Amyloid Formation by Transthyretin: From Protein Stability to Protein Aggregation

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Abstract: In recent years the issues of protein stability, folding and aggregation have become central in several pathological conditions and in particular in amyloid diseases. Here, we review the recent developments on the molecular mechanisms of amyloid formation by transthyretin (TTR), in particular, in what concerns to protein conformational stability, protein folding and aggregation.

Transthyretin has been implicated in pathologies such as senile systemic amyloidosis (SSA), familial amyloid polyneuropathy (FAP) and familial amyloid cardiomyopathy (FAC) which are characterized by extracellular deposition of insoluble amyloid fibrils. SSA is generally a mild disorder and affects predominantly individuals over 80 years of age. In contrast, FAP is an autosomal dominant lethal disease, characterized by peripheral neuropathy, which may affect individuals from their twenties. While in SSA WT-TTR and its fragments are the major constituents of the amyloid fibrils, in FAP and FAC the amyloid fibrils are mostly constituted by variants of TTR. Today, more than 80 amyloidogenic TTR mutations throughout the TTR sequence are known.

Transthyretin is a homotetrameric protein found in the plasma and in the cerebral-spinal fluid, it is synthesized in the liver and in the choroid plexus of the brain, it has a total molecular mass of 55kDa and a high percentage of $\beta$-sheet. Current views on amyloid fibril formation by TTR state that, depending on the protein variant or solution conditions, the native tetrameric protein might dissociate to non-native or partially unfolded monomeric (or even dimeric) species with a high tendency for ordered aggregation into soluble oligomers which grow into insoluble oligomers and eventually mature amyloid fibrils. Thus, issues such as dissociation thermodynamics and dissociation kinetics of the native tetrameric TTR and thermodynamic stability and conformational fluctuations of the non-native TTR molecular species are essential in determining the amyloidogenic potential of different TTR variants. In addition, several other cellular and tissue factors must be involved in modulating the penetrance and age of onset of amyloid pathologies by TTR.

Key Words: Transthyretin, Amyloid, Familial Amyloidotic Polyneuropathy, Protein Stability, Protein Aggregation, Misfolding.

INTRODUCTION

Over the years, transthyretin (TTR) has been implicated in several amyloid pathologies. Professor Corino de Andrade first identified, in Portugal, a “peculiar form of peripheral neuropathy, characterized histologically by generalized amyloidosis, involving specially the peripheral nerves”, and manifested by early impairment of pain and heat sensations in the lower limbs and progressing to a general lowering of the state of health, gastro-intestinal disorders, premature impotence, disorders of the sphincters and other symptoms, and having a high mortality rate [1]. Following this early work, Costa and collaborators [2] identified TTR as the major protein component of the amyloid fibrils in portuguese patients with Familial Amyloidotic Polyneuropathy (FAP). Subsequently, the genetic defect in this autosomal dominant disease was identified as a single point mutation in the TTR gene leading to a protein variant with methionine replacing a valine at position 30 (Val30Met-TTR) [3, 4]. The same mutation has also been identified in FAP patients from northern Sweden [5, 6], Japan [7] and some other minor foci worldwide. Although a single origin for the mutation was initially considered, haplotype analysis strongly suggests that the same mutation may have independently arisen in different populations [8, 9].

Human TTR is a homotetrameric protein with 127 aminoacids per subunit, 55 kDa of total molecular mass, and a high percentage of $\beta$-sheet (Fig. (1)). The tridimensional crystal structure [10-12] the gene sequence [13, 14] and the gene localization in chromosome 18 [14] are well established. The name transthyretin derives from the two known functions of the protein: thyroxine transport and retinol (vitamin A) transport. Thyroxine binding to TTR shows
negative cooperativity [15] and the crystal structure of TTR reveals a large hydrophobic central channel, formed at the interface between two dimers, where two thyroxine binding sites are located [16]. Retinol is transported bound to the retinol binding protein (RBP) in association with TTR. The crystal structure of the complex between TTR and RBP has been solved and revealed a stoichiometry of 2RBP:1TTR [17, 18].

Transthyretin in humans is mostly found in the plasma, and represents a high proportion (around 25%) of the total protein in the cerebral-spinal fluid (CSF) [19]. The major sites of transthyretin biosynthesis are the liver and the choroid plexus of the brain and they seem to form independent TTR pools. The TTR turnover in vivo is relatively rapid with a half-life in the plasma of approximately 2 days.

Today, more than 80 TTR mutations are identified1. Only a small proportion of the known mutations are apparently non-amyloidogenic. The majority is related to amyloid deposition affecting predominantly the peripheral nervous system and the heart. The most common form of TTR-related polyneuropathy is due to the Val30Met mutation. Clinical manifestations, age of onset and penetrance vary among different human populations, but generally the disease is characterized by early impairment of temperature and pain sensation in the feet, progressing to autonomic dysfunction, and leading in many cases to death. Most TTR mutations produce similar clinical manifestations, involving peripheral and autonomic neuropathy, and frequently additional involvement of other organs such as the heart, gut, kidneys, and the vitreous of the eye. This is the case, for example, of Leu55Pro-TTR, which produces a more aggressive form of the disease with an early age of onset and cardiac involvement [20].

Some amyloidogenic TTR mutations produce somewhat different phenotypes, with clinical manifestations dominated by, for example, cardiomyopathy (Val122Ile-TTR) [21, 22], vitreous opacities (Tyr69His-TTR), carpal tunnel syndrome (Tyr114His-TTR) [23], or even leptomeningeal amyloid (Asp18Gly-TTR) [24]. The Val122Ile-TTR mutation is associated with the most common form of cardiac amyloidosis, and is more common in the United States among individuals of African descent [25, 26]. It is not yet known why different TTR mutations may apparently produce different clinical manifestations, but potential differences in the molecular properties of each TTR variant as well as differences in tissue and cellular factors among different human populations may play a critical role.

