molten globule-like partially folded intermediate is likely involved in the formation of fibril nucleus and in the subsequent propagation of fibrils.

Interestingly, α-synuclein mutants associated with familial early-onset of PD have been shown to be more aggregation-prone in vitro than the wild-type protein [22,176–180]. However, neither the natively unfolded nor the partially folded intermediate conformations were affected by the familial PD point mutations [178,179]. Based on these observations, it has been concluded that the effect of the enhanced aggregation of these mutants is attributed to the increased propensity of their partially folded intermediates to aggregate, rather than to any changes in the monomeric natively unfolded species [178,179].

A common property of the interior of all cells is the high concentration of macromolecules present. This excluded volume effect of macromolecules (proteins and RNA) makes most of the interior space of a cell unavailable to other macromolecules. This has major consequences on the aggregation of proteins. For example, macromolecular crowding modeled by high concentrations of various polymers (proteins, polysaccharides and polyethylene glycols) dramatically accelerated α-synuclein fibrillation in vitro [181,182]. The stimulation was observed in the presence of high concentrations of both charged and neutral polymers, and the magnitude of the accelerating effect depended on the nature of the polymer, its length and concentration [181].

2.4. Tau-protein

Tau, a microtubule assembly protein isolated from brain microtubules, represents a family of isoforms, which migrate as close bands of 55–62 kDa in SDS gel electrophoresis. Heterogeneity is explained in part by alternative mRNA splicing leading to the appearance of one, two, three or four repeats in the C-terminal region [183]. Posttranslational phosphorylation of tau is an additional source of microheterogeneity [184]. In vitro, tau binds to microtubules, promotes microtubule assembly, and affects the dynamic instability of individual microtubules [185–190]. In situ, tau is highly enriched in the axons [191]. On the basis of its in vitro activity and its distribution, it is believed that tau regulates the organization of neuronal microtubules. Interest in tau dramatically increased with the discovery of its aggregation in neuronal cells in the progress of AD and various other neurodegenerative disorders, especially frontotemporal dementia [192,193]. In these cases, specific tau-containing neurofibrillary tangles (paired helical filaments) are formed [194]. Hyperphosphorylation was shown to be a common characteristic of pathological tau [195]. Hyperphosphorylated tau isolated from patients with AD was shown to be unable to bind to microtubules and promote microtubule assembly. However, both of these activities were restored after enzymatic dephosphorylation of tau protein [196–200].

During brain development, tau is phosphorylated at many residues, including sites phosphorylated with GSK-3β, cdk 5, and MAPK [201]. In vitro, tau can be phosphorylated on multiple sites by several kinases (for a review, see Ref. [202]). Most of the in vitro phosphorylation sites of tau are located within the microtubule interacting region (repeat domain) and sequences flanking the repeat domain. Many of these sites are also phosphorylated in PHF-tau [203,204]. In fact, 10 major phosphorylation sites have been identified in tau isolated from PHFs from patients with AD [203]. All of these sites are located in regions flanking tau's repeat domain and constitute recognition sites for several AD diagnostic antibodies, which may point to an important role for these phosphorylation sites for AD pathogenesis. Hyperphosphorylation was shown to be accompanied by the transformation from the unfolded state of tau into a partially folded conformation [205,206], accelerating dramatically the self-assembly of this protein into paired helical filaments in vitro [197]. To analyze the potential role of tau hyperphosphorylation in tauopathies, mutated tau proteins have been produced, in which all 10 serine/threonine residues known to be highly phosphorylated in PHF-tau were substituted for negatively charged residues, thus producing a model for a defined and permanent hyperphosphorylation-like state of tau protein [207]. It has been demonstrated that, like hyperphosphorylation, glutamate substitutions induce compact structure elements and SDS-resistant conformational domains in tau protein, as well as lead to the dramatic acceleration of its fibrillation [207].

2.5. Prothymosin α

This 109-residue protein is very acidic, containing ~ 50% aspartic and glutamic acids, no aromatic or cysteine residues, and very few large hydrophobic aliphatic amino acids [208]. Because of these features, prothymosin α, pTα, adopts a random coil-like conformation with no regular secondary structure (Refs. [208,209], Table 1). However, at acidic pH pTα adopts a partially folded conformation [209]. Interestingly, it has been recently shown that at low pH (below pH 3, i.e. under conditions favoring the formation of the pre-molten globule-like conformation Ref. [209]), prothymosin α is capable of relatively fast formation (lag time ~ 100 min) of regular elongated fibrils with a flat ribbon-like structure 4–5 nm in height and 12–13 nm in width [41].

