Sri Lankan J. Biol. 2026, 11 (1): 18-33

ISSN: 2513-2245
DOI: https://doi.org/10.4038/sljb.v11i1.185

Review Open Access

From Protein Folding to Misfolding: Pathways to Disease and Therapeutic Directions

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Proteins are complex macromolecules that perform a variety of biological functions in the living cell only when they are in their native state. Anfinsen's experiment shows that the native structure of a protein depends on the primary structure of the protein. The native state of protein is attained by protein folding, and this process is initiated by different intra- and intermolecular forces. Thermodynamic stability plays a key role in guiding protein folding, as proteins continuously shift and adjust their structure until they reach their most stable form. Molecular chaperones, cellular enzymes, and other cellular mechanisms drive protein folding. Despite all the guiding factors, proteins may misfold and form toxic aggregates of amyloid fibrils, and it can develop neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and prion diseases. Recent studies show that the incidence of neurodegenerative diseases associated with protein folding is becoming a threat to global health. Unfortunately, these diseases are incurable, and therapeutic approaches are currently being researched by scientists to mitigate this issue. Thus, study of protein folding and misfolding remains a key area of research in the bio-medical field.

Received: 29 Nov 2024

Accepted: 10 Feb 2025

Keywords:
Amyloid fibril;
Native state;
Neurodegenerative diseases;
Protein folding;
Protein misfolding.

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1. Introduction

Proteins are precisely constructed complex macromolecules that are present in every living organism, with ribosomes responsible for protein synthesis through major steps of transcription and translation (Carter and Houlihan, 2001). Proteins are responsible for diverse and highly specific functions in cells, ranging from acting as building blocks of hair to regulating neural activity in the brain. All these specific biological functions can only be attained if the protein is in the three-dimensional (3D) conformation or native conformation. Proteins predominantly attain their 3D conformation through a process called "Protein folding," where amino acids interact with each other through various kinds of interactions such as hydrophobic interaction, hydrogen bonds, disulfide bonds, and ionic bonds in a thermodynamically stable state (Blanco and Blanco, 2022). The amino acid sequence in the peptide chain controls the way protein folds and this can be explained by Anfinsen's experiment on protein folding (Szilágyi *et al.*, 2007). However, the exact mechanism of protein folding is very complex and still not fully understood. Proteins can sometimes misfold due to various factors, rendering them biologically inactive. Our body has an active biological system to repair the misfolded protein to properly folded protein by providing a

suitable microenvironment or special systems to eliminate the misfolded protein from the cell. If these misfolded proteins are not regulated or eliminated by the body, they may become toxic.

The pathological consequence of protein misfolding is referred to as protein aggregation, which leads to the formation of insoluble amyloid fibrils. Aggregation of misfolded protein causes neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and prion diseases. These diseases have the potential to cause toxicological effects ranging from insomnia to brain death (Prasansuklab and Tencomnao, 2013). Currently, no definitive cure exists for these disorders, and available treatments primarily alleviate symptoms without addressing the underlying pathology. Therefore, a comprehensive understanding of protein folding, misfolding, and their pathological implications is crucial for the development of future therapeutic strategies.

This review explores the fundamental principles of protein folding and misfolding. Additionally, it discusses the pathological implications of protein misfolding-related diseases, current therapeutic strategies to mitigate their effects, and potential future directions in treatment development.

2. Protein structure

Protein structure can be classified into four categories: primary, secondary, tertiary, and quaternary structures. The primary structure is basically a polypeptide chain consisting of a linear amino acid sequence. Polypeptide chains are formed from the N-terminus to the C-terminus, complying with the ribosome's synthesis direction (Carter and Houlihan, 2001). The polypeptide chain is formed by peptide bonds between the amino acids. But primary structures are non-functional. It should transform into a 3D form, such as a secondary structure, to be functional. Alpha helices and beta-pleated sheets are the major secondary forms of protein. Hydrogen bonding between the carbonyl oxygen and amide hydrogen is essential for the formation of various helical structures. Specifically, the hydrogen bond formed between the peptide C=O group and the N-H group of the fourth amino acid in the sequence stabilizes the helical conformation. A helix is formed for every 3.6 amino acids (Littlechild, 2013). Typically, the alpha helix adopts a right-handed twist (Doig *et al.*, 2005), while left-handed alpha helices are rare. This is primarily due to the prevalence of L-amino acids in proteins, whose molecular structure promotes a right-handed twist, thereby maximizing hydrogen bond formation and stabilizing the alpha helix (Vasudevan and Vaidyanathan, 2016). R groups of amino acids reside on the outside of the helix and perpendicular to the axis of the helix. The zigzag polypeptide chains can be positioned side by side to form a structure that looks like a sequence of pleats. This arrangement is called beta sheet, where hydrogen bonds are formed between C=O and N-H of adjacent segments of the polypeptide chain (Kuhlman and Bradley, 2019). Polypeptide chains in a sheet might be antiparallel or parallel. If adjacent polypeptide chains run in the same direction (both chains extending from the N-terminus to the C-terminus), they are considered parallel. If they run in opposite directions, they are antiparallel. However, the secondary structure alone does not confer biological functionality. Consequently, the protein further folds into a tertiary structure, which represents the three-dimensional conformation of the polypeptide chain in its native, functional state. The amino acid sequence in the polypeptide chain of the tertiary structure determines the distinct 3D structure. Interactions between the functional groups will stabilize the tertiary structure of proteins. Hydrophobic interaction, disulfide bonds, hydrogen bonds, and ionic bonds are the major interactions that stabilize the tertiary structure of the protein (Blanco and Blanco, 2022). Some proteins are functional in their tertiary structure, while others require additional organization into a quaternary structure. For instance, the myoglobin protein is biologically functional in its tertiary form, whereas hemoglobin functions in its quaternary form (Ruiz-Larrea, 2002). The quaternary structure consists of two or more distinct polypeptide chains, which may be identical or different, and is stabilized by the same forces that stabilize the tertiary structure, primarily non covalent interactions.