A few cases of homozygosity for the Val30Met mutation are known, but do not lead to a more severe form of the disease [27]. Of particular significance is the existence of heterozygous individuals with amyloidogenic and non-amyloidogenic mutations, usually in different alleles. At least in two such cases (Thr19Met/Val30Met and Arg104His/Val30Met) the non-amyloidogenic mutation gives some protection against the disease, with the subjects presenting close to normal phenotypes [28-30]. Interestingly, it was recently identified a compound heterozygous TTR (Thr59Lys/Arg104His) in a patient of Chinese ancestry, who was diagnosed with ATTR [31]. In this case the protection given by the Arg104His mutation did not overcome the influence of the amyloidogenic mutation Thr59Lys.

TTR has also been identified as the causative agent of Senile Systemic Amyloidosis (SSA). This is mostly an asymptomatic condition, characterized by amyloid deposition in many organs, and affecting approximately 25% of the people over 80 years old [32]. Some individuals are affected by heavy deposition of amyloid in the myocardium, which may lead to cardiomegaly and congestive heart failure [33-36]. Interestingly, in SSA the amyloid fibrils are mostly constituted by WT-TTR and proteolitic fragments starting at positions 46, 49 and 52 of the WT-TTR sequence [37]. It is not clear if the proteolitic cleavage occurs before or after amyloid fibril formation, but the presence of a widely variable proportion of full length TTR in the fibrils suggests that proteolysis is a secondary event [37].

The development of SSA in older individuals may raise the question of which protective mechanisms are responsible for avoiding amyloid formation by WT-TTR in younger individuals. General mechanisms of protein degradation and protein turnover, for example, may be less efficient in older persons. We may even find that some of these mechanisms are also responsible for the different ages of onset and the different penetrance rates among different individuals and populations in all TTR-related amyloidosis.

Another interesting issue raised by WT-TTR and SSA has to do with cytotoxicity. Do different TTR variants produce molecular species with different cytotoxic potential? Or is the development of clinical symptoms more dependent on the amount of toxic species produced by different TTR variants? These are questions to be answered in years to come.

TRIDIMENSIONAL STRUCTURE

TTR is a tetrmeric protein composed of identical subunits. Each monomer has a β-sandwich fold composed of two four-stranded β-sheets labeled DAGH and CBEF (Fig. 1). The β-sheets from two monomers associate edge-to-edge to form a dimer, composed of two extended β-sheets formed by strands DAGH’G’A’D’ and CBEF’E’B’C’.

Association of two dimers through hydrophobic contacts and hydrogen bonds between the AB and GH loops, as well as, strand H form the soluble and functional tetrameric protein.

The X-ray crystallographic structure of human TTR was initially reported by Colin Blake [10] and over the years the tridimensional structures of several TTR variants were determined with the aim to identify potential structural changes responsible for amyloid formation. Currently, more than 50 structures of TTR are available in the Protein Data Bank, including structures of very high resolution, structures from different animal species, several natural and synthetic amyloidogenic and non-amyloidogenic TTR variants, and TTR structures with the natural ligands thyroxine and RBP bound, as well as, several structures with synthetic small molecules bound. In all the cases studied to date, human

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1 For recent reviews on TTR and known TTR mutations see references [89-94]. Additionally, see the following web sites: http://gaia.ibmc.up.pt/~mjsaraiv/ttrmut.html; http://www.bumc.bu.edu/Departments/PageMain.asp?Page=5530&DepartmentId=354 and http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=176300
TTR shows the same global fold. A detailed analysis of 23 TTR structures concluded that in most cases only minor structural changes are observed, and the small structural differences observed between amyloidogenic variants and WT-TTR are not significantly bigger than what is observed, for example, when two independently determined structures of WT-TTR are compared [12]. However, two exceptions should be considered. These are the cases of two highly amyloidogenic TTR variants, the naturally occurring mutant L55P-TTR and the synthetic triple mutant Gly53Ser-Glu54Asp-Leu55Ser-TTR. In both cases, different packing interactions in the crystal and conformational changes in the region of β-strand D were observed.

In the case of Leu55Pro-TTR, the protein crystallizes with eight monomers per asymmetric unit and the small β-strand D adopts a coil conformation, becoming part of a long loop that connects β-strands C and E [38]. This long loop is located at the edge of the subunit β-sandwich and consequently may facilitate the exposure of a new region of the molecule to the solvent. Additionally, the hydrogen

![Fig. (1). Schematic views of the crystal structure of tetrameric WT-TTR (Protein Data Bank code 1TTA [11]), emphasizing the interactions between subunits. (A) and (B) are Molscript [97] representations of the tetramer (B) and the crystallographic dimer (A) showing the identification of the β-strands (arrows). (C) and (D) show the native inter-subunit contacts at the monomer-monomer interface of the dimer (C) and at the dimer-dimer interface of the tetramer (D). Polar aminoacid residues are represented in red tones and hydrophobic residues are represented in yellow tones. In (C) it is represented the surface of one subunit and the residues of the other subunit across the monomer-monomer interface. In (D) it is represented the surface of the crystallographic dimer and the residues of the other dimer across the dimer-dimer interface. (C) and (D) were created with the program VMD [98].](image-url)
bonds between the AB-loop of one dimer and strand H of the other dimer are significantly longer than those observed in WT-TTR, which may indicate a potentially less stable tetramer in Leu55Pro-TTR [38].

