

Tetramer Dissociation and Monomer Partial Unfolding Precedes Protofibril Formation in Amyloidogenic Transthyretin Variants*

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Amyloid fibril formation and deposition is a common feature of a wide range of fatal diseases including spongiform encephalopathies, Alzheimer's disease, and familial amyloidotic polyneuropathies (FAP), among many others. In certain forms of FAP, the amyloid fibrils are mostly constituted by variants of transthyretin (TTR), a homotetrameric plasma protein. Recently, we showed that transthyretin in solution may undergo dissociation to a non-native monomer, even under close to physiological conditions of temperature, pH, ionic strength, and protein concentration. We also showed that this non-native monomer is a compact structure, does not behave as a molten globule, and may lead to the formation of partially unfolded monomeric species and high molecular mass soluble aggregates (Quintas, A., Saraiva, M. J. M., and Brito, R. M. M. (1999) *J. Biol. Chem.* 274, 32943–32949). Here, based on aging experiments of tetrameric TTR and chemically induced protein unfolding experiments of the non-native monomeric forms, we show that tetramer dissociation and partial unfolding of the monomer precedes amyloid fibril formation. We also show that TTR variants with the least thermodynamically stable non-native monomer produce the largest amount of partially unfolded monomeric species and soluble aggregates under conditions that are close to physiological. Additionally, the soluble aggregates formed by the amyloidogenic TTR variants showed morphological and thioflavin-T fluorescence properties characteristic of amyloid. These results allowed us to conclude that amyloid fibril formation by some TTR variants might be triggered by tetramer dissociation to a compact non-native monomer with low conformational stability, which originates partially unfolded monomeric species with a high tendency for ordered aggregation into amyloid fibrils. Thus, partial unfolding and conformational fluctuations of molecular species with marginal thermodynamic stability may play a crucial role on amyloid formation *in vivo*.

Amyloid fibril formation has been associated with several pathological states including spongiform encephalopathies, Alzheimer's disease, Parkinson's disease, type II diabetes and Familial Amyloidotic Polyneuropathies (FAP).¹ Recently, several proteins were found to form amyloid fibrils in partially unfolding non-native conditions (1, 2). However, only some proteins seem to form amyloid *in vivo*. Transthyretin (TTR) is such a protein and has been implicated in pathologies such as senile systemic amyloidosis (SSA) and familial amyloidotic polyneuropathy (FAP), which are characterized by systemic extracellular deposition of insoluble amyloid fibrils, mostly constituted by TTR or its variants (3, 4). While SSA is generally a mild disorder and predominantly affects individuals over 80 years of age, FAP is an autosomal dominant lethal disease and may affect individuals from their twenties. In SSA, intact normal TTR and its fragments constitute the amyloid fibrils. In FAP, the amyloid fibrils are mostly constituted by variants of TTR. More than 80 TTR mutations have been reported, many of them amyloidogenic (5). One of the most common amyloidogenic TTR variants is V30M-TTR, and L55P-TTR is the variant associated with one of the most aggressive forms of FAP.

TTR is a homotetrameric protein with a total molecular mass of 55 kDa, 127 amino acid residues per subunit, and a high percentage of β -sheet. TTR is synthesized mainly in the liver, choroid plexus, and retina (6, 7). However, amyloid deposits in FAP occur extracellularly mostly in the peripheral nerves but also in the heart, kidneys, vitreous, and other organs. Thus, it is of the utmost importance to elucidate the pathways of synthesis, folding, and trafficking of TTR in order to select the proper therapeutic approaches.

To date, a lot of emphasis has been placed on the role of a low pH environment, for example found in the lysosomes, in determining the conditions for amyloid formation by TTR *in vivo* (8). However, we recently showed that the TTR tetramer dissociates to a non-native monomer at pH 7 and near physiological ionic strengths and that monomeric species may self-assemble into high molecular mass aggregates (9, 10). This allowed us to propose a model for amyloidogenesis by TTR under commonly observed physiological conditions and, thus, consistent with extracellular amyloid deposit formation (10).

Here we show, based on aging experiments and thermodynamic stability measurements at pH 7.0 that there is a clear

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¹ The abbreviations used are: FAP, familial amyloidotic polyneuropathy; GdnHCl, guanidinium chloride; NOMO, non-native monomer; SSA, senile systemic amyloidosis; PUFs, partially unfolded monomeric species; TTR, transthyretin; L55P-TTR, transthyretin with Leu at position 55 replaced by Pro; T119M-TTR, transthyretin with Thr at position 119 replaced by Met; V30M-TTR, transthyretin with Val at position 30 replaced by Met; WT, wild type.

