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REVIEW

Mechanisms of Amyloid Fibril Formation

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Abstract—Amyloid and amyloid-like aggregates are elongated unbranched fibrils consisting of β-structures of separate monomers positioned perpendicular to the fibril axis and stacked strictly above each other. In their physicochemical properties, amyloid fibrils are reminiscent of synthetic polymers rather than usual proteins because they are stable to the action of denaturing agents and proteases. Their mechanical stability can be compared to a spider's web, that in spite of its ability to stretch, is stronger than steel. It is not surprising that a large number of diseases are accompanied with amyloid fibril depositing in different organs. Pathologies provoked by depositing of incorrectly folded proteins include Alzheimer's, Parkinson's, and Huntington's diseases. In addition, this group of diseases involves mucoviscidosis, some types of diabetes, and hereditary cataracts. Each type of amyloidosis is characterized by aggregation of a certain type of protein that is soluble in general, and thus leads to specific distortions of functions of the corresponding organs. Therefore, it is important to understand the process of transformation of "native" proteins to amyloid fibrils to clarify how these molecules acquire such strength and what key elements of this process determine the pathway of erroneous protein folding. This review presents our analysis of complied information on the mechanisms of formation and biochemical properties of amyloid fibrils.

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The paradigm that one polypeptide chain can accept only one unique three-dimension conformation became history long ago [1]. Convincing evidence of this are prions, which illustrate that because of conformational changes, one protein can form various structures including amyloids and amyloid-like fibrils. This became especially relevant when it was clarified that incorrect packing of some proteins could be a reason for pathological aggregations and cause development of many neurodegenerative diseases such as Alzheimer's and Parkinson's, type II diabetes, amyotrophic lateral sclerosis (ALS), frontotemporal degeneration (FTD), Huntington's disease, etc. [2-5]. It should be noted that not all amyloids and different amyloid-like fibrils are associated with neurodegenerative diseases, and this property is inherent in a great number of proteins. However, in spite of such diversity of proteins, at first glance the formed amyloid fibrils are similar and look like antiparallel β-structures stacked in a pile perpendicular to the fibril axis [6]. Electron microscopy and X-ray analysis have revealed a more accurate description of fibrils: amyloids are antiparallel β-sheets folded in a helix with a cylinder-like cavity formed within the latter [7]. Amyloid-like structures are formed by yeast prions Sup35 that are rich in asparagines and glutamines. Nevertheless, more profound analyses have revealed a number of differences between amyloid fibrils depending on the protein composition and conditions under which they are formed. For example, α-synuclein, aggregates of which are specific for Parkinson's disease, forms single cylinders consisting of β-sheets, while fibrils of Aβ-fragments specific for Alzheimer's disease consist of two- or three-cylinder β-sheets [7].

The first amyloid depositions were detected in tissues upon iodine staining more than 150 years ago [8]. The depositions were stained blue analogous to starch grains in plants. Though the result lead to the erroneous conclusion of the existence in humans of starch grains like in plants (hence the name "amyloid" appeared), during the following 100 years data were accumulating on the occurrence of amyloids in different tissues. At the time of the discovery of amyloid depositions, researchers could use

Abbreviations: ALS, amyotrophic lateral sclerosis; ApoE, apolipoprotein E; APP, amyloid precursor protein; CJD, Creutzfeldt–Jakob disease; EWSR, Ewing sarcoma breakpoint region 1; TAF15, TATA box binding protein (TBP)-associated factor; FTD, frontotemporal degeneration; FUS, fused in sarcoma; PSF, PTB-associated splicing factor; SAP, serum amyloid P component; TDP-43, TAR DNA-binding protein-43; TIA1, cytotoxic granule-associated RNA binding protein.

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only a light microscope and various stains, and during the following "descriptive" period the revealed depositions were classified depending on the site of location, the extent of staining with a certain reagent, and the clinical case in which the amyloid depositions were found. Therefore, in the 1920s it was known that amyloids were stained with Congo red and therefore they could be attributed to active substances, and the extent of staining indicated their amount in the sample [9, 10]. For example, Congo red was once used as a test for amyloidosis, when the stain was applied intravenously, and after a specified time the portion of bound stain in serum was estimated [11, 12]. It was accepted that the estimate was proportional to the total amount of amyloids in the blood. Though the use of the test was discontinued because of the risk of developing anaphylactic shock, it was believed that the binding of amyloid aggregates is a specific peculiarity of the stain, and it was used for isolation of fibrils from depositions upon autopsy. However, at present the accurate mechanism of binding with Congo red remains unclear [13, 14]. Another frequently used stain for revealing amyloidosis is thioflavin T (ThT), which when incorporated in fibrils significantly increases the fluorescence quantum yield with a shift to longer wavelength of the spectrum. However, not all amyloid formations are stained with these preparations, including protein MAVS that is active in antiviral immunity [15], α-synuclein in Parkinson's disease [16], τ-protein in Alzheimer's disease [17], FUS in amyotrophic lateral sclerosis [18], and others. This can be explained by the fact that in water the short-wavelength excitation spectrum overlaps insignificantly the absorption spectrum of the stain, i.e. only weak absorption occurs on maximal excitation. Meanwhile, for a long-wavelength excitation spectrum, thioflavin T fluoresces in the same spectral range as the stain incorporated in fibrils, the solution of the stain and amyloid fibrils being usually a mixture of unbound stain and stain intercalated in the fibrils [19]. It was demonstrated that thioflavin T can bind to aromatic amino acid residues located in a particular order in β-sheets [20]. Although thioflavin T is widely used as a fluorescence probe, no agreement has been achieved on the mechanism of thioflavin T incorporation into amyloid fibrils [19].