In the case of the triple mutant Gly53Ser-Glu54Asp-Leu55Ser-TTR, a three-residue shift in β-strand D is observed, changing the normal register of the β-sheet and the normal pattern of hydrogen bonds between β-strands D and A. This shift, the β-slip, affects residues 50 to 63 in the CD-loop, D-strand and DE-loop [39]. New packing interactions between tetramers are also observed in the crystal. These involve the β-slip region of one subunit from one tetramer and the RBP binding site from a second tetramer, which led the authors to propose a model for amyloid formation based on aggregation of tetrameric TTR [39]. However, this model is not consistent with most of the experimental data on amyloid fibril formation by TTR, as we shall see later in this review.

Recently, the X-ray crystal structure of the hybrid variant Val30Met/Thr119Met-TTR, isolated from human plasma, has also been solved, and shows enhanced intra-subunit and inter-subunit interactions, when compared with Val30Met-TTR, in a pattern very similar to what is observed for Thr119Met-TTR [40]. This might explain in part why the mutation Thr119Met seems to protect carriers of the Val30Met mutation to develop FAP. Determination of new very high resolution structures of amyloidogenic and non-amyloidogenic TTR variants might help us understand to what extent these subtle structural changes of the native state influence the global stability of the protein and eventually determine the amyloidogenic potential of the variant.

An interesting topic of consideration is the distribution and incidence of amyloidogenic and non-amyloidogenic mutations over the protein sequence. In contrast to what might be expected, amyloidogenic mutations are not restricted to the most rigid or inner regions of the protein, or even the subunit interface regions. In fact, the amyloidogenic mutations are distributed over the entire TTR sequence [41]. The regions with the lowest concentration of amyloidogenic mutations are the N- and C-terminals, the α-helix, part of the EF-loop, and the FG-loop. The majority of non-amyloidogenic mutations are located in the flexible regions of the N- and C-terminals, the FG-loop, and the surface-exposed saddle (Fig. (1B)) [41]. It is also interesting to point out that the same position in the TTR sequence may give rise to amyloidogenic and non-amyloidogenic variants, depending on the aminoacid replacement. Ala109 is such an example, giving rise to non-amyloidogenic variants in Ala109Thr and Ala109Val, and to an amyloidogenic variant in Ala109Ser.

**PROTEIN STABILITY**

In recent years, amyloid diseases have also been called “protein misfolding disorders”, which could be interpreted as the inability of a protein to fold correctly. This terminology should be used with care because most amyloid diseases are probably not due to errors in the normal folding pathway of a protein, but due to decreased protein conformational stability, partial unfolding events or even conformational fluctuations of the native structure ensemble, which allows the protein to more easily search alternative stable conformational states. Thus, we would prefer to use the expression “protein conformational disorders”.

Most amyloidoses known are characterized by the extracellular deposition of amyloid fibrils [42]. At least in the case of these extracellular amyloid diseases, amyloid formation is probably a consequence of the post-folding fate of a protein in the extracellular environment. Thus, issues such as protein stability, conformational fluctuations, protein unfolding pathways and protein turnover must be of particular relevance in amyloid formation.

In the case of TTR, an oligomeric protein, the issue of protein stability by itself has several levels of potential importance (Fig. (2)). In fact, in this context protein stability includes dissociation equilibrium, dissociation kinetics, conformational stability and unfolding kinetics of the native tetramer and other non-native species. In order to fully understand the molecular mechanisms of amyloid formation, all these components of the protein stability issue have to be independently and controllably studied. However, in some instances, it is not obvious which components are playing a role.

**Conformational Stability and Conformational Fluctuations of Tetrameric TTR**

It has long been known that WT-TTR is highly stable to chemical unfolding [43]. More recently, micro-calorimetric work [44] and pressure-induced unfolding experiments followed by NMR [45] confirmed in a more quantitative way this earlier view. In fact, the micro-calorimetric studies showed that, at pH 7.0 and relatively high protein concentrations (6.2 µM tetramer) both amyloidogenic and non-amyloidogenic variants of TTR showed very high transition temperatures (6.2 µM tetramer) both amyloidogenic and non-amyloidogenic variants of TTR showed very high transition temperatures (Tm) for thermal unfolding. The four variants studied, V30M-, L55P-, T119M- and WT-TTR showed transition temperatures between 92 and 101 °C, at 2 atm of pressure [44]. These authors also showed that the thermal unfolding of tetrameric TTR followed a two-state mechanism, with an equilibrium between native tetramer (N4) and unfolded monomer (U) (Eq. 1).

\[
N_4 \rightleftharpoons 4U \quad (\text{Eq. 1})
\]

Interestingly, it was also pointed out that, although the amyloidogenic variants are less stable than WT- and T119M-TTR, the relative conformational stability of tetrameric TTR does not perfectly correlate with the amyloidogenic potential of the four TTR variants studied [44].

Pressure-induced unfolding experiments of tetrameric WT- and V30M-TTR, followed by NMR, also showed that, at neutral pH and high protein concentrations (0.14 to 0.17
mM), these proteins unfold following a simple two-state equilibrium between native tetramer and unfolded monomer (Eq. 1) [45]. The authors pointed out that the native tetrameric form of WT-TTR is highly stable ($\Delta G_{\text{unf}} = 104$ kJ/mol, at 37 °C and pH 7.1), but the conformational stability is reduced by 18 kJ/mol in the amyloidogenic variant V30M-TTR [45].