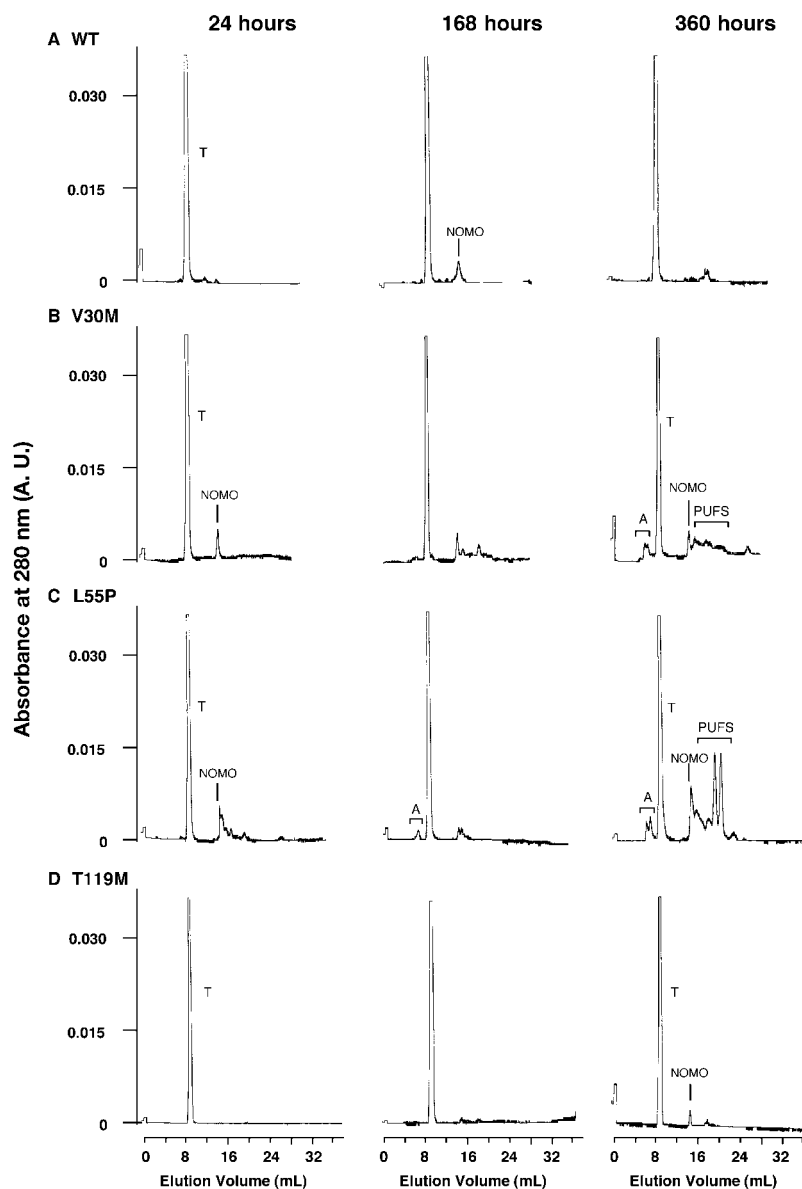


FIG. 1. Gel filtration chromatograms of WT-TTR (A), V30M-TTR (B), L55P-TTR (C), and T119M-TTR (D) monitoring tetramer aging in solution at pH 7.0 and 37 °C. All chromatograms were run at a flow rate of 0.4 ml/min. Initial tetramer concentrations were 2.3 μ M for WT-TTR, 2.2 μ M for V30M-TTR, 1.6 μ M for L55P-TTR, and 2.0 μ M for T119M-TTR. The labels in the chromatograms have the following meaning: T, tetramer; NOMO, non-native monomer; PUFS, partially unfolded monomeric species; A, soluble aggregates.

correlation between the stability of the non-native monomer formed upon tetramer dissociation, aggregate formation, and the amyloidogenic potential of four different TTR variants: WT-, V30M-, L55P-, and T119M-TTR. We also show that tetramer dissociation and monomer partial unfolding seem to precede amyloid protofibril formation. Thus, partial unfolding and conformational fluctuations of marginally stable TTR non-native monomers play a key role in the molecular events leading to amyloid fibril assembly.

EXPERIMENTAL PROCEDURES

Materials—Guanidinium chloride, thioflavin-T, and all other chemicals were of the highest commercially available purity and were purchased from Sigma Chemical Company, St. Louis, MO.

Protein Sample Preparation—Recombinant WT-, V30M-, L55P-, and T119M-transferrin were produced in an *Escherichia coli* expression system (11) and purified as described previously (12). Tetrameric and monomeric forms of all TTR variants were isolated by gel filtration chromatography (10). Protein concentrations were determined spectrophotometrically at 280 nm, using an extinction coefficient of 7.76×10^4 M⁻¹ cm⁻¹, based on a 55 kDa molecular mass for TTR ($A_{280}(1\%) = 14.1$ mg⁻¹ ml cm⁻¹) (13). When needed, TTR samples were reconstituted by ultrafiltration on a Centricon-3 microconcentrator (from Amicon Inc.) with a molecular mass cutoff of 3 kDa.

Aging Studies—Aging studies of tetrameric TTR variants were performed incubating for up to 4 weeks TTR samples at 37 °C in chroma-

tography buffer with 0.05% sodium azide in order to prevent microbial growth. Samples were withdrawn at different times for analysis by gel filtration chromatography and further processing.

Gel Filtration Chromatography—Gel filtration chromatography experiments were performed on an Amersham Pharmacia Biotech FPLC Superdex-75 HR column, coupled to a high precision pump P-500, a UV detector equipped with a deuterium lamp, and an integrator from Konik Instruments. The column was allowed to equilibrate with 3–5 column volumes of chromatography buffer (20 mM sodium phosphate buffer, 150 mM sodium chloride, pH 7.0). Final chromatography runs were performed at a flow rate of 0.4 ml/min. Apparent molecular masses were calculated by interpolation on an elution volume versus log(molecular mass) calibration curve for four protein standards: bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and apoferritin (6.5 kDa).

Thioflavin-T Assay—Thioflavin-T assays (14) were performed adding a freshly prepared stock solution of thioflavin-T in 20 mM sodium phosphate buffer, 150 mM sodium chloride, pH 7.0, to the TTR samples under study to a final thioflavin-T concentration of 10 μ M. Fluorescence measurements were performed on a SPEX Fluorolog spectrofluorometer equipped with a thermostated cell compartment at 25 °C and using a 1.0 \times 0.4 cm pathlength fluorescence cuvette. Fluorescence emission spectra were obtained with an excitation wavelength of 450 nm and excitation and emission slits of 1 and 1.5 nm, respectively.

Synthetic amyloid fibrils were produced by lowering the pH of a 2 μ M solution of L55P-TTR to pH 5.0 and incubating at room temperature for 1 week.

