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Trends of Protein Aggregation in Neurodegenerative Diseases

Abdulkali Agbas

Abstract

Protein aggregation trends in neurodegenerative diseases are largely unmapped due to the complex nature of protein-protein interactions and their regulatory machineries such as protein proteolytic systems. Since the protein aggregation process in humans is a slow process, early determination of the patients that will develop neurodegenerative diseases later in life is critical in terms of starting effective treatment, which will reduce the expensive health care. In this chapter, I will discuss the nature of protein aggregation of signature proteins and the status of protein proteolytic systems such as proteasome and autophagosome in Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, frontotemporal lobar degeneration, Huntington's disease, and prion disease under the light of recent studies including our new findings.

Keywords: protein aggregation, protein misfolding, neurodegenerative disease, aging, proteinopathy, amyloid plaque

1. Introduction

Extracellular deposits of protein aggregates are often relevant to human diseases in general. Protein aggregates are the product of misfolded proteins that escape from protein quality checkpoints such as the chaperon/chaperonin system, heat shock proteins (Hs90, Hs70, etc.), proteasomes, and the autophagosome system. They are mostly insoluble and tend to form amyloid plaques over time. In this chapter, I will review trends of protein aggregation in the most studied neurodegenerative diseases such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), frontotemporal lobar degeneration (FTLD), prion, Huntington's disease, etc.

1.1 An overview for protein-folding

Biological self-assembly of proteins in a compact three-dimensional (3D) structure is the universal example of how the functional proteins can be separated from other biomolecules. This feature provides a functional advantage for proteins. 3D folding brings functional groups to close proximity creating a space where chemical reactions can occur; hence, the protein molecule becomes functional. Properly folded proteins need to maintain their stability which requires naturally interacting partners during their life term [1]. Failure of this native environment-protein interaction can lead to a wide variety of pathological conditions called *proteinopathy*.

Approximately 30 or more structurally different proteins have the potential to form an amyloid structure. Although there is no obvious homology in their primary structure, they all share a beta-pleated sheet (β -structure) as a polymer scaffold [3].

1.2 Energy landscape in protein-folding

Proteins in their native state, under the physiological conditions, are in a low energy state which provides thermodynamic stability [4]. With a large number of permutations, a systematic search for a stable polypeptide chain requires an enormous length of time ($\sim 1.6 \times 10^{15}$ trillion years). This makes it clear that the protein-folding process does not involve sequential steps. Cyrus Levinthal's calculation known as *Levinthal's paradox* reveals that proteins do not follow a folding process by trying every possible conformation; instead, they follow a partially defined pathway consisting of intermediates between fully denatured protein and its native structure (**Figure 1**) [1]. Two basic questions have not yet been answered: (i) what determines the correct folding state from the intermediate stage and (ii) how is the energy landscape unique to a specific protein-folding? Folding characteristics of small proteins (~ 100 amino acid residue) provide invaluable information about the amino acid sequence and energy landscape. A specific mutation in an amino acid sequence may provide critical information about the folding and unfolding kinetics [5]. Therefore, the energy landscape of certain signature proteins in neurodegenerative diseases may provide some critical information about the trends of such proteins that misfold and form aggregate. The problem lies on how to study the specific energy landscape of such proteins that are obtained from the patients (AD, ALS, PD, Creutzfeldt-Jakob disease, and Huntington's disease), which will predict the aggregate formation of the proteins. This will help in designing new drugs that either postpone or eliminate such aggregate formations; consequently, treatment options for neurodegenerative diseases may be possible.

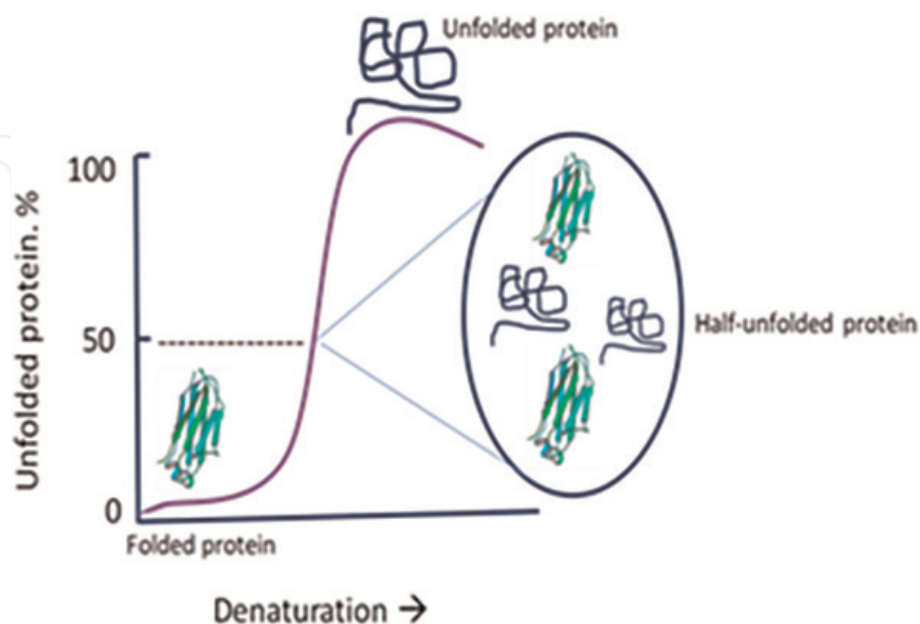


Figure 1.

Components of a partially denatured protein solution. In a half-unfolded protein solution, half of the protein species are fully folded and the other half are unfolded. This is an experimental condition; therefore, it is not known whether same or similar condition is existing in biology. The image is redrawn from 6th Edition of Biochemistry [1].

