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Review

Structural Biology

# What drives an amyloid protein precursor from an amyloidogenic to a native-like aggregation pathway?

A Quintas*

## Abstract

**Introduction**

Conformational disorders such as Alzheimer's, Parkinson's, familial amyloidotic polyneuropathy and spongiform encephalopaties are a consequence of protein misfolding and aggregation predominantly in the form of amyloid fibrils. These pathologies represent a major health problem, which most probably will overwhelm the health systems of developed countries in the near future. Significant progress has been made recently to understanding the underlying mechanism of protein misfolding and aggregation. The current picture of protein aggregation is a phenomenon resulting from protein conformational fluctuations leading to misfolded intermediates prone to form non-native interactions with other intermediates, resulting in amyloid fibril formation. Fortunately just a small group of proteins are associated with human conformational disorders. The primary causes that lead this group of proteins to misfolding and aggregation are point mutations, protein over-expression and failure of protein quality-control system. Beside amyloid formation, there are other types of aggregation available to a misfold-disease-related polypeptide chain in the protein-free energy landscape. Among them, native-like aggregation is becoming a widely studied topic of research. This aggregation type, simultaneously straightforward and ubiquitous, seems to be involved concurrently in the pathway of amyloid fibril formation and disruption. In this review, the pathways of misfold and aggregation of a protein are accessed along with the primary causes that turn a native soluble protein into amyloid fibrils or native-like aggregate. In addition, an insight into the biophysical and biochemical aspects fundamental to amyloid fibril formation and native-like aggregation is provided. Finally some clues are presented about what makes a protein follow an amyloidogenic or native-like aggregation pathway.

**Conclusion**

More laboratory data should be gathered about the structure, stability, dynamics and aggregation kinetics, in order to get a clearer picture of the biophysical mechanisms underlying native-like aggregation.

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## Introduction

Globular proteins rapidly fold into a well-defined three-dimensional structure after synthesis in a cell endoplasmic reticulum (ER). However, under some conditions proteins do not fold correctly into their native structure. This malfunction might result in a set of maladies called protein conformational disorders or misfolding diseases[1]. These pathologies are called this due to the structural modifications that can occur during the lifetime events of a protein. Amyloidoses are subset of misfolding diseases, comprising pathologies such as Alzheimer's, Parkinson's, familial amyloidotic polyneuropathy and spongiform encephalopaties, which result from misfolding of protein precursors and amyloid fibril formation.

One exquisite and contemporary way of describing the different three-dimensional states accessible to a polypeptide chain is the protein-free energy landscape[2]. This has allowed scientists to grasp the protein folding, misfolding and aggregation mechanisms[3].

The features of a protein-free energy landscape depend mostly on the amino acid sequence, post-translational modifications, ligands, cofactors and environmental conditions[4,5]. After being synthesized a polypeptide chain usually folds into its native state, the lower free energy value of the landscape[2] (Figure 1). However, under physiological conditions, a small group of peptides and proteins evolve into non-native folds, partially or total unfolds and aggregates[6]. This can be seen as a modification in the shape of proteins free energy landscape (Figure 2).

The primary causes driving this reshaping are point mutations or protein over-expression[7]. Point mutations result in protein sequence modifications favouring fibrillation by crowding protein partially unfolded states, which are prone to form aggregates[8,9]. In that case, the protein free energy landscape is reshaped in such a way that it stabilizes protein-folding intermediates (Figure 2). Consequently, the increment on the concentration of intermediates can lead to aggregation. On the other hand, protein over-expression increases the concentration of polypeptide chains driving the polypeptide chain into an aggregation pathway as well. It is noteworthy that folding of a monomeric protein is a zero-order reaction whereas aggregation is at least a first-order reaction[10]. This means that increasing protein concentration may force a protein into an aggregation route.