2.6. Apolipoprotein C-II

Human apolipoprotein C-II is a plasma protein consisting of 79 amino acid residues, which is associated with

lipoproteins. The function of this protein is to activate lipoprotein lipase. The structure of apoC-II in the presence of the lipid mimetic, SDS, reveals three regions of well-defined amphipathic α-helix with loosely defined intervening regions that may reflect flexible hinge regions [210]. Such amphipathic α-helical regions are characteristic of the exchangeable apolipoprotein family and are crucial for the reversible exchange of apolipoproteins between lipoprotein classes. Under lipid-free conditions, human apolipoprotein C-II lacks ordered structure and forms amyloid ribbons after incubation under these conditions for several days [211–217]. Fibril formation was dramatically accelerated by the addition of phospholipids in sub-micellar concentrations, which were shown to induce partial folding of the protein into a pre-molten globule-like intermediate [211]. In contrast, the fibrillation of apolipoprotein C-II was completely inhibited in the presence of micellar phospholipids, i.e. under conditions favoring α-helical conformation [211].

2.7. Yeast prions

There are at least two natural genetic elements, the [*PSI*$^+$] and [*URE3*] factors, in budding yeast *Saccharomyces cerevisiae* that exhibit non-Mendelian inheritance but can be ''cured'' by treatment of the cells with low concentrations of the protein denaturant guanidine hydrochloride and reappear without introduction of new DNA [218]. The [*PSI*$^+$] factor, a protein-based genetic element (prion) of *S. cerevisiae*, is a type of epigenetic inheritance in which changes in phenotype are transmitted through self-perpetuating, conformationally altered forms of cellular proteins [219–221]. The inheritance of [*PSI*$^+$] from mother to daughter cells is based on the transmission of conformational information from ordered nonfunctional Sup35p aggregates (amyloid-like fibrils) to the soluble, functional Sup35p, a subunit of the polypeptide chain release complex that is essential for translation termination [222,223]. In vitro, the prion-determining region of Sup35p, NM, retains a random coil-rich conformation in solution for hours before it converts to a structure with all the characteristics of amyloid fibers [224–226]. It has been shown that factors influencing conformational flexibility of Sup35/NM influence amyloid propagation in vitro: elevated temperatures, chemical chaperones and certain mutations in NM increased or changed its structural content and modulated aggregation [226]. Multiple Sup35p mutants that either are poorly recruited into, or cause curing of, wild-type amyloid in vivo have been identified [221]. In vitro, these mutants showed markedly decreased rates of amyloid formation. It has been shown that the acceleration of Sup35p fibril formation is determined by the acceleration of slow conformational changes rather than by providing stable nuclei. Strikingly, inhibitory mutations map exclusively within a short glutamine/asparagine-rich region of Sup35p, and all but one occur at polar residues. Even after replacement of this region with polyglutamine, Sup35p retains its ability to form fibrils [221]. This suggests similarities between the prion-like propagation of [*PSI*$^+$] and polyglutamine-mediated pathogenesis of several neurodegenerative diseases (see below).

The [*URE3*] element is another factor of *S. cerevisiae* that propagates by a prion-like mechanism and corresponds to the loss of the function of the cellular protein Ure2 [227,228]. It has been demonstrated that the N-terminal region of the protein is flexible and unstructured, while its C-terminal region is compactly folded [229–231]. The overexpression of full-length Ure2p in wild-type *S. cerevisiae* strains induced a 20- to 200-fold increase in the frequency with which [*URE3*] arose. On the other hand, expression of just the N-terminal 65 residues of Ure2p increased the frequency of [*URE3*] induction 6000-fold [229]. Without this ''prion-inducing domain,'' the C-terminal domain performed the nitrogen regulation function of Ure2p, but could not be changed to the [*URE3*] prion state [229]. Thus, the N-terminal fragment (residues 1–65) is the prion-inducing domain, which can propagate [*URE3*] in the absence of the C-terminal part of the molecule. In contrast, the C-terminal part of Ure2p cannot be converted to the prion (inactive) form without the prion-inducing domain covalently attached [230]. These observations emphasize a key role of the disordered N-terminal domain in the fibrillation of Ure2 protein.