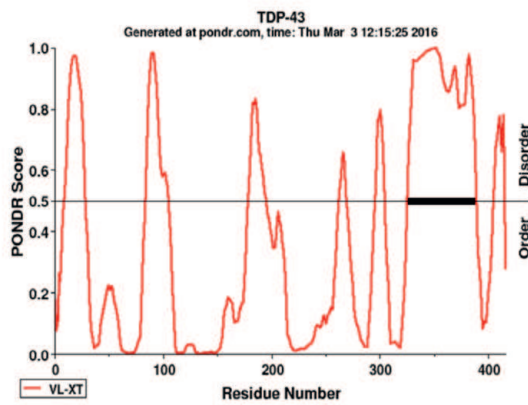
1.3 Protein misfolding in the cell

Although protein-folding principles are universal, the protein-folding environment needs to be taken into consideration in order to comprehend the protein-misfolding event. Some protein-folding is co-translational; they are initiated before leaving the ribosomal machinery upon completion of primary structure [6]. Most proteins undergo proper folding process in the cytoplasm after they leave the ribosome “quality control checkpoints” and began to interact with chaperones and heat shock proteins (HSPs). Recent studies reveal that molecular chaperones are essential not only in preventing misfolding but also in rescuing misfolded proteins even in their early stage of aggregation enabling them to have a “second chance” to fold correctly; this process requires ATP [7, 8]. Increased concentration of chaperone molecules and HSPs during cellular stress supports the notion that ATP is required [9]. Chaperonins, a subclass of chaperones, are the preferred molecules participating in the protein-folding process [10–12]. A possible chaperonin-naïve protein adverse interaction may very well initiate protein misfolding that will lead to protein aggregation. There are other proteins that complete their folding process in certain organelles such as the endoplasmic reticulum (ER) and mitochondria after being translocated into these organelles [7, 8]. The ER contains a large repertoire of molecular chaperons and folding catalysts [13, 14], making this organelle a major folding site and also the source of misfolded protein-related diseases [15]. Such organelles may utilize internal signals that allow certain proteins to penetrate into cell organelles to complete their folding. Wang et al. [16] recently demonstrated that a nuclear protein transactive response DNA-binding protein 43 kDa (TDP-43) penetrates into mitochondria using such internal signals and binds, preferably mitochondria-transcribed mRNA that encodes respiratory complex-I subunits (ND3 and ND6). This subsequently interferes with the proper assembly of complex-I and mitochondrial functions causing them to be impaired. The mitochondrial Hsp60/Hsp10 chaperonin system is essential for proper folding of proteins that are transported from cytosol to the inside of mitochondria via porins, and any mutation on this mitochondrial chaperonin system could be associated with neurodegenerative diseases [17].

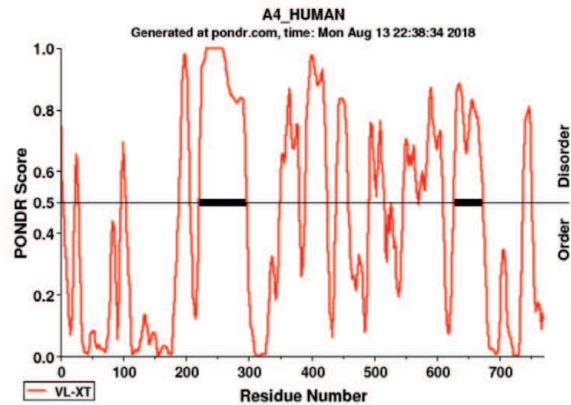
Many misfolded proteins that escaped the “quality control checkpoints” have exposed regions that are normally buried in the hydrophobic core of the protein. Such regions could inappropriately interact with other macromolecules within the crowded bioenvironment of the cytosol [18]. This leads to the initiation of protein aggregation that may be the foundation of protein-relevant disease, proteinopathy. The readers should have a broad perspective of diverse process like translocation across the membranes, trafficking, secretion, the immune response, and regulation of the cell cycle that are dependent on the protein-folding mechanism [19]. Any failure of proper folding or the escape from quality control checkpoints gives rise to cell malfunctioning and, hence, to development of a proteinopathy [20, 21]. Protein propensity can determine the probability of misfolded protein that has a relatively higher extracellular milieu. Such protein propensity can be analyzed by employing Predictors of Naturally Disordered Regions (PONDR) analysis. A few representatives of neurodegenerative disease hallmark proteins’ PONDR[®] analysis were performed based on their primary amino acid sequence, and the results were shown in **Figure 2**. All four proteins possess reasonably high levels of disorder region that makes the protein a good candidate to undergo aggregation process once the necessary environmental conditions are achieved.

Protein misfolding is likely to initiate the formation of the seed for aggregation. Therefore, researchers have studied the protein-folding chaperone machinery and HSPs in the context of neurodegenerative disease [22]. Mutant Cu/Zn superoxide

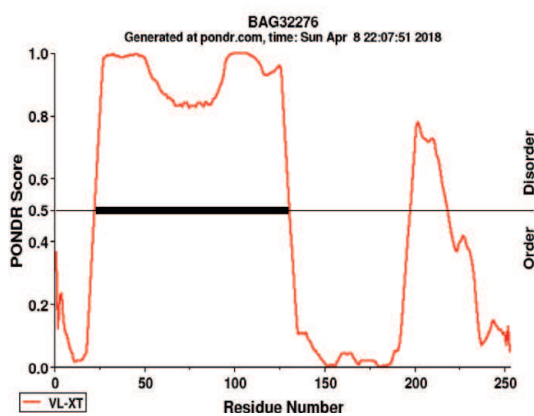
TDP-43



APP



Prion



α -synuclein

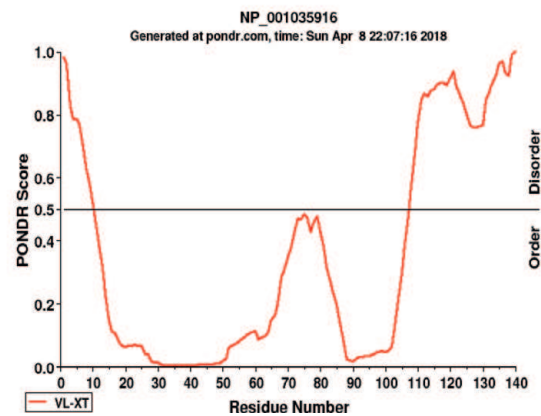


Figure 2.