FORMATION OF AMYLOIDS BY THE PRION MECHANISM

Prions are proteins able to accept different conformations including amyloid fibrils that can serve as a matrix and "infect" other proteins, both within and between cells and between organisms [21-23]. Originally, prions were studied in connection with infectious diseases such as Kuru, Creutzfeldt–Jakob disease (CJD), scrapie, bovine spongiform encephalopathy, fatal familial insomnia, in which a prion protein was the pathological agent [24]. However, later it was found that yeast prions assist them in adaptation to diverse conditions of the environment [25]. In mammals they can both cause different diseases, sometimes even leading to death, as well as performing useful functions, for example, activate cell-based innate immunity (protein MAVS), provide for long-term memory (protein CPEB) [26, 27], and form stress granule assemblies under conditions unfavorable for the cell [28]. Many proteins contain prion-like domains that endow the protein with properties close to those of prions, including the ability for self-assembly. For example, the amino acid composition of prion-like domains of proteins fused in sarcoma (FUS), TAR DNA-binding protein-43 (TDP-43), and cytotoxic granule-associated RBA-binding protein (TIA1) is similar to prion domains of yeast proteins such as Sup35 and Ure2 [29]. The latter two proteins are characterized by a high content of polar amino acid residues, for example, asparagine, glutamine, tyrosine, and glycine. These domains enable the proteins to pass from the unfolded three-dimensional structure to intermediate states, which in their turn are predisposed to different conformational transitions including the formation of amyloid fibrils [18, 30, 31]. These proteins are generally in a dynamic equilibrium between two forms: unfolded soluble monomers and oligomers close to molten ones. Such oligomers can be involved in diverse conformational states (Fig. 1). According to one scenario, they can organize in structured amyloidogenic oligomers, then transform into pathological aggregates of a non-amyloid type or amyloid fibrils. The latter can serve as a "matrix" for incorrect folding by the prion type. According to another scenario, protein molecules can form amorphous aggregates consisting of both soluble monomers and molten oligomers organized as dynamic cross-β-structures with properties of fluids, and can also form gel-like structures [32-34]. The hydrogel state is crucial for the formation of various non-membrane structures such as stress granules, ribonucleic complexes, etc. [31, 32, 35, 36]. In other words, prion-like domains are required for aggregation at the stage of protein conformational transition from the fluid state to the gel-like state in order to perform some vital functions, but under certain conditions they can irreversibly transform into amyloid fibrils.

FORMATION OF AMYLOIDS BY THE PRION MECHANISM RESULTING IN PATHOGENESIS

Recently, a new concept on the development of some neurodegenerative diseases has appeared. It includes the idea of the transition and multiplication of protein aggregates from cell-to-cell, from one cortex lobe to another during the disease progression. It has been shown both *in vitro* and *in vivo* that in different neurological diseases, pathological proteins can invade into a cell, find proteins similar to themselves, and then serve as a "matrix" for