2.8. Polyglutamine repeats

There are eight hereditary diseases, including Huntington's disease, in which the genetic expansion of a CAG repeat in an open reading frame leads to neurodegeneration [232,233]. The neurotoxicity in these diseases was shown to be due to a toxic gain of function associated with the expansion of the CAG_N-encoded polyglutamine (polyGln) repeat. The mechanistic hypothesis linking CAG repeat expansion to toxicity involves the tendency of longer polyGln sequences, regardless of protein context, to form insoluble aggregates [234–242]. To help evaluate various possible mechanisms, the biophysical properties of a series of simple poly(Gln) peptides have been analyzed. The circular dichroism spectra of poly(Gln) peptides with repeat lengths of 5, 15, 28 and 44 residues were shown to be nearly identical and were consistent with a high degree of random coil structure, suggesting that the length dependence of disease is not related to a conformational change in the monomeric states of expanded poly(Gln) sequences [240]. In contrast, there was a dramatic acceleration in the spontaneous formation of ordered, amyloid-like aggregates for poly(Gln) peptides with repeat lengths of greater than 37 residues. Poly(Gln) peptides form morphologically small, finely divided aggregates significantly faster than assembly into large aggregates, suggesting a possible explanation for why disease pathology does not always correlate with the observable neuronal intranuclear inclusions [240].

2.9. Short peptides

Many short peptides derived from both disease-related (e.g. see Refs. [46,243–251]) and disease unrelated proteins (e.g. see Refs. [20,252–259]) or synthesized de novo [37,260,261] have been shown to form amyloid-like fibrils in vitro. The majority of these peptides were devoid of ordered structure in solution.

2.10. Long soluble homopolypeptides

It has been recently reported that polyamino acids such as poly-L-lysine, poly-L-glutamic acid and poly-L-threonine are able to form amyloid fibrils under appropriate solvent conditions [262]. For example, incubation of poly-L-lysine solutions at pH 11.1 readily induced the formation of large quantities of fibrillar aggregates. Based on these observations, it has been assumed that the process of fibril formation does not require the presence of specific side chain interactions or sequence patterns, and primarily originates from main chain interactions [262].

3. Conformational prerequisites for amyloidogenesis

The data presented above on the structural analysis of the early events during the fibrillation of several proteins and polypeptides have demonstrated the critical role of substantially unfolded conformations as fibril precursors. The question then arises as to the nature of this amyloidogenic conformations. Globular proteins under equilibrium conditions may have at least two different partially folded conformations, the molten globule and its precur-