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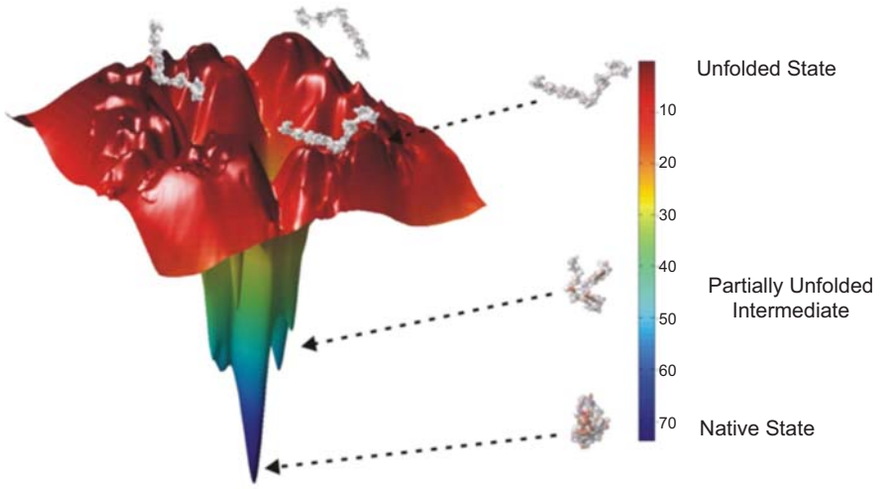


***Figure 1:*** Schematic representation of protein free-energy landscape. Native state corresponds to bottom of the funnel (lowest free-energy value). Partially fold intermediates have higher free-energy values.

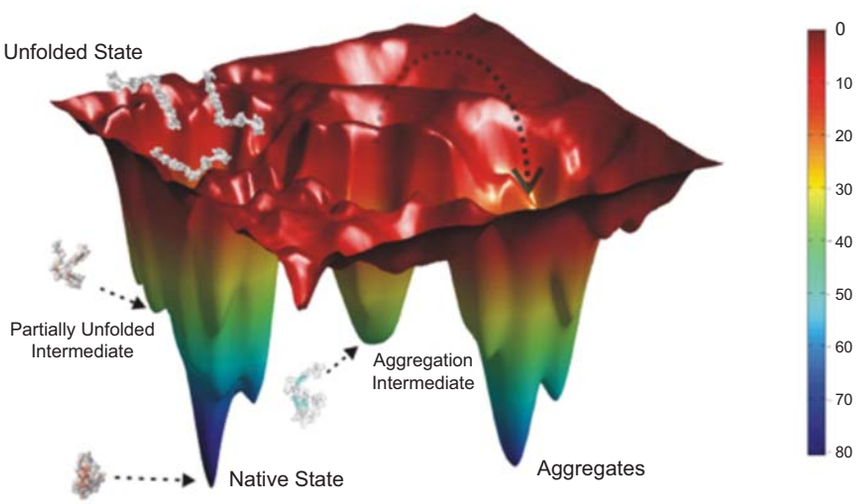


***Figure 2:*** Schematic representation of protein free-energy landscape where aggregation is an accessible pathway. The reshaping of the free-energy landscape occurs due to point mutation, covalent modification and oxidative stress, among others.

Beside mutation and protein over-expression, oxidative stress, activation of signalling pathways from quality control systems and post-translation modifications may act as inductors of protein aggregation[11]. The accumulation of toxic aggregates might damage the cells and progress to intra- or extra-cellular amyloid deposits[1].

Recent studies with the amyloidogenic proteins β-2-microglobulin, insulin and stefin B have shown that native-like aggregation might be associated with conformational diseases[12-14]. Indeed, some authors suggest that amyloidogenesis under physiological conditions follows a native-like folding aggregation[12], whereas others argue that it must follow a misfolding pathway[13,14]. In spite of the lack of consensus about native-like aggregation, these investigations highlight the diversity of routes that might happen in conformational disorders. Despite this, it must be emphasized that native-like aggregates per se have been associated with cellular toxicity[15]. This meaningful research is opening new avenues into the understanding of non-amyloid aggregation types in human diseases. The aim of this review was to discuss what drives an amyloid protein precursor from an amyloidogenic to a native-like aggregation pathway.

## Conformational disorders and amyloid fibril formation

From the tens of thousands of human proteins just 40 are currently known to be associated with amyloid formation. Amyloid fibrils are well-organized protein aggregates that bind to congo red and thioflavin dyes. Morphologically, these fibrils have a variable length and a diameter between 6 and 12 nm, displaying a characteristic cross-β structure perpendicularly oriented to the fibber axis[16,17]. The amyloid fibril low-resolution crystal structure shows a periodic β-sheet stacking stabilized by hydrogen bonds forming a packed zipper structure, the core of the fibre[18].