Prediction analysis of some of the signature proteins for neurodegenerative diseases. PONDNR[®] score predicts the disorder probability for a given protein or polypeptide based on the amino acid sequence. Disordered regions are defined as the entire proteins or regions of proteins that lack a fixed tertiary structure. These figures represent disordered regions of major proteins in neurodegenerative diseases. The black rectangle on the 0.5 line indicates the region that was visible in the crystal structure with this protein bound to its binding partner (www.molecularkinetics.com; main@molecularkinetics.com) under license from the WSU Research Foundation. PONDNR[®] is copyright C_1999 by the WSU Research Foundation, all rights reserved).

dismutase (SOD1^{G93A}) abundant in motor neurons and HSP interactions was studied. The proposal was made and experimentally demonstrated that mutant SOD1 binding to HSPs (Hsp70 and Hsp25) makes this chaperone unavailable for their anti-apoptotic functions and eventually leads to motor neuron death [23]. Our laboratory has also demonstrated both in vitro and in vivo that mutant SOD1 failed to bind calcineurin (CaN) in a fashion that CaN lost its activity [24]. This failed interaction may yield the accumulation of hyper-phosphorylated protein aggregations [25] since CaN is one of the Ser-/Thr-specific phosphatase that removes the phosphate from proteins [26]. Under the light of existing literature, it is now known that a number of diseases such as AD, PD, prion disease, and typ. 2 diabetes are directly relevant to aberrant proteins that escaped from chaperone quality check system and form insoluble aggregates [21, 27–29].

1.3.1 Amyloid formation

Filament-like (fibrous) protein aggregates are generally referred to as amyloid. The word *amyloid* indicates a starch-like compound. It is an accepted term for a group of conformational disorders. About 30 or so proteins have the tendency to form amyloid structure, and they are involved in the well-defined amyloidosis

(*amyloidosis*: abnormal proteins called amyloids buildup in the tissue). Although there is no consensus homology in their amino acid sequence and molecular details of amyloid fibrils have some commonalities, among them are as follows: (i) all share β -sheet as a polymer scaffold; (ii) all show specific optical behavior on binding dye molecule Congo red, displaying long-unbranched and often twisted structures; and (iii) a characteristic cross-beta X-ray fiber diffraction pattern [3, 30]. Sequence characteristics of certain regions, especially at either N- or C-terminal, may predict the protein propensity to form amyloid fibrils. PONDR[®] (**Figure 2**) analysis shows known neurodegenerative disease protein's tendency to form amyloid fibers. The idea that the relative aggregate rates for a wide range of polypeptides and proteins correlate with the physicochemical features of the molecules such as charge, secondary structure propensities, and hydrophobicity [31] was experimentally supported.

It is now known that polypeptides or proteins that have propensity for β -pleated structure have a tendency to form amyloid plaques. These β -pleated-enriched proteins fall to the lowest energy level in the energy landscape (**Figure 3**), and they are more hydrophobic. Consider a globular protein; the main polypeptide chain and hydrophobic regions are buried in the core of the protein. When these regions are exposed to more hydrophilic environment due to partial unfolding caused by low pH, proteolytic fragmentation, etc., conversion to amyloid fibrillation becomes possible [9]. Amyloid fibril formation takes years before it reveals clinical manifestation, and the fibril formation follows a lag phase followed by a period of rapid growth [32, 33]. The fibril structure is measurable and determinable by laboratory techniques; however, it requires postmortem tissues. It is now critical to develop some approaches that utilize less invasively obtained biosamples (e.g., blood) so that protein fibrillation may be monitored and fibril formation can be restrained with at early stages as part of early treatment option.

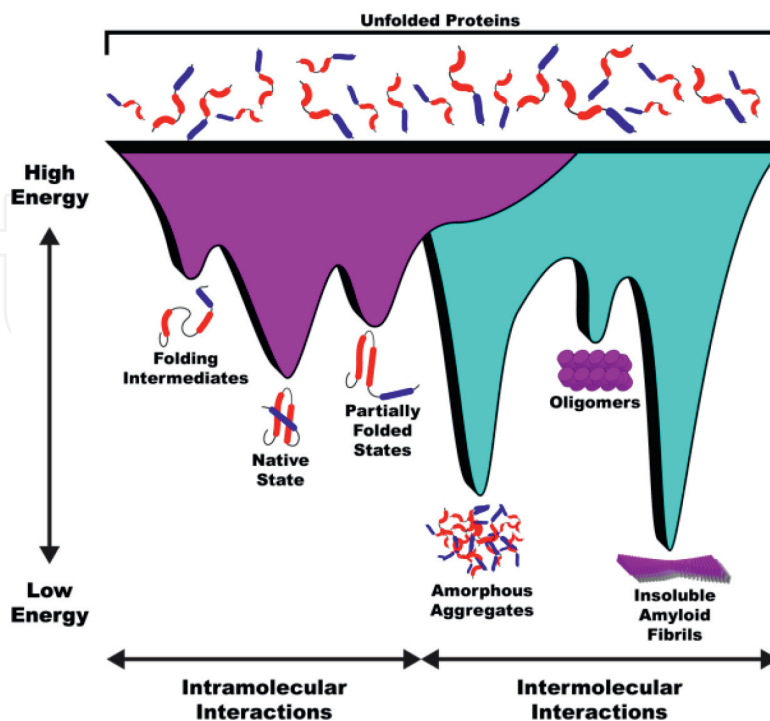


Figure 3. Energy landscape of protein folding and aggregation. The purple surface shows possibility of the conformations leading to the thermodynamically balanced state (native state). Cyan-colored area of the landscape indicates the conformations moving toward to amorphous aggregates of insoluble amyloid fibrils (adopted and redrawn, Vabulas et al. [34]).

