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Viewpoint

Can Prions Carry Biological Information?

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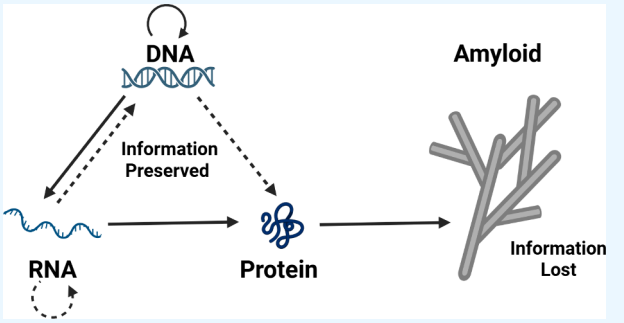
ABSTRACT: The discovery of the structure of DNA and the elucidation of the molecular mechanisms of replication, transcription, and translation are the foundations of modern biology and medicine. However, in the early 80s, the prion hypothesis introduced a new system of biological information transfer that does not rely on DNA; it introduced the concept of conformational propagation through templating. Unlike the molecular biology revolution, which was based on detailed molecular structures and mechanisms, the prion hypothesis was postulated in the absence of clear molecular structures or mechanisms. In this Viewpoint, we highlight 10 points in which the prion hypothesis contradicts the molecular, structural, and mechanistic experimental evidence accrued since its inception four decades ago. Alternatively, we postulate that an extension of the thermodynamic hypothesis of protein folding (Anfinsen's dogma) to the state of proteins at high concentration (supersaturation) is better suited for explaining the different facets and pathways of protein aggregation.



The prion hypothesis introduced a new system of biological information: conformational information transfer through templating. Unlike normal protein folding, which takes place spontaneously based on the primary sequence information of the protein (the thermodynamic hypothesis of protein folding or Anfinsen's dogma), the amyloid conformation in diseases such as Creutzfeldt-Jakob disease is postulated to require a conformational template, a prion, which acts as a template to "imprint" its corrupt conformation on similar proteins and become self-propagating.[1,2] Experimentally, the prion hypothesis is based on one phenomenon: seeding. This is when an amyloid fibril fragment, a seed, is added to a concentrated solution of other proteins, and it catalyzes their transformation into amyloids. However, for decades, it has remained unclear what exactly is the conformational information carried by prions and what is the molecular mechanism of templating. More recently, and thanks to a better understanding of the structure of amyloid fibrils via cryoelectron microscopy, the term "conformational information" has come to designate the specific 2D cross-sectional shape, i.e., the particular cross-sectional pattern of folds and turns of protofilaments and fibrils (Figure 1A).[3] In this framework, a prion, which is an amyloid fibril fragment, templates its cross-sectional shape on incoming protein molecules binding to its tip during elongation. The incoming molecules must accommodate the cross-sectional shape of the fibril by binding in a parallel, in-register manner (i.e., the N and C termini align in the same direction and each amino acid of the incoming molecule stacks on top of the identical residue at the tip of the fibril).[4] This is also the underlying principle behind the concept of prion "strains", where different cross-sectional seed shapes are postulated to imprint their distinctive pattern of folds and turns on incoming protein molecules, leading to different disease phenotypes.[3] Similarly, prion propagation occurs via breakage or fission of a fibril with a particular cross-sectional shape into smaller fragments or seeds, which then template their shape onto incoming protein molecules. Thus, in its modern formulation, the prion templating and propagation of conformational information are based on elongation and fission.

However, for the elongation and fission mechanism to preserve and propagate specific cross-sectional shape information sustainably across generations of fibrils and across different cells, tissues, and hosts, it must fulfill some basic criteria:

1. Incoming proteins must only bind to the fibril tip to accommodate its specific cross-sectional shape.
2. Incoming proteins must have the same sequence as the protein in the fibril tip to enable parallel, in-register stacking.