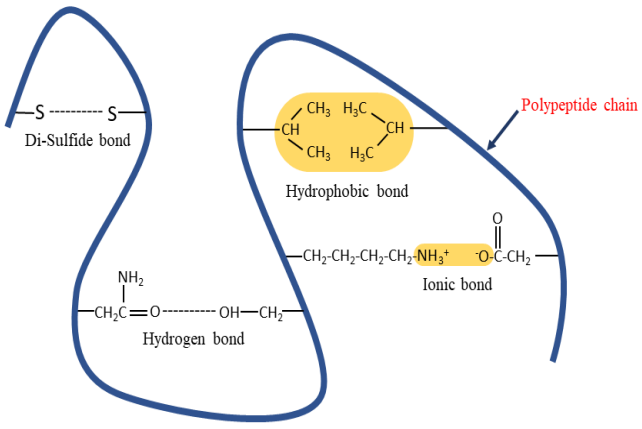

Figure 01: Different type of bonds in protein

3. Driving force for the protein folding

Protein folding is initiated by different types of mechanisms such as interaction between molecules, thermodynamic driving force, enzymatic driving force, and chaperones present in the cell. These mechanisms altogether contribute to the proper folding of protein.

3.1 Molecular interactions

Hydrophobic interactions, van der Waals forces, ionic interactions, and hydrogen bonds all play significant roles in protein folding and the stabilization of tertiary and quaternary structures (Blanco and Blanco, 2022). However, hydrophobic interactions are the primary driving force behind protein folding (Shakhnovich, 2006). Nonpolar, hydrophobic residues in polypeptide chains tend to avoid the aqueous cellular environment. Consequently, these residues cluster together in the interior of the protein, minimizing interaction with water. This phenomenon drives the initial collapse of the polypeptide chain into a compact structure, facilitating the formation of a stable protein conformation (Zhou *et al*., 2004). This compact structure of the 3D model is supported by hydrogen bonds between the backbone carbonyl oxygen and amide hydrogen atoms, which stabilize alpha helices and beta sheets of the secondary structure. Hydrogen bonds also occur between side chains. When a protein approaches its 3D conformation, van der Waals forces act between the atoms of the protein chain, helping to tightly pack the protein structure (Schaeffer and Daggett, 2011). Ionic bonds or salt bridges can develop between the oppositely charged side chains of polypeptide chains, such as aspartate and lysine. All these interactions make a way for protein folding. But protein folding is a completely random process where every atom in the protein chain tries every possible interaction to form a biologically active 3D structure. However, thermodynamically stable proteins can only act as native proteins. To attain thermodynamic stability, folded proteins should have the lowest Gibbs free energy (Shirdel and Khalifeh, 2019).

3.1.1 Anfinsen's experiment

Christian Anfinsen, who is a Nobel prize winner, proposed a theory that a protein's three-dimensional structure is determined by the amino acid sequence in the primary structure (Szilágyi *et al.*, 2007). To test this, Anfinsen unfolded a protein and observed whether it would refold correctly in a test tube.

To conduct this experiment, Anfinsen used ribonuclease A, an enzyme that breaks down RNA, as the protein sample. He prepared two test tubes containing ribonuclease A and added a reducing agent (β-mercaptoethanol) and 8 M urea to each sample. The reducing agent broke the disulfide bonds that stabilize the ribonuclease A structure, while the urea unfolded the protein by disrupting the hydrogen bonding network and suppressing the forces driving protein folding.

In the first sample, Anfinsen removed the urea through dialysis and then added an oxidizing agent. This oxidizing agent reformed the disulfide bonds that were broken by the reducing agent, and the sample regained 90% of its original enzymatic activity. This result demonstrated that the protein had mostly refolded into its correct three-dimensional structure.

In the second sample, Anfinsen reversed the final two steps, first oxidizing the protein and then removing the urea by dialysis. This sample retained only 1–2% of its original enzymatic activity, indicating that the protein did not refold correctly. The presence of urea prevented the formation of hydrogen bonds, continuing the unfolding process and impeding proper protein folding.

Simultaneously, oxidizing agents can induce the formation of disulfide bonds within the protein, potentially locking the protein into a non-native

conformation. Therefore, the order of breaking and regenerating disulfide bonds is crucial, as these bonds stabilize the final structure of the protein. Anfinsen ultimately demonstrated that the correct formation of disulfide bonds is essential for proper folding.

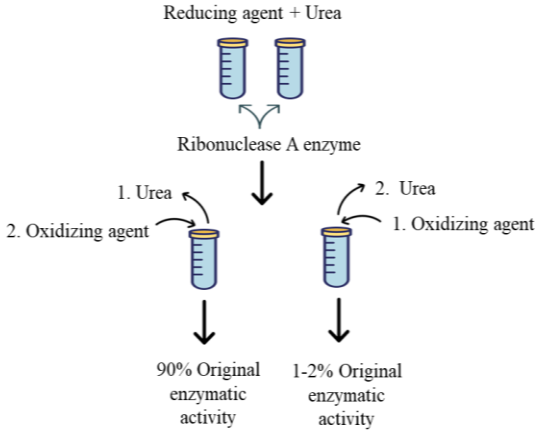

Figure 02: Anfinsen's experiment

However, the formation of disulfide bonds is determined by the amino acid sequence in the primary structure. This supports the idea that the information required for a protein to fold correctly is encoded within its primary structure.