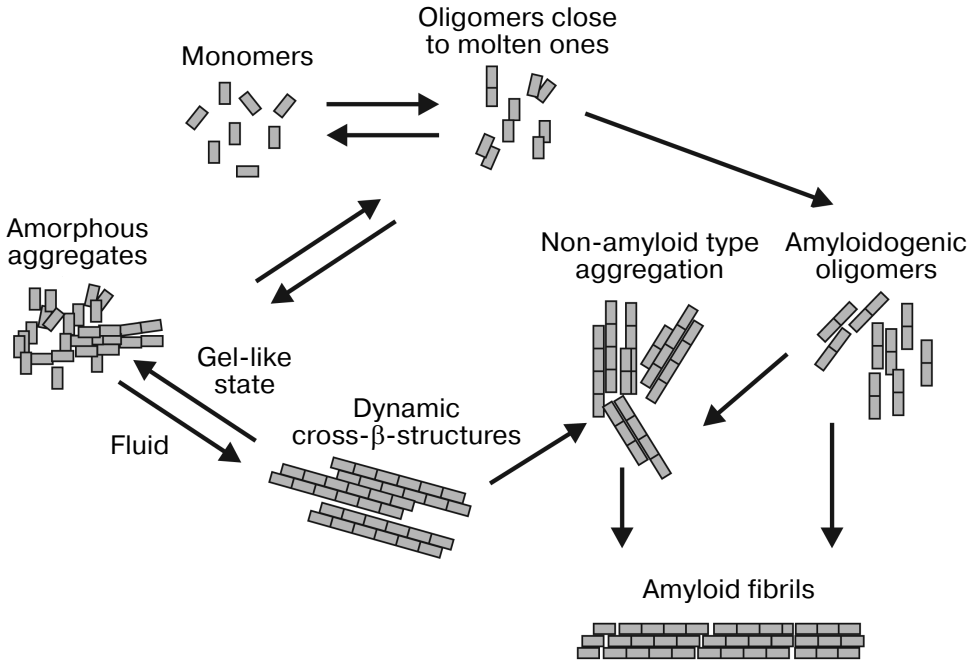

Fig. 1. Schematic representation of prions and prion-like domains that can lead to different conformational states and scenarios (modified and adapted from [37]).

pathological aggregation [23, 38-41]. Thus, pathologic affection of cells spreads from the lesion focus. It should be noted that the cases when the disease broke out in different cortex lobes do not necessarily exclude the prion-like mechanism of its excrescence. In such a case, every lesion focus would spread the disease independently from the others. But what is the molecular basis for neurodegeneration spreading by the prion mechanism? How can these proteins acquire the conformation when the modified protein can serve as a "matrix" for "healthy" proteins, transforming the latter into an aggregated state?

The formation of insoluble β-amyloid fibrils in brain tissues accompanies many neurodegenerative diseases such as Alzheimer's disease, Down's syndrome, and others. The Aβ-peptide is formed as a result of proteolytic cleavage of the amyloid precursor protein (APP). The mRNA of APP frequently undergoes alternative splicing, and so it has several isoforms. In general, proteins perform important physiological functions, and the APP gene is expressed in almost all cells. A change in APP proteolysis results in accumulation of Aβ-fragments that associate in amyloid fibrils [42].

When prion-like domains were discovered in TDP-43 and FUS [18, 30, 31, 43], it became clear that mutations in these proteins can lead to pathological states. Thus in protein TDP-43, mutations Q331K and M337V located in the prion-like domain directly enhance incorrect folding. In protein FUS, mutations associated with amyotrophic lateral sclerosis (ALS) are positioned in the nuclear localization signal (NLS), which causes the protein to accumulate in the cytoplasm, thus facilitating pathological aggregation. Normally, the FUS protein is localized in the nucleus, though it sometimes moves to the cytoplasm and back [44-49]. Along with TDP-43 and FUS, prion-like domains have been revealed in 40 other RNA-binding proteins. It is not surprising that they will be also related to the development of ALS and similar neurodegenerative diseases. As has been determined, other members of the family such as TAF15 (TATA box-binding protein (TBP)-associated factor) and EWS (Ewing sarcoma breakpoint region 1) are also connected with ALS and FTD. Moreover, in the case of FTD another RNA-binding protein, PSF (PTB-associated splicing factor), is anomalously accumulated in the cytoplasm of oligodendrocytes and forms an insoluble structure [50]. Mutations in TIA1, which is an important protein for the formation of stress granules, have been detected in Welander distal myopathy (a slowly progressing muscle dystrophy) [51]. In RNA-binding proteins with prion-like domains, other mutations associated with different neurodegenerative diseases were also revealed [52]. This suggests the important role of this domain in pathogenesis.

FORMATION OF AMYLOIDS BY THE PRION MECHANISM IS REQUIRED FOR NORMAL CELL FUNCTIONING

Living organisms widely use the property of protein molecules to form amyloid structures for various specific

purposes. Normally, some organisms form amyloid fibrils for performing various functions. One of the best-studied examples of such functional amyloids is protein curlin, which is used by *E. coli* to colonize inert surfaces and is a mediator upon binding with proteins of other organisms. Like other amyloid structures, such fibrils are 6-12 nm thick in diameter, have a large portion of β-structure (as shown with CD), and bind thioflavin T and Congo red [53]. Another example is the bacterium *Streptomyces coelicolor*, which due to the formation of amyloid fibrils with chaplin proteins form hyphae used for spreading spores [54].

In these examples, the process of amyloid nucleation that will initiate aggregate growth depends on the ambient conditions and is controlled by a definite cascade of reactions. Controlled formation of functional amyloid aggregates occurs in mammals as well. For example, melanosomes are organelles differentiating into melanocytes responsible for melanin biogenesis in skin cells, containing fibril formations on which melanin granules are formed. Such fibril formations have much in common with amyloids; they are formed from a proteolytically cleaved domain of the membrane protein Pmel17 [55].