Along with morphological studies, the understanding of amyloid fibril formation kinetics is fundamental. Several models have been proposed to explain amyloid fibril formation kinetics. The classical kinetic view for amyloidogenesis describes amyloid fibril formation as a nucleation-dependent process with three distinct steps: (i) the lag phase, which is associated with the formation of the seed; (ii) the elongation phase, where the fibril growth takes place; and (iii) the final steady-state phase[19].

The mechanism by which the nucleation process starts usually involves the native polypeptide



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precursor undergoing conformational changes that lead to the formation of a partially unfolded β-sheet-rich intermediate prior to aggregation and nucleus formation[20]. These conformational modifications depend on the initial structural features of the native precursor. The elongation phase occurs through addition of monomeric units to the nuclei or stacking of small fibrils.

All major protein-fold motifs and protein hierarchical levels are represented in amyloid precursor proteins[6]. In this review oligomeric, monomeric and intrinsically unstructured proteins (IUP) amyloid precursor are discussed independently.

*Oligomeric protein as amyloid precursors*

Familial amyloidosis polyneuropathy (FAP), senile systemic amyloidosis (SSA) and amyotrophic lateral sclerosis (ALS) are conformational diseases involving multi-subunits of all-beta native-state precursors. In FAP and SSA the precursors are transthyretin variants and wild-type transthyretin (TTR) and in ALS the precursors are superoxide dismutase variants (SOD).

Human TTR is a homo-tetrameric protein with an eight-stranded β-sandwich motif in each subunit. Wilde-type TTR is associated with SSA, a degenerative disorder affecting predominantly individuals aged above 80 years. Variants of TTR are associated to FAP, an autosomic dominant degenerative disease. Depending on the TTR variant, the disease can have an earlier onset. In FAP, the physiological model for amyloid fibril formation establishes that amyloid formation by TTR is triggered by irreversible tetramer dissociation to a compact non-native monomer. Depending on its thermodynamic stability, the non-native monomer originates partially unfolded species with a high tendency for ordered aggregation into amyloid fibrils[8,21,22]. Interestingly, the amyloidogenic potential of TTR variants seems to correlate to their tendency to produce partially unfolded monomeric species[8]. There is very little knowledge regarding amyloid fibril formation from oligomeric proteins, and the few models found in the literature indicate that dissociation and thermodynamic instability of the resulting monomers are the primary causes behind the formation of amyloid fibrils (Figure 3).

*Monomeric proteins as amyloid precursors*

A group of monomeric proteins or its variants, such as lysozyme, cystatin C, immunoglobulin light chain, prolactin, insulin, lactoferrin and γ-crystallin, suffer conformational changes prior to amyloid fibril formation[23]. Human lysozyme is used as an example of monomeric amyloid protein precursor.

Human lysozyme is a small monomeric protein that belongs to the α+β motif with two structural domains, an alpha-domain with four alpha-helices and one $3_{10}$ helix, and a beta domain, which consists mainly in an anti-parallel β-sheet. Several lysozyme variants are associated with a familial non-neuropathic amyloidosis, which eventually forms amyloid deposits in the liver, spleen and kidneys[24,25]. Comparative conformational stability studies between wild-type lysozyme and its amyloidogenic variants have shown that the native states of pathogenic variants are significantly less stable when compared to the wild-type protein[26]. Experimental data have shown that a lesser conformational stability of lysozyme variant correlates to a more amyloidogenic behaviour[27]. The suggested molecular mechanism of fibrillation of amyloidogenic variants of human lysozyme points to the native states in dynamic equilibrium with partially unfolded species. In turn, the partially unfolded intermediates of lysozyme can undergo self-association, leading to formation of β-sheet ordered aggregates and, eventually, amyloid fibrils[27].