3.2 Thermodynamic aspect of protein folding

Protein folding is a spontaneous and complex mechanism that is governed by thermodynamics. Thermodynamic stability is one of the primary factors that directs protein folding since proteins constantly move and modify themselves until they achieve their most stable state (Sorokina *et al.*, 2022). The thermodynamic analysis of protein folding involves the free energy, enthalpy, and entropy of the system. All these factors mentioned earlier can be put in a Gibbs free energy equation (Benítez and Jiménez, 2025).

$$\Delta \mathbf{G} = \Delta \mathbf{H} - \mathbf{T}\Delta \boldsymbol{S}$$

The amount of energy available to perform work in a system is known as the free energy (ΔG). A negative ΔG indicates that the protein will fold spontaneously, whereas a positive ΔG indicates that the protein will not fold spontaneously (Chong and Ham, 2014). The heat that a system absorbs or releases throughout a process is known as its enthalpy (ΔH). When new bonds form between atoms, the system often releases energy (negative ΔH). Breaking existing bonds typically requires energy input (positive ΔH). In protein folding, enthalpy change indicates bond formation between amino acid residues. As mentioned earlier, these bonds can be hydrophobic, van der Waals, ionic, and hydrogen bonds (Shakhnovich, 2006). Entropy (ΔS) represents the degree of molecular disorder or arrangement. A negative entropy value indicates an ordered molecular arrangement, whereas a positive entropy value signifies a high degree of disorder. An unfolded protein has positive conformational entropy, as its amino acid chain can adopt multiple configurations, resulting in significant disorder (Bachmann and Kiefhaber, 2005). Upon folding into its functional three-dimensional structure, the protein's conformational freedom is reduced, leading to a decrease in entropy. Consequently, spontaneous and continuous folding and unfolding do not occur, allowing the protein to attain a stable conformation.

According to thermodynamic principles, protein folding is favored when the Gibbs free energy change (ΔG) is negative. A more negative ΔG increases the likelihood of spontaneous protein folding. For this to occur, enthalpy change (ΔH) should be negative, and entropy change (ΔS) should be positive. However, protein folding involves a transition from a highly disordered, unfolded state to a more ordered, folded state, resulting in a negative ΔS for the protein itself. Since a negative ΔS increases ΔG, protein folding may not appear thermodynamically favorable. This is where the role of water becomes crucial. Water molecules, due to their polar nature, form extensive hydrogen bonds with each other. In the unfolded state, hydrophobic amino acid side chains are exposed to the aqueous environment, leading to interactions between the protein's hydrophobic groups and the hydrogen-bonded water molecules. This forces the surrounding water molecules into an ordered arrangement, restricting their natural movement and creating an energetically unfavorable state. To minimize this unfavorable condition, hydrophobic amino acid side chains aggregate inward, burying themselves within the protein's core. This process, known as the hydrophobic effect, results in the release of ordered water molecules, increasing the overall entropy of the system. The increase in solvent entropy compensates for the decrease in protein entropy, ultimately driving spontaneous protein folding (Zhou *et al*., 2004). This aggregation of hydrophobic residues expels water from the protein's interior. Once free from interacting with the hydrophobic groups, these water molecules become more disordered (higher entropy) as they re-enter the bulk solvent. This increase in entropy among water molecules is a key factor in the thermodynamic driving force behind protein folding (Plotkin and Onuchic, 2002).

The critical point here is that the increase in entropy of the water molecules (positive ΔS) can balance the decrease in entropy of the protein itself (negative ΔS) (Szilágyi *et al.*, 2007). If an increase in water entropy is greater than the loss in protein entropy, the overall ΔG becomes negative, thereby favoring the folded state.

3.3 Enzymatic driving force

There are two types of enzymes that regulate the protein folding pathways: Protein disulfide isomerase (PDI) and Peptide prolyl cis-trans isomerase (PPI) (Fu *et al.*, 2020; Schmid *et al.*, 1993). PDI facilitates the interchange of disulfide bonds and the elimination of folding intermediates, which have inappropriate cross-links. PPI catalyzes the interconversion of cis and trans isomers in pro-peptide bonds. For example, Proline is a unique amino acid that restricts the peptide bond rotation, if bond rotation is restricted, it may limit fast protein folding. PPI enzymes can switch the conformation of this bond, allowing the protein to adopt different conformations during folding (Nagradova, 2007).

3.4 Chaperones assisted protein folding

As soon as proteins are synthesized by the ribosome, it takes some time for the protein to fold. During this time interval, those unfolded proteins may aggregate

due to the hydrophobic interaction between each other. In these cases, specialized proteins called molecular chaperones guide the unfolded polypeptides to the proper folding process by giving a suitable microenvironment. Molecular chaperones are protein that interacts with misfolded proteins and stabilizes or help the protein to acquire its functionally active conformation, without being present in its final structure (Hartl *et al.*, 2011). Chaperons, which are involved in protein folding, are known as Stress proteins or heat-shock proteins (HSP). Chaperones are generally classified based on their molecular weight into categories such as HSP40, HSP60, HSP70, HSP90, HSP100, and small heat shock proteins (sHSPs) (Macario *et al*., 2013). Among these, the major chaperones involved in protein folding include HSP70, HSP60 (chaperonins), and HSP100, each performing distinct functions: HSP70 acts as a holdase, preventing premature aggregation; HSP60 facilitates proper folding as a foldase; and HSP100 functions as a disaggregase, aiding in the refolding or degradation of misfolded proteins (Fernandez-Funez *et al*., 2016). As a newly synthesized protein emerges from the ribosome, it initially exists in an unfolded state without a defined structure. The intracellular environment is highly crowded, with proteins occupying approximately 15–35% of the total cellular volume (Brown, 1991). This dense environment increases the likelihood of interactions between unfolded proteins. Due to their exposed hydrophobic regions, these unfolded proteins can undergo hydrophobic interactions with adjacent polypeptides, promoting aggregation. Consequently, unfolded or partially folded proteins may cluster together, forming aggregates that can be detrimental to cellular function.

Holdase chaperones, such as HSP70, recognize and bind to the hydrophobic regions of unfolded or partially folded proteins, preventing aggregation and cellular damage (Sučec *et al*., 2021; Sharma et al., 2009). This binding process occurs without ATP (Ben-Khoud *et al*., 2023). Holdases temporarily stabilize proteins until foldase chaperones become available to facilitate proper folding. If conditions remain unfavorable, the holdase continues to prevent aggregation until folding is possible or the protein is targeted for degradation.