Long-term memory is also provided by the principle of fibril formation in which protein CPEB (an RNA-binding protein capable of controlling local translation of mRNAs in dendrites) plays an essential role. This protein can stimulate mRNA polyadenylation, and its aggregation activates translation of the "silent" mRNA accumulated in synaptic end-feet [56]. The N-domain of CPEB is rich in asparagines and glutamines, which is specific for prion-like domains.

Thus, protein MAVS located on the surface of mitochondrial membranes activates innate antiviral cell immunity. When aggregated, it can interact with cytoplasmic receptors recognizing patterns specific for most pathogens, which activates a cascade of reactions leading to the synthesis of β-interferon [57, 58]. This protein can also aggregate by the prion mechanism.

These examples demonstrate that even in highly organized organisms, the formation of amyloids located in a strictly defined place and rigidly controlled can be beneficial physiologically for performing specific and specialized biological functions.

BIOCHEMICAL CHARACTERISTICS OF AMYLOID FIBRILS

In the first stages of studies, chemical analysis of amyloids was complicated because both in human and animal samples the formed fibrils are frequently coated with other tissue components resulting in their nonsusceptibility to the extraction procedure. More vigorous methods of extraction, such as the use of strong bases and acids, destroy amyloid structures. The crucial point in chemical identification of amyloid proteins was the fact that intact amyloid fibrils can be quantitatively extracted from human and animal tissues using a distillate and buffers with low ionic strength [10]. The procedure is as follows: the tissue samples are repeatedly extracted with a saline solution. When the supernatant stops showing significant optical density at 280 nm, the granules are kept in the distillate until the formation of an opalescent solution that upon precipitation with salt leaves a precipitate of pure fibrils associated with Congo red when viewed in an electron microscope. Then the fibrils can be denatured in 8 M urea or 6 M guanidine chloride and layered onto a chromatography column for further purification to analyze the amino acid sequence [59, 60]. It is evident that any product soluble in the saline solution will be lost upon washing, and so will impede the determination of critically important molecules that lead to amyloidogenesis or toxicity. The chemical identification of specific precursors allowed the development of immunohistochemical reagents that can identify the chemical nature of amyloids in tissue samples without chemical extraction.

The first identified and chemically characterized amyloid was an aggregate of light chains of human immunoglobulin isolated from tissues of a patient with so-called primary amyloidosis and was not associated with any other disease [61]. Subsequent studies showed that the same class of proteins is a precursor of amyloid depositions in multiple myeloma [62]. L-chains and their fragments included in amyloid have a similar amino acid sequence with circulating in serum and/or monoclonal light chains isolated from the same patient. The depositions in tissues consist of intact L-chains and/or C-terminal fragments of such chains, which shows that the fragments *in vivo* subjected to proteolysis are more amyloidogenic than intact chains or the intact chain depositions are the object of proteolysis *in situ* [63]. Biosynthetic experiments with bone marrow cells revealed that in some cases truncated L-chains can be products of the synthesis, but these experiments were not strict enough to exclude the hypothesis of a fast postsynthetic digestion [64]. Subsequent experiments demonstrated that fragments of some isolated L-chains obtained by treatment with proteases *in vitro* formed amyloid-like fibrils in a test tube [59, 65]. The basic result of these experiments is the finding that the amyloidogenic potential is associated with the amino acid sequence of the protein.

The following observations revealed some structural peculiarities rendering some members of a given class of proteins more amyloidogenic as compared to other members [66]. Experiments with recombinant versions of the variable region of the amyloidogenic IgL-chain and non-amyloidogenic IgL-chain (obtained from an amyloidogenic molecule by mutating some amino acids) also showed differences that can lead to the propensity to form fibrils [67]. From the biophysical point of view, the mul-

tiplicity of sequences of amino acid residues in L-chains brings about four potentially soluble forms when the concentration of any single L-chain molecule increases by cloning upon the immune response during the disease.

Some human L-chains of immunoglobulins (about 15% of all possible versions of L-chains, predominantly from the λ-class) are exceptionally amyloidogenic and form deposits leading to dysfunction of many organs, especially kidney, heart, stomach, and peripheral nerves [63].

Other L-chains can form non-fibrillar aggregates, and this is a menace for organs just like the fibril-forming L-chains [63]. Cases of depositions of the two types of the same protein in one person are rare, though having been registered, and this suggests that the conformation acquired *in vivo* can be associated with the environment of the affected tissue [68].

Still other L-chains have a specific Bence-Jones feature (aggregation at specific temperatures, usually 56°C, with subsequent dissociation at higher temperatures), and this is associated with aggregation in renal tubules where the protein concentration is higher while pH is lower, and this can lead to kidney dysfunction [69].