1.3.2 Proteolysis-generated toxic protein species

Thermodynamic stability of a protein and its conformational kinetic determines the state of proper folding. Amyloid fibrils maintain the thermodynamically stable conformation in a highly organized hydrogen-bonded structure that is insoluble in aqueous media. This structure takes many years to progressively build up in tissues. Cellular homeostasis recognizes this event as toxic event and begins to encapsulate the amyloid fibrils in a plaque formation as part of the cellular defense mechanism. This plaque formation slows down and can eliminate further growth of the subsequent conversion of additional quantities of the same protein into amyloid fibrils [9]. However, readers should be aware that there are some naturally occurring nonpathogenic amyloid-like fibril formations such as the nanostructure of certain bacteria [35] and the mammalian melanocyte integral membrane protein [36]. The pathogenesis of amyloidogenic proteinopathy may be initiated with amyloidogenic peptide fragments by one or more proteases [37]. Human amyloid pathologies known to require proteolytic processing of a precursor protein include AD where A β peptide fragments are liberated from a large APP precursor protein by β - and γ -secretases [38]. A new potential biomarker for AD TDP-43 [39] may be involved in activating β -secretase that will generate A β peptide fragments [2]. **Figure 4** illustrates a simplified diagram of APP processing [2]. Modulation of A β generation by bio-metals was studied in both cell-free and cell-based assays. It was found that zinc (Zn²⁺) ion induces APP-C99 dimerization, which prevents APP cleavage by γ -secretase and A β production [40]. The same group reported that copper (Cu²⁺) ion was a γ -secretase inhibitor affecting APP processing [40]. These findings may suggest that the metal dyshomeostasis is a critical issue in generation of toxic protein species.

1.3.3 Proteasome malfunctioning

The β -sheet structure-enriched amyloid fibril formation relevant to protein aggregation is tightly controlled by molecular chaperones and the proteasome machinery. The proteasome is a large multisubunit complex that can be analogous to a food waste disposer. The proper function of such system is absolutely necessary for maintaining cell homeostasis [41].

It is expected that any proteasomal abnormalities may contribute to misfolding and protein aggregation diseases [42, 43]. In a pilot study, we observed that proteasome activity levels were reduced in plasma/platelet obtained from AD and ALS patients (**Figure 5**), while TDP-43 protein levels were increased in platelets

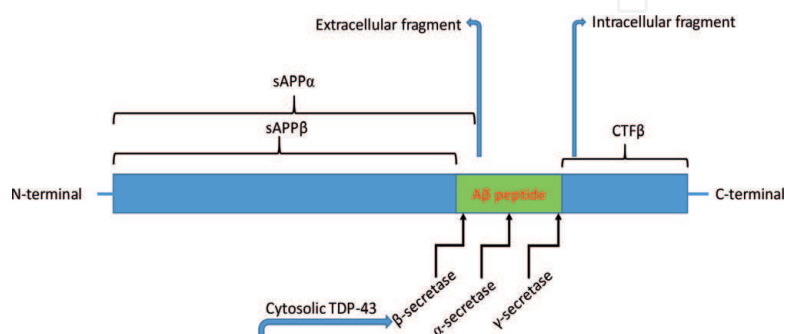


Figure 4. Simplified diagram of APP structure and processing. APP undergoes sequential proteolysis by β -secretase, α -secretase, and γ -secretase for the release of A β from the neuronal plasma membrane. TDP-43 has been shown to increase intraneuronal A β accumulation via increased β -secretase activation (adapted and modified from Ref. [2]).

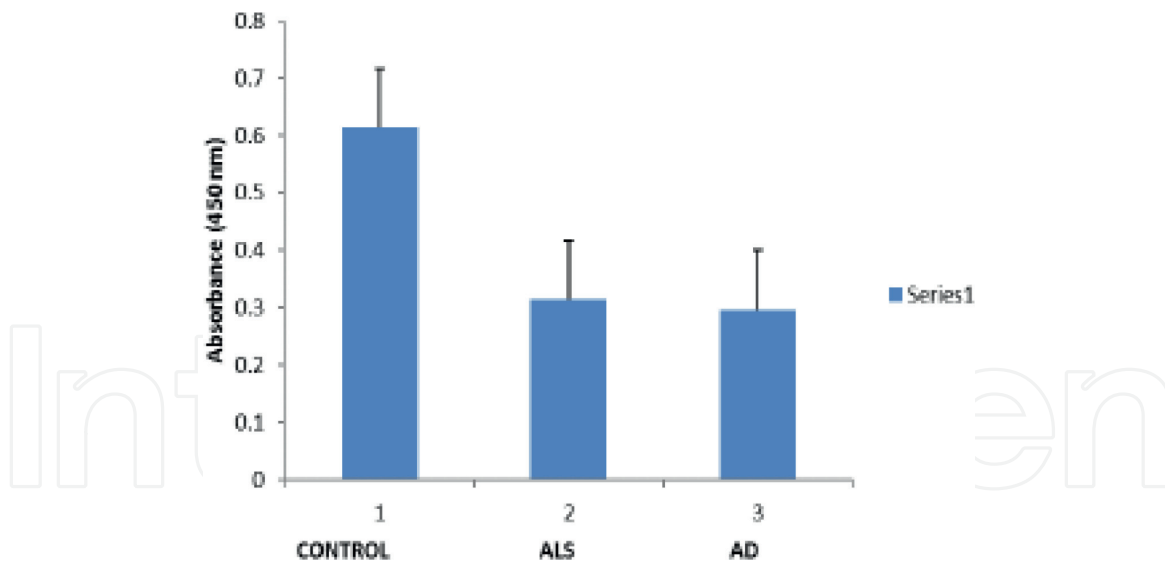


Figure 5.

Proteasome activity measurements in plasma. Extracellular proteasome activity levels were measured by proteasome 20S assay fluorogenic system (Enzo Biochem Inc. cat#BML-AK740-0001) designed to measure chymotrypsin-like protease activity of purified 20S proteasome. The detection of proteolytic activity is based on the release of three fluorogenic peptides. About 50% decrease in proteasome activity were observed in AD and ALS patient plasma ($n = 3$) (unpublished data).