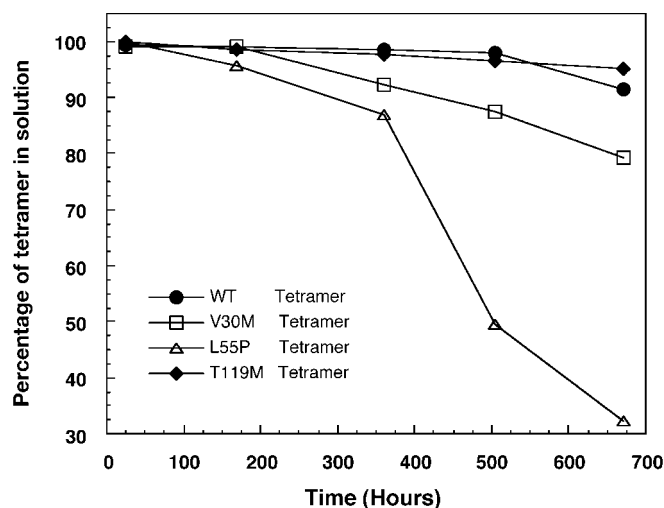


FIG. 2. Percentage of TTR tetrameric species in solution during tetramer aging at pH 7.0 and 37 °C for WT-, V30M-, L55P-, and T119M-TTR, monitored by gel filtration chromatography.

Electron Microscopy—High molecular mass soluble aggregates of L55P-TTR were isolated by gel filtration chromatography at a final concentration of ~20 µg/ml. For negative staining, 5 µl of these preparations were adsorbed to glow-discharged carbon collodion film on 200-square mesh copper grids for 1 min. The grids were washed with deionized water and stained with 0.75% uranyl formate (Polysciences, Inc). Specimens were viewed and images recorded with a Hitachi H-7000 electron microscope operating at 100 kV.

Conformational Stability Measurements—Native tetrameric forms of TTR dissociate to a non-native monomeric species, as previously described (10). The conformational stability of these non-native monomeric forms of WT-, V30M-, L55P-, and T119M-TTR variants was measured in GdnHCl-induced protein unfolding experiments at 25.0 °C and followed by intrinsic tryptophan fluorescence changes. Fluorescence measurements were performed on a SPEX Fluorolog spectrofluorometer equipped with a thermostated cell compartment at 25 °C and using a 1.0 × 0.2 cm pathlength fluorescence cuvette. Fluorescence emission spectra were obtained with an excitation wavelength of 280 nm and excitation and emission slits of 1.5 and 2 nm, respectively.

Due to the difficulty in isolating and concentrating the non-native monomeric species, unfolding experiments were carried out by titrating monomeric TTR samples (~2 µM) in 20 mM sodium phosphate buffer, 150 mM sodium chloride at pH 7 and 25 °C, with a 8 M GdnHCl stock solution prepared in the same buffer. After each addition of GdnHCl, several fluorescence measurements were performed in order to evaluate when the solutions reached the equilibrium. It was found that a 0.5-h incubation was enough to reach equilibrium for all GdnHCl concentrations. However, all the samples were allowed to equilibrate 1.5 h before the final fluorescence measurements. All fluorescence measurements were corrected for protein dilution produced during the titration.

Equilibrium denaturation curves were constructed using the intensity of fluorescence emission at 380 nm, with excitation at 280 nm. Excitation at 295 nm, to excite just tryptophan residues, produced curves of lower intensity but no significantly different shape. Denaturation curves were analyzed according to a two-state unfolding model and using the linear extrapolation method (15) in a non-linear least squares fitting procedure (16). Non-linear least squares fitting of the experimental fluorescence data was performed with the program Kaleidagraph (Synergy Software, Pennsylvania) and yielded values for $\Delta G^\circ(\text{H}_2\text{O})$, the conformational stability, and m , the dependence of ΔG° on denaturant concentration. C_m , the denaturant concentration at the midpoint of the unfolding transition was calculated as $C_m = \Delta G^\circ(\text{H}_2\text{O})/m$.

RESULTS

We have previously shown that the transthyretin tetramer in solution dissociates to a non-native monomer at nearly physiological conditions (9, 10). We have also shown that the extent of tetramer dissociation is not very different among the TTR variants studied (WT-, V30M-, L55P-, and T119M-TTR) after a 24-h incubation, at 25 °C (9). Here, based on aging experiments

of the native tetrameric forms of several TTR variants at pH 7.0 and 37 °C and stability measurements of the non-native monomeric forms of these TTR variants, we put forward additional hypothesis for the mechanisms of amyloid formation by TTR.

Aging of Tetrameric TTR—Fig. 1 shows gel filtration elution profiles of the tetrameric forms of WT-, V30M-, L55P- and T119M-TTR, as a function of incubation time at 37 °C and pH 7.0 after initial tetramer isolation. Several molecular species can be identified in the chromatograms. The major species correspond to the tetramer, with an elution volume of 8.8 ml and an apparent molecular mass of 60 kDa. Depending on the incubation time at 37 °C and the TTR variant under analysis, a complex mixture of molecular species is observed with elution volumes around 20 ml, corresponding to a non-native monomer (elution volume = 15.2 ml) and several partially unfolded monomeric species, as was previously shown by fluorescence and ANS-binding (10). In fact, the tetramer initially dissociates to small amounts of the non-native monomer, which then interconverts to the partially unfolded monomeric species. Additionally, in some chromatograms of the more amyloidogenic TTR variants (V30M- and L55P-TTR, Fig. 1, B and C), species with elution volumes around 6.5 ml and apparent molecular masses higher than 70 kDa, the resolving limit of the column, are observed.

Fig. 1 clearly shows two distinct patterns for TTR tetramer aging, one pattern for WT- and T119M-TTR and another for V30M- and L55P-TTR. For amyloidogenic TTR variants (V30M- and L55P-TTR, Fig. 1, B and C, respectively) there is an initial dissociation of the tetramer to the non-native monomer within the first 24 h. This is followed by interconversion to several partially unfolded monomeric species with elution volumes around 20 ml and finally formation of high molecular mass soluble aggregates with elution volumes around 6.5 ml. The amount and variety of partially unfolded monomeric species and aggregates formed correlates with the amyloidogenic potential of the TTR variant (Fig. 1, B and C). In fact, the presence of partially unfolded monomeric species and aggregates in L55P-TTR occurs at an earlier stage and to a larger extent (Fig. 1C) than in V30M-TTR (Fig. 1B). On the contrary, for WT-TTR (Fig. 1A) and the non-amyloidogenic variant T119M-TTR (Fig. 1D) aggregate formation is not evident up to 360 h at 37 °C and at the physiologically relevant TTR concentrations used. Additionally, the amount of partially unfolded monomeric species is much lower in these proteins, and they appear later in the aging process when compared with the amyloidogenic variants. In fact, for T119M-TTR partially unfolded monomeric species are almost non-observable. From the analysis of Fig. 1, it is also evident that formation of high molecular mass aggregates is preceded by formation of partially unfolded monomeric species.