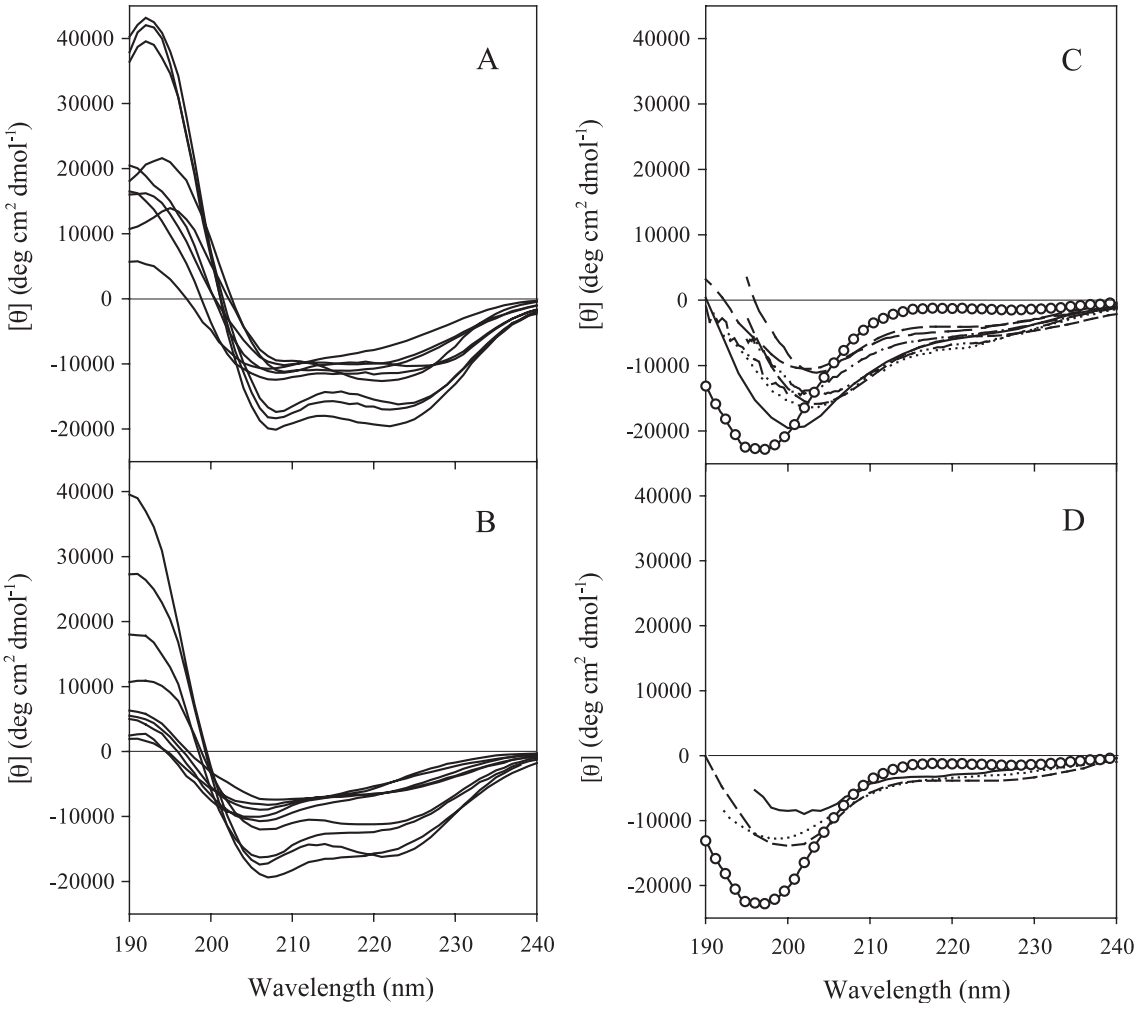

Fig. 3. Representative far-UV CD spectra of proteins in their native (A), molten globule (B), pre-molten globule (C) and spectra of proteins in their amylodogenic conformation (D). Data for native and molten globule states are from [273]. Pre-molten globules are: SNase, pH 2.0, 500 mM NaCl (solid); apomyoglobin, pH 2.0, 500 mM NaCl (dot); nucleoporin Nup2 (short dash); de novo protein albebetin (dash-dot-dot); phosphodiesterase γ-subunit at 80 °C (long dash); caldesmon at 85 °C (dash-dot) and C-domain of calreticulin (medium dash). Amylodogenic conformations are: *S*-carboxymethyl-α-lactalbumin (solid); α-synuclein at pH 3.0 (dot); and prothymosin α at pH 3.0 (short dash). Spectrum of random coil (prothymosin α, pH 7.5) is shown for comparison in plots C and D as line with circles.

sor, the pre-molten globule [263–267]. The structural properties of the molten globule are well known and have been reported in a number of reviews (e.g. see Ref. [264]). Molten globules have an increased affinity for hydrophobic fluorescence probes (such as 8-anilinonaphthalene-1-sulfonate, ANS). Although lacking significant tertiary structure, the molten globule is globular, has a high content of secondary structure, a native-like topology and has hydrodynamic dimensions relatively close to those of the native protein [264].

The pre-molten globule is also a denatured state. It is characterized by considerable secondary structure, although much less than that of the molten globule. The pre-molten globule state is considerably less compact than in the molten globule state, but it is still more compact than the corresponding random coil. Pre-molten globule conformations can interact with the hydrophobic fluorescent probe ANS, although more weakly than in the corresponding molten globule state. This means that at least some hydrophobic clusters are already formed in the pre-molten globule state, although there is no globular structure. The pre-molten globule and the molten globule are separated by an all-or-none phase transition, reflecting the fact that these partially folded intermediates represent discrete thermodynamic (phase) states [137,263, 268–272].

