The amyloidogenic potential of transthyretin variants correlates with their tendency to aggregate in solution

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Abstract Amyloid fibril formation and deposition are the basis for a wide range of diseases, including spongiform encephalopathies, Alzheimer's and familial amyloidotic polyneuropathies. However, the molecular mechanisms of amyloid formation are still poorly characterised. In certain forms of familial amyloidotic polyneuropathy (FAP), the amyloid fibrils are mostly constituted by variants of transthyretin (TTR). V30M-TTR is the most frequent variant, and L55P-TTR is the variant associated with the most aggressive form of amyloidosis. Here, we report gel filtration chromatography experiments to characterise the aggregation states of WT-, V30M-, L55P-TTR and a non-amyloidogenic variant, T119M-TTR, in solution, at nearly physiological pH. These studies show that all four protein tetramers dissociate to monomer upon dilution, in the submicromolar range, at pH 7.0. The amyloidogenic proteins V30M- and L55P-TTR show a complex equilibrium between monomers, tetramers and high molecular weight aggregate species. These aggregates dissociate directly to monomer upon dilution. This study shows that the tendency to form aggregates among the four studied proteins correlates with their known amyloidogenic potential. Thus, the amyloidogenic mutations could perturb the structure and/or stability of the monomeric species leading initially to the formation of soluble aggregates and at a later stage to insoluble amyloid fibrils.

Key words: Transthyretin; Amyloid; Amyloidosis; Amyloidogenesis; Familial amyloidotic polyneuropathy; Protein stability; Gel filtration chromatography

1. Introduction

Transthyretin (TTR) is a homo-tetrameric protein with a total molecular weight of 55 kDa and 127 amino acid residues per subunit, found in the cerebrospinal fluid and in the plasma. TTR transports thyroxine and retinol in association with the retinol-binding protein. In certain forms of familial amyloidotic polyneuropathy (FAP), characterised by early impairment of temperature and pain sensation in the feet, and evolving to autonomic dysfunction with generalised amyloidosis [1], the amyloid fibrils are mostly constituted by variants of TTR [2]. Among these variants, V30M-TTR is the most frequent [2], and L55P-TTR is the variant associated with the most aggressive form of amyloidosis, characterised by an early age of onset, between 15 and 20 years old [3]. T119M-TTR was originally described in a kindred without amyloidosis [4] and has now been found frequently in several populations, including the Portuguese population [5]. This variant is non-amyloidogenic and it is thought to protect against FAP individuals who also carry the V30M mutation [6]. Recent studies have suggested that acid-induced partial denaturation of TTR is sufficient to effect amyloid fibril formation by self-assembly of a denaturation intermediate [7,8], and that T119M-TTR is more stable toward acid-induced fibril formation than WT-TTR, in contrast with V30M-TTR which is less stable [9].

Comparison between the crystal structures of WT- and V30M-TTR showed a very similar global fold for both proteins with the tetramer having a central cylindrical cavity where thyroxine binds. Each monomer of TTR is a flattened β-barrel with residue 30 in the interior. Substitution of valine 30 by methionine forces the β-sheets of the monomer approximately 1 Å apart, resulting in the distortion of the thyroxine-binding cavity [10,11]. However, the small differences between the crystal structures of WT- and V30M-TTR have not clearly pointed out the causes for the amyloidogenicity of V30M-TTR. Preliminary X-ray diffraction studies [12] of L55P-TTR showed that the asymmetric unit contains eight monomers instead of two monomers as in the WT- and V30M-TTR asymmetric units.

In order to evaluate the aggregation states of WT-, V30M-, L55P- and T119M-TTR, in solution, we have carried out a comparative study by gel filtration chromatography [13,14]. This study showed that, at approximately physiological pH, WT- and T119M-TTR have a very low tendency to form aggregates, and V30M-TTR and L55P-TTR have a high and a very high tendency to form aggregates, respectively. These different tendencies to form soluble aggregates correlate with the amyloidogenic potential of each one of the four variants studied.