Another fraction of L-chains of human immunoglobulin probably does not precipitate in physiological conditions and does not aggregate *in vivo* [70]. The exact structural features responsible for the wide multiplicity of biophysical peculiarities of proteins so similar under physiological conditions are not known.

It is no surprise that taking into account the homology of Ig domains, rare cases have been reported when solitary heavy chains served as precursors for amyloid aggregates upon monoclonal B-cell production [71]. The initial non-immunoglobulin amyloid was isolated from depositions in a monkey with chronic inflammation. The protein has an amino acid sequence dissimilar either to the sequence of an amyloid formed by immunoglobulin L-chains or to the sequence of any other protein sequenced to date [72]. At the same time, a similar protein was isolated and sequenced from the kidney of an Israeli citizen with a periodic disease and secondary (or associated with inflammation) amyloidosis [60]. The two proteins had similar amino acid sequence, it being identical from the N-terminus and varying amino acids not deviating from the frequency characteristic of residues of normal polymorphisms. Later it was found that the given protein also forms amyloids under long-term rheumatoid arthritis (another chronic inflammatory disease) and is a basic component of fibrils in murine and rat models [73].

More than 30 amyloid proteins are now recognized by the International Nomenclature Committee on Amyloidosis [33, 50, 74, 75]. The precursors of most of these proteins are apolipoproteins A (1, 2), serum amyloid A, as well as different proteohormones and immunoglobulins [76]. It has been found that depositions contain also other molecules such as apolipoprotein E (ApoE), SAP (serum amyloid P component), and the proteoglycan perlecan [77-80]. Under AA-amyloidosis (see below), fibrils are generated from the cleaved product SAA whose concentration increases upon development of chronic inflammations and other systemic diseases [81, 82]. This protein is involved in lipometabolism in macrophages participating in inflammations and can also realize other functions, for example, play a part in atherogenesis associated with inflammation [74]. SAA was the first of the five proteins (the other proteins being IAPP, Aβ-peptide, prion, and protein Bri2 whose depositions were found at fatal familial insomnia [83-86]) in which amyloid depositions provide evidence of a normally functioning soluble precursor, identified either after preparation of appropriate antibodies to fibrils and the binding of these antibodies to normal protein, or after identification using cDNA of the corresponding gene in normal tissues.

However, of more importance is that these investigations allowed developing isolation methods that can be used for studying fibrils from any tissue or organism; for example, the methods were used to isolate proteins from congophilic vascular and cerebral deposits of Aβ [87, 88]. Extracellular molecules of matrix and membrane components like laminin and tenascin were also present in some depositions. Such molecules may contribute to accumulation and formation of depositions in tissues. It has been proposed that additional molecules can play a part in stabilization of fibril structure or provide conditions for enhancement of fibrogenesis.

EXPERIMENTAL MODELING OF AMYLOIDOSIS IN ANIMALS

Since the 1960s, animal models have become one of the means to study amyloidosis. By introducing a particular dose of casein or Freund's adjuvant into mice or rabbits, an acute inflammatory process was provoked which, in turn, was concomitant with accumulation of deposits stained with Congo red in the liver, kidneys, and spleen [89]. It was found that as a result of inflammation, the generation of cytokines promotes formation of the amyloid precursor serum amyloid A (SAA) [90]. This model completely mirrors human AA amyloidosis generated during inflammations and is frequently used for studying pathogenesis. A high percentage of amyloidosis in humans is associated with AA amyloidosis, so-called secondary or reactive amyloidosis, when as a response to any chronic inflammation, acute phase proteins (precursors of serum amyloid A – SAA) are synthesized in the liver [76]. Another interesting feature of the model is that spleen extracts of an already infected animal, when transferred to another animal, can competitively accelerate the process of fibril deposition. Such acceleration was explained by the presence of fibril fragments that can serve as beginnings for genesis and deposition of newly

formed amyloid precursors [91]. By associating monomers to the termini, these seeds can form elongated protofibrils, which later can form amyloid fibrils. Of interest is that the murine model of amyloidogenesis can be activated by incorporating seeds of xenogenous origin, which shows that the seeds have comparable structural elements [92, 93]. A similar phenomenon was found for the model of spontaneous amyloidogenesis in SAMP1 mice. This line is characterized by the existence of an isoform of protein ApoA2(C) with the following substitutions: Gln for Pro in position 5, Ala for Val in position 26, and Met for Val in position 38 [94]. The rate of amyloid accumulation in the animals is directly proportional to their age and, as has been revealed, amyloid accumulation in young animals can be accelerated by introduction of preparations obtained from aged animals [95, 96]. These models are characterized by the requirement of a sufficient amount of the amyloid precursor, caused genetically in one case (ApoA2-AA amyloidosis) and by artificially stimulated inflammation in the other case of AA amyloidosis. Mutual effect of ApoA2-AA is also likely upon either inhibition or acceleration of fibril formation [97].