![Diagram](image)

**Fig. (2).** Diagram representing some of the possible routes for inter-conversion between different TTR molecular species in solution. With all likelihood, not all these molecular species will be present simultaneously in solution. However, depending on the TTR variant or the solution conditions, most of these molecular species have already been observed experimentally. And these molecular species are equilibrium species. The picture could become more complicated if kinetic intermediates were considered. This clearly demonstrates the complexity in defining and studying TTR stability because it relates to issues such as: thermodynamics and kinetics of tetramer and dimer dissociation; tetramer, dimer and monomer conformational stability; and tetramer, dimer and monomer unfolding kinetics.

Results of chemically-induced unfolding experiments of TTR are more difficult to interpret. It has been reported that tetrameric WT-TTR denatures at concentrations of 4 to 6 M guanidinium chloride (GdmCl), and refolding and reconstitution exhibits hysteresis [46]. However, recently, the authors evaluated the denaturation energetics of several TTR variants, using urea-induced unfolding experiments, and reported curves with transitions at urea concentrations around 3 M [47] which is quite unexpected for tetrameric TTR, at the protein concentrations used (1.8 µM tetramer), knowing that urea is a less effective denaturant than GdmCl. However, as the authors mentioned, the reported curves reflect the slow urea-induced dissociation of tetrameric TTR followed by tertiary structural changes of the resulting species. Thus, these urea-induced denaturation curves can not be taken as a direct measurement of the conformational stability of tetrameric TTR, but they probably report more closely on tetramer dissociation.

Recent results of GdmCl-induced unfolding experiments of tetrameric WT-, V30M- and L55P-TTR suggest that the amyloidogenic variants follow alternative equilibrium unfolding pathways, when compared with WT-TTR. This observation might have physiological relevance because it shows different tendencies of these TTR variants to produce different intermediate molecular species in their unfolding mechanism [48].

It is thus clear that more quantitative studies on the conformational stability of different TTR variants are needed in order to properly evaluate how important is the unfolding equilibrium of the native tetramer in producing the molecular species responsible for amyloid formation in vivo. It becomes also clear that it is of the utmost importance to clearly separate the issues of dissociation and unfolding and the issues of equilibrium and kinetics.

In addition to the issue of conformational stability, it is also important to point out that some amyloidogenic TTR variants, even in the tetrameric form, do show conformational fluctuations that expose protein surfaces characteristic of the fibrillar forms. Thus, some amyloidogenic variants may have a native state compatible with a wider range of accessible conformations than WT-TTR. Apparently, this is the case of synthetic mutants such as TTRdel53-55 (with residues 53, 54 and 55, belonging to $\beta$-strand D, deleted) or Gly53Ser-Glu54Asp-Leu55Ser-TTR (TTRs53-55) [49] and Tyr78Phe-TTR [50]. Using monoclonal antibodies (mAbs) raised against the TTRs53-55 mutant, the authors identified new epitopes in these highly amyloidogenic mutants, which are not exposed in native WT-TTR or even in native V30M-TTR. Interestingly, these mAbs also reacted with ex vivo amyloid fibrils from the vitreous body of the eye. The epitopes for two of these monoclonal antibodies were mapped to two regions of the TTR sequence comprising aminoacid residues 39-44 ($\beta$-strand C) and 56-61 (loop DE), suggesting loss of structure in $\beta$-strands C and D [49]. These data also indicate that, in the amyloid fibrils, the region corresponding to $\beta$-strands C and D may be exposed to the solvent. Total or partial unfolding of strands C and D may then expose new protein surfaces for inter-subunit interaction and TTR aggregation.

Comparative quantitative analysis of the Circular Dichroism (CD) far-UV spectra for V30M-, L55P-, T119M- and WT-TTR showed that the L55P-TTR tetramer, the most amyloidogenic of the four variants analyzed, loses more than 20% of the regular secondary structure, in solution, at pH 7.0, when compared with WT-TTR [48]. This is additional evidence for significant conformational fluctuations of the native tetrameric structure in the more amyloidogenic TTR variants. Evidence of larger conformational fluctuations in variant TTR than in WT-TTR has also been obtained from studies of susceptibility to thiol conjugation [51]. Hydrogen
exchange studies, at pH 7, followed by NMR, also show a larger conformational plasticity for L55P-TTR when compared with WT-TTR, T119M-TTR or even V30M-TTR [52]. These data also show that the fastest amide exchange rates in the TTR \( \beta \)-sandwich are associated with residues belonging to strands C and D, and to a lesser extent strand F, in the four variants studied.

**Dissociation of Tetrameric TTR**

Oligomeric proteins create additional problems to the issue of protein conformational stability. In fact, the equilibrium between native protein (\( N_n \)) and unfolded monomer (\( U \)) (Eq. 2c) may be subdivided in two different processes, the dissociation equilibrium between the native oligomer (\( N_n \)) and \( n \) subunits (\( S \)) (Eq. 2a), and a second equilibrium corresponding to the unfolding of the subunits (Eq. 2b):

\[
N_n \rightleftharpoons nS \quad \text{(Eq. 2a)}
\]
\[
S \rightleftharpoons nU \quad \text{(Eq. 2b)}
\]
\[
N_n \rightleftharpoons nU \quad \text{(Eq. 2c)}
\]

Depending on the protein being studied and the method used to study the conformational stability, the two stages of the equilibrium above may or may not be observed experimentally. In fact, several studies on thermal unfolding of oligomeric proteins by differential scanning calorimetry (DSC) do show a simple two-state equilibrium between native oligomer and unfolded subunit, which means that at least in some cases, dissociation and unfolding do occur simultaneously. Additionally, the species indicated as \( S \), in the equations above, may have a structure very similar to the native subunit in the oligomeric protein, or alternatively a conformationally altered or partially unfolded structure.

