Trapping the Monomer of a Non-amyloidogenic Variant of Transthyretin

EXPLORING ITS POSSIBLE USE AS A THERAPEUTIC STRATEGY AGAINST TRANSTHYRETIN AMYLOIDOGENIC DISEASES*

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Transthyretin (TTR) is a 127-residue homotetrameric β-sheet-rich protein that transports thyroxine in the blood and cerebrospinal fluid. The deposition of fibrils and amorphous aggregates of TTR in patients’ tissues is a hallmark of TTR amyloid disease. Familial amyloidotic polyneuropathy is a hereditary form of TTR amyloidosis that is associated with one among 80 different variants of TTR. The most aggressive variants of TTR are V30M, L55P, and A25T, and the propensity to undergo aggregation seems to be linked to tetramer stability. T119M is a very stable, non-amyloidogenic variant of TTR. Here we show that the combination of high hydrostatic pressure with subdenaturing concentrations of urea (4 M) at 1 °C irreversibly dissociates T119M into monomers in less than 30 min in a concentration-dependent fashion. After pressure and urea removal, long lived monomers are the only species present in solution. We took advantage of the slow reassociation kinetics of these monomers into tetramers to produce heterotetramers by mixing the T119M monomers with the tetramers of the aggressive mutants of TTR. Our data show that T119M monomers can be successfully incorporated into all of these tetramers even when the exchange is performed in a more physiological environment such as human plasma; these monomers render the resultant heterotetramers less amyloidogenic. The data presented here are relevant for the understanding of T119M folding and association reactions and provide a protocol for producing T119M monomers that function as inhibitors of TTR aggregation when incorporated into tetramers. This protocol may provide a new strategy for treating TTR diseases for which there is no therapy available other than liver transplantation.

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2 The abbreviations used are: TTR, transthyretin; FAP, familial amyloidotic polyneuropathy; HHP, high hydrostatic pressure; M-TTR, monomer of wt TTR; wt, wild-type; T4, thyroxine; bis-ANS, bis-(β-anilino-naphthalene-1-sulfonate); VBO, 2-[(3,5-dichlorophenyl)amino]benzoic acid; MES, 4-morpholineethanesulfonic acid; HPLC, high pressure liquid chromatography; FITC, fluorescein isothiocyanate.

Transthyretin (TTR) is a 55-kDa homotetrameric protein composed of identical 127-residue subunits with a predominately β-sheet structure (1). TTR is found in human plasma (0.1–0.4 mg/ml) and cerebral spinal fluid (0.017 mg/ml). The plasma form serves as a secondary carrier for thyroxine (T4) and binds to retinol-binding protein (1), whereas the form that resides in the cerebral spinal fluid is the primary T4 transporter (2).

Wild-type TTR is responsible for senile systemic amyloidosis, a disease that affects 10% of people over 80 years old and is characterized by heavy amyloid deposits in the heart (3). Around 80 point mutants of TTR have been described thus far that are involved in familial amyloidotic polyneuropathy (FAP), familial amyloidotic cardiomyopathy, and central nervous system amyloidosis (4). In general, patients with the familial form of the disease experience the first symptoms by the time they reach their second or third decade with peripheral neuropathy, cardiomyopathy, and leptomeningeal deposition (5).

Among the variants of TTR identified worldwide, V30M and L55P are the most important because of their high frequency of occurrence and the aggressiveness of the symptoms they evoke. A25T is one of the most unstable known tetramer of TTR that is involved in central nervous system amyloidosis (6). The non-amyloidogenic variant T119M has been described as an interallelic trans-suppressor variant in compound heterozygotes and has a high frequency of occurrence in the Portuguese population. In heterozygotic individuals, this variant, which forms a highly stable tetramer (7), ameliorates the effects of the pathogenic mutation, reducing the severity of the symptoms (8, 9). This variant also presents an enhanced T4 binding capacity. X-ray crystallography studies have revealed that the substitution of methionine for threonine at position 119, a residue located in the T4-binding channel, allows for closer contact between the hormone and the protein and, therefore, the increased T4 binding affinity of T119M (9, 10). Additionally, T119M carriers have higher TTR serum levels because of the slow clearance rate of this variant from serum in contrast to V30M, which is cleared even faster than the wild-type protein (11).