Foldases (HSP60) are located inside the mitochondria (Cheng *et al*., 1990). They differ from holdases in that they are ATP-dependent chaperones (Ben-Khoud *et al.*,2023). They bind to ATP molecules and use the energy released from ATP hydrolysis to drive their protein-folding functions (Ostermann *et al.*, 1989). Foldases /chaperonins assemble into a barrel-shaped complex that encloses the unfolded protein and misfolded proteins within its cavity (Bhakta *et al.*, 2024). This barrel-shaped encapsulation is called an "Anfinsen cage". This cage provides a suitable microenvironment for protein folding. Inside the cage, weak bonds, such as hydrogen bonds and ionic bonds, are broken, causing the unwinding of misfolded protein (Motojima, 2015). Proteins undergo conformational exploration within the HSP60 chamber until they attain their native structure, after which they are released to perform their specific mitochondrial functions. Despite the actions of holdases and foldases, proteins may misfold due to errors in synthesis, cellular stress, or genetic mutations (Valastyan and Lindquist, 2014). Misfolded proteins can aggregate, disrupting cellular processes and potentially leading to disease. Disaggregases recognize these protein aggregates and use ATP hydrolysis to unwind misfolded chains, providing an opportunity for refolding. This allows holdases to rebind the unfolded proteins, preventing further aggregation. HSP100 is the predominant chaperone with disaggregase activity (Kumar *et al*., 2015).

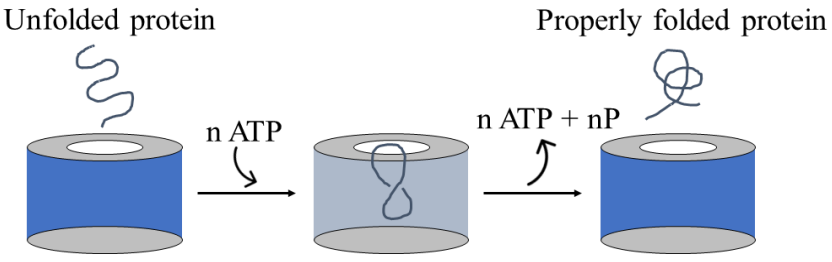

Figure 03: Simplified view of Anfinsen cage mediated protein folding

4. Protein misfolding

Protein folding is a spontaneous process in which synthesized polypeptide chains explore numerous conformations to achieve their native structure. Properly folded proteins exhibit high stability and solubility, preventing aggregation by sequestering hydrophobic regions and a significant portion of the peptide backbone within their interior. However, proteins may deviate from the correct folding pathway, resulting in misfolding. This misfolding can be triggered by factors such as cellular stress, genetic mutations, or intrinsic instability of the protein conformation.

In vitro studies suggest that stressful conditions such as extreme pH values, high temperature, pressure, and organic co-solvents encourage protein misfolding (Campioni *et al.*,2010). When it comes to *in vivo* studies, factors such as mutations or truncations induce the protein misfolding inside the cell. DNA stores the genetic code for protein synthesis in the form of codons (Meisenberg and Simmons, 2012). These codons specify the order of amino acids. The amino acid sequence in the peptide chain regulates the proper protein folding. DNA mutation can change the order of specific amino acid sequences of protein folding, which leads to protein misfolding. The conformational stability of proteins plays a crucial role in preventing misfolding and aggregation. When a protein's conformational stability is compromised, it is more likely to misfold and aggregate. Misfolded proteins are non-functional and toxic, posing a threat to cellular health. To prevent their accumulation, misfolded proteins must either be refolded into their native conformation or eliminated.

Chaperone-assisted protein folding can facilitate refolding, but if this process is unsuccessful, protein quality control systems are activated to eliminate the misfolded proteins. The Ubiquitin-Proteasome System (UPS) and autophagy are key defense mechanisms for eliminating misfolded proteins. In eukaryotic cells, the UPS serves as the primary pathway: misfolded or damaged proteins are tagged with ubiquitin molecules, marking them for degradation by the proteasome, which breaks them down into smaller peptides (Li *et al.*, 2022). Alternatively, in the autophagy process, misfolded proteins are transported to the lysosome for degradation. If neither refolding nor degradation occurs, misfolded proteins can aggregate, forming toxic protein clumps that disrupt cellular function.

5. Protein misfolding diseases

Protein misfolding diseases arise primarily through two mechanisms: the loss of functional proteins and the toxic effects of protein aggregation (Koszła and Sołek, 2024). In the first mechanism, misfolded proteins lose their functional capacity, which can lead to disorders like Fabry disease**,** Gaucher's disease**,** Retinitis Pigmentosa 3 (RP3)**,** and Cystic fibrosis**.** For example, mutations in the GLA gene cause misfolding of α-galactosidase in Fabry disease**,** impairing the breakdown of globotriaosylceramide (Gb3) and leading to accumulation in tissues such as the kidneys and heart. Similarly, in Cystic fibrosis**,** mutations in the CFTR gene cause the misfolding of CFTR protein, disrupting chloride ion transport and causing thick mucus accumulation in the lungs. These misfolding events contribute to symptoms such as respiratory infections and organ dysfunction (Koszła and Sołek, 2024).

The second mechanism involves the toxic effects of protein aggregation, which play a critical role in Alzheimer's disease**,** Huntington's disease**,** Parkinson's disease**,** and prion diseases (Koszła and Sołek, 2024)**.** Aggregation of amyloid-beta proteins in Alzheimer's leads to plaque formation, disrupting neuronal communication and causing cognitive decline. Similarly, Huntington's disease involves the aggregation of Huntingtin proteins with polyglutamine expansions, while in Parkinson's disease**,** α-synuclein aggregates into Lewy bodies, impairing neuron function. Prion diseases are characterized by the accumulation of misfolded prion proteins, leading to severe neurodegeneration. These diseases, resulting from either functional loss or aggregation-induced toxicity, demonstrate the diverse impact of protein misfolding on human health. Table 01 summarizes the causes, mechanisms, symptoms, and relevant references for various protein misfolding diseases.