During the course of the aging experiments we have also monitored the relative amount of tetrameric species in solution. Fig. 2 shows the disappearance of tetrameric species in solution, monitored by gel filtration chromatography for several TTR variants when incubated at 37 °C and pH 7.0 for up to 670 h. WT- and T119M-TTR show only a very slight decrease in the relative amount of tetramer in solution over this period of time. On the contrary, V30M- and L55P-TTR show a significant decrease in the relative amount of tetramer in solution. This decrease is much more significant for L55P-TTR, the variant that produces the most aggressive form of amyloidosis *in vivo*. These data for the decrease of tetramer in solution at 37 °C and pH 7.0 directly correlates with the appearance of several monomeric species and aggregates in solution, as shown in Fig. 1. It is interesting to note that the time course for the disappearance of tetramer among the soluble species and the simultane-

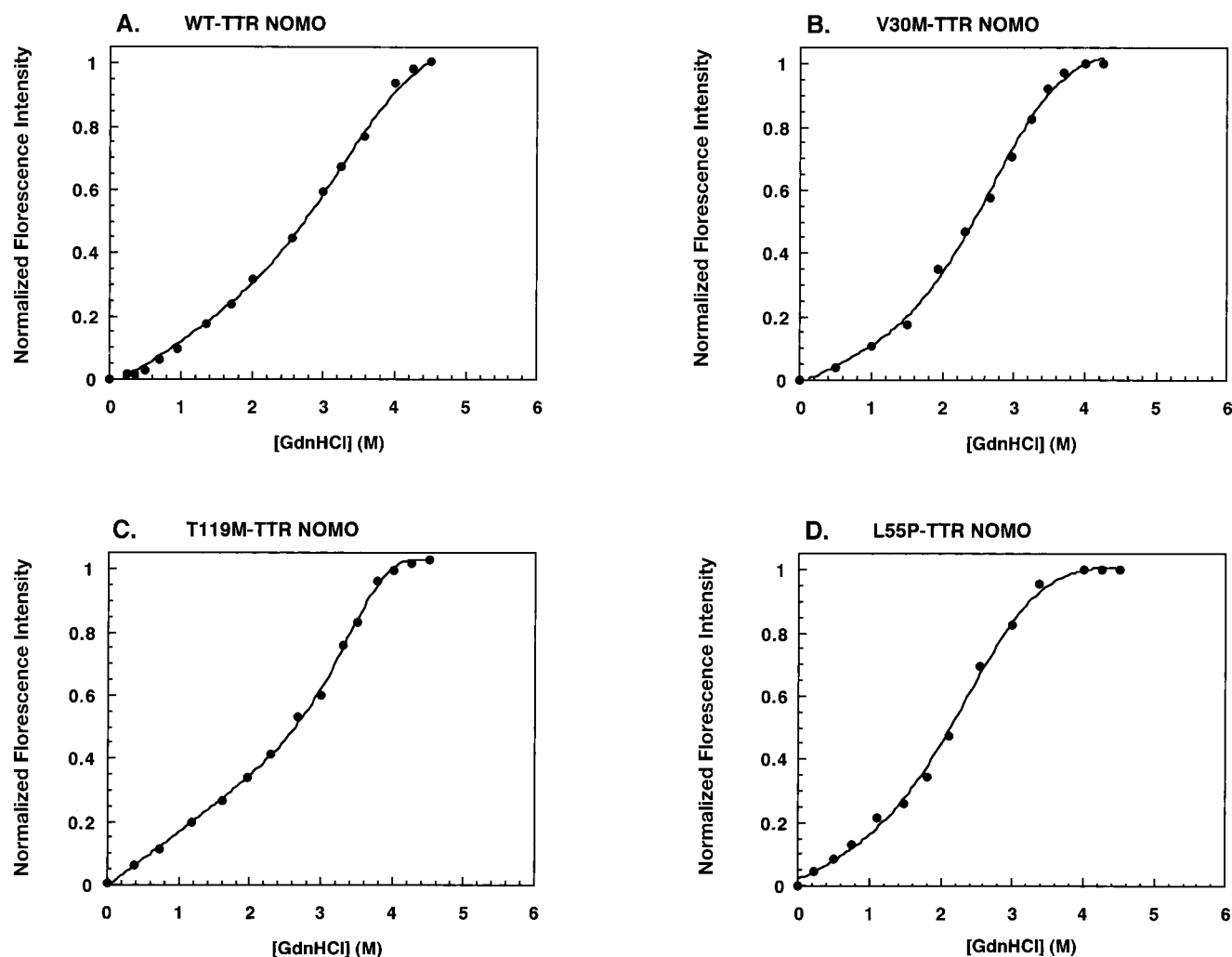


FIG. 3. Guanidinium chloride equilibrium denaturation curves of non-native TTR monomers at pH 7.0 and 25 °C monitored by intrinsic tryptophan fluorescence changes: WT-TTR (A), V30M-TTR (B), T119M-TTR (C), and L55P-TTR (D). The curves are non-linear least squares fits of an equation representing the entire denaturation curve, based on a two-state model and using the linear extrapolation method, to the experimental fluorescence data (16).

TABLE I

Stability parameters for non-native monomers of several TTR variants obtained from a direct fit of the experimental data in Fig. 3 to a two state unfolding model (16)

Non-Native Monomeric TTR	$\Delta G^\circ (\text{H}_2\text{O})^a$	$\Delta\Delta G^\circ (\text{H}_2\text{O})^b$	C_m^c	m^d
	kcal mol ⁻¹	kcal mol ⁻¹	M	kcal mol ⁻¹ M ⁻¹
WT-TTR	3.4 ± 0.5	—	3.4	1.0 ± 0.2
V30M-TTR	2.9 ± 0.4	-0.5	2.9	1.0 ± 0.2
L55P-TTR	2.5 ± 0.5	-0.9	2.5	1.0 ± 0.2
T119M-TTR	5.8 ± 0.4	2.4	3.6	1.6 ± 0.1

^a $\Delta G^\circ (\text{H}_2\text{O})$ is the free energy of unfolding in the absence of denaturant.