Several studies have identified the major contributions of single amino acid substitutions on the thermodynamic stability, rate of tetramer dissociation, and amyloidogenicity of TTR (12–14). In a recent report, Hammarstrom et al. (7) observed that T119M dissociates 40-fold slower and resembles 90–200-fold slower than the wt TTR. These slow rates of folding and unfolding allow the protein to be very stable and resist
Production of Heterotetramers as a Strategy to Prevent FAP

High urea concentrations for long periods of time. For example, after 96 h of incubation in the presence of 8 M urea, only 40% of the population of T119M tetramers dissociate into monomers (7). This high thermodynamic stability displayed by T119M tetramers has been used to explain why this variant protects against FAP disease: the formation of an amyloidogenic species, be it a monomer (15), a dimer (16), or an altered tetramer (17–19), would be disfavored.

Damas and co-workers (9) resolved the atomic structure of T119M unbound and bound to T4 by x-ray analyses. It was clear from their data that the enhanced structural stability displayed by T119M is mainly due to new H-bonds within each monomer and between the monomers as well as to changes in the overall protein conformation that are provoked by Met-119, which extends into the T4-binding channel. No major structural changes, however, were observed in the structure of T119M that could explain its diminished amyloidogenicity.

High hydrostatic pressure (HHP) has been used successfully to denature and dissociate proteins, protein-DNA complexes, virus particles (20), and, more recently, protein aggregates and amyloid fibrils (21). In addition, we have shown recently that a cycle of compression-decompression is the tetramer of TTR; TTR (L55P and V30M) form fibrils under mild conditions (pH 5.0) (22). In addition, we have shown recently that in the presence of 8 M urea, only 40% of T119M tetramers dissociate into monomers (7). This high thermodynamic stability displayed by T119M tetramers has been used to explain why this variant protects against FAP disease: the formation of an amyloidogenic species, be it a monomer (15), a dimer (16), or an altered tetramer (17–19), would be disfavored.

Production of Heterotetramers as a Strategy to Prevent FAP

In the present study, we combined HHP with subdenaturing pressures to facilitate the dissociation of T119M into monomers, and then after decompres- sion and urea dilution, folded monomers were recovered. T119M monomers presented a thermodynamic stability similar to that of the wt monomer, suggesting that most of the differences in stability between the tetramers of T119M and wt TTR can be attributed to the intersubunit contacts inside the tetramers and not to the intrasubunit contacts of the monomers. Our data also show that T119M monomers can be successfully incorporated into amyloidogenic tetramers even when the exchange is performed in a more physiological environment, such as the plasma; these monomers render the resultant heterotetramers less amyloidogenic. The data presented here are relevant for understanding the T119M folding and association reactions and provide a protocol for producing stable T119M monomers that function as inhibitors of TTR aggregation when incorporated into tetramers; this protocol may be a new strategy for treating TTR diseases.

EXPERIMENTAL PROCEDURES

Chemicals—All reagents were of analytical grade. Bis(8-anilinonaphthalene-1-sulfonate) (bis-ANS) was purchased from Molecular Probes (Eugene, OR). 2-[3,5-Dichlorophenyl]amino]benzoic acid (VBO) (23), an analog of diclofenac, was dissolved in DMSO to yield a stock solution of 2 mM. Distilled water was filtered and deionized through a Milli-Q water purification system (Millipore Corp., Bedford, MA). The high pressure experiments were performed in the following buffer: 50 mM MES, 100 mM KCl, pH 5.0 (buffer A). Urea, when necessary, was added to this buffer. The experiments performed at pH 7.0 were performed in 50 mM Tris-HCl, 100 mM KCl (buffer B). We emphasize that MES and Tris buffers were chosen for the pressure experiments because their pH does not change significantly under high pressure. The ΔV for the protonic ionization of MES and Tris is positive and equal to 3.9 and 4.3 cm3/mol, respectively (24).

Protein Purification—Recombinant wt TTR, A25T, V30M, L55P, monomer of wt TTR (M-TTR), and T119M were expressed and purified as described previously (25). Protein concentration was determined using an extinction coefficient of 7.76 × 104 M−1 cm−1 at 280 nm. The engineered M-TTR has already been described (26); it has two point mutations (F87M and L110M) that prevent its tetramerization.