2. Materials and methods

Recombinant WT-, V30M-, L55P- and T119M-TTR were produced in an *Escherichia coli* expression system [15] and purified as described previously [16]. To date, structural [10,11] and functional studies [16] have not shown any significant differences between TTR from human plasma and recombinant sources. All the TTR samples for gel filtration chromatography were diluted more than 10 times in gel filtration chromatography buffer, 20 mM sodium phosphate buffer, 150 mM sodium chloride, pH 7.0. Protein concentrations were determined spectrophotometrically, at 280 nm, using an extinction coefficient of

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Abbreviations: TTR, transthyretin; V30M-TTR, transthyretin with valine at position 30 replaced by a methionine; L55P-TTR, transthyretin with leucine at position 55 replaced by a proline; T119M-TTR, transthyretin with threonine at position 119 replaced by a methionine; WT, wild type; Tris, tris(hydroxymethyl)aminomethane

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7.76×10^4 M^{-1} cm^{-1} based on a 55 kDa molecular weight for TTR ($A_{280}(1\%) = 14.1$ mg^{-1} ml cm^{-1}) [17].

Gel filtration chromatography was performed on a Pharmacia FPLC Superdex-75 HR column, coupled to a Pharmacia high precision pump P-500 and a UV detector, equipped with a deuterium lamp, and an integrator from Konik Instruments. The column was allowed to equilibrate with 2–5 column volumes with chromatography buffer, and was frequently cleaned with 0.5 M NaOH, and always before a new TTR variant was injected. Different concentrations of transthyretin variants, each in 100 μl, were injected using a 100 μl loop. Final runs were performed at a flow rate of 0.4 ml/min. Apparent molecular weights were calculated by interpolation on a elution volume versus log(molecular weight) calibration curve for four protein standards: bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa) and aprotinin (6.5 kDa).

All chemicals were of the highest purity commercially available and were purchased from Sigma Chemical Company.

3. Results

Several concentrations of wild type and mutant transthyretins were prepared in chromatography buffer from an initial pure concentrated batch in 10 mM Tris-glycine, pH 8.8. Final dilutions were allowed to incubate at room temperature for 24–48 h before being applied to the gel filtration chromatography column. Fig. 1 shows the gel filtration elution profiles of WT-, V30M-, L55P- and T119M-TTR, at pH 7.0, at high (~3 μM) and low concentration (~0.3 μM). Peaks corresponding to different molecular species in solution are observed with apparent molecular weights in the order of 60 kDa for the tetramer (elution volume = 8.8 ml), 5.9 kDa for the monomer (elution volume = 15.2 ml), and aggregates of molecular weight higher than 70 kDa, the resolving limit of the column. For 3 μM WT-TTR (Fig. 1A), most of the protein is in the tetrameric form. Dilution to 0.3 μM (Fig. 1A) leads to dissociation of a significant amount of the tetramer to monomer. A similar behaviour is observed for T119M-TTR (Fig. 1D), however a slightly higher ratio of tetramer to monomer is observed at 0.3 μM (Fig. 1D). In the case of V30M-TTR, at 3 μM (Fig. 1B) most of the protein is in the tetrameric form, but significant amounts of high molecular weight aggregates are observed. Upon dilution to 0.3 μM these aggregates are not observed and a mixture of tetramer and monomer is obtained (Fig. 1B). For L55P-TTR, at 1.5 μM (Fig. 1C) a similar amount of aggregate and tetramer is