^b $\Delta\Delta G^\circ (\text{H}_2\text{O})$ is the difference between the free energy of unfolding of each variant and WT-TTR ($\Delta G^\circ (\text{H}_2\text{O})_{\text{variant}} - \Delta G^\circ (\text{H}_2\text{O})_{\text{WT-TTR}}$).

^c C_m , the denaturant concentration at the midpoint of the unfolding transition, was calculated as $C_m = \Delta G^\circ (\text{H}_2\text{O})/m$.

^d m is the ΔG° dependence on denaturant concentration.

ous appearance of monomeric and aggregate species in solution correlates with the amyloidogenic potential of the TTR variants studied. These data suggest that the source of amyloidogenic intermediates is the dissociation of tetrameric TTR to a non-native monomer and that this process might be kinetically controlled.

Conformational Stability of the Non-native TTR Monomer—To address the importance of the non-native TTR mono-

mer in the formation of partially unfolded monomeric species and aggregates, we have characterized the conformational stability of the non-native monomer for WT-, V30M-, L55P-, and T119M-TTR. Fig. 3 shows the equilibrium denaturation curves at pH 7.0 and 25 °C using guanidinium chloride as denaturant. The free energy of unfolding ($\Delta G^\circ (\text{H}_2\text{O})$) was directly obtained by a nonlinear least-squares fit of the experimental fluorescence data to a two-state model for monomer unfolding (15, 16). GdnHCl-induced denaturation of the non-native monomer was found to be reversible for all the variants studied, as judged by gel filtration chromatography and fluorescence experiments. Application of GdnHCl-unfolded monomeric TTR samples to the gel filtration column produced a chromatographic species with the same elution volume and fluorescence properties of the non-native monomer.

Table I shows the values obtained from the curves in Fig. 3 for $\Delta G^\circ (\text{H}_2\text{O})$, the conformational stability, m , the dependence of ΔG° on denaturant concentration, and C_m , the denaturant concentration at the midpoint of the unfolding transition. The lower $\Delta G^\circ (\text{H}_2\text{O})$ values obtained for the amyloidogenic variants indicate a decrease in the conformational stability of the non-native monomer in these variants, when compared with the non-native monomer of WT-TTR (Table I). Additionally, the conformational stability of the T119M-TTR non-native monomer is significantly higher than that of the wild type. The

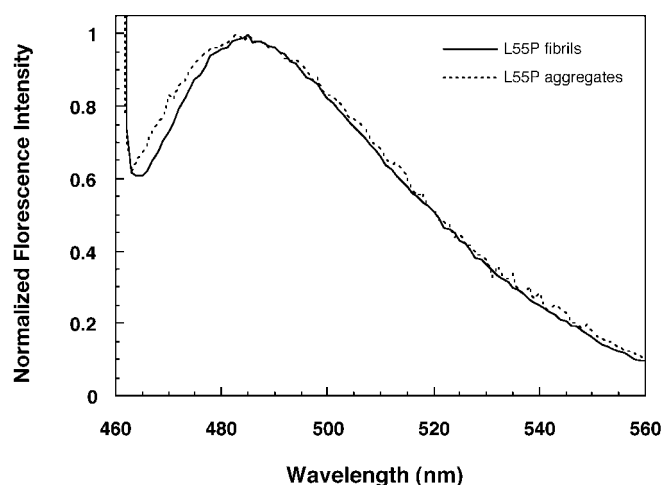


FIG. 4. Fluorescence emission spectra of thioflavin-T in the presence of L55P-TTR fibrils (solid line), and L55P-TTR high molecular mass soluble aggregates (broken line) formed at pH 7.0 and 37 °C. Excitation wavelength was 450 nm. Total protein concentration was in both cases ~ 0.1 mg/ml.

decreasing values of the conformational stability of the non-native monomers studied (T119M-TTR > WT-TTR > V30M-TTR > L55P-TTR) completely agree with the increasing amyloidogenic potential of these TTR variants (T119M-TTR < WT-TTR < V30M-TTR < L55P-TTR). Moreover, the denaturation midpoint, C_m , also shows this dependence, *i.e.* the variants showing the lower C_m value are those with highest amyloidogenic potential (Table I). It is worth noting that the significantly higher conformational stability shown by the T119M-TTR non-native monomer is mostly due to a higher m value for this monomer (Table I). The m value is believed to reflect the extent of newly exposed surface area upon unfolding (17). Thus, the higher m value observed for the unfolding of T119M-TTR non-native monomer might indicate a more compact structure in this monomer.

The results on conformational stability of the non-native monomers explain the observations obtained in the aging experiments shown in Fig. 1. The number and amount of partially unfolded monomeric species observed in solution for the four studied TTR variants seems to be directly related with the conformational stability of the non-native monomers. TTR variants with less stable non-native monomers produce the largest amount and variety of partially unfolded monomeric species in the aging experiment.

Characterization of the High Molecular Mass Soluble Aggregates—In order to characterize the nature of the high molecular mass soluble aggregates formed in the aging experiment (Fig. 1) we carried out thioflavin-T assays (14) in soluble aggregates, isolated by gel exclusion chromatography, and amyloid fibrils of L55P-TTR (Fig. 4). Thioflavin-T is known to undergo a red shift of its fluorescence excitation and emission maxima upon binding to amyloid fibrils. In the absence of amyloid fibrils thioflavin-T has a low fluorescence quantum yield with excitation and emission maxima at 350 and 438 nm, respectively. In the presence of amyloid fibrils the fluorescence quantum yield increases significantly and the excitation and emission maxima are shifted to 450 and 482 nm, respectively (14).