Spectroscopic Measurements under Pressure—We used a high pressure cell equipped with optical windows that has been described elsewhere (27); it was purchased from ISS Inc. (Champaign, IL). Fluorescence spectra were recorded on an ISS K2 spectrophotometer (ISS Inc.). The kinetic experiments under pressure were performed by increasing the pressure quickly to the mentioned values (in less than 1 min); the spectroscopic measurements were then collected over time. In the experiments where pressure was titrated, pressure was increased in steps of 200 bars. At each step, the sample was allowed to equilibrate for 15–20 min prior to making measurements. The high pressure cell was equipped with a circulating bath, which allowed us to control temperature during compression.

Tryptophan emission spectra were obtained by setting the excitation to 280 nm and collecting the emission in the 300–400-nm range. The mean energy of the fluorescence emission at pressure p evaluated by the center of spectral mass (νp) is given by Equation 1,

\[ \nu_p = \frac{\sum_i \nu_i F_i}{\sum_i F_i} \]  

Eq. 1

where \( F_i \) is the fluorescence emitted at wavelength \( \nu_i \). The degree of dissociation (α) is related to \( \nu_p \) by the expression

\[ \alpha = \frac{(\nu_f - \nu_p) / (\nu_i - \nu_p)}{(\nu_f - \nu_i) / (\nu_f - \nu_p)} \]  

Eq. 2

where \( \nu_i \) and \( \nu_f \) are the initial and final values of the center of spectral mass in nm, respectively, and \( \nu_p \) is the center of spectral mass at pressure p.

The light scattering increase was measured to evaluate the aggregation of the protein. Light scattering was measured by exciting the samples at 320 nm and collecting the scattered light from 315 to 325 nm. Bis-ANS and VBO spectra were recorded by exciting the sample at 360 and 320 nm and collecting emission from 400 to 600 nm and from 450 to 600 nm, respectively.

The experiments shown in Figs. 1–5 were repeated at least twice, and there was less than 10% discrepancy among them. For experiments shown in Figs. 6 and 7, the error bars represent the S.D. of three independent measurements.

Thermodynamic Parameters—The standard volume change of folding (ΔV) for the M-TTR and for the T119M monomers...
and the unfolding equilibrium constant (Ku) were determined from the thermodynamic relation

\[
\ln \left( \frac{\alpha_p}{(1 - \alpha_p)} \right) = \frac{p(\Delta V/RT)}{R} + \ln Ku
\]

(Eq. 3)

where \(\alpha\) is the extent of reaction, \(p\) correspond to a given pressure, \(R\) is the gas constant, and \(T\) the temperature in K at which the experiment was performed. The \(\Delta V\) is calculated from the slope of the curve, and Ku is the intercept on the y axis.

Circular Dichroism Measurements—Circular dichroism measurements were performed in a Jasco-715 spectropolarimeter (jasco) using a 1.0-mm-path length quartz cuvette. The buffer used for circular dichroism measurements was 25 mM Tris-HCl, 100 mM KCl, pH 7.0, with or without 4 M urea. Data were averaged for five scans at a speed of 100 nm/min collected in 0.2-nm steps. The base lines (buffer alone) were subtracted from the corresponding spectra.

Fibril Formation—Aggregation was performed by incubating TTR in 20 mM acetate buffer, pH 4.4, at 37 °C for 72 h. Fibrillation of TTR was evaluated by Congo red binding according to Klunk et al. (28). Briefly the samples were centrifuged at 17,000 \( \times \) g for 30 min, and the pellet was resuspended in 5 mM potassium phosphate, 150 mM NaCl, pH 7.4, and incubated with 10 \( \mu \)M Congo red. Then absorbance was recorded at 540 and 477 nm (28). The increase in absorbance at 400 nm was also used to evaluate the extent of fibril formation (data not shown because of their similarity with Congo red).

The HHP protocol to induce fibril formation was also used (17). Briefly TTR was compressed at 3,000 bars for 60 min in buffer A at 37 °C to induce the structural modifications that are necessary to trigger aggregation. After removing the pressure, the light scattering was measured as stated above to evaluate the extent of fibril formation.