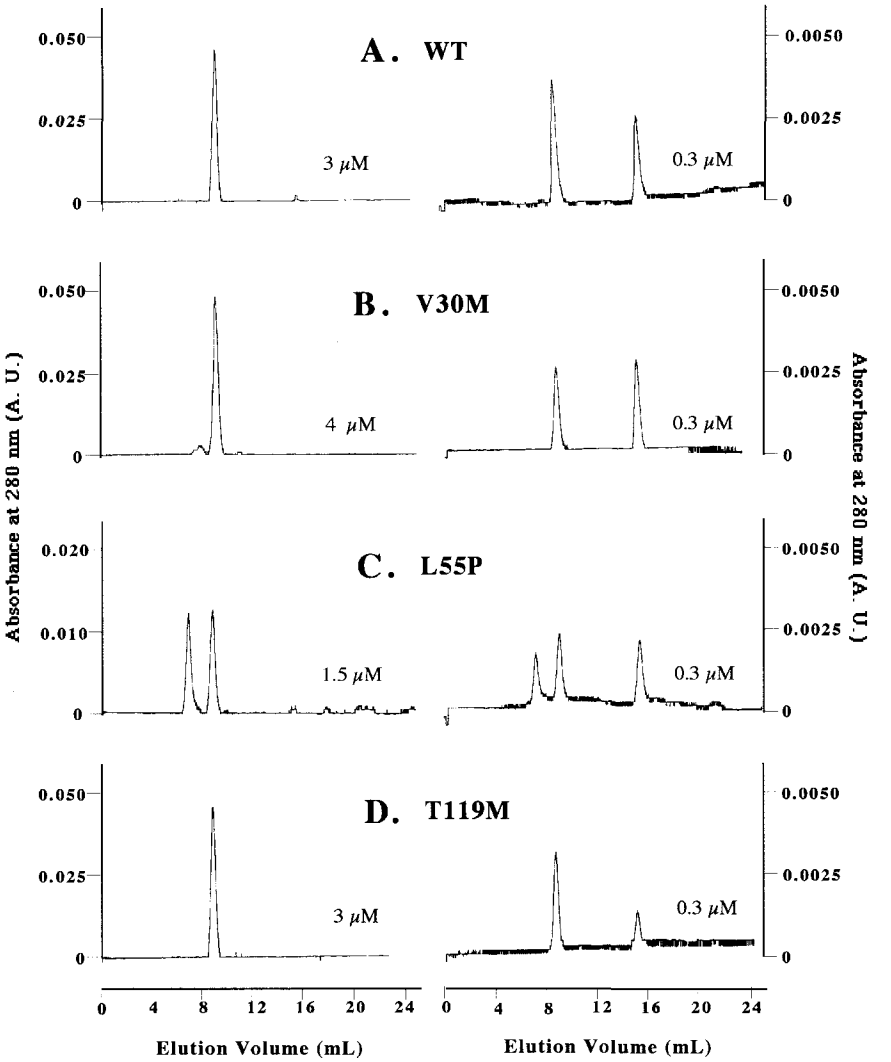

Fig. 1. Gel filtration chromatograms of WT-TTR (A), V30M-TTR (B), L55P-TTR (C) and T119M-TTR (D). Applied protein concentrations are indicated. Before being applied to the column, samples were allowed to equilibrate at room temperature and at their final concentrations, for at least 24 h. All chromatograms were run at a flow rate of 0.4 ml/min.

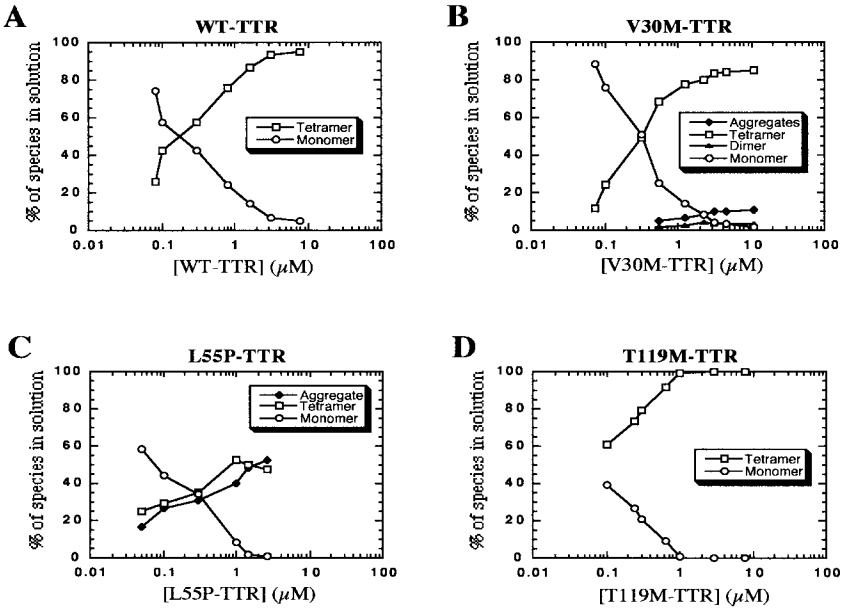

Fig. 2. Percentage of TTR molecular species in solution as a function of protein concentration, at pH 7, from gel filtration chromatography: (A) WT-TTR, (B) V30M-TTR, (C) L55P-TTR and (D) T119M-TTR.

observed. At 0.3 μM, three large peaks are observed corresponding to aggregate, tetramer and monomer. This behaviour for all four proteins is highly reproducible as long as the solution conditions are maintained. The chromatograms are also indicative of slow equilibrium between the different molecular species. In fact, short incubation times after dilution produce chromatographic profiles more characteristic of the initial conditions. Thus, for all chromatograms shown we waited until equilibrium was reached before injecting the samples in the gel filtration chromatography system.