Fig. 4 shows the response of thioflavin-T in the presence of L55P-TTR high molecular mass soluble aggregates, isolated by gel filtration chromatography, and in the presence of L55P-TTR fibrils as a control prepared as described under “Experimental Procedures.” At the excitation wavelength used (450 nm), thioflavin-T does not show any significant fluorescence in phosphate buffer, at pH 7.0. However, in the presence of L55P-

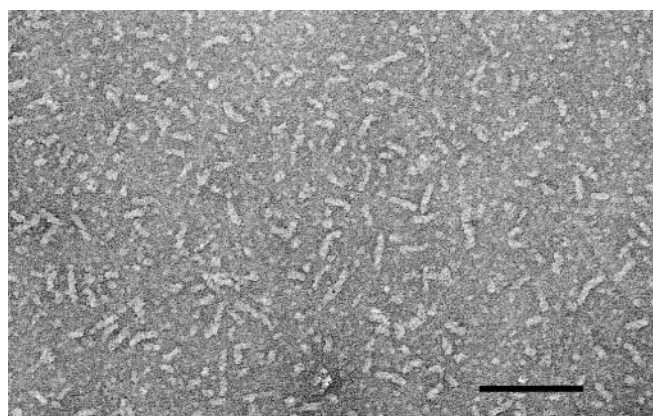


FIG. 5. Electron micrograph of negatively stained L55P-TTR high molecular mass soluble aggregates isolated by gel filtration chromatography and produced by incubating tetrameric L55P-TTR at 37 °C and pH 7.0, for ~ 2 weeks. The scale bar represents 100 nm.

TTR high molecular mass soluble aggregates, fluorescence is observed with an emission maximum of 482 nm, exactly the same emission maximum observed in the presence of L55P-TTR fibrils used as a control. This result clearly shows that the nature of the molecular interaction between thioflavin-T and the L55P-TTR soluble aggregates is similar to the one occurring between thioflavin-T and TTR fibrils (Fig. 4) or thioflavin-T and amyloid fibrils of other origins. We note, however, that the absolute fluorescence intensity enhancement, per mass of protein, observed in the presence of L55P-TTR soluble aggregates is smaller than in the presence of TTR fibrils (data not shown). This may be the result of the smaller size of the soluble aggregates when compared with the mature fibrils.

To further characterize the nature of the soluble aggregates, we studied the morphology of the L55P-TTR high molecular mass soluble aggregates by electron microscopy (EM). The electron micrographs (Fig. 5) revealed short unbranched fibrillar structures with 8–9 nm diameter and variable length ranging from 25 to 75 nm. The diameter of these fibrillar structures is similar to the diameter observed by EM of amyloid fibrils isolated from the kidneys of patients with V30M-TTR FAP (18). Additionally, mature amyloid fibrils vary widely in length. This might indicate that the mature TTR fibrils are formed by end-growth or by end-to-end association of initial short protofibrils of similar width as the final fibrils.

Taken together, the results from electron microscopy and thioflavin-T assays clearly indicate that the TTR soluble aggregates, readily formed by amyloidogenic TTR variants at pH 7.0, represent an initial stage of amyloid fibril assembly.

DISCUSSION

In recent years, the molecular mechanisms of amyloid formation have been the focus of wide attention due to their implication in several human and animal pathologies. Recently, we showed that transthyretin, the homotetrameric protein responsible for pathologies such as SSA and FAP, may dissociate in solution to a non-native monomer even under close to physiological conditions of temperature, pH, ionic strength, and protein concentration. We also showed that this non-native monomer is a compact structure, does not behave as a molten globule, and may lead to the formation of partially unfolded monomeric species and high molecular mass soluble aggregates (10).

Here, based on aging studies of the tetrameric forms as well as on thermodynamic stability studies of the monomeric forms of amyloidogenic and non-amyloidogenic TTR variants, we show that TTR variants with the least thermodynamically