Size Exclusion Chromatography—High performance liquid chromatography was carried out in a TSK3000 column at room temperature using an HPLC system (Shimadzu SPD-10A). The system was equilibrated in buffer B. A flow rate of 1 ml/min was used. Sample elution was monitored by Trp fluorescence at 330 nm and absorbance at 280 nm. For T119M labeled with FITC, the elution was monitored by FITC fluorescence (excitation at 494 nm and emission at 520 nm).

Production of Heterotetramers by HHP Treatment—Mono-
mers of T119M were mixed with tetramers of V30M in a ratio of 2:1 in buffer B. This solution was compressed at 3,000 bars at 25 °C for 60 min to allow for the complete dissociation of V30M tetramers (18). After releasing the pressure, samples were kept at 4 °C for 24 h to allow for complete subunit exchange. This solution was injected into the HPLC system, and the peak corresponding to the tetramer was collected, concentrated, and used to evaluate the aggregation rate using the HHP protocol described under “Fibril Formation.”

Production of Heterotetramers at Ambient Pressure—Het-
erotetramers were produced as described previously (29). Briefly a solution of T119M monomers, obtained by the HHP treatment in the presence of 4 M urea, was concentrated in a Centricon (cutoff, 5 kDa; Millipore Corp.) up to \( \sim 400 \mu\)M. Then this concentrated solution of monomers was rapidly diluted to 8 \( \mu\)M in 11 ml of 10 mM phosphate, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0, in the presence of a 1 \( \mu\)M concentration of the amyloidogenic variants, A25T, L55P, or V30M (ratio of 2:1 (w/w) T119M monomer:variant tetramer). The mixtures were dialyzed for 24 h at 4 °C against the mentioned buffer to remove residual urea and to allow for subunit exchange (incorporation of T119M monomers into the amyloidogenic variants of TTR). Then this solution was transferred to 37 °C for 72 h to induce aggregation after being concentrated to \( \sim 21 \mu\)M and mixed with equal volumes of 200 mM acetate, pH 4.4. After acetate addition, the concentrations of amyloidogenic variant and T119M tetramers were 3.5 and 7 \( \mu\)M, respectively. As a control, the same protocol was repeated replacing T119M monomers with T119M tetramers where we expected no subunit exchange. The extent of aggregation after subunit exchange was compared with those displayed by solutions containing 3.5 \( \mu\)M A25T, L55P, or V30M alone, which represents 100% aggregation for each variant.

Sample Labeling—The amyloidogenic variant (A25T, L55P, or V30M) at 200 \( \mu\)M was incubated with 2 mM acrylodan in buffer B for 90 min at 4 °C. Then the free probe was removed by extensive washing in a Centricon (cutoff, 30 kDa) at 25 °C. The labeling efficiency was estimated by measuring the absorption at 372 nm (\( \epsilon = 1.64 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \)) and 280 nm (\( \epsilon = 7.76 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \)) for all variants used, the extent of labeling was higher than 75%. A similar protocol was used for labeling T119M with FITC except that in this case we used the buffer 10 mM phosphate, 100 mM KCl, pH 7.0. Labeling efficiency was calculated by absorption at 494 nm (\( \epsilon = 6.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \)).

Trans-suppression in Human Plasma—T119M monomers at 400 \( \mu\)M were diluted to 28 \( \mu\)M in human plasma containing a 3.5 \( \mu\)M concentration (2:1, w/w) of the amyloidogenic variants A25T, L55P, or V30M, which had been previously labeled with acrylodan. The samples were incubated at 4 °C for 24 h to allow subunit exchange. Then to induce protein aggregation, a small volume of 0.5 M HCl was added to change the plasma pH from 7.3 to 4.4. After 72 h of incubation at 25 °C, the appearance of aggregates and the extent of remaining acrylodan-labeled tetramers were estimated by measuring the fluorescence intensity of this band on native PAGE (10%) under UV light. The bands were quantified by Image J (30). The fraction of remaining tetramers was calculated by dividing the fluorescence intensity of the band corresponding to the acrylodan-labeled tetramers incubated at pH 4.4 for 72 h by the intensity of this band before acidification (soluble tetramers). The same protocol was performed with TTR variants in the absence of T119M monomers or in the presence of T119M or wt tetramers (same ratio: 2:1, w/w). Electrophoresis was performed at 4 °C with 100 V and protection from light.