However, there is an overwhelming amount of experimental evidence showing that both criteria are not fulfilled during amyloid growth:

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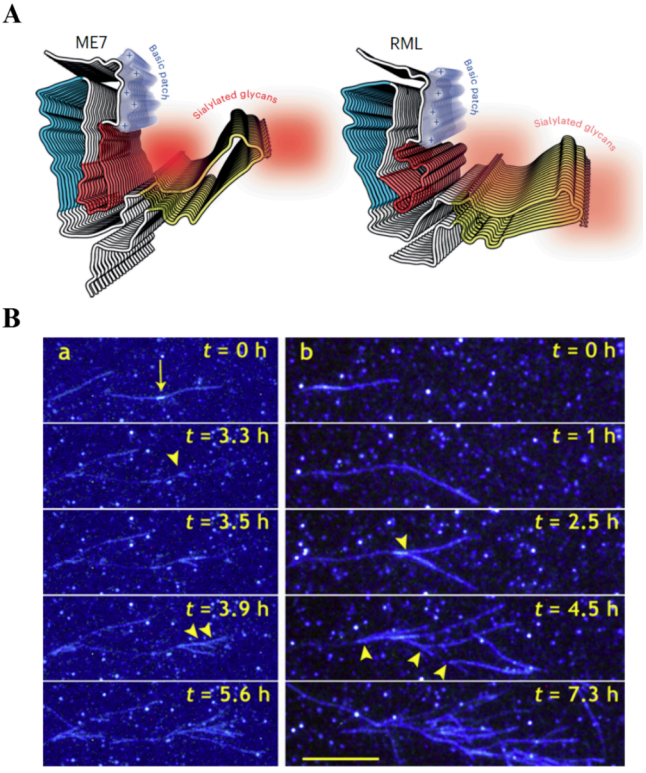

Figure 1. Immediate branching during amyloid growth, precluding cross-sectional shape templating even for one generation of fibrils. (A) 2D cross-sectional shape of two PrP amyloid protofilaments that is proposed to denote and propagate different prion strains via elongation. Reproduced with permission from ref 3. Copyright 2023 The Authors, published by Springer Nature. (B) Total internal reflection fluorescence microscopy (TIRFM) images obtained following the growth over time of glucagon amyloid fibrils by branching. The bars represent 10 μm. Reproduced with permission from ref 5. Copyright 2009 Cell Press.

1. Amyloid growth cannot maintain elongation even for one generation of fibrils because the process is immediately taken over by *branching* (Figure 1B), where new fibrils grow as branches on the lateral surface of the parent fibril, a process termed *secondary nucleation*.[5,6] The lateral surface of the fibril bears no resemblance to its cross-sectional shape (the conformational information) and cannot engage in a parallel, in-register protein binding.
2. Seeds of one protein can induce amyloid aggregation of another protein without the sequence homology needed for parallel-in register elongation, a process termed *cross-seeding*.[7] In this case, heterologous seeds act mainly as catalytic surfaces that do not relay any conformational information.[8]

Based on these two points alone, the prion hypothesis as it pertains to sustained conformational templating and propagation via elongation and fission is not supported by experimental evidence. Yet there are additional fundamental problems with the prion hypothesis, including the following:

3. There is no thermodynamic incentive for a protein molecule to exit its stable native conformation to mold itself on a fibril's tip.
4. There is no mechanism by which seeds can go around in solution "fishing" for similar protein molecules to mold it into their shape.
5. No machinery has ever been found that could restrict amyloid growth to tip elongation and prevent branching to preserve the cross-sectional template information.
6. While parallel in-register is the most common β-sheet stacking architecture of amyloids, amyloids with anti-parallel and out-of-register architecture have been experimentally found.[9]
7. In a process termed *heterogeneous nucleation*, amyloid growth can be initiated by any surface including lipid membranes, polysaccharides, nucleic acids, nanoparticles, air−water interfaces,[10] and viruses, which all lack any conformational templating information.[11]
8. In a process termed *homogeneous nucleation*, amyloids form spontaneously at high protein concentrations without any template.[12]
9. Amyloid formation is strictly dependent on protein concentration and does not take place under dilute conditions no matter how many seeds or other catalysts are present.
10. Different fibrillar cross-sectional shapes (polymorphs) depend on environmental conditions and not on the shape of the seed.[13−15]

The prion hypothesis, postulated long before the mechanistic and structural information outlined above was known, is currently in conflict with the experimental evidence accrued since its inception. It mischaracterized the phenomenon of protein phase transition, where no information transaction is involved, as replication. While prion "strains" have been proposed to explain different disease phenotypes in animal models, the "strainness" of prions has been characterized using imperfect surrogate biomarkers such as differential resistance to proteolysis.[16] With the increasing availability of detailed amyloid fibril structures via cryoelectron microscopy, it is still to be seen whether these assumptions would hold on the structural biology level.