Table 01: Various protein misfolding diseases, their causes, mechanisms, symptoms, and relevant references

Disease	Cause of Disease	Mechanism	Symptoms/Effects	References
Fabry Disease	Misfolded α-galactosidase due to mutations in GLA gene	Loss of functional enzyme leads to lipid accumulation (globotriaosylceramide) in tissues like kidneys, heart, and nervous system	Burning pain in hands/feet (acro paresthesia), kidney dysfunction, cardiac issues, dark red skin spots (angiokeratomas)	Lenders and Brand, 2021
Gaucher's Disease	Misfolded β-glucocerebrosidase due to mutations in GBA gene	Accumulation of glucocerebroside in macrophages leads to the formation of Gaucher cells in spleen, liver, bone marrow	Enlarged spleen and liver, bone pain, fatigue, anemia, and bruising	Stirnemann *et al*., 2017
Retinitis Pigmentosa 3 (RP3)	Misfolded rhodopsin due to mutations in the rhodopsin gene	Accumulation of misfolded rhodopsin in photoreceptor cells leads to cellular stress, apoptosis, and gradual retinal degeneration	Night blindness, progressive loss of vision, ultimately leading to blindness in extreme cases	Ferreira, 2005
Cystic Fibrosis	Misfolded CFTR protein due to mutations in CFTR gene	Defective chloride ion channel causes thick, sticky mucus buildup in lungs and pancreas, impairing normal function	Respiratory infections, lung damage, malabsorption, nutritional deficiencies	Lukacs and Verkman, 2012
Alzheimer's Disease	Aggregation of amyloid-beta proteins	Amyloid-beta fibrils form plaques that disrupt normal neuronal communication, contributing to cognitive impairment	Memory loss, confusion, personality changes, and other cognitive deficits	Alaei *et al*., 2021
Huntington's Disease	Aggregation of Huntingtin with polyQ expansion	Misfolded Huntingtin proteins form aggregates that affect neurons, leading to motor and cognitive dysfunction	Uncontrolled movements (chorea), cognitive decline, psychiatric symptoms	Alaei *et al*., 2021
Parkinson's Disease	Aggregation of α-synuclein	α-synuclein aggregates into Lewy bodies within neurons, impairing normal function	Tremors, rigidity, bradykinesia, postural instability	Alaei *et al*., 2021
Prion Diseases (e.g., Creutzfeldt-Jakob Disease)	Aggregation of prion proteins	Misfolded prion proteins aggregate in the brain, causing neurodegeneration and spongiform encephalopathy	Progressive neurological decline, memory loss, personality changes, loss of motor function	Alaei *et al*., 2021
Sickle Cell Anemia	Misfolded hemoglobin due to mutations in HBB gene	Aggregated hemoglobin S forms polymer fibers, distorting red blood cells and leading to blockages in blood flow	Painful episodes (vaso-occlusive crises), anemia, organ damage (heart, spleen, kidneys)	Valastyan and Lindquist, 2014

5.1 Structure of amyloids

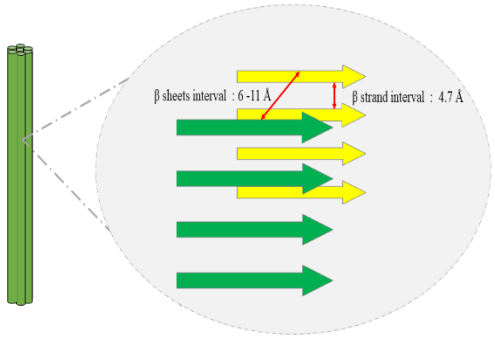

Figure 04: Schematic representation of an amyloid fibril

Amyloid deposits, initially believed to be related to cellulose, were shown by Friedrich and Kekulé in 1859 to be protein aggregates (Sipe and Cohen, 2000). Under electron microscopy, amyloids are characterized by long, thin, unbranched filaments with a diameter of approximately 6-12 nm. X-ray fiber diffraction studies reveal that amyloid fibrils are elongated, unbranched structures composed of intermolecular β-sheets. These β-sheets consist of β-strands aligned parallel to each other, while the β-sheets themselves are oriented perpendicular to the fibril axis, forming cross-β sheets. The β-sheets are stacked and interconnected through hydrogen bonds. Meridional reflections in X-ray analysis indicate that the spacing between β-strands within a sheet is approximately 4.7 Å. Equatorial reflections reveal that the distance between stacked β-sheets ranges from 6 to 11 Å (Greenwald and Riek, 2010). This distinctive diffraction pattern serves as a hallmark of amyloid formation (Figure 04).

5.2 *Pathological properties of amyloid fibrils*

Amyloid fibrils exhibit several pathological properties, including their role as templates for fibril aggregation, which can seed the native protein to adopt a fibrillar state, promoting further aggregation. This templating mechanism is implicated in diseases such as mad cow disease in cattle, scrapie in sheep, and chronic wasting disease in deer (Xu *et al*., 2023). Additionally, amyloid fibrils are more thermodynamically stable than their native protein counterparts, which contributes to the stability of the aggregates. These fibrils are insoluble, metabolically stable, and resistant to protease degradation, making them difficult to denature under both *in vitro* and *in vivo* conditions.

Another notable property of amyloid fibrils is their mechanical rigidity and surface charge-mediated activity. The hydrogen bonds between the β-sheets in the fibrils impart stiffness, enabling amyloid fibrils to damage and deform cell membranes. For example, β-2-microglobulin (β-2m) fibrils have been shown to cause damage to liposomes (Milanesi *et al*., 2012). Moreover, amyloid aggregates possess an increased surface area, which allows them to bind with other unfolded proteins with a higher affinity, a phenomenon referred to as "boosted partner binding activity" (Xu *et al*., 2023).