RESULTS

The High Pressure-induced Denaturation of T119M in the
Presence of Subdenaturing Concentrations of Urea Is
Irreversible—We have shown previously that the variants
of TTR, namely L55P, V30M, and T119M, present different levels of thermodynamic stability against HHP: at 1 °C, L55P < V30M < wt \( \ll T119M \). Under the conditions we used in this first study (pH 7.5 or 5.0 and 1 or 37 °C), the tetramers of T119M were unusually highly resistant to the high pressure...
Production of Heterotetramers as a Strategy to Prevent FAP

treatment (18). Its partial dissociation-denaturation has been shown previously to occur only at high urea concentrations (8 M) for prolonged incubation times (96 h (7)).

To find an experimental condition under which the quaternary and tertiary structures of T119M could be perturbed more easily and rapidly, we performed several pressure titration curves at pH 5.0 and 37 °C in the presence of increasing subdenaturing concentrations of urea, ranging from 2 to 4.5 M (Fig. 1A, hollow symbols). We followed the changes in the center of spectral mass of Trp fluorescence emission to evaluate the extent of dissociation-unfolding of T119M; because TTR has two Trp residues per monomer, they have been used as suitable sensors of the changes in tertiary structure of TTR (12, 17, 18, 31). As seen in Fig. 1A, at 37 °C, pressure titration in the presence of increasing concentrations of urea promoted a progressive but partial shift to the red of the maximum emission of Trp, suggesting an incomplete denaturation event. Even in the presence of 4.5 M urea and 3.0 kilobars, the maximum emission of Trp reached only 346 nm, a value compatible with a large but incomplete exposure of the Trp residues to the aqueous environment.

Because it has previously been shown that low temperatures facilitate the dissociation of TTR (7, 17, 18), we performed a pressure titration curve at 1 °C in the presence of 4 M urea, aiming to destabilize the tetrameric structure of T119M even more (Fig. 1A, filled circles). Under these conditions, the maximum emission of the Trp shifted to 350 nm, suggesting that the Trps were fully exposed to the aqueous environment. Thus, to be dissociated and unfolded in a short time frame, the tetramers of T119M need a combination of HHP, urea, and low temperature. As shown in Fig. 1A by the isolated symbols on the left, however, after decompression in any condition, the maximum emission of Trp did not return to its original value (342 nm), indicating that the structural perturbation induced by HHP in the presence of urea is irreversible. As shown previously, even after 96 h in the presence of 8 M urea, denaturation of T119M is only partial (40%) and does not reach equilibrium (7).

To address the time dependence of the HHP-induced dissociation of T119M in the presence of urea, kinetic experiments were performed at 3,000 bars (1 °C at pH 5.0); the data are shown in Fig. 1B. As expected, the higher the concentration of urea added to the medium, the faster the dissociation of the tetramers: in the presence of 4 M urea, the complete exposure of Trp to the red (351 nm) took ~20–30 min, whereas at 4.5 M urea, this transition took around 5 min. Again the process was irreversible as indicated by the lack of recovery of the initial value of the Trp maximum emission (isolated symbols on the left).

The dissociation of oligomeric proteins such as TTR is expected to be accompanied by dependence on protein concentration (32). To investigate whether the reaction under question was indeed the dissociation of T119M tetramers into monomers or the denaturation of the monomers inside the tetramer, we followed the shift in Trp emission as a function of time in the presence of 4 M urea at 1 °C at two protein concentrations, 4 and 16 μM (Fig. 1C). As expected for a dissociation reaction, the dissociation of T119M was slower at 16 μM than at 4 μM, confirming that the tetramers of T119M are indeed being irreversibly dissociated and unfolded.