Amyloidogenic Behavior

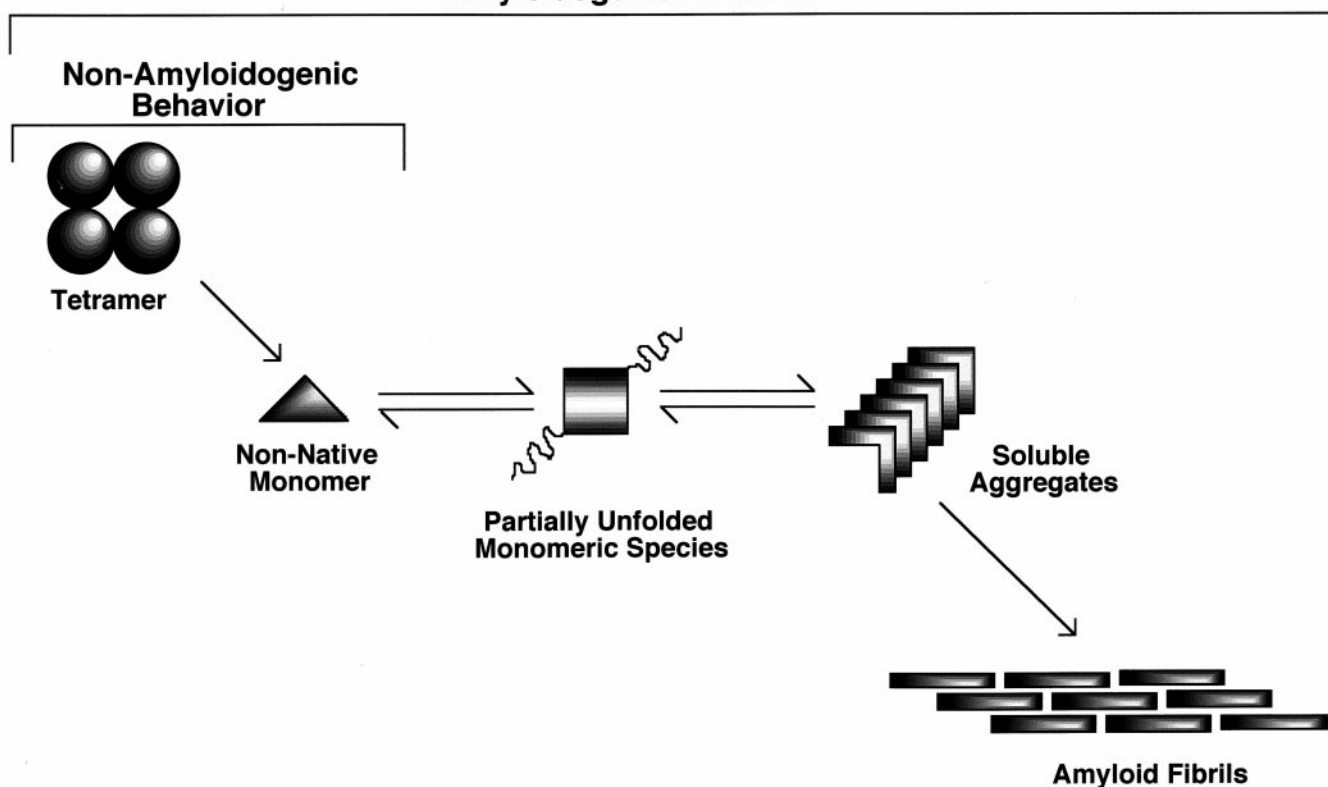


FIG. 6. **Model for amyloid fibril formation by TTR at commonly encountered physiological conditions.** At 37 °C and pH 7, tetrameric TTR may dissociate to a non-native monomer, which in turn depending on its conformational stability, may undergo partial unfolding that leads to aggregate formation and eventually amyloid fibril assembly. Both amyloidogenic and non-amyloidogenic TTR variants dissociate to a non-native monomer. However only the amyloidogenic variants produce large amounts of partially unfolded monomeric species as a consequence of the marginal conformational stability of the non-native monomer.

stable non-native monomer produce the largest number and amount of partially unfolded monomeric species and soluble aggregates (Fig. 1 and Table I). Additionally, the soluble aggregates formed by the amyloidogenic TTR variants show morphological and thioflavin-T fluorescence properties characteristic of amyloid (Figs. 4 and 5). In particular, the observed diameter of the aggregates (8–9 nm) is very close to what has been previously observed for mature V30M-TTR *ex-vivo* fibrils (18). This indicates that the soluble aggregates isolated in the aging experiments represent an early stage of amyloid fibril formation, *i.e.* protofibrils. Therefore, TTR amyloid fibril assembly may proceed initially by end-elongation of small protofibrils followed by end-association of these protofibrils, a mechanism similar to what has been proposed for the assembly of A β 40 amyloid fibrils (19, 20).

Recently, we showed by differential scanning calorimetry (DSC) of WT-, V30M-, L55P-, and T119M-TTR that the tetrameric forms of these variants are highly stable to thermal unfolding (21). Additionally, the observed small differences in conformational stability do not agree with the relative amyloidogenic potential of the TTR variants studied (21). Thus, differences in the conformational stability of tetrameric TTR do not seem to justify the amyloidogenic character of some TTR variants.

The aging and stability data presented here points out new clues on the mechanisms of amyloid formation by TTR and allowed us to refine our previously proposed model for amyloid fibril formation by TTR (10). At pH 7 and 37 °C the TTR tetramer may dissociate to a non-native monomer (Fig. 6). Depending on its conformational stability, the non-native monomer may undergo partial unfolding, which leads to the forma-

tion of aggregates with amyloid character and eventually amyloid fibrils (Fig. 6). Previously, we considered the possible existence of a complex equilibrium between the non-native monomer, partially unfolded monomeric species, and soluble aggregates (10). However, the data presented here identifies the partially unfolded monomeric species as the probable source of aggregate formation. The aging experiments show the appearance of partially unfolded monomeric species preceding the formation of amyloid aggregates. Additionally, comparison of the stability data for the non-native monomers of the four TTR variants studied shows that the variants with the unfolding equilibrium most shifted away from the non-native monomer produce the largest amount of aggregates. This clearly indicates that at least some of the partially unfolded monomeric species are intermediates in the pathway leading to amyloid fibril formation. In fact, if the amyloid aggregates were formed directly from the non-native monomer we would expect that higher conformational stabilities of the non-native monomer would produce larger amounts of aggregates, which is not what we observe.

Our model also allows for discrimination between amyloidogenic and non-amyloidogenic behavior. In both cases, non-native monomers are formed upon tetramer dissociation. However, while highly amyloidogenic TTR variants produce large amounts of partially unfolded monomeric species and aggregates, non-amyloidogenic variants do not (Fig. 6). Thus, the different *in vivo* behavior between amyloidogenic and non-amyloidogenic TTR variants may be mostly mediated by the conformational stability of the non-native monomers formed upon tetramer dissociation. In fact, the higher *m* value we obtained in the conformational stability studies of the non-

native monomer of T119M-TTR, a non-amyloidogenic variant, may indicate a better structured and more compact monomer in this case. It is also important to remember that due to the logarithmic dependence of ΔG° on the equilibrium constant, the apparent small differences in conformational stability observed between non-native monomers of TTR variants are translated into significant differences in the equilibrium constants for unfolding. For example at 25 °C, while for the WT-TTR non-native monomer ~320 molecules are unfolded per 100,000 molecules, in L55P-TTR as much as 1500 molecules are unfolded per 100,000 molecules and in T119M-TTR only 6 molecules are unfolded per 100,000 molecules. This reveals that a low conformational stability of the non-native monomer produced from tetramer dissociation may be the molecular key for amyloidogenesis by TTR. Recent studies also revealed correlations of this type between the amyloidogenic potential and the conformational stability of variants of immunoglobulin light-chain variable domains (22) and the conformational stability of variants of the B1 Ig-binding domain of protein G (23).