Alternatively, amyloid formation can be understood as a phase transition (similar to crystallization, for example), which results from the increased probability of forming intermolecular bonds due to molecular proximity at higher protein concentrations (supersaturation). From this perspective, Anfinsen principles (the thermodynamic hypothesis) still apply to the amyloid phenomenon, where the amyloid cross-β conformation (protein monomers stacking on top of each other at a 4.8 Å distance in long β-sheet ladders, which pair together via interdigitating side chains forming the so-called dry steric zipper[17]) is spontaneously adopted under supersaturated conditions because it is more thermodynamically favorable (see Thermodynamic Forces Driving Protein Folding at Different Concentrations). In this case, no template is needed, just a nucleation catalyst, which can be any surface that lowers the thermodynamic barrier to the phase transition. Thus, instead of expanding the prion hypothesis to accommodate even more diseases such as Alzheimer's and Parkinson's,[1,2] we suggest extending Anfinsen's thermodynamic hypothesis to include protein folding into the amyloid conformation under supersaturated conditions. This classical biophysical framework fits better with the experimental evidence as it includes seeding (via homologous or heterologous seeds) without excluding other pathways of amyloid induction that do not involve a proteinaceous seed component (e.g., lipid/microbial catalyzed nucleation, protein overexpression). Such a framework frees the phenomenon from the unnecessary constraints of templating, enabling a better understanding of

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the etiological factors triggering amyloid formation and hopefully opening novel avenues for therapeutic interventions.

Thermodynamic Forces Driving Protein Folding at Different Concentrations. Both native folding and cross-β amyloid folding processes can be described by the same Gibbs free energy equation, where $-\Delta G$ means that a process is spontaneous. Folding is driven by the enthalpy of the folded state, the entropy of the folded state, and the entropy of the water molecules constituting the solvent environment. The equation can be described as follows:

$$\Delta G_{\text{fold}} = \Delta H_{\text{fold}} - T(\Delta S_{\text{protein}} + \Delta S_{\text{solvent}}) \quad (1)$$

where ΔG_{fold} is the free energy change for folding, ΔH_{fold} is the enthalpy change for folding, T is the temperature, $\Delta S_{\text{protein}}$ is the change in protein entropy after folding, and $\Delta S_{\text{solvent}}$ is the change of solvent entropy after folding. In dilute conditions, ΔG_{fold} describes native folding, while in highly concentrated supersaturated conditions, ΔG_{fold} describes folding into the cross-β conformation and amyloid formation.

Native folding is mostly driven by the hydrophobic effect, which relies on the increased entropy of water during protein folding.[18] This increase in the entropy of water compensates for the decrease in protein entropy due to folding. At high concentrations, cross-β amyloid folding is also driven by an increase in the entropy of water, which results mainly from the expulsion of water molecules during the formation of the dry steric zipper.[17] Furthermore, amyloid formation is exothermic[19,20] (with $-\Delta H$) and involves the formation of chains of very stable intermolecular hydrogen bonds within the β-sheet ladders. Consequently, both enthalpic and entropic contributions make the ΔG_{fold} of amyloid formation negative.