To characterise in more detail the equilibria in solution for all four proteins, we ran gel filtration experiments at up to eight different protein concentrations varying between 0.1 and 10 μM. Fig. 2 shows that, for all proteins, dilution leads to dissociation of the tetramer to monomer, with a decrease in the amount of tetramer corresponding to an increase in the amount of monomer. However, in the case of V30M-TTR (Fig. 2B) and L55P-TTR (Fig. 2C), two amyloidogenic variants, a significant amount of aggregate species is also observed. In L55P-TTR, the protein that produces the most aggressive form of amyloidosis, the aggregate species are observed for a wide range of protein concentrations, and in much higher amounts than in the case of V30M-TTR (Fig. 2B,C). There is no evidence for any significant amount of aggregate species in WT- or T119M-TTR, in the protein concentration range studied (Fig. 2A,D). Additionally, it seems that the T119M-TTR tetramer is slightly more stable to dissociation than the other three proteins.

In order to probe the nature of the aggregate species in V30M- and L55P-TTR we collected and reinjected, at several different incubation times and at two different protein concentrations, the aggregate peaks. Fig. 3 (A, B and C) shows a time course for the dissociation of the aggregate to monomer in V30M-TTR, at low initial protein concentration (~0.03 μM). Two main peaks are observed for elution volumes of 7.7 ml and 15.2 ml, corresponding to the initial aggregate and monomer, respectively. Two main peaks, at exactly the same elution volumes, are also observed at higher initial protein concentration (~0.18 μM) (Fig. 3D). In both cases, no tetramer was observed. Determining the dissociation equilibrium from tetramer to monomeric species is very slow, and since even at early time points (Fig. 3A) no tetramer is observed, we conclude that the aggregate apparently dissociates directly to monomer.

4. Discussion

Gel filtration chromatography experiments with WT-TTR show that the native tetrameric form dissociates to monomer upon dilution, in the sub-micromolar range. There is no evidence for aggregates at pH 7 and at sub-micromolar protein concentrations. T119M-TTR tetramer also dissociates to monomer upon dilution, but apparently at a slightly lower protein concentration, indicating a slightly higher stability to dissociation in this variant. The tetrameric form of the amyloidogenic variant V30M-TTR dissociates to monomer with traces of dimer present. In this amyloidogenic variant a significant amount of aggregates with molecular weights higher than 70 kDa is observed, in the low micromolar concentration range. Apparently, these aggregates are in a slow equilibrium with the monomeric form of the protein, as we have shown by collecting and reinjecting the aggregates. In L55P-TTR, the presence of aggregate species is even more prevalent than in V30M-TTR. For this variant the tetramer also dissociates to monomer in the sub-micromolar range, but there is a large amount of aggregates in equilibrium, in solution, over a wide range of concentrations. This agrees with the preliminary X-ray data showing eight monomers instead of two monomers in the asymmetric unit [12].

To date, most of the mechanisms proposed for amyloid fibril formation by TTR are based on the assumption that the protein has to be in contact with a low pH medium which induces tetramer dissociation [7] and partial monomer unfolding [8]. Our results indicate that, even at pH 7.0, tetramer dissociation for the four proteins occurs around a similar, but not identical, sub-micromolar concentration range, with