Potentially, either of these partially folded conformations (the molten globule or the pre-molten globule) may play a role as the crucial amyloidogenic species. Data considered in our review are consistent with the assumption that the amyloidogenic species is significantly unfolded, and structurally closer to the pre-molten globule than to the molten globule state. Fig. 3 visualizes this assumption by representing far-UV CD spectra of several ‘‘normal’’ proteins in their native (Fig. 3A), molten globule (Fig. 3B) or pre-molten globule state (Fig. 3C) and far-UV CD spectra of some well-characterized amyloidogenic intermediates (Fig. 3D). Fig. 3C and D shows the close similarity of spectral properties for the proteins from last two groups, pre-molten globules and amyloidogenic intermediates. The most characteristic feature of their spectra is a large negative ellipticity at 200 nm and low ellipticity in the vicinity of 222 nm, reflecting considerably unfolded structure. We have used this observation to further demonstrate that the conformation of the critical amyloidogenic intermediates is equivalent to that of known pre-molten globules, using far-UV CD data, where available. Fig. 4 compares the data measured for the amyloidogenic conformations of 11 proteins (SH3-domain [27], cytochrome c_{552} [39], monellin [29], MAP [35], SMA [26], α-LA [45], PGK [34], amylin [20], prothymosin α [41], Aβ [160] and α-synuclein [24]) with those retrieved for the four basic protein conformations, native, molten globule, pre-molten globule, and unfolded states. The figure clearly shows that amyloidogenic intermediates overlap the conformational space of pre-molten globules. In this plot, data for the pre-molten globule and unfolded states are taken from Ref. [11]; data for molten globules are from Ref. [273]; for native proteins two sets of data were distinguished: native forms of the amyloidogenic globular proteins (SH3-domain [27], cytochrome c_{552} [39], monellin [29], MAP [35], immunoglobulin light chain SMA [26], PGK [34], human lysozyme [124], transthyretin [83], α-

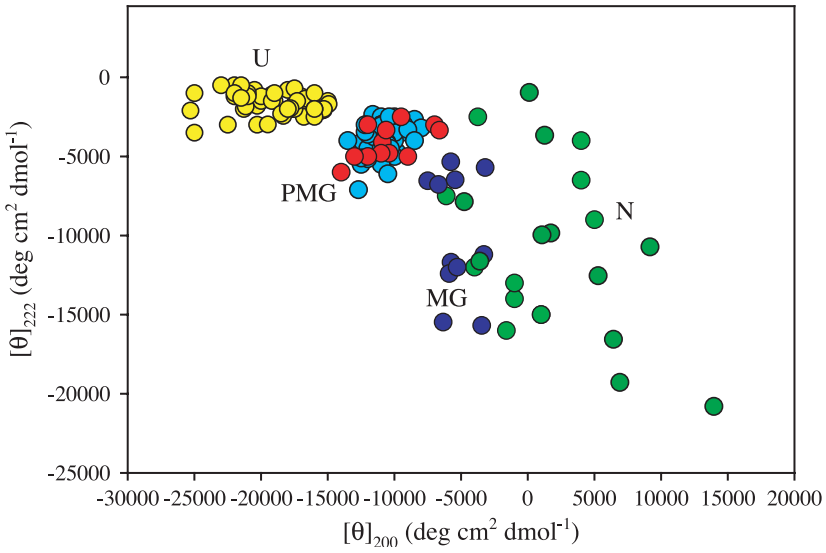

Fig. 4. Amyloidogenic intermediates can be distinguished by their secondary structure properties. The figure compares the far-UV CD spectral parameters measured for the amyloidogenic conformations of 11 proteins to those of proteins in other defined conformations. The amyloidogenic intermediates, shown as red circles, are for amylin [20], SH3-domain [27], cytochrome c_{552} [39], monellin [29], MAP [35], the immunoglobulin light chain SMA [26], α-LA [45], PGK [34], prothymosin α [41], Aβ [160] and α-synuclein [24]. The other basic protein conformations shown (see text) are the native (green, see below) and molten globule states (blue circles), pre-molten globule-like natively unfolded proteins (cyan circles), and random coil-like natively unfolded proteins (yellow circles). Data for the pre-molten globule and unfolded states are taken from Ref. [11]; data for molten globules are from Ref. [273]; the set of native proteins consists of two parts, native forms of the amyloidogenic globular proteins (light green circles) and native forms of proteins able to form molten globules (dark green circles) [273].