5.3 Mechanism of amyloid aggregate formation

Amyloid formation can occur via two primary mechanisms: nucleation-dependent and nucleation-independent. The nucleation-dependent mechanism, also known as nucleation elongation polymerization, involves three stages: the nucleation phase, elongation phase, and stationary phase (Ferrone, 1999). In the nucleation phase, misfolded protein monomers interact to form small clusters, creating critical nuclei. These nuclei serve as intermediates for continuous monomer addition, leading to oligomer formation. The nucleation phase is rate-limiting (Almeida and Brito, 2020) but can be accelerated by pre-formed aggregates, which act as templates in a process called seeding. Seeding can be homologous, where the monomer and seed are identical, or heterologous, where they differ, with homologous seeding being more efficient. Heterologous seeding, however, is implicated in neurodegenerative diseases (Morales *et al.*, 2009; Spires-Jones *et al*., 2017).

In the elongation phase, oligomers attract more monomers, forming prefibrillar structures that further grow into protofibrils, which are thermodynamically stable. This phase is faster than nucleation (Almeida and Brito, 2020). In the stationary phase, monomer addition slows as almost all monomers are incorporated into aggregates, stabilizing protofibrils into mature amyloid aggregates with a characteristic cross-β structure. These aggregates are insoluble and more stable than earlier intermediates.

The nucleation-independent mechanism bypasses the formation of critical nuclei, with monomers directly attaching to form aggregates. This process is faster than the nucleation-dependent mechanism (Subedi *et al*., 2022).

5.4 Alzheimer's disease

Alzheimer's disease affects nearly 45 million people worldwide and causes death (Ma *et al*., 2022). The main cause of Alzheimer's disease (AD) has not been fully found. There are several theories and hypotheses proposed by scientists to describe the disease condition. Out of all, the amyloid cascade hypothesis

is the primary model to describe AD pathogenesis. According to this hypothesis, the accumulation of amyloid beta protein (Aβ) in the brain is the major reason for Alzheimer's disease (Maltsev *et al.*, 2014). Aβ-protein is synthesized from the Amyloid precursor protein (APP), which is a single-pass transmembrane protein. APP proteins are concentrated in neural synapses of the brain. Thus, it is highly connected to brain development and memory power. If there is any mutation in the APP gene, it increases the risk of Aβ-protein misfolding. Specifically, Aβ40 and Aβ42 may accumulate to form oligomers, and they further aggregate to form senile plaques they can damage the peripheral neurons. Oligomers penetrate through the cell membrane of the neurons in the brain, especially in the cerebral cortex (Prasansuklab and Tencomnao, 2013). Ultimately, it causes cell dysfunction and drives to Alzheimer's disease. Alzheimer's disease symptoms include emotional fluctuations, sleep difficulties, behavioral abnormalities, and cognitive impairment. When Alzheimer's disease advances to late stages, it can produce severe symptoms such as multi-organ failure because of neuronal necrosis or even brain death (Prasansuklab and Tencomnao, 2013).

5.5 Prion disease

Prion diseases are transmissible neurodegenerative conditions affecting both animals and humans. Stanley B. Prusiner introduced the term "prion," defining it as a "small proteinaceous infectious particle resistant to inactivation by most procedures that modify nucleic acids" (Aguzzi and Calella, 2009). These diseases are characterized by the misfolding of cellular prion protein (PrPC) into an insoluble, protease-resistant form known as scrapie prion protein (PrPSc). This misfolding can result from genetic mutations in the prion protein gene (PRNP) or exposure to external PrPSc sources (Wright et al., 2018).

PrPSc induces the conversion of normal PrPC into the abnormal PrPSc form, which differs structurally. While PrPC contains about 45% helical structure and less than 5% beta sheets, PrPSc consists of around 30% helices and 50% beta sheets, with the majority of helices converting to beta-sheets (Riek *et al*., 1996). This beta-sheet dominance makes PrPSc insoluble and resistant to proteinase K (PK) degradation (Yuan *et al*., 2006). The accumulation of PrPSc leads to amyloid aggregates in the brain, contributing to diseases like Kuru, Creutzfeldt-Jakob disease (CJD), variant Creutzfeldt-Jakob disease (vCJD), and fatal familial insomnia (FFI). These diseases are associated with specific brain regions: Kuru affects the cerebellum, CJD the cerebral cortex, and FFI the thalamus.

Prion transmission was first documented with Kuru in Papua New Guinea, where it spread through ritual cannibalism (Aguzzi and Calella, 2009). Bovine spongiform encephalopathy (BSE), or "mad cow" disease, is another prion disease, primarily affecting cattle. It is transmitted via contaminated cattle feed containing infected animal remnants. Cows with BSE exhibit aggression, difficulty walking, weight loss, and eventual death. Other animals, including sheep, goats, and deer, are also susceptible to prion diseases (Iwasaki, 2017).

Variant Creutzfeldt-Jakob disease (vCJD) in humans is linked to the consumption of BSE-infected beef. vCJD is rare but causes depression, anxiety, behavioral changes, dysesthesia (itching and burning sensations), and later, dementia and impaired coordination (Ritchie *et al*., 2021). Currently, there is no effective treatment for vCJD, and once symptoms appear, no therapy can halt its progression.

5.6 Parkinson's disease

Under normal conditions, brain cell signaling is regulated by neurotransmitters, with dopamine playing a crucial role. In Parkinson's disease, dopamine regulation is impaired due to insufficient levels of the neurotransmitter, affecting cell signaling in the brain (Gómez-Benito *et al*., 2020). Alpha-synuclein, a protein encoded by the SNCA gene, regulates dopamine release. When alpha-synuclein misfolds, it forms amyloid fibrils, which aggregate into amyloid deposits in dopamine-producing neurons, eventually leading to the formation of Lewy bodies. These Lewy bodies damage neurons, contributing to dopamine deficiency (Xu and Pu, 2016). As a result, patients experience difficulty in controlling muscle movements, which manifests as slow movements, postural rigidity, and imbalance. If left untreated, the disease progresses, potentially leading to severe neuronal damage and dementia-like symptoms.