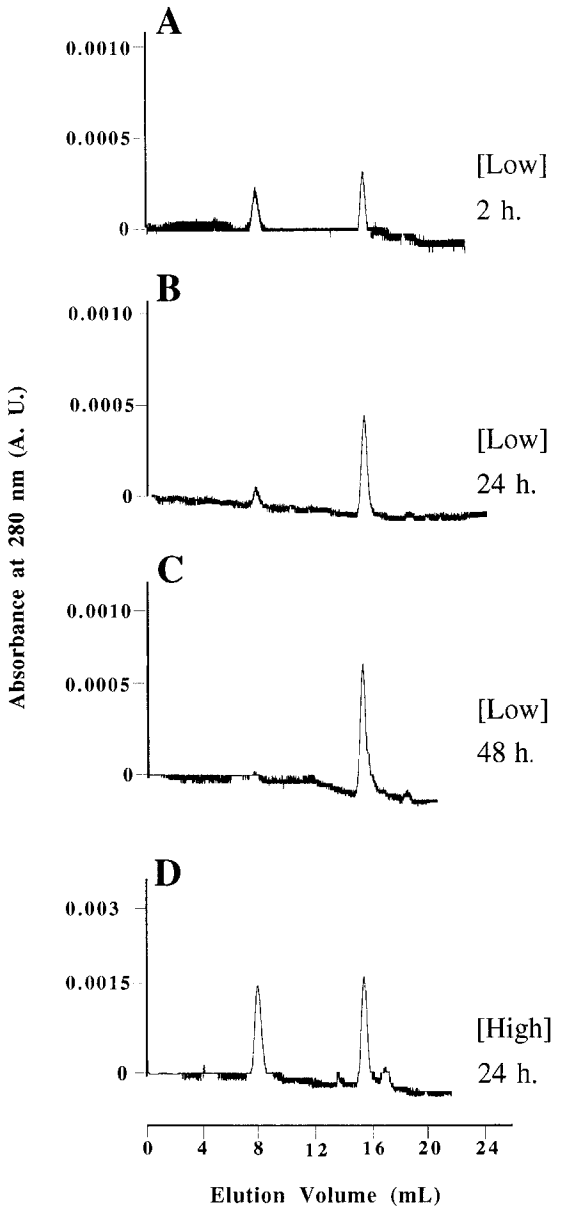

Fig. 3. Time course of V30M-TTR aggregate dissociation, at pH 7.0, followed by gel filtration chromatography. The sample, at a low initial protein concentration (~0.03 μM), was applied to the column (A) 2 h, (B) 24 h and (C) 48 h after the aggregate had been collected from the initial chromatography. A second gel filtration chromatography experiment was performed at a higher initial protein concentration (~0.18 μM) and the sample was applied to the column 24 h after the aggregate had been collected from the initial chromatography (D).

apparently a slightly higher tetramer stability to dissociation in T119M-TTR. However, the tendency for aggregate formation is significantly different in the four proteins: L55P-TTR ≫ V30M-TTR ≫ WT-TTR > T119M-TTR. This decreasing tendency for aggregate formation among the four proteins correlates with their decreasing amyloidogenic potential. In fact, L55P-TTR produces the most aggressive forms of FAP, followed by V30M-TTR. WT-TTR does not produce FAP, but it is responsible for an amyloidosis with a late onset – senile systemic amyloidosis. T119M-TTR is particularly interesting from a clinical point of view because subjects carrying genes for V30M-TTR and T119M-TTR do not develop FAP, which would indicate an almost 'anti-amyloidogenic' function of T119M-TTR. This could be explained by the formation of mixed V30M/T119M-TTR tetramer proteins with a slightly higher stability to dissociation and a decreased tendency for aggregate formation due to the presence of T119M-TTR subunits. In fact, our results seem to indicate a higher stability to dissociation of the T119M-TTR variant. This is also corroborated by recent results by Alves et al. [18] who compared different serum mutant TTRs on their resistance to dissociation into monomers by 4 M urea isoelectric focusing. A higher tetrameric stability of TTR was found in heterozygotic carriers of the T119M variant in contrast to a lower resistance to urea dissociation found for V30M heterozygotic carriers. Compound heterozygotes for the two variants presented a pattern similar to the normal individuals [18].

In conclusion, it seems that, even at physiological pH, tetramer stability to dissociation could play some role in the amyloidogenic potential of TTR, but more importantly the structure and/or dynamics of the monomeric species seem to play crucial role in aggregate formation and potentially amyloidosis. Some of the TTR mutations could perturb the structure and/or stability of the monomeric species to an extent that could lead initially to the formation of soluble aggregates and at a later stage to insoluble amyloid fibrils.

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