Independently of the existence or not of a stable intermediate in the equilibrium unfolding pathway of an oligomeric protein, the dissociation stage of this equilibrium is highly dependent on protein concentration, which is not the case for simple unfolding equilibria in monomeric proteins. For example, in the case of a tetramer dissociating to four monomers with dissociation constant of the order of 1.7X10\(^{-18}\) M\(^2\), for a total protein concentration of 10 \( \mu M \)\(_{\text{tetramer}}\), a tetramer-to-monomer ratio of 9.5 \( \mu M \)\(_{\text{tetramer}}\) to 2 \( \mu M \)\(_{\text{monomer}}\) is observed at the equilibrium. However, for a total protein concentration of 0.2 \( \mu M \)\(_{\text{tetramer}}\) a very different molar ratio of 0.055 \( \mu M \)\(_{\text{tetramer}}\) to 0.55 \( \mu M \)\(_{\text{monomer}}\) is observed.

In this example, a dilution of 50 times in the total protein concentration leads to a complete change in the proportion of tetramer-to-monomer in solution. However, not only the relative proportion of different molecular species is important but also the concentration of each one of the species. Thus, in oligomeric proteins it is crucial the relationship between the physiological level of the protein concentration and the respective equilibrium dissociation constant, to determine the amounts of different molecular species present. This however is only physiologically relevant if the dissociation kinetics is significantly faster than the turnover of the native protein. If the dissociation kinetics is much slower than the protein turnover in vivo, then the protein never reaches its dissociation equilibrium, or by other words, the oligomeric protein is kinetically stabilized. This in fact might be the case for many oligomeric proteins [53].

In the case of TTR, it was found that solution conditions which favored amyloid formation, such as low pH, also favored dissociation of tetrameric TTR to monomers [54]. Later it was reported that even at neutral pH and at physiologically relevant protein concentrations, TTR dissociation to monomeric species could be experimentally observed [55]. For example, WT-TTR showed 50% of tetramer dissociation at protein concentrations around 0.2 \( \mu M \), which is a concentration in the range found for example in the cerebral spinal fluid. The dissociation was slightly shifted towards the monomer in the case of V30M-TTR and L55P-TTR, and towards the tetramer in the case of T119M-TTR. More recently, Kelly and collaborators used urea-induced dissociation curves to compare the behavior of several TTR variants [47]. This study showed the following dependence towards urea-induced tetramer dissociation: V30M-TTR ≅ L55P-TTR < WT-TTR ≅ V122I-TTR ≅ T119M-TTR, with V30M and L55P-TTR more prone to dissociation, but with V122I-TTR, an amyloidogenic variant, having a tendency for dissociation very similar to WT-TTR.

The studies just mentioned show that tetramer dissociation thermodynamics can not fully justify the observed spectrum of amyloidogenic potential in TTR variants. In fact, studies on the kinetics of urea-induced tetramer dissociation were reported [47, 56] and showed that amyloidogenic TTR variants such as V122I-TTR and L55P-TTR have urea-induced dissociation rate constants (extrapolated to 0 urea concentration) 2 and 9 times larger than WT-TTR. However, V30M-TTR has a urea-induced dissociation rate slightly slower than WT-TTR. These results clearly show that dissociation kinetics and dissociation thermodynamics of tetrameric TTR are both implicated in determining the amyloidogenic behavior of TTR variants, but thermodynamics and kinetics of dissociation do not have correlated variations among TTR variants.

**Conformational Stability and Conformational Fluctuations of Monomeric TTR Species**

We have just seen in this review that the thermodynamics and the kinetics of TTR tetramer dissociation do not seem to completely justify the amyloidogenic potential of some of the TTR variants known. In fact, several reports have pointed out the need for tetramer dissociation, but also monomer partial unfolding, in order to form amyloid fibrils [57-61]. Kelly and collaborators have stressed the need of a low pH environment, around pH 4.5, in order to form amyloid fibrils. However, other authors have shown that, in highly amyloidogenic TTR variants, amyloid fibrils are readily formed even at neutral pH [59, 62, 63]. Both at low and neutral pH, it was reported that tetramer dissociation and monomer partial unfolding were required for amyloid fibril formation. From ageing studies of several TTR variants, at pH 7 and 37 °C, it was shown that monomer unfolding preceded fibril formation [59, 60]. In this study it was also shown that the conformational stability of the monomeric species of T119M-, WT-, V30M- and L55P-TTR correlated quite well with the amyloidogenic potential of each one
these TTR variants. While the monomer of T119M-TTR was reported as having a conformational stability of 5.8 kcal/mol, L55P-TTR has only a marginal stability of 2.5 kcal/mol [59, 60].

It seems that the unfolding thermodynamics (conformational stability) of the monomeric species play an important role in determining the amyloidogenic potential of TTR variants. To this we may add, depending on the TTR variant, the contribution of the dissociation equilibrium and/or the dissociation kinetics of the tetramer. For example, the conformational stability of the monomer of V122I-TTR is very close to WT-TTR, but the variant protein has faster dissociation kinetics [47]. On the contrary, the amyloidogenic V30M-TTR variant has dissociation kinetics slightly slower than WT-TTR [47], but higher dissociation tendency [55] and lower conformational stability of the monomer [59, 60]. Thus, these three factors: i) unfolding thermodynamics of the monomeric species, ii) dissociation thermodynamics of the tetramer and iii) dissociation kinetics of the tetramer, may prove crucial in producing the necessary amounts of intermediate molecular species leading to aggregation and amyloid formation.