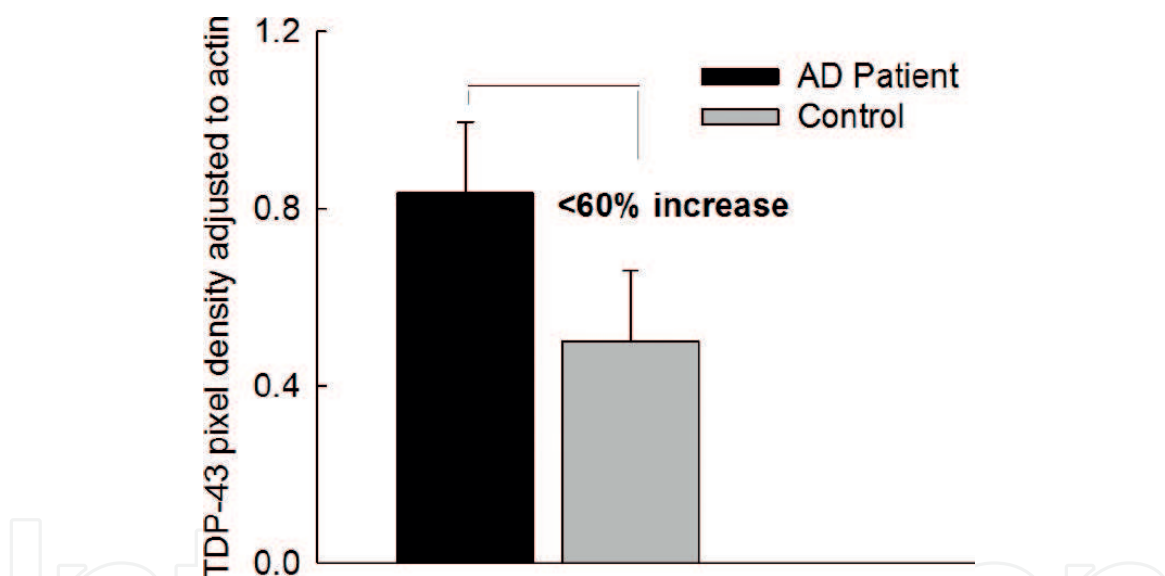


Figure 6.

Platelet lysate TDP-43 profile. TDP-43 protein levels were determined in platelet lysate by Western blotting method with a TDP-43 Ab (1:1000 dilution). Blood samples were obtained from AD patients ($n = 3$) and age-matched healthy subject ($n = 3$). Approximately <60% increase were observed in TDP-43 levels in AD patients (the figure was reproduced by permission [39]).

obtained from AD patients (**Figure 6**). This suggests that proteasome machinery was either malfunctioning or overwhelmed due to massive protein aggregation. Proteasome activity measurements in human plasma were successfully performed as a useful potential marker for various malignant and nonmalignant diseases [44]. Amyloid fibril formation that leads to abnormal protein aggregation may result in two functional consequences: (i) a toxic gain of function and (ii) a loss of function of the protein in question. Although the mature and organized protein fibrils are usually benign [32, 45], it is not well documented how disordered amyloid fibrils are being converted to malfunctioned protein species. One thought would be that the nonnative hydrophobic surfaces of the aberrant protein's interaction with cell membrane or other cellular components may initiate cell death [46].

2. Trends for misfolded proteins lead to neurodegeneration

Insoluble extracellular protein deposits in various human diseases have been recognized for a long time. Many proteins that have a tendency to be misfolded do form aggregates that initiate cellular dysfunction [47]. This section discusses how the proteins involved in neurodegenerative disease have a tendency to misfold. If we comprehend the biochemical and biophysical behavior of the proteins and their misfolding features, we will have a better understanding of proteinopathy and relevance to neurodegenerative diseases.

2.1 Common protein behavior in aggregation

In the last 25 years or so, many diseases have been linked to protein-misfolding cases, although their etiology of such diseases is different. In this section, the focus will be on neurodegenerative diseases because of the following reasons: (i) they are progressive, (ii) early diagnosis for these diseases are not available yet, (iii) they are not effectively treatable, and (iv) they inflict enormous personal, societal, and economic burdens. Some of them are aging relevant such as AD and PD; some of them are not like ALS, Creutzfeldt-Jakob disease (mad cow disease), and other human prion diseases (e.g., variant Creutzfeldt-Jakob diseases, Gerstmann-Straussler-Scheinker syndrome, fatal familial insomnia, and kuru).

Specific polypeptides that go into aggregation are different in each amyloidosis; however, there is a common feature in the behavior of these proteins; they all present enriched β -sheet structure. Such proteins are normally soluble in cytosol and in extracellular environment; however, somehow they progress into β -sheet-enriched insoluble filamentous polymers [47]. Not all β -structure-enriched insoluble filaments are an amyloid in nature. For example, some forms of SOD1-ALS are a conformational disease which involves amorphous aggregation of misfolded SOD1 [16, 48]. That is to say, the common structural motif in all amyloid fibers consists of cross- β -sheets. It is not uncommon that normally soluble proteins can undergo β -sheet-enriched conformational rearrangement and they tend to be more insoluble in nature. The concept of the β -sheet-enriched protein aggregation now becomes a common trend for polypeptide chains regardless of amino acid sequence [27]. The causes for the initiation of protein aggregation are not well documented; however, oxidative stress-induced reactive oxygen species (ROS) may be involved due to the role of ROS in several pathological disorders and aging [49, 50]. For example, glycine (Gly) residues are particularly susceptible for loss of a hydrogen ion, which results in the formation of Gly radical on the protein backbone which destabilizes protein structure [51]. Consequently, buried hydrophobic regions of the protein are exposed to the aqueous environment, and β -sheet structure formation is enhanced. These newly formed β -sheet structures link with that of neighboring structure which leads to the formation of a “seed” that eventually produces an aggregate [1].