Structurally, the constraints of protein chemistry, which allow for either α-helix or β-sheet conformations, also contribute to the favorability of the cross-β conformation at supersaturation. In dilute conditions, the protein molecule is free to adopt the combinations of α-helices or β-sheets dictated by intramolecular interactions based on its primary sequence. In supersaturated conditions, however, a β-sheet conformation is the only stereochemically accessible option that maximizes molecular packing and intermolecular hydrogen bonds in order to form a stable structure. Since peptides in the helical conformation cannot be packed any closer than 8 Å, it is necessary for peptide monomers to undergo conformational transition from the helical conformation to the β-sheet conformation during amyloid formation, which reduces the interpeptide distance to 4.8 Å, enabling maximum interpeptide hydrogen bonding.[21] This geometric stereochemical limitation is in good agreement with the interstrand distance (4.8 Å) typically observed in the X-ray diffraction of amyloid fibrils and explains the striking similarity of the cross-β conformation among amyloids originating from peptides and proteins of different sequences. Moreover, additional stability can come from side chain interactions, which can be maximized by parallel in register stacking, which makes this stacking architecture the most common.[17] Furthermore, interdigitation or mating between β-sheet ladder pairs is based on the complementarity of the physical properties of the side chains of each ladder: hydrophobic or polar side chains tend to mate with counterparts of similar properties, and ladders of positively charged amino acids tend to mate with ladders of negatively charged ones.[17] Since side chain net charge and polarity are dependent on environmental conditions such as pH and ionic strength of the solution, differential ladder mating, and hence fibrillar polymorphism, is also dependent on environmental conditions.

Despite the similar thermodynamic driving forces for native folding and cross-β amyloid folding, amyloid formation is also a phase transition, where soluble, freely moving protein molecules condense into solid fibrils. Thus, while adopting the cross-β conformation favorable under supersaturated conditions, forming the first piece of solid amyloid, the *nucleus*, requires breaking the bonds between water molecules in the bulk of the solution to create a new interface between the amyloid solid phase and water. This adds an interfacial energy cost or barrier, termed the *nucleation barrier*,[22] which keeps supersaturated protein solutions in a metastable state, unable to proceed to phase transition and adopt the cross-β conformation. According to classical nucleation theory, overcoming this barrier requires the formation of a nucleus of a certain size (of radius r). Below this radius, nuclei will dissociate back to monomers, and only above it will the system proceed into phase transition into a solid.[23] With the addition of the nucleation barrier, the free energy equation of amyloid formation becomes as follows:

$$\Delta G_{\text{fold}} = \Delta H_{\text{fold}} - T(\Delta S_{\text{protein}} + \Delta S_{\text{solvent}}) + 4\pi r^2\sigma \quad (2)$$

where ΔG_{fold} describes folding into the cross-β amyloid conformation, r is the radius of the nucleus, and σ is the surface tension of the interface between the nucleus and the solvent.

However, anything that helps a nucleus to form without having to insert itself between water molecules will facilitate overcoming the nucleation barrier and catalyze amyloid formation. This catalysis is usually achieved by introducing a surface into the system. A nucleus can start to form on this *nucleation surface*, bypassing the interfacial energy cost required for breaking bonds between water molecules. This adds a new term to the equation, according to the spherical cap approximation model,[23] which is dependent on the wetting angle (θ) between the protein and the surface. The higher the affinity, the lower the wetting angle, θ, which will lead to more significant reduction of the nucleation barrier.

$$\Delta G_{\text{fold}} = \Delta H_{\text{fold}} - T(\Delta S_{\text{protein}} + \Delta S_{\text{solvent}}) + \left(4\pi r^2\sigma\frac{2 - 3\cos\theta + \cos^3\theta}{4}\right) \quad (3)$$

Surface assisted nucleation is termed *heterogeneous nucleation*, and nucleation catalyzing surfaces can be non-proteinaceous, like membranes, nanoparticles, or viruses. It can also be preformed amyloid fragments or seeds, eliciting *seeding*, which mainly acts as nucleation surfaces. This is why seeds of one protein can catalyze the amyloid formation of another protein of a different sequence by acting as a catalytic surface, a phenomenon known as *cross-seeding*.[8] Although heterogeneous nucleation is much more common, at very high concentrations, a nucleus can spontaneously form in a process called *homogeneous nucleation*. Once the first nucleus is formed and the first fibrils start to grow, the growing fibrillar surface itself acts as a nucleation surface for the heterogeneous nucleation of new nuclei, a process called *secondary nucleation*, leading to immediate branching. This autocatalytic chain reaction, where fibrils catalyze the formation of other fibrils, proceeds, consuming the soluble monomer population and leading to a dramatic and abrupt decrease in monomer concentration.[24,25]

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Notes

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The authors declare the following competing financial interest(s): K.E. cofounded REGAIN Therapeutics and is co-inventor of the patent Compositions and methods for treatment and/or prophylaxis of proteinopathies.

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