Tetramer dissociation and monomer unfolding are key events on amyloid formation by TTR. However, from a structural point of view, a question remains: how much monomer unfolding is required for aggregation and amyloid formation to occur? Based on deuterium-proton (D/H) exchange experiments of WT-TTR at pH 4.5 followed by NMR, it was reported that the exchange rates of amide protons were increased in 13 residues, when compared with similar experiments performed at pH 5.75 [95]. Interestingly, 12 of these 13 residues belong to strands C, B, E and F which form one of the sides of the β-sandwich in the TTR subunit (Fig. (1)), indicating an increase in mobility of the CBEF β-sheet, in conditions that favor amyloid formation [95].

Very recently, molecular dynamics simulations of WT-V30M- and L55P-TTR subunits, at 300 K, indicated that the monomeric form of WT-TTR shows a more compact packing between the CBEF and DAGH β-sheets than the V30M-TTR and L55P-TTR monomers [64]. Additionally, some of the hydrogen bonds between strands D and A, A and G, and C and B, show lower persistence during the simulation in L55P-TTR than in WT-TTR. These data show increased mobility in the strand-D/strand-C edge of the subunit β-sandwich in L55P-TTR, but does not predict the same behavior observed by NMR which shows increased mobility at the CBEF β-sheet, at low pH [95].

PROTEIN AGGREGATION AND FIBRIL FORMATION

In recent years, evidence has been accumulating that pre-fibrillar oligomeric states of several amyloidogenic proteins are highly cytotoxic. In the light of these observations, it is crucial for the understanding of the mechanisms of pathogenesis in amyloid diseases, and for the development of appropriate therapeutic strategies, to characterize the structural identity of the intermediates and the kinetic mechanisms of protein aggregation and fibril assembly.

In the absence of a high resolution structure of amyloid fibrils, recent efforts have been made to determine the tridimensional arrangement of the TTR subunits in the fibrils, based on site-directed spin labeling studies [65, 66] and hydrogen/deuterium exchange studies by NMR [67]. Using a series of cysteine substituted TTR mutants derivatized at the sulphydryl group with a nitro oxide spin label and estimating inter-nitroxide distances, Yeates and co-workers proposed a head-to-head/tail-to-tail arrangement of the subunits in the fibril [66]. In this model, the inter-subunit contact between β-strands F and F’, observed in the native dimer, is maintained and a new inter-subunit interface is formed in order to build the extended cross-β structure characteristic of amyloid. The authors proposed that the new inter-subunit contact is formed between β-strands B and B’, associated in an anti-parallel arrangement. This implies displacement of strands C and D from the sheet edges, in order to expose strand B (see Figs. (1) and (3)). This conformational change in the C-D region was confirmed by the authors using mutants with spin labels incorporated in strand-C. The observed inter-subunit contacts are consistent with the formation of a continuous inter-subunit β-sheet with
strands arranged in the order (BEFF’E’B’BEFF’E’B’). However, no information was obtained for inter-subunit contacts involving strands of β-sheet DAGH, the other side of the subunit β-sandwich.

Hydrogen/Deuterium exchange studies, followed by NMR, in amyloid fibrils of Y114C-TTR, an highly amyloidogenic TTR variant, allowed to define a core region of the fibril [67]. Briefly, the study allowed that H/D exchange proceeded for 10 minutes in the fibrillar state, followed by solubilization of the fibrillar material in SDS and NMR analysis of the post-trap H/D exchange rates. Extrapolation of the intensity decay curves to time zero, allowed determination of the H/D exchange protection factors in the fibrillar state. Even from a somewhat small set of amide NMR assignments, it was possible to identify a core of amide hydrogen nuclei protected from exchange with the solvent. The protected amides belonged to residues in β-strands B, E, F, G and H. Most residues in strand C did not have protected amides, indicating that these are exposed to the solvent. No assignments were obtained for residues in strand A, thus no conclusions could be drawn for this β-strand. These data indicate that strands B, E and F in one of the β-sheets, and strands G and H in the other β-sheet of the native TTR subunit β-sandwich are if not totally, partially maintained in the fibrils. These results completely agree with the model proposed based on site-directed spin labeling studies [65, 66].

In a recent study, upon the analysis of more than 75 β-sheet protein structures, it was suggested that most β-sheet proteins avoid edge-to-edge aggregation by several negative design strategies [68]. In the case of TTR, the native dimer is formed by edge-to-edge aggregation of two subunits, through inter-strand H-bonds between strands H and H’, and F and F’ (Fig. 1). However, more extensive edge-to-edge aggregation between TTR subunits may be avoided by some of the negative-design strategies observed by Jane and David Richardson, such as the presence of very short and very twisted β-strands. This is the case of strand D and strand C, at the opposite sheet-edges from strands H and F, in the TTR subunit. Partial unfolding and displacement of strands D and C allows exposure of strands A and B and should readily lead to amyloid fibril formation mediated by interactions between strands A and A’, and B and B’, at one edge, and strands H and H’, and F and F’, at the other edge of the TTR subunit, as suggested by Serag and collaborators [66] and Olofsson [67] (Fig. 1). In fact, we have mentioned earlier in this review that deletion or multiple substitutions in strand-D produces highly amyloidogenic TTR variants [49]. Additionally, proteolysis patterns of TTR amyloid indicate that strands C and D might be solvent exposed in the fibrillar state [69, 70]. Recent X-ray spectroscopy data also demonstrated that cysteine 10 is more exposed and oxidized in the fibrils than in the native tetrameric form of TTR [71]. This again might indicate conformational changes in the C-D region allowing Cys10 to be more exposed due to the displacement of strands C and D and part of the loop DE.