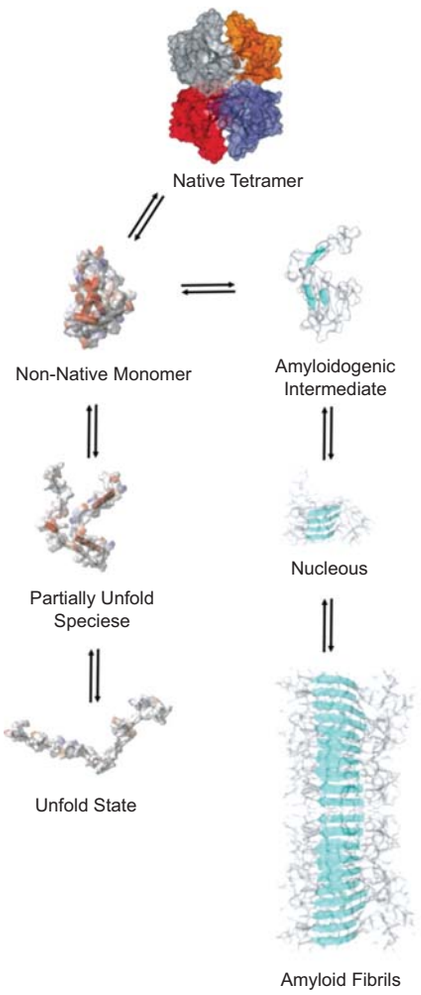


***Figure 3:*** Schematic representation of pathways for amyloid fibril formation from a homo-tetrameric protein precursor according to Quintas et al.[8]. The oligomeric protein in its native state dissociates into a non-native monomer. The non-native monomeric species formed from dissociation may undergo several conformational changes due to a lack of conformational stability. As a result of hydrophobic exposition, these species may associate to form aggregates that eventually form amyloid fibrils.



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Figure 4 presents possible amyloidogenic paths from native structure to different putative intermediates and, finally, to amyloid fibril formation.

*Intrinsically unfolded protein as amyloid precursors*

After ribosomal synthesis, the folding pathway of globular proteins is overcome with a delicate balance between the hydrophobic effects, non-covalent interactions such as hydrogen bonds and configurational entropy. The later is the negative counter-balance of forces involved in protein folding. However, IUP show a low overall hydrophobicity and a large net charge. Consequently, the major driving effect in the folding pathway is configurational entropy, which may surpass the hydrophobic effect. This balance impels the polypeptide chain into a native disordered state[28]. Although the general underlying molecular mechanisms of amyloidogenesis for globular proteins are associated with protein misfolding, IUP must go through a partial folding in order to undergo aggregation and amyloid fibril formation[29].

The triggering cause for partial folding and amyloid fibril formation seems to be related to (i) natural propensity to form β-sheet intermediates; (ii) covalent modification, which may lead to the development of local structure; or (iii) protein overexpression, which may lead to aggregation simultaneously with β-sheet formation[30].

α-synuclein is an IUP associated with Parkinson's disease (PD), a movement disorder characterized by degeneration of dopaminergic neurons in the *substantia nigra* in the brain[31]. α-synuclein amyloid fibril formation seems to occur in the form of partially folded intermediate, pre-molten globule-like structure, the first step for fibrillization[31] (Figure 5).

**Conformational disorders and native-like aggregation**

A key question regarding pathologies involving protein aggregation and deposition is the mechanism by which such protein precursors are transformed from their native state into high-ordered aggregates. There are two major pathways that have been identified till date. The amyloidogenic route, as previously

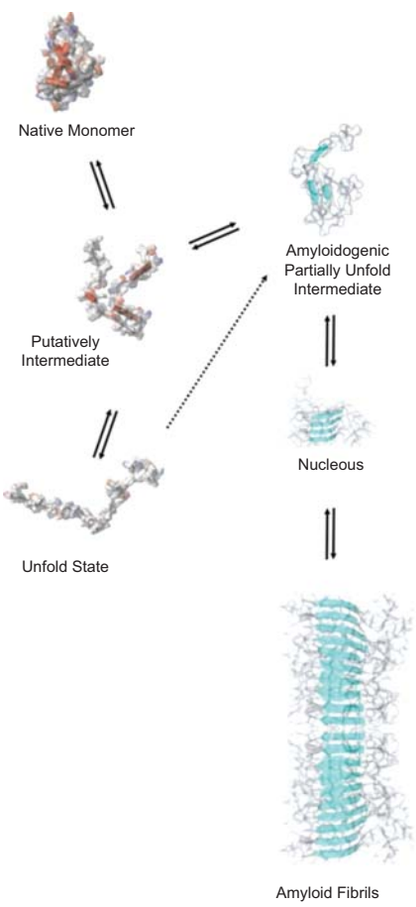


***Figure 4:*** Schematic representation of molecular pathways for amyloid fibril formation from a monomeric protein precursor. Due to its lesser conformational stability, monomeric protein in its native state can populate partial unfolded states that in turn can form amyloidogenic intermediates with high propensity for aggregation. Eventually, these intermediates lead to amyloid fibril formation.