2.2 Milestones for aggregate formation

Another characteristic in protein-folding disorders is a prolonged period in aggregate/plaque formation before clinical manifestation becomes obvious [47]. In aging-dependent neurodegenerative diseases such as AD and PD, aggregate/plaque formation is a lengthy process, while in ALS, SOD1, and TDP-43, aggregates are formed in the middle [52] or even younger ages [53]. One explanation for a lengthy process would be that the initial nucleation of a misfolded protein is very small and energetically stays in the upper level of the energy landscape (see **Figure 3**).

Therefore, the growth of the protein aggregate seed is kinetically unfavorable; hence, recruitment of new aberrant proteins for aggregate formation takes a longer period of time [54, 55]. Once the nucleation achieves a critical mass, the fibril formation and subsequent plaque formation become accelerated. This is why clinical manifestation of amyloidoses relevant to neurodegenerative disease mostly appears in old age. This is mostly true for sporadic AD; however, in familial AD, which represents only 5–10% of the total AD population, the disease onset tends to occur at the middle age (50 and above). In Down syndrome, β -amyloid precursor protein (APP), is encoded on chromosome 21 [47]. Patients with trisomy 21 develop abundant A β aggregates in the brain at younger age. Therefore, the lifelong aggregate formation is inevitable in Down syndrome patients, which supports the notion that Down syndrome patients are at high risk in developing AD. In the USA, it is estimated that more than a quarter million individuals live with Down syndrome and all will develop AD pathology as early as in their 30s [56]. In summary, protein aggregate formation starts at an early stage of life. This process is quicker in individuals with genetic conditions. Others display clinical signs at an old age as part of aging process.

2.3 Regional protein aggregation

Cellular misfolded proteins are inclined to accumulate in nearby organs, in a preferred cell type in a particular tissue [57], and in a particular cell organelle [58]. Although these proteins are distributed in systemic circulation, high concentrations can be maintained in organs. For example, A β fragments deposition in brain regions of AD patient, SOD1 and TDP-43 accumulation in spinal cord of ALS patients, and α -synuclein plaques in brain regions (neocortex, hippocampus, substantia nigra, thalamus, and cerebellum) of PD patients. Appearance of signature proteins (i.e., SOD1, TDP-43, α -synuclein, A β fragments, etc.) in systemic circulation supports the development of a surrogate biomarker in the blood when tissue sampling is not accessible [39].

It is interesting to note that misfolded aberrant proteins interact with apoptotic proteins in organ-specific organelle. Pasinelli et al. have demonstrated that anti-apoptotic protein Bcl-2 binds to detergent-insoluble mutant SOD1 (SOD1^{G93A}) protein aggregates that are present in mitochondria from the spinal cord but not in the liver in both mice and humans [58]. This observation suggests that misfolded aberrant protein functions are location specific. Valentine has reviewed studies on mutant SOD1 fragmentation in the Golgi apparatus, which may reveal early molecular signals before the onset of ALS symptoms [59]. There are more emerging studies in which emphasizing the region-specific protein aggregation can be considered a discriminatory signature in neurodegenerative diseases [60].

2.4 Systemic amyloidosis

The circulating proteins that have the potential to form extracellular amyloid deposits in multiple organs have been recently reviewed [47]. Local production of amyloid and non-amyloid protein species achieves the critical concentration for oligomerization and fibrillogenesis in specific organs. For example, A β deposition appears specifically in the brain tissue in AD. The Cu/Zn superoxide dismutase (SOD1) and TDP-43 deposition are measurable in brain and spinal cord tissues in ALS. These signature proteins can also be measurable in systemic circulation (**Table 1**).

We have recently published a paper describing platelet TDP-43 measurements as a proxy for brain tissue TDP-43 levels in AD patients [39]. This approach will aid

| Clinical syndrome | Fibril species |
|---|---|
| Alzheimer's disease | A β fragments (A β 36-43) in brain |
| Spongiform encephalopathies (mad cow disease) | Prion protein fragments in brain |
| ALS | Spinal cord and brain region (movement control region.) |
| Hereditary cerebral hemorrhage with amyloidosis | Amyloid β -peptide or cystatin C |
| Huntington disease | HTTExon1 |
| Parkinson's disease | α -synuclein |

Table 1.
Extracellular fibril types in disease

to monitor the progress of aberrant protein aggregation in neurodegenerative diseases.

In age-related neurodegenerative diseases, it is quite common to observe β -sheet-enriched protein aggregates that are mostly detergent-insoluble. These insoluble protein aggregates tend to accumulate inside the cell; however, ultra-structure analysis of these aggregates may not be the same as that of extracellular amyloid fibrils. The commonality of these aggregate-forming proteins was discussed earlier (Section 1.3.1). Therefore, it is reasonably acceptable to classify the aberrant protein aggregation-related neurodegenerative diseases as a special form of amyloidosis [47].

Protein misfolding and subsequent aggregation are central in neurodegenerative diseases; however, the protein behavior in forming aggregates is somehow disease specific. In case of the α -synuclein, this protein is natively folded and normally water soluble in the cell. In normal health conditions, α -synuclein participates in the maintenance of synaptic vesicle supplies at the presynaptic terminals [61]. In PD, this protein misfolds and accumulates in spherical filamentous structures called Lewy bodies. This encapsulated structure forms particularly in dopaminergic and noradrenergic brainstem neurons and causes premature cell death [62, 63]. Therefore, Lewy bodies become a signature pattern for PD. Polyglutamine repeat of corresponding proteins that are produced as the result of different mutant genes becomes a distinct pattern in Huntington's disease and several forms of familial spinocerebellar ataxia [64]. AD is the only brain disorder that displays the accumulation of amyloid forming proteins both extracellularly (A β fragments) and intracellularly (hyper-phosphorylated tau) [47]. The question would be whether hyper-phosphorylated tau neurofibrillary tangles or A β accumulation initiate AD. The amyloid cascade hypothesis has been the most studied model of molecular pathogenesis in AD. This is a long-debated issue in the scientific community which has polarized into two schools of thought: "Baptists" that believe A β accumulation is the starting event or "Tauist" that believes that tau-relevant neurofibrillary tangles are the initiators for AD [65]. However, inherited mutations in tau protein do not directly lead to AD; yet, it causes another devastating disorder, FTL with PD [66]. It is now more probable that inherited mutations in APP or in one of the APP cleaving proteases (e.g., presenilin/ γ -secretase) cause aggressive early onset forms of AD [67].