Protein ApoE is no less important in the formation of amyloid fibrils. The progeny produced by cross breeding of *apoe*-(ApoE–/–) knockout animals with transgenic mice with hyperexpression of mutant human protein APP-(PDAPP+/+) contained much less cerebral Aβ deposits compared to the transgenic parents (PDAPP+/+ ApoE+/+) [98, 99]. As a result of these experiments, it was suggested that protein ApoE might be used for the treatment of Alzheimer's disease [100]. However, further experiments with *apoe* knockout mice demonstrated that such animals are sensitive to amyloidosis caused by IAPP to the same extent as the wild-type mice. There are conflicting data on the increase or decrease in depositions in inflammation-induced AA amyloidoses [101-103]. It was shown that protein SAP *in vitro* inhibits cleavage of amyloid fibrils not because of direct inhibition of the process, but rather because of direct binding to fibrils, thus shielding potential cleavage sites from proteases [80]. The *sap* knockout mice had a lower rate of amyloidogenesis in response to inflammatory stimulation, which agrees with the above observations, but these experiments do not indicate that protein SAP is required for fibrogenesis [104, 105]. The absence of sustainable models with knockout of the gene encoding perlecan prevents experiments like those with SAP protein. At the same time, the observations of expression of the gene encoding perlecan before the production of amyloid depositions in a murine model of AA-amyloidosis in tissues, as well as the experiments *in vitro*, demonstrated the acceleration of fibrillogenesis by the action of both the core protein perlecan (a heparan-sulfate proteoglycan) and associated with it chains of heparan sulfate and chondroitin sulfate. In particular, the formation of amyloids in transgenic mice with hyperexpression of heparanase was inhibited. Moreover, other sulfated glucosoaminoglycans, such as heparin, dermatan sulfate, dextran sulfate, and pentosan, augmented the formation of amyloid fibrils [106-109].

The use of these models shows that homomolecular aggregation associated with nucleation and the subsequent growth of fibrils occurs in connection with heteromolecular interactions, some of which are amyloidogenic and others are anti-amyloidogenic.

Monitoring of these models permitted posing the important question whether all amyloids are prion-like (or possess infectious activity) and whether this phenomenon can hold true for some forms of amyloids causing neurodegenerative diseases capable of transmission over the whole organism [110]. However, one should take into account the difference between cell-to-cell transmission of amyloids in a restricted space where autologous proteins and genetic compatibility for direct cell-to-cell transmission exist and the spread of infectious diseases from the environment, which imposes strict requirements on the amount and quantity of interactions between the seed and further monomers of the aggregate. The difference between prions existing predominantly as fibrils and infectious prions as well as the visible interleaving of the aggregation and neurotoxicity phases during crisis may reflect in some way acquisition or selection of conformational compatibility required for infectivity or toxicity [111, 112].

CHARACTERIZATION OF KINETICS OF AMYLOIDOGENESIS

To make the description of amyloidogenesis complete, it is necessary to find all possible conformational states of the protein during its aggregation as well as possible oligomeric structures formed during amyloidogenesis. Moreover, the description would not be complete without a corresponding determination of kinetic and thermodynamic parameters for all potential forms and states of the protein in the course of aggregation. It should be noted that these requirements include also the analysis of aggregation on the molecular level for subsequent revealing of the regions vital for the whole process of amyloidogenesis. It is accepted that this process has a nucleation stage (see Fig. 2). The aggregation of protein monomers into a fibril can be divided in two stages: the lag-time when seeds are generated, and the time of transition of all monomers into an aggregate. Notice that the latter phase varies depending on the protein and can be either linear or exponential [113]. Reactions having a nucleation stage have been studied quite well from the theoretical and experimental points of view for a wide range of substances, mostly in connection with crystallization of large and small molecules [114].