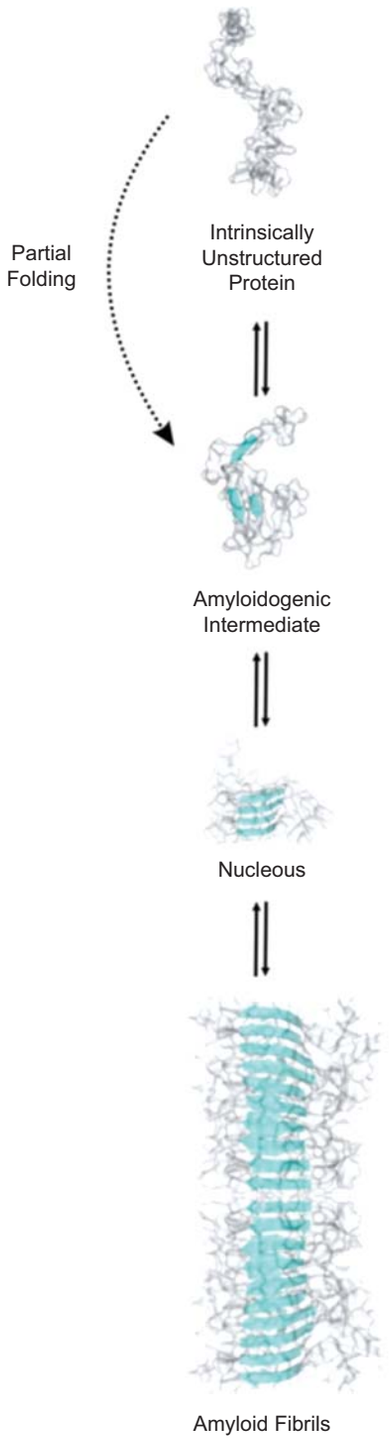


***Figure 5:*** Schematic representation of the molecular pathways of amyloid fibril formation from an IUP precursor. The IUP partial folds into a β-sheet intermediate. This may starts to aggregate and eventually progress to amyloid fibrils.



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mentioned, is where aggregation starts from misfolded conformational states of proteins, and the native-like aggregation pathway is where aggregation of normally globular proteins may occur directly from native states via mutations, thermal fluctuations, protein concentration or *post*-translational modifications[13,32,33]. Unlike amyloidogenesis, native-like aggregation pathways do not present an unfolding specie to trigger oligomerization[33,34]. In fact, native-like aggregation seems to proceed by stacking of near-native protein intermediates towards oligomeric species of finite size[32,33] (Figure 6).

In humans, native-like aggregation has been described in a very small group of proteins. Among them β-2-microglobulin is associated with dialysis-related amyloidosis, factor VIII to haemophilia A, and insulin to insulin-injection amyloidosis[12,13,35]. One study with β-2-microglobin suggests that amyloid fibril formation occurs through self-assembly of native-like intermediates[12]. Pisal et al. showed that native-like aggregates of factor VIII are significantly more immunogenic than the non-aggregated monomeric form[36]. Oliveira et al. showed that post-folding modification of insulin with methylglyoxal inhibits formation and growth of insulin amyloid fibrils, blocking the seeding nuclei[13]. Interestingly, β-2-microglobulin is an all-beta motif protein whereas insulin is an all-alpha motif protein. This suggests that depending on the protein precursor fold class, native-like aggregation may proceed to amyloid-like fibrils.