What are the initiation factors in sporadic AD which makes about 5–10% of all AD cases? Not much is known so far. A new player in AD field is TDP-43 protein which induces intramural A β accumulation via increasing β -secretase (BACE-1) [2]. As it can be seen in **Figure 4**, TDP-43 acts on an upstream in the APP structure and may induce β -secretase. A ripple effect may induce to generate toxic A β fragments.

Levels of γ - and β -secretase activities are greater in brain tissue samples from AD patients than non-demented control subjects [68, 69]. Experimental studies conducted on 3xTg-AD (swAPP, PS1-M146V, tau-P301L) demonstrate that BACE-1 activity levels were elevated in the brain tissue and γ -secretase inhibitors reduced the BACE-1 activity, suggesting that γ -secretase mediates oxidative stress-induced expression of BACE-1 resulting in excessive A β production in AD [70]. Extracellular cleavage of APP by BACE-1 creates a soluble extracellular fragment and a cell membrane-bound fragment referred to as C99. Cleavage of C99 within its trans-membrane domain by γ -secretase releases the intracellular domain of APP and produces A β . Since γ -secretase cleaves APP closer to the cell membrane than BACE1 does, it removes a fragment of the A β peptide. Initial cleavage of APP by α -secretase rather than BACE-1 prevents eventual generation of A β [71]. It is clear that the most notable neurodegenerative diseases (i.e., AD, ALS, FTLN, and PD) share a common prominent pathological feature, TDP-43 proteinopathy. This issue has been recently reviewed, and possibility of targeting TDP-43 as a common therapeutic approach to formulate a treatment for neurodegenerative diseases was discussed [72]. In our laboratory, we are also working on an assay methodology that uses peripheral blood-derived platelet TDP-43 profile that may help for early diagnosis of such diseases so that early treatment options could be available [39].

2.5 Aging and protein aggregation

Aging is the normal biological process that includes increased protein misfolding and aggregation process due to either reduced levels of quality control checkpoints such as chaperone system or proteasome complex. The proteins that form orderly (amyloid fibers) and disorderly (amorphous aggregates and plaques) show some common and essential biochemical and biophysical features that were discussed earlier. Therefore, such age-related neurodegenerative diseases may be considered a special form of amyloidoses [47]. The biochemical processes in aging are contributing to free radical-induced protein oxidation; hence, unnatural disulfide bridge formations can contribute oxidized protein aggregation as well [73]. Dismutase metalloenzyme (e.g., SOD1 and SOD2) activity levels are also reduced in aging [74, 75]. This contributes the inefficient removal of reactive oxygen ions; hence, more protein oxidation events take place, and subsequently protein aggregation occurs. Principal unanswered questions about these neurodegenerative disorders remain: how precisely native soluble proteins undergo partial unfolding, and does aberrant refolding produce highly stable polypeptide polymers? We can still make some predictions about the propensity of a given protein through PONDR[®] analysis. This publicly available online prediction program (www.pondr.com) can be utilized for predicting which regions of the protein will be more susceptible for disorderliness which may increase the chance of aberrant protein refolding [76, 77]. It is clear that time and supraphysiological concentrations of predictable pro-aggregate proteins are two important parameters in aggregation process. Other factors such as oxidizable amino acids (e.g., cysteine and methionine), population, local pH, and higher hydrophobic propensity of the protein help the oligomerization process. Equilibrium between natively folded protein and aberrant-folded protein can last for a long period of time. One approach would be that molten globule-like intermediates have persistent structure in unstable α -structure. Stable β -structure of the protein provides a template (seed) for the recruitment of additional peptide chains through physical interaction of those two structural regions of the protein. Finally, new hydrogen bonds form and stabilize the protein in an insoluble amyloid fibril [78, 79]. On the other hand, fibril deposition is not a necessary feature in prion disease. NMR structure of a domain of prion protein (PrR(121–231)) indicates that

mutated amino acids in prion protein are involved in the maintenance of the hydrophobic core [80]. Exactly, how prion conversion propagates? The disease is currently under study [46]. As mentioned in Section 1.3, the ER contains a large repertoire of molecular chaperones and folding catalysts [13, 14]. The ER protein-folding system is also affected by aging process, and less fold-assisting proteins would be available; hence, unfolded and misfolded protein levels would be expected to be high. Two of the unconventional ER chaperone molecules are calnexin (Cnx) and calreticulin (Crt) [81–83] as cited in [84]. In a pilot study, we demonstrated that Cnx levels were reduced in aging rat brain as well as in neuronal cell culture (Figure 7). This observation supports the other works published in literature stating that protein-folding mechanisms are less efficient; therefore, aberrant-misfolded proteins rise and form aggregates.

2.6 Misfolded aberrant proteins cause cell dysfunction

Dynamic equilibrium between misfolded and natively folded proteins may be shifted in favor of protein misfolding and oligomerization in proteinopathy. No definite amyloid fibrils are seen in diseases, suggesting that smaller diffusible toxic protein species consisting of dimers, trimers, tetramers, and large oligomers may be involved in cell cytotoxicity [47]. Therefore, forming a plaque or aggregate may be considered a defense mechanism of cell against recruitment of more toxic protein species. The aggregate in plaque no longer poses toxicity for cell life; however, having such foreign structure in the cytosol or in extracellular milieu brings some serious problems in cell homeostasis. For example, the plaques are insoluble and indestructible by ubiquitinated proteasome system. The increased population of such aggregates may overwhelm or even block the proteasome machinery. Therefore, proteasome either slows down or becomes less functional. We have observed such reduced proteasome activity in AD and ALS cases (Figure 5).