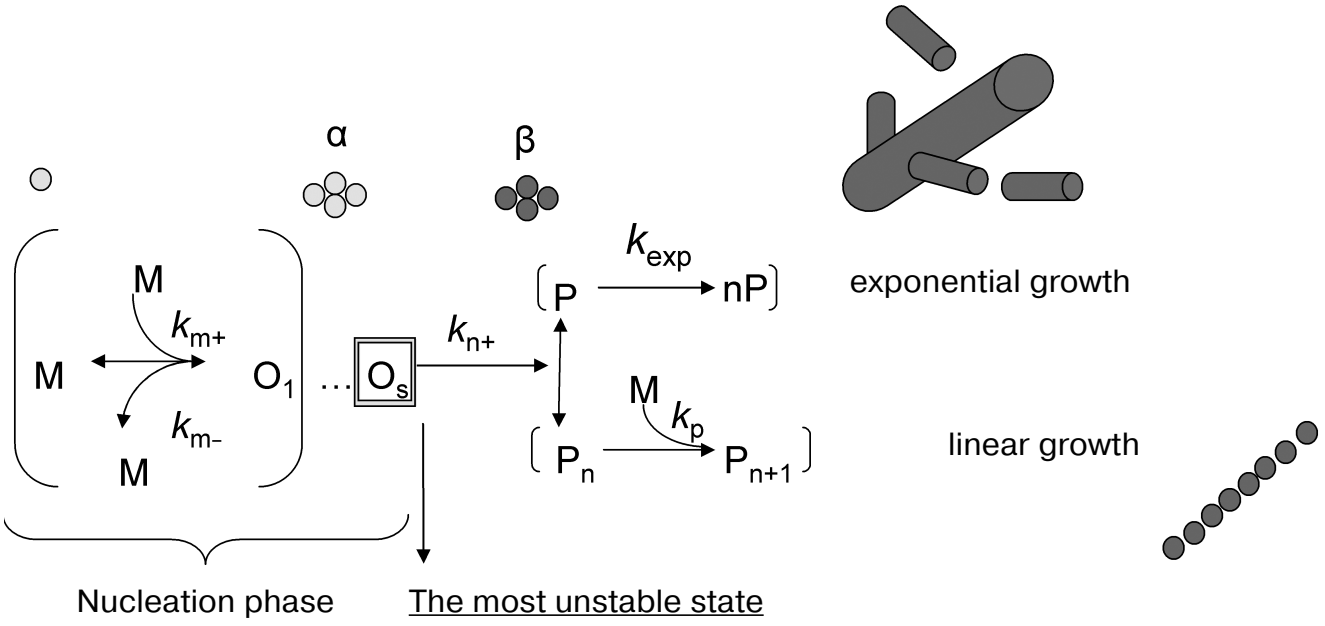

Fig. 2. Schematic representation of amyloidogenesis. k_{m+} is the rate constant of monomer association with the oligomer; k_{m-} is the rate constant of monomer dissociation from the oligomer; k_{n+} is the rate constant of transition of the seed (an oligomer consisting of *s* monomers) from state α to state β; k_p is the polymerization rate constant; k_{exp} is the rate constant of exponential increase of the ends of a growing fibril.

Like for other processes with a nucleation phase (including crystallization), the addition of primes from the seeds already formed during amyloidogenesis practically levels off the lag-period because the aggregation rate is no longer limited by the nucleation stage [115, 116]. It was demonstrated that introduction of particular mutations to aggregating proteins or certain changes in experimental conditions could also level off the lag-period, assuming that nucleation is not a limiting stage of the process any longer [117-119]. The absence of a lag-period is not necessarily related to the fact that the description of the aggregation mechanism with a nucleation stage is no longer applicable. Rather, it shows that the time required for fibril formation is quite large relative to the rate of seed formation, and so nucleation is not already a rate-limiting stage during the transition of the protein from the soluble form to an amyloid. Though no fibrils appear in the lag-period, it is clear that this is an essential stage for the formation of different oligomers including those that would serve as a seed for the formation of mature fibrils.

The efficiency of the fibrils formed in stimulation of aggregation as a seed can strongly decrease on an increase in the differences of the primary structure [120-122]. It was demonstrated for the example of immunoglobulin domains with different primary structure that co-aggregation of various types of domains does not occur when the identity of the protein primary structure is below 30-40% [121]. Bioinformatics analysis of homologous sequential domains in large multidomain proteins revealed identity of less than 40%, which may be evidence of the primary structure of such proteins being composed to avoid aggregation.

During recent decades, significant attempts have been made to identify, isolate, and describe oligomeric particles formed in solution before fibrils appear. This interest in oligomers can be explained by two reasons: apparently, the formation of such particles is a vital stage in amyloidogenesis, and one has every reason to believe that oligomers are the greatest menace for pathogenesis, i.e. they are toxic. As an example, we can take the formation of amyloids by the Aβ-peptide. The aggregation of this peptide is preceded by the development of a number of metastable non-fibril formations observed using the AFM and TEM techniques [123-126]. Some of the formations look like small spherical beads 2-5-nm in diameter, and others look like small beads on a string with individual beads having also the diameter of 2-5 nm, and the third form ring structures that have evidently appeared as a result of closure of the structures similar to the beads on a string. All these assemblies, called protofibrils by the authors who were the first to detect them [123-126] should not be confused however with protofilaments that are singular threads of mature fibrils. Protofibrils, which consist of the Aβ peptide, can bind Congo red and thioflavin T [126], contain a large portion

of β-structure and are composed of about 20 molecules forming small spherical particles.