A very interesting finding by Deva et al. show that native-like aggregation may inhibit ribosomal protein S6 from *Thermus thermophilus* to form amyloid. However, a very high protein concentration must be reached. According to the authors[33], the off-pathway towards native-like aggregates overrules amyloid fibril formation at high protein concentration. It

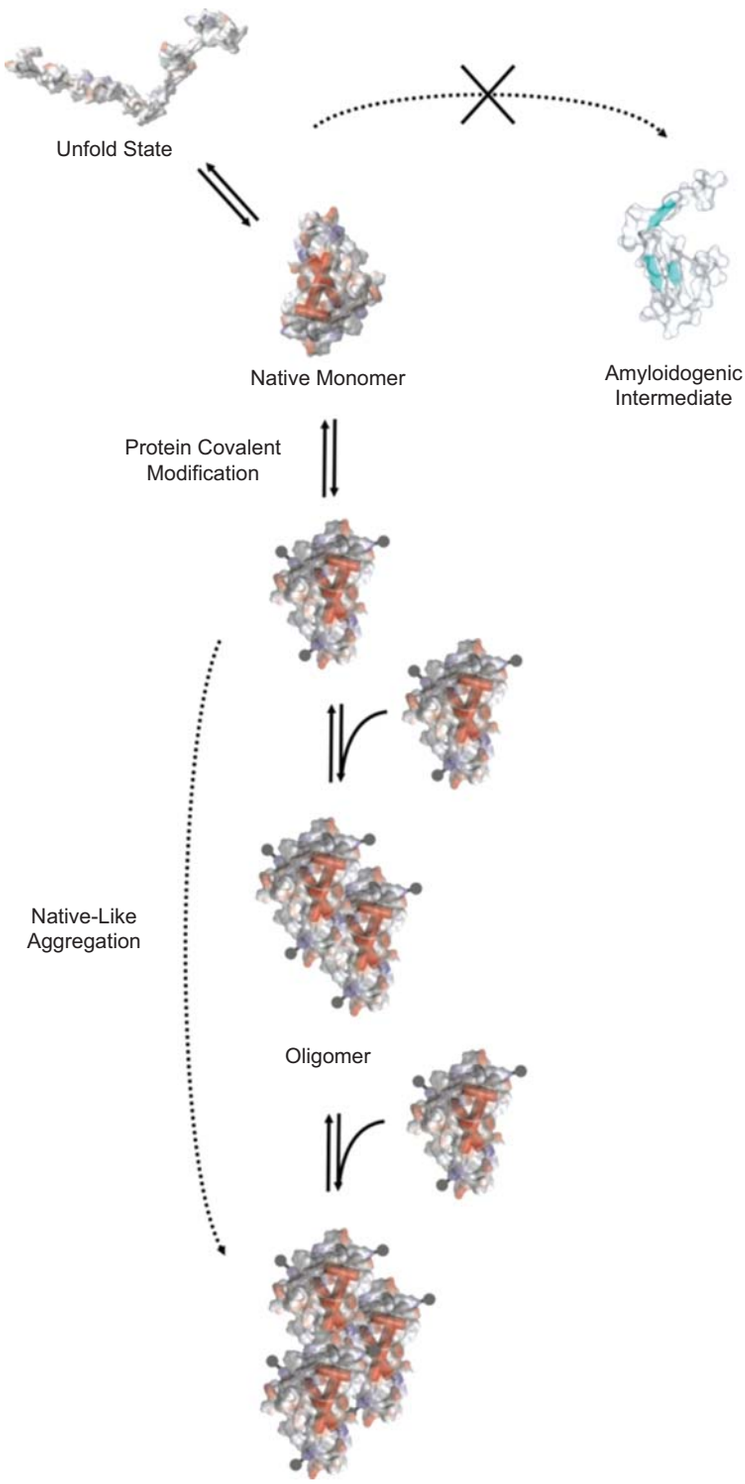


***Figure 6:*** Native-like aggregation model. The native protein can primarily follow two different pathways. It can undergo a rapid equilibrium with a partial unfolded monomeric form, or it can irreversibly go through an aggregation pathway by sequential addition of glycated monomers in each step of the reaction, as represented by the curve arrows. Based on Oliveira et al.[37].



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seems that partial unfold intermediate formation makes the amyloidogenic pathway less favourable with the increment protein concentration[33].

The association between native-like aggregation and human disorders suggests that this is more relevant than was thought.