Here, we show that the more amyloidogenic TTR variants produce a larger number and amount of partially unfolded monomeric species. The presence of a critical amount of these less structured species in solution seems to trigger the formation of TTR soluble aggregates with amyloid character. Thus, partially unfolded monomeric species seem to be the building blocks of the soluble aggregates, protofibrils, and eventually amyloid fibrils. In fact, Kirschner and co-workers (24), based on x-ray fiber diffraction studies on biopsy samples of kidney and vitreous human TTR amyloid fibrils, proposed a fibril structure composed of four protofilaments of axially arrayed TTR monomers. Additionally, the crystal structure of L55P-TTR showed a tubular structure formed by association of TTR monomers (25). In a recent study, using constructed mutants, it was shown that dimers resulting from disulfide-linked monomers could not form amyloid *in vitro* but under reducing conditions readily formed thioflavin-T positive fibrils (26). These data suggest the formation of amyloid fibrils from monomeric species, which supports our model (Fig. 6). Upon aggregation the TTR partially unfolded monomeric species may reacquire structure stabilized by intermolecular interactions. Observations agreeing with this possibility were reported in a study with staphylococcal nuclease, which showed that the amount of secondary structure in a monomeric partially folded intermediate doubled upon formation of soluble aggregates and the propensity to aggregate decreased with increasing structural content of the partially folded intermediate (27).

Considering the results presented here, we propose that amyloid formation by TTR is triggered by tetramer dissociation to a compact non-native monomer, which, depending on its

thermodynamic stability, originates less structured monomeric species with a high tendency for ordered aggregation into amyloid fibrils through specific intermolecular contacts. Therefore, the amyloidogenic potential of some TTR variants could be related to higher tendencies to produce partially unfolded monomeric species, which reacquire β -sheet structure upon aggregation, forming the characteristic cross- β structure of amyloid fibrils. Thus, partial unfolding and conformational fluctuations of molecular species with marginal thermodynamic stability may play a crucial role in amyloid formation *in vivo*.

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REFERENCES

- Chiti, F., Webster, P., Taddei, N., Clark, A., Stefani, M., Ramponi, G., and Dobson, C. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 3590–3594
- Dobson, C. M. (1999) *Trends Biochem. Sci.* **9**, 329–332
- Saraiva, M. J. M., Birken, S., Costa, P. P., and Goodman, D. S. (1984) *J. Clin. Invest.* **74**, 104–119
- Saraiva, M. J. M. (1996) *J. Peripheral Nervous System* **1**, 179–188
- Connors, L. H., Richardson, A. M., Theberge, R., and Costello, C. E. (2000) *Amyloid* **7**, 54–69
- Soprano, D. R., Herbert, J., Soprano, K. J., Schon, E. A., and Goodman, D. S. (1985) *J. Biol. Chem.* **260**, 11793–11798
- Stauder, A. J., Dickson, P. W., Aldred, A. R., Schreiber, G., Mendelsohn, F. A., and Hudson, P. (1986) *J. Histochem. Cytochem.* **34**, 949–952
- Kelly, J. W. (1996) *Curr. Opin. Struct. Biol.* **6**, 11–17
- Quintas, A. L., Saraiva, M. J. M., and Brito, R. M. M. (1997) *FEBS Lett.* **418**, 297–300
- Quintas, A., Saraiva, M. J. M., and Brito, R. M. M. (1999) *J. Biol. Chem.* **274**, 32943–32949
- Furuya, H., Saraiva, M. J. M., Gawinowicz, M. A., Alves, I. L., Costa, P. P., Sasaki, H., Goto, I., and Sakaki, Y. (1991) *Biochemistry* **30**, 2415–2421
- Almeida, M. R., Damas, A. M., Lans, M. C., Brouwer, A., and Saraiva, M. J. M. (1997) *Endocrine* **6**, 309–315
- Raz, A. and Goodman, D. S. (1969) *J. Biol. Chem.* **244**, 3230–3237
- Naiki, H., Higuchi, K., Hosokawa, M., and Takeda, T. (1989) *Anal. Biochem.* **177**, 244–249
- Pace, C. N. (1986) *Methods Enzymol.* **131**, 266–280
- Santoro, M. M., and Bolen, D. W. (1988) *Biochemistry* **27**, 8063–8068
- Myers, J. K., Pace, C. N., and Scholtz, J. M. (1995) *Protein Sci.* **4**, 2138–2148
- Tawara, S., Araki, S., Toshimori, K., Nakagawa, H., and Ohtaki, S. (1981) *J. Lab. Clin. Med.* **98**, 811–822
- Walsh, D. M., Lomakin, A., Benedek, G. B., Condron, M. M., and Teplow, D. B. (1997) *J. Biol. Chem.* **272**, 22364–22372
- Harper, J. D., Wong, S. S., Lieber, C. M., and Lansbury, P. T., Jr. (1999) *Biochemistry* **38**, 8972–8980
- Shnyrov, V. L., Villar, E., Zhadan, G. G., Sanchez-Ruiz, J. M., Quintas, A., Saraiva, M. J. M., and Brito, R. M. M. (2000) *Biophys. Chem.* **88**, 61–67
- Rafen, R., Dieckman, L. J., Szpunar, M., Wunschil, C., Pokkuluri, P. R., Dave, P., Stevens, P. W., Cai, X., Schiffer, M., and Stevens, F. J. (1999) *Protein Sci.* **8**, 509–517
- Ramirez-Alvarado, M., Merkel, J. S., and Regan, L. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 8979–8984
- Inouye, H., Domingues, F. S., Damas, A. M., Saraiva, M. J., Lundgren, E., Sandgren, O., and Kirschner, D. A. (1998) *Amyloid* **5**, 163–174
- Sebastião, M. P., Saraiva, M. J., and Damas, A. M. (1998) *J. Biol. Chem.* **273**, 24715–24722
- Redondo, C., Damas, A. M., Olofsson, A., Lundgren, E., and Saraiva, M. J. M. (2000) *J. Mol. Biol.* **304**, 461–470
- Uversky, V. N., Segel, D. J., Doniach, S., and Fink, A. L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 5480–5483

Tetramer Dissociation and Monomer Partial Unfolding Precedes Protofibril Formation in Amyloidogenic Transthyretin Variants

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