Similar spherical and bead-like protofibril formations were also detected in other systems including α-synuclein [127], amylin [128], immunoglobulin light chains [129], transthyretin [130], poly-Q [128], β_2-microglobulin [131], lysozyme [132], acylphosphatase from *Sulfolobus solfataricus* [133], and the SH3 domain [134]. These formations can be characterized as particles rich in β-structure with sufficiently high orderliness allowing for binding Congo red and thioflavin T. A peculiarity of the formations is the affinity of specific antibodies to protofibrils obtained from different sources, the absence of affinity to monomer units and mature particles, which suggests the presence of definite similar structural elements in soluble oligomer formations.

In some cases protofibrils may be off pathway aggregates [131, 135], but there are data showing that protofibrils represent a state that the protein overcomes during amyloidogenesis [116, 123]. It was found that the protofibril → mature fibril transition of peptide (109-122) from Syrian hamster prion protein proceeds through the adjustment of originally non-aligned β-regions forming a potential fibril [136]. This alignment includes isolation of β-regions with their subsequent inclusion into the potential fibril; however, internal rearrangement of β-regions is also possible, which is realized subject to conditions [137]. Summarizing the above, it can be stated that independent of the particular role of protofibrils in the formation of amyloid fibrils, the determination of the mechanism of formation of such oligomer particles as well as their structure is extremely important especially because it is believed that amyloid fibrils are the basic toxic formations involved in neurodegenerative diseases.

Further investigations of oligomer particles preceding the formation of fibrils have become feasible through the method of photoinduced coupling of unmodified proteins (PICUP). Thus, it has been found that soluble oligomers formed by the Aβ-peptide (both versions 1-40 and 1-42) exist in dynamic equilibrium with the monomer form. These oligomers consist of 2-4 monomers for Aβ 1-40 and 5-6 monomers for Aβ 1-42 and, as shown by CD methods, are relatively unstructured [138]. The interest in oligomer formations of Aβ was driven, in particular, by detection of such particles in the brains of patients with Alzheimer's disease [139] and also in lysates and the solution concentrate with cells expressing the precursor protein of Aβ [140, 141].

Region NM of yeast prion Sup35p is liable also to fast formation of unstructured oligomers. Transition with a growing β-structure takes place only after the formation of such oligomers, and then after transformation they can serve as seeding for fibrils [116]. The transition can occur due to covalent dimerization of NM molecules if the residues in the head of particle N (25-38) are cross-linked. Moreover, provided the oligomers are kept in an oxygen-enriched medium, intramolecular disulfide bridges form more promptly for the molecules in which cysteines are in the N region. These results apparently show that the interaction of the two head regions of molecule N generates a seed for an amyloid-like structure within the aggregate.

Similar behavior was detected for aggregation of denatured yeast phosphoglycerate kinase at low pH using dynamic light scattering and CD spectroscopy [142].

In this case, stabilization of β-structure occurred with growing dimensions of the aggregate. When a critical mass is accumulated, oligomers stick together and form short irregular protofibrils similar to those formed by Aβ and α-synuclein [142]. Moreover, the unfolding of the SH3 domain of bovine phosphatidylinositol kinase leads to fast formation of unstructured particles of different dimensions, which then transform sequentially into thin irregular protofibrils that bind thioflavin T [134]. So, the experimental data show that structured protofibrils can form either by sticking to each other or through rearrangement of small relatively unstructured oligomers formed at the very beginning of aggregation.

CONCLUSION

Many natural proteins can form amyloid fibrils. Nevertheless, the molecular mechanism underlying the formation of amyloids is still unclear. This is because a vast number of factors can affect the conformational transition from a "native" protein into pathological aggregates, including high protein concentration, specific proteolytic cleavage, mutations, interaction with ligands, and many other factors not yet determined. At present, many questions concerning the process of protein aggregation remain unanswered. A striking circumstance is that amyloidogenesis is specific for all living beings from microorganisms to humans. Based on this, it can be concluded that formation of amyloid fibrils has been tested by evolution and has preserved a conservative structure. The unique physicochemical properties of amyloids and the fact that many proteins form them with an ability to regulate the process as well as its being widespread in nature highlight the biological requirement for such formations. In spite of a great variety of neurodegenerative and other diseases associated with the formation of pathological protein aggregations, it can be proposed that the ability of proteins to form amyloid aggregates is connected first of all with multiple functions of proteins, and therefore the library of proteins generating "functional" amyloids will widen. Further studies are required to clarify the detailed mechanism of fibril amyloidogenesis and to reveal certain determinants affecting the kinetics of the process. But first and foremost is determination of possible key factors for controlling the process of aggregation to prevent development of pathologies and diseases caused by them,

or on the contrary to use them for amelioration of health, for example, for improving antiviral immunity or memory strength.

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