The stacking of conformationally altered TTR subunits in the formation of the fibrils was previously proposed, based on X-ray fiber diffraction studies [96]. Additional evidence for TTR subunit stacking in amyloid TTR was recently put forward by studies on the kinetics of fibril formation, followed by scanning transmission electron microscopy and mass-per-length analysis [63]. This study showed that in vitro assembled TTR fibrils are polymorphic and they are composed by two, three, four and five elementary protofilaments intertwined. Each protofilament contains two twisted β-sheets and seems to be a single vertical stack of structurally modified TTR monomers [63]. A similar model for amyloid fibril assembly was previously proposed for immunoglobulin light-chains [72] and was very recently proposed for α-synuclein, insulin and the B1 domain of protein G [73].

Although it is feasible that TTR amyloid fibrils may be formed by dimeric units, preserving the edge-to-edge inter-subunit native contacts between strands H and H’ and strands F and F’, it is interesting to note that dimeric species have not been commonly observed in solution, both in physiological conditions [55] or even at low pH [54, 57]. Until now, TTR dimeric species in solution have been observed for highly amyloidogenic artificially engineered mutants [62] or in solution conditions such as 0.1% SDS used in non-native gel electrophoresis (SDS-PAGE). Additionally, temperature-induced [44] and pressure-induced [45] equilibrium unfolding studies of tetrameric TTR show a two-state equilibrium between native tetramer and unfolded monomer without any significant participation of other intermediate species. This may indicate that in order to unfold and displace strands C and D of the TTR subunit, we must have a conformationally stable species competent for highly ordered and extended self-association. Knowing that the native tetrameric TTR has high conformational stability [44, 45], it is reasonable to assume that the low conformational stability displayed by the non-native monomer, formed upon tetramer dissociation, plays a central role on amyloid fibril formation, allowing the search for conformations compatible with the structure of amyloid [59]. Upon partial unfolding, the non-native monomer could re-associate in a head-to-head/tail-to-tail arrangement, recreating the native dimeric interface between strands F/F’ and H/H’, and forming a new interface between strands A/A’ and B/B’.

Eventually, more important than discussing the possibility of forming TTR amyloid fibrils from dimeric units [62, 65] or from monomeric units [55, 57, 59, 74], it is to determine the relevant pathways for amyloid formation in vivo. In fact, the observations above may be perfectly consistent with each other. It is today clear that breakdown of the TTR tetramer integrity is required for amyloid formation. In WT-TTR and naturally occurring amyloidogenic TTR variants, tetramer unfolding and dissociation produces monomeric species. The re-association of partially unfolded monomeric species may form amyloid aggregates and fibrils, accompanied by partial refolding of the subunits and rebuilding of a inter-subunit interface very similar to the native inter-strand contacts F/F’ and H/H’.

In the case of the highly amyloidogenic strand-D mutants [62] or in the case of cross-linked dimers subjected to partially unfolding conditions, such as pH 4.4 [65], dissociation and partial unfolding of the tetramer leads to the formation of partially unfolded dimers which re-associate to form amyloid fibrils, maintaining the native inter-subunit
interface contacts F/F’ and H/H’. Thus, these observations do not seem to contradict each other. Amyloid TTR may be formed by dimeric units in solution conditions where dimers are favored or in some mutants where the dimer-dimer native interface has been weakened. However, for most naturally occurring TTR variants, it seems that tetramer dissociation leads to the formation of monomeric units that partially unfold and eventually re-associate to form amyloid fibrils.

**TTR DEPOSITION IN TISSUES**

In recent years, several reports appeared implicating non-fibrillar deposits of amyloidogenic proteins as cytotoxic species in pathologies such as light chain deposition disease (LCDD) [75], Alzheimer’s disease [76], pancreatic amyloid [77], and familial British dementia [78], among others. Concerning TTR and FAP, it is interesting to mention here that early observations by Coimbra and Andrade (1971) [79], based on electron microscopy of nerve biopsies of FAP patients, at different stages of disease progression, already suggested that “nerve fiber changes preceded interstitial amyloid deposition” and that fibrillar amyloid deposits did not seem to be the cause of degeneration of neighboring nerve fibers. This clearly raises the need for identification of the TTR molecular species most likely to be responsible for cytoxicity.

The form in which TTR is deposited in the nerves of FAP patients prior to major nerve fiber degeneration was recently investigated in nerve biopsies from asymptomatic V30M-TTR carriers and FAP patients in different stages of disease progression [80]. TTR deposition was studied by immunohistochemistry and presence of amyloid was accessed by Congo red staining. Individuals lacking amyloid deposition and not evidencing a reduction in the number of nerve fibers, when compared to normal individuals, were classified as FAP 0. The subsequent stages in disease progression presented amyloid deposition and varied in the severity of nerve fiber reduction, and were classified as FAP 1, 2 and 3, respectively. In the stage prior to loss of unmyelinated and myelinated fibers, and major nerve fiber degeneration (FAP 0), despite the absence of Congo red birefringence, the hallmark of amyloid, TTR was present in all 12 cases studied, as revealed by immunohistochemistry with an anti-TTR antibody. These experiments revealed immunolabeling extracellularly, in the proximity of Schwann cells, in a non-fibrillar form as shown in Fig. (4). TTR immunolabeling of nerves in FAP patients with overt disease, clearly demonstrated a fibrillar structure of the deposited material. Thus, TTR can deposit early, in an asymptomatic phase of FAP, in a non-fibrillar or pre-fibrillar form.