5.7 Huntington's disease

When the gene code for the Huntingtin (HTT) protein is mutated, it induces the HTT protein to misfold and causes amyloid aggregation. Patients who are diagnosed with Huntington's disease have 36 to 55 cytosine, adenine, and guanine (CAG) repeats in the mutated HTT gene. Mood changing, depression, imbalance, involuntary movements (chorea), slurred speech, difficulty in swallowing and weight loss are the major symptoms of Huntington's disease (Ajitkumar and De Jesus, 2024).

6. Approaches to treat protein misfolding diseases

Between 1990 and 2019, Alzheimer's disease and other dementias saw a growth from 147.95% to 160.84%, marking a significant increase in global burden (Li *et al*., 2022). This rise has been more pronounced in high-SDI (sociodemographic index) regions, particularly among the elderly, who require focused care and attention. The aging population necessitates prioritizing interventions aimed at reducing dementia risk factors, along with developing action plans to combat the growing prevalence of Alzheimer's disease. Similarly, the global burden of Parkinson's disease has doubled in the last generation due to the increasing number of older individuals. Statistical analysis reveals that Parkinson's disease cases increased from 3.5 million in 1990 to 6.1 million in 2016 (Dorsey *et al*., 2018). A comprehensive understanding of protein folding mechanisms, misfolding, and amyloid fibril aggregation pathways is critical for the development of targeted therapies aimed at mitigating neurodegenerative diseases. Early diagnosis remains essential in preventing further brain cell damage and limiting the progression of these diseases. However, no effective treatment has been identified for Alzheimer's disease, with current medications only slowing symptom progression. As a result, ongoing research is crucial to address this growing concern.

As previously discussed, Alzheimer's disease is primarily caused by the aggregation of Aβ-amyloids in the brain. In response, anti-Aβ immunotherapy has been explored, where antibodies are used to neutralize and prevent the formation of toxic Aβ aggregates. Despite the absence of a cure for Alzheimer's disease, diet control and lifestyle modifications have been shown to potentially help in preventing its onset. Curcumin, for example, has demonstrated anti-amyloidogenic properties by inhibiting amyloid formation and reducing plaque accumulation *in vivo* (Kundu *et al*., 2020). Ceylon cinnamon extract has also been found to inhibit tau aggregation, with Cinnamomum tree extract, particularly cinnamon bark oil, exhibiting anti-amyloidogenic and anti-fibril formation activities, especially against tau 187 amyloid-forming (Peterson *et al*., 2009).

Prion diseases, on the other hand, are particularly challenging as there is no cure; they can only be prevented. One of the major causes of bovine spongiform encephalopathy (BSE) in cattle is inferior quality cattle feed, making the regulation of cattle feed standards essential to prevent prion transmission. Additionally, avoiding the consumption of meat infected with BSE can help prevent the development of Variant Creutzfeldt-Jakob Disease (vCJD) in humans (Aguzzi and Calella, 2009).

For Parkinson's disease, Zinc (Zn^{2+}) ions have been found to promote chaperone (HSP) activity, which helps prevent the aggregation of α-synuclein, the key protein implicated in Parkinson's disease (Al-Harthi *et al*., 2022). Methylthioninium, an effective inhibitor of α-syn fibrillar aggregation both *in vitro* and *in vivo*, has shown potential in preventing disease progression in transgenic mouse models. NPT200-11 is another inhibitor that stabilizes α-synuclein conformers, preventing its aggregation and promoting proper protein folding (Fields *et al*., 2019).

In the case of Huntington's disease, where no definitive treatment exists, gene silencing techniques are being explored. Recent research suggests that silencing the mutated HTT gene can help restore its normal function without destroying the gene, offering a promising therapeutic approach (Ajitkumar and De Jesus, 2024).

7. Techniques to study protein folding

Fluorescence spectroscopy is a valuable technique for detecting amyloid fibrils by combining a UV light source with a highly sensitive spectrometer. Certain amino acids, particularly aromatic amino acids like tryptophan, tyrosine, and phenylalanine, exhibit intrinsic fluorescence properties. Among these, tryptophan is the most commonly used due to its high quantum yield, which results in high absorptivity and strong fluorescence emission (Ghisaidoobe and Chung, 2014). This makes tryptophan an excellent indicator for analyzing both native and aggregated protein structures. Upon exposure to UV light, the tryptophan molecules absorb photons and transition to an excited state. As they return to their ground state, they emit fluorescence within the 300–350 nm wavelength range (Bashir *et al*., 2024). A spectrometer analyzes this emitted fluorescence, allowing the determination of tryptophan concentration and enabling the identification of protein aggregates containing tryptophan residues.

Fluorescence dye binding is another method used to detect amyloid fibrils via fluorescence spectroscopy. These dyes preferentially bind to the hydrophobic regions of proteins, which are exposed during aggregation. Common dyes employed for detecting amyloid fibrils include Thioflavin T (ThT), 1-anilinonaphthalene-8-sulfonate (ANS), 4,4′-bis-1-anilinonaphthalene-8-sulfonate (Bis-ANS), Nile Red, and SYPRO Orange (Oshinbolu *et al*., 2018).

High-pressure NMR spectroscopy is a useful tool for studying protein folding phenomena. Under high pressure, proteins transition to a more compact and

organized "folded" form. By applying varying pressure values at different stages, the protein folding pathway can be investigated, and the conformations of intermediate states can be analyzed (Szilágyi *et al*., 2007). This method enables the observation of the entire range of protein conformations, from fully folded to unfolded. Additionally, hydrogen/deuterium exchange (HDX) combined with mass spectrometry can provide insights into the structural and kinetic features of distinct conformational states. Pulse-labelling HDX-MS, in particular, is valuable for evaluating folding kinetics and exploring complex folding scenarios (Szilágyi *et al*., 2007a).