Another interference of such toxic protein species is that they nonspecifically bind to receptors and channel proteins on the plasma membrane, thus interfering with numerous cell-signaling events [16, 85]. Mitochondrion homeostasis should be

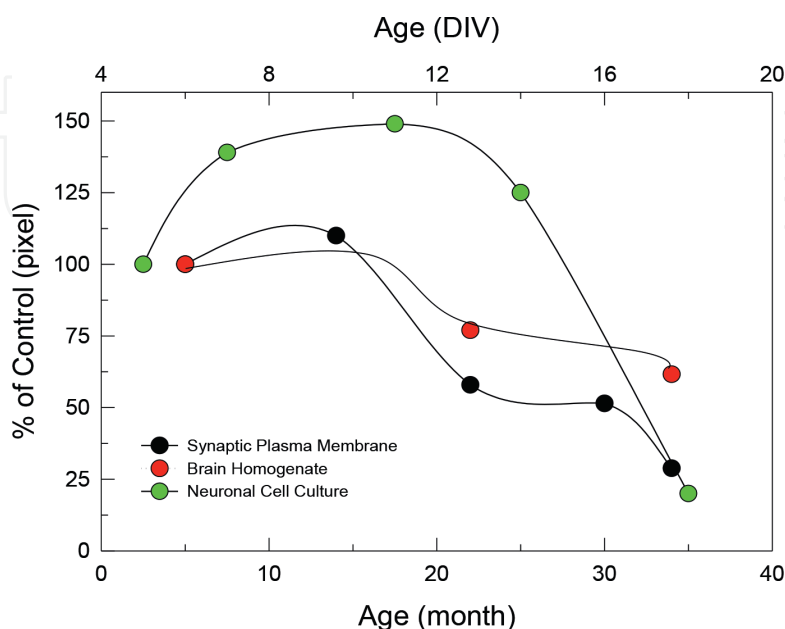


Figure 7. Calnexin protein levels in aging. Calnexin protein levels were analyzed by Western blotting. Cnx levels were reduced during the aging process in total brain homogenate, synaptic plasma membrane, and neuronal cell culture. All samples were prepared from rat ($n = 3$) (unpublished data).

taken into consideration as well. Many mitochondrial proteins are nuclear-coded and transported into mitochondria [86]. These proteins are being transferred into mitochondria via outer membrane pores. Although these pores are specific, such small toxic protein species can nonspecifically bind the pores and slow down the protein entries into mitochondria if not completely block. Consequently, energy production mechanism of mitochondria may be compromised. Such hypothetical ideas need to be experimentally tested and should provide more convincing data about the cytotoxic effects of such protein species. Misfolded protein species get involved in apoptosis induction. Pasinelli et al. have reported that anti-apoptotic protein Bcl-2 interacts with both *wt* and mutant SOD1 (SOD1^{G93A}) [58]. This interaction induces apoptotic cascade because SOD1^{G93A} mitochondria triggers apoptosis more strongly than the cytosolic mutant SOD1 [87].

3. Therapeutic approach to protein-misfolding disease

Proper protein-folding in the cell occurs either in the cytoplasm or within the secretory pathway. Dobson has reviewed this concept in detail [9]. The readers are referred to this review to attain more in-depth understanding of the protein-folding relevant issues. The trend of misfolding protein increases when part of the polypeptide chain does not participate in a proper folding process. However, there are mechanisms that are available for aiding protein-folding. Chaperones are the molecules that collaborate with misfolded proteins to give a polypeptide chain several opportunities to fold. ATP-dependent chaperone molecules are critical for ensuring accuracy in proper folding [88]. The lumen of the ER also participates in protein-folding process by modifying the secretory proteins while they are still associated with the ER [89]. Any of these mechanistic failures contribute misfolded protein accumulation. Despite the attempt to rescue misfolded proteins that are destined to form insoluble aggregates, *proteasomes*, protein aggregate removal machinery, degrade such proteins from the cell so that cell homeostasis be maintained.

Several other protein misfolding-relevant diseases are caused by conformational modifications in extracellular milieu. Protein quality control-check systems of the cell (chaperones and heat shock protein family) cannot be linked to such misfolded protein population because such aggregation formation does not take place in cytosol. However, recent studies demonstrate the presence of extracellular proteasome machinery [92–94]. It is not clear yet whether these extracellular proteasomes are in the same categories in that of cytosolic since a recent report demonstrated that such extracellular proteasomes structurally differ from their cytosolic counterparts [94].

The major representatives of such disorders are the amyloidoses, in which protein aggregation in the extracellular space is associated with the presence of malfunctioned protein molecules [37, 90]. The chaperone-like small molecules may have the potential to be included in the treatment options for amyloidoses [91]. The more knowledge we attain on how chaperones and heat shock proteins interact with protein-folding process the better design for small molecules would be feasible.

Folding process of proteins is an environment-dependent physicochemical process. Some proteins have a folding issue where protein-folding takes place (i.e., lysosomal enzymes) while the others are efficiently folded in the ER but misfolded and misassembled at the destination (i.e., amyloidogenic proteins). This knowledge is helping industry-academia partnership for developing pharmacological intervention that reduces the mutant protein production, increases the rate of clearance of misfolded/mildly aggregated proteins, and increases the native stability of the proteins.

4. Conclusions

The majority of non-treatable neurodegenerative diseases are related to misfolding protein-induced aggregation and insoluble plaque formation. This process is very slow which makes early diagnosis of neurodegenerative diseases almost impossible at this time. However, some predictive studies may help to identify the proteins that have a tendency to form amyloid plaques. To know protein behavior in various physiological conditions and environmental factors will contribute to designing disease-specific drugs that interfere the aggregation formation in neurodegenerative diseases.

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Conflict of interest

The author declares no conflict of interest.


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