Whether TTR aggregates and/or TTR amyloid fibrils produce cellular toxicity has been investigated in an *in vitro* Schwannoma cell culture system measuring Caspase-3 activation. Activation was observed only with TTR preparations containing pre-fibrillar aggregates, whereas soluble tetrameric TTR and mature fibrils did not produce statistically significant activation of Caspase-3, indicating that non-fibrillar TTR aggregates are toxic to cells [80]. Similar results, related to apoptotic responses induced by pre-fibrillar aggregates of TTR have been obtained with a human neuroblastoma cell line [81].

Fig. (4). Electron micrograph of TTR immunogold labeled nerves from a FAP patient with 80,000X magnification. Arrowheads show pre-fibrillar deposits and arrows small fibrils. Scale bar: 100 nm.

Semi-quantitative analysis of immunohistochemical images for immunoreactive interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), macrophage colony-stimulating factor (M-CSF) and nitric oxide synthase (iNOS), in FAP nerve biopsies, compared with age-matched control individuals, demonstrated expression of these proteins localized to endoneurial axons. Although normal nerve showed virtually no detectable antigen in control assays, FAP 0 individuals (i.e. before amyloid was present) already displayed increased cytokine and iNOS expression, also evident in FAP 1 to 3 individuals. The pattern of expression appeared juxtaposed to deposits of TTR and presented an increase by approximately 3-fold, compared with controls [80]. Thus, a sustained pathogenic inflammatory and oxidative stress response is an early event in FAP, possibly leading to neurodegeneration, even in the absence of overt fibril deposition.

**FINAL REMARKS**

Until now, the only therapeutic approach to TTR amyloidosis known to be effective is liver transplantation. Circulating plasma TTR is synthesized in the liver. Thus, replacement of the liver carrying the mutated gene by a normal organ allows for replacement of the mutated circulating TTR by normal TTR. Follow up of the transplanted patients shows a clear slow down, if not stoppage, in the progression of the disease. However, structural damage to the peripheral nervous system is not reversed. This suggests that liver transplantation should be carried out soon after the first symptoms develop.

Although liver transplantation has been somewhat of a success story in improving prognosis for ATTR patients, the invasiveness of the procedure, the expense, and shortage of organs, makes this a far from ideal solution. Thus, several researchers have been concentrating in the development of therapeutic drugs based in two main ideas: i) stabilization of
the native tetrameric form of TTR; ii) solubilization of amyloid fibrils and pre-fibrillar aggregates. A significant effort has been devoted to the search and rational design of compounds that might decrease the tendency for tetramer dissociation, for example, through binding at the thyroxine binding sites of TTR. Several classes of compounds have been tested for their binding affinity to TTR and some look promising as lead compounds in the development of a therapeutic drug [82-87]. Recently, was also reported the action of tetracyclines and IDOX (4'-iodo-4'-deoxyoxorubicin) in the solubilization of Leu55Pro-TTR fibrils [88].

Vital importance in this last approach, it is the confirmation that the TTR species generated upon fibril disruption are not cytotoxic.

The development of novel therapeutic approaches against ATTR strongly depends on the precise identification of the aggregation state and structure of the most pathogenic TTR molecular species and the mechanism of their cytotoxic action in the tissues. If in fact the pre-fibrillar forms of aggregated TTR are pathogenic, therapeutic approaches initially planned to act in the destruction of mature amyloid fibrils might reveal themselves more detrimental than initially hoped because they might increase the amount of aggregated, non-fibrillar material in the tissues. Thus, the detailed characterization of the initial stages in amyloid formation is revealing itself of the utmost importance.

As reviewed here, evidences from different laboratories point out that several issues are crucial in the process of formation of amyloidogenic molecular species of TTR. Tetramer dissociation thermodynamics, tetramer dissociation kinetics and unfolding thermodynamics (conformational stability) of monomeric (or even dimeric) species are central issues in determining the amyloidogenic potential of different TTR variants. These protein related factors must be complemented by other cellular and tissue factors in order to justify different target tissues, penetration rates and ages of onset in TTR amyloid diseases.

Interestingly, the short half-life of TTR in the plasma and simultaneously the slow rate of amyloidosis development in vivo points towards an important role for long-living non-native molecular species in the formation and growth of the TTR amyloid fibrils.

Many questions still have to be answered and will remain at center stage in the near future, in order to have a detailed molecular picture of amyloid formation by TTR and its pathogenic action. Some of the questions to pursue are: How much protein unfolding is necessary to produce the amyloidogenic structures able to self-associate and form the aggregates and fibrils? What is the aggregation state of the most pathogenic TTR species? What is the molecular mechanism of citotoxicity of these species? How can different TTR variants produce somewhat different pathological symptoms? Which cellular and tissue factors are important in determining penetration rates and ages of onset in TTR-dependent amyloidosis?

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ABBREVIATIONS

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<thead>
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<th>Symbol</th>
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<tr>
<td>ATTR</td>
<td>Amyloid TTR</td>
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<td>CD</td>
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<td>FAP</td>
<td>Familial amyloidotic polyneuropathy</td>
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<td>GdmCl</td>
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<td>MCSF</td>
<td>Macrophage colony-stimulating factor</td>
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<td>NMR</td>
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<td>Polyacrylamide gel electrophoresis</td>
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<td>RBP</td>
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<td>Senile systemic amyloidosis</td>
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