### What drives an amyloidogenic protein into a native-like aggregation pathway?

Recent observations have shown that amyloid proteins, such as β-2-microglobulin, alpha-synuclein, insulin and ribosomal protein S6, can follow a native-like aggregation route[12,13,33,37]. Some of these proteins when covalently modified or in very high protein concentration environment change from an amyloid fibril formation pathway to an native-like aggregation pathway[13,33,37]. The native-like aggregation pathway seems to compete with the amyloid fibril pathway.

Oliveira et al. have investigated the effects of methylglyoxal modification in insulin structure and fibril-forming properties. Circular dichroism studies showed that insulin glycation leads to native-like aggregation and that insulin fibril formation is substantially reduced. In effect, the authors suggest that glycation impairs insulin conformational alterations to the extent that they convert into an amyloidogenic intermediate, inhibiting the fibrillation process by blocking the formation of the seeding *nuclei*. Ultimately, fibril formation is reduced due to a lack of critical concentration of seeds[13].

Deva et al. has shown a similar result in different environmental conditions with ribosomal protein S6[33]. The authors state that when the concentration of ribosomal protein S6 is too high, the tendency is to form native-like aggregates instead of amyloid fibrils. According to the authors, amyloid fibrillation pathway demands a structural rearrangement of the monomeric specie into a pre-aggregation monomer before it starts to aggregate. This means there is a zero-order kinetics step before the aggregation into amyloid takes place in such a way that at very high concentration of protein, native-like aggregation, a pure first-order step, is favoured[33].

Recently, a model for native-like aggregation of methylglyoxal-glycated proteins has been proposed[32]. Native-like aggregation occurs due to localized protein structural changes leading to a decrease on the conformational stability of the modified protein. Interestingly, the decrease in the stability of monomeric specie is counterbalanced by the formation of native-like aggregates that are thermodynamically more stable. The same study observed that the formation of glycated cytochrome c unfolded species is an off-pathway of the native-like aggregation route. The authors suggest that glycation of amyloidogenic proteins may lead to a shift from an amyloidogenic pathway to a native-like aggregation through a process that is thermodynamically and kinetically favoured[32].

Taking together the observations previously presented, what emerges is a preliminary picture of the driving forces that make an amyloidogenic protein follow a route of native-like aggregation. The first step into the native-like aggregation route points to the inhibition of amyloidogenic intermediate formation. This may happen due to hindrance of amyloidogenic sequences to aggregate after post-folding modification of the protein precursor. The second step is the overcoming of protein free-energy landscape barrier into native-like aggregation. This might happen through a significant increment in protein concentration or by reducing the activation energy between native-states and native-like aggregates. The second step can happen independently of the first step.

## Discussion

The authors have referenced some of their own studies in this review. The protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed.

Understanding how amyloidogenic proteins aggregate and form amyloid is a key issue in bringing light to understanding human conformational diseases and designing novel therapeutic approaches. Amyloid fibril formation usually occurs through one of the following events: (i) defective protein folding pathway and formation of misfolding intermediates, (ii) partial unfolding of the native state due to lack of protein stability, (iii) defective folding due to overcrowding of polypeptide chains in the endoplasmic reticulum and (iv) overwhelming of the cell-folding quality-control systems[8,19,38]. Overall, the underlying molecular mechanisms of aggregation into amyloid fibrils imply conformational changes of protein, disruption of native non-covalent interactions[35] and formation of aggregation-prone non-native intermediates[21].

Until recently amyloid fibril formation has been seen as a unique aggregation pathway in human conformational disorders. However, native-like aggregation may play a significant role in physiological processes. The biophysical mechanism underlying this process is not yet fully understood. However, the consensus is that native-like aggregation seems to proceed by stacking of near-native protein intermediates towards oligomeric species of finite size. In addition, experimental data suggest that the native-like aggregation pathway competes with the amyloid fibril pathway. Covalent modifications such as glycation can prevent the formation of amyloidogenic intermediates, inhibiting the amyloidogenic pathway.

## Conclusion

More laboratory data should be gathered about the structure, stability, dynamics and aggregation kinetics in



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order to get a clearer picture of the biophysical mechanisms underlying native-like aggregation.

Understanding what makes a protein follow an amyloid or a native-like aggregation pathway is essential to opening new avenues in therapeutic approaches to conformational diseases.

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