Chemically induced nuclear polarization (CIDNP) pulse-labelling techniques are useful for identifying tryptophan and tyrosine residues in molten globule states (Szilágyi *et al*., 2007). NMR spectroscopy also plays a significant role in understanding protein folding mechanisms, particularly in examining the characteristics of intermediate folding states, such as molten globules (Szilágyi *et al*., 2007). Furthermore, Fourier transform infrared (FTIR) spectroscopy has advanced our understanding of protein misfolding and aggregation in neurodegenerative disorders, such as Alzheimer's, Parkinson's, and Huntington's diseases. FTIR works by analyzing key vibrational modes, including Carbon–Oxygen (C=O) stretching, Carbon–Nitrogen (C≡N) triple bond stretching, and Nitrogen–Hydrogen (N-H) bending, all of which are involved in protein structure alterations during aggregation (Bashir *et al*., 2024).

8. Future direction and challenges

The mechanisms underlying protein aggregation and its connection to neurodegenerative diseases remain incompletely understood. This knowledge gap significantly hampers the development of definitive treatments for amyloid-based disorders. Currently, there is no precise treatment for amyloid-related neurodegenerative diseases, prompting scientists to explore natural inhibitors present in foods. However, the efficacy of these natural inhibitors is often moderate, and their potential has not been extensively tested. Furthermore, although various amyloid fibrils exist, they do not exhibit substantial structural differences, which presents a challenge in the discovery of new, more effective inhibitors. To address these issues, large-scale platforms for analysis are required, yet the recent decision by major pharmaceutical companies like Pfizer to exit the neuroscience field represents a significant setback for future progress.

At present, only therapeutic medications are available to manage these disorders, but these treatments do not guarantee consistent success. It is estimated that research on Alzheimer's disease medications fails 99.6% of the time (Chung, Lee, and Lee, 2018). Despite these challenges, ongoing research continues to offer hope for future advancements. The rising incidence of neurodegenerative disorders, coupled with increased life expectancy, has driven therapeutic research forward. However, existing diagnostic methods, such as enzyme-linked immunosorbent assays (ELISA) and immunohistochemistry, tend to be expensive, time-consuming, and suffer from limited antibody specificity. Recently, researchers at the University of Minnesota have developed a more efficient method to detect abnormal proteins, utilizing Real-Time Quaking-Induced Conversion (RT-QuIC) assays, which promise a quicker and more effective means of identifying misfolded proteins (Christenson *et al*., 2023).

The advancement of artificial intelligence (AI) and computational modeling has also significantly improved the study of protein folding and misfolding, with the potential to accelerate drug discovery efforts. For instance, understanding the structure of SARS-CoV-2 viral proteins enabled scientists to devise innovative vaccines. Over the last six decades, experimental techniques like X-ray crystallography and cryo-electron microscopy (cryo-EM) have facilitated the discovery of over 190,000 protein structures (EMBL Communications, 2024). Recently, Demis Hassabis, John Jumper, and David Baker were awarded the 2024 Nobel Prize for their groundbreaking work on solving the "protein folding" problem using AI. They achieved this through the development of AlphaFold, a deep learning-based program created by DeepMind, which predicts the three-dimensional structures of proteins based solely on their amino acid sequences (Zimmer, 2024). This development could be pivotal in advancing our understanding of protein misfolding in neurodegenerative diseases and may lead to more effective therapeutic interventions in the future.

9. Conclusion

The formation of amyloid fibrils caused by protein misfolding is a major factor for neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington and prion diseases. For a protein to be biologically active, it must be properly folded. Protein folding is driven by different intermolecular and intramolecular forces and stabilized by achieving the lowest energy state. Despite the cell's sophisticated machinery to ensure proper folding, proteins occasionally misfold, leading to aggregation and subsequent cellular toxicity. These aggregates are highly toxic and often linked with neurodegenerative diseases, emphasizing the urgent need for therapeutic interventions.

Unfortunately, there is no defined treatment to cure these diseases. While significant progress has been made in understanding protein folding, many mysteries remain. A deeper understanding and continuous research regarding the role of the cellular environment in protein folding and the precise mechanisms of protein misfolding are crucial for developing effective therapies for protein misfolding diseases.

Declaration of completing interest

Authors declare that they have no conflict of interest.

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Abbreviation	Meaning
3D	Three-dimensional
AD	Alzheimer's disease
ANS	1-anilinonaphthalene-8-sulfonate
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Aβ- protein	Amyloid beta protein
B2M	Beta-2-microglobulin
Bis-ANS	4,4′-bis-1-anilinonaphthalene-8-sulfonate
BSE	Bovine spongiform encephalopathy
CFTR	Cystic fibrosis transmembrane conductance regulator
CIDNP	Chemically induced nuclear polarization
CJD	Creutzfeldt-Jakob disease
Cryo-EM	Cryo-electron microscopy
DNA	Deoxyribonucleic acid
FFI	Fatal familial insomnia
FTIR	Fourier transform infrared spectroscopy
Gb3	Globotriaosylceramide
GBA gene	Glucosylceramidase beta acidic gene
GLA gene	Galactosidase alpha gene
HD	Huntingtin disease
HDX	Hydrogen/deuterium exchange
HSP	Heat-shock proteins
HTT	Huntingtin
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PDI	Protein disulfide isomerase
PK	Proteinase K
PolyQ	Polyglutamine
PPI	Peptide prolyl cis-trans isomerase
PRNB	Prion protein gene
PrP^{C}	Cellular prion protein
PrP^{Sc}	Protease-resistant scrapie prion protein
RNA	Ribonucleic acid
RP3	Retinitis pigmentosa 3
RT-QuIC	Real-time Quaking-induced conversion
SDI	Sociodemographic index
SNCA gene	Synuclein alpha gene
ThT	Thioflavin T
UPS	Ubiquitin-Proteasome System
vCJD	Variant Creutzfeldt-Jakob disease