LA [45], prion protein [70], β-lactoglobulin [44]), and native forms of proteins able to form molten globules [273]. That all the amyloidogenic conformations are grouped with the set of data for the pre-molten globule proteins is an extremely important observation, which unequivocally demonstrates the importance of this conformation for protein fibrillogenesis. Moreover, this observation suggests that all pre-molten globule-like natively unfolded proteins (see Ref. [11] for a list of these proteins) may easily form fibrils.

Results of recent studies on Raman optical activity, ROA, spectra and NMR analysis provide good evidence that proteins previously thought to be in a statistical coil state may in fact be flickering in and out of metastable poly-(L-proline) II, PPII, helical conformation [274–276]. PPII helices have even been hypothesized to be a major component of protein denatured states [277–282]. PPII conformations might be transiently populated in natively unfolded proteins as well; for example, ROA spectra of α-synuclein suggest that this protein may contain some PPII conformation [282]. The overall importance of this conformation has recently become apparent [280,283], and may play a central role in numerous processes including signal transduction, transcription, cell motility, and the immune response [280]. The PPII helix is believed to be the dominant conformation for many proline-rich regions of proteins [284], although many sequences not rich in proline also adopt this structure [274,285,286]. For example, around 2% of all residues in known protein structures were found in PPII helices at least four residues long [287,288]. It is difficult to exclude the possibility that the pre-molten globule conformation described here as a major amyloidogenic conformation is also enriched in PPII helices. For example, it has recently been shown that the hydrated α-helix in human lysozyme readily undergoes a conformational change to PPII structure on heating, i.e. under conditions favoring fibrillation [289]. It has been assumed that this conformational change may be a key step in the conversion of α-helix into β-sheet associated with the formation of amyloid fibrils in this protein. Furthermore, since the PPII helix is extended, flexible, lacks intrachain hydrogen bonds and is fully hydrated in aqueous solution, it has the appropriate characteristics to be implicated as a critical conformational element in conformational diseases [289].

4. Protein aggregation and fibrillation as a particular case of colloidal assembly

It is of interest to note that the processes of protein aggregation and fibrillation are consistent with a colloidal assembly mechanism [290–292]. In fact, molecular assembly occurs as a result of intermolecular forces, which are determined by the osmotic second virial coefficient, B_{22}, a measure of non-ideal solution behavior that arises from two-body interactions. Positive B_{22} values indicate overall dominance of repulsive forces between proteins, where protein–solvent interactions are favored over protein–protein interactions [293], whereas negative B_{22} values reflect attractive forces between proteins, with protein–protein interactions being favored over protein–solvent interactions. It has been shown that the effectiveness of protein crystallization and salting out is determined by B_{22} values. For example, solutions that promote protein crystallization are characterized by a narrow range of moderately negative B_{22} values [293]. Furthermore, proteins are soluble in solutions wherein B_{22} values are greater than this range, whereas in solutions with B_{22} values below the range proteins tend to form amorphous precipitates with native protein structures (i.e. "salting out") [290].

In contrast to crystallization and salting out (processes, which do not generally affect the native structure of a protein molecule), aggregation is a more complicated phenomenon. In fact, protein aggregation is known to be accompanied by large changes in the secondary and tertiary structures of the protein, with increased non-native intermolecular β-sheet content compared to that for protein in the native state [49]. Thus, the intrinsic conformational stability of the protein native state plays an important role in its propensity to aggregate (see above). In other words, non-native aggregation of a protein involves at least two processes—conformational changes of the protein native state to the amyloidogenic conformation, and assembly of protein molecules into higher order aggregates, and their energetics are controlled by conformational stability and colloidal stability, respectively [290].

5. Conclusion

Substantial data now indicate that relatively unfolded and highly flexible conformations (notably distinct from the native, molten globule-like and completely unfolded conformations), as found in the pre-molten globule, are key species in the fibrillation process. As comparable aggregation behavior has been observed for both globular and natively unfolded proteins, both disease-associated and disease-unrelated, we assume that such relatively unfolded conformations are a structural prerequisite for fibril formation in all systems.

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