FIGURE 1. HHP induces dissociation-denaturation of TTR T119M in the presence of subdenaturing concentrations of urea. A, the center of spectral mass of Trp emission was followed as a function of pressure at 37 °C in the presence of 2 (●), 3 (□), 4 (○), or 4.5 M (▲) urea or in the presence of 4 M urea at 1 °C (●). The inset shows the emission spectra of Trp before pressure in the presence of 4 M urea, pH 5.0, at 1 °C (continuous black line); at 3,000 bars (dashed line); or after returning to atmospheric pressure (gray line). [Protein] = 2 μM in all measurements. B, kinetics of the pressure-induced dissociation-denaturation of T119M (2 μM) at pH 5.0 at 1 °C as followed by the shift in the center of spectral mass of Trp emission in the presence of 1 (●), 3.5 (▲), 4 (○), or 4.5 M (▲) urea. In C, 4 (●) or 16 μM (○) T119M was subjected to 3,000 bars at 1 °C at pH 5.0 in the presence of 4 M urea, and the center of spectral mass of Trp emission was measured as a function of time. The isolated symbols on the left in all panels represent the center of mass values achieved after decompression; the incomplete recovery of the initial value suggests that there is irreversibility in the structural perturbations induced by HHP. Excitation, 280 nm; emission, 300–400 nm. a.u., arbitrary units.
Production of Heterotetramers as a Strategy to Prevent FAP

T119M was incubated in the presence of 5 μM bis-ANS and subjected to increasing pressures in the presence of 4 M urea. The extent of bis-ANS binding was evaluated by measuring the bis-ANS spectral area (excitation, 360 nm; emission, 400–600 nm). Inset, bis-ANS emission spectra at atmospheric pressure (●), 1,654 bars (○), and 3,120 bars (□) and after atmospheric pressure return (●, superimposed with ○). C, extent of reaction (χ) as a function of pressure calculated from Equation 3 using the center of spectral mass data from Fig. 1A (Δ), the VBO data from Fig. 2B (●), the bis-ANS data from Fig. 2B (○), and the center of mass of Trp emission obtained from the experiment performed in the presence of VBO (×). The isolated symbols on the left present the fluorescence measurements obtained after decompression. a.u., arbitrary units.

FIGURE 2. Following dissociation-denaturation of T119M induced by HHP in the presence of 4 M urea at 1 °C by VBO and bis-ANS binding. A, 3 μM T119M was incubated in the presence of 10 μM VBO, a fluorescent probe that binds to the T4-binding channels. When free in solution, VBO has no fluorescence emission, whereas upon protein binding its quantum yield increases considerably (inset, filled squares). Upon compression, the VBO spectral area decreases, suggesting that the T119M tetramers have dissociated. The inset shows the fluorescence emission spectra of VBO at 0 bar (●), 2,350 bars (○), 2,750 bars (▲), and 3,120 bars (□) and after returning to atmospheric pressure (●, superimposed with ○). Excitation, 320 nm; emission, 450–600 nm. B, 1 μM VBO dropped considerably (Fig. 2A). Note that after decompression the emission spectrum of VBO remained low (inset, spectrum in filled circles, which is under the spectrum in hollow squares and the isolated symbol in the left of A), indicating that after decompression tetramers do not reassemble.

To obtain additional insights into the tertiary structure content of the species formed under and after HHP treatment, bis-ANS binding experiments were performed (Fig. 2B). Bis-ANS is a hydrophobic probe that binds specifically to apolar pockets on proteins, which are present, for instance, in partially folded species (33). In the case of TTR, as shown previously, the tetramers bind ANS compounds in the two T4-binding channels present in the tetramer (Ref. 34 and Fig. 2B, inset). Fig. 2B shows that upon compression at pH 5.0 at 1 °C, in the presence of 4 M urea, the fluorescence intensity of bis-ANS decreases to very low values, confirming that the T4-binding sites are being disrupted by the pressure treatment (Fig. 2B, inset). In addition, because the spectrum of bis-ANS under pressure was identical to its spectrum when free in water (Fig. 2B, inset), we can conclude that under pressure in the presence of 4 M urea T119M tetramers dissociate, and the isolated monomers are completely unfolded. When pressure is released, bis-ANS binding remains very low (isolated symbol on the left), confirming that the dissociation and denaturation processes are irreversible.

Fig. 2C shows the extent of reaction (χ; dissociation-denaturation) as a function of increasing pressure in the presence of 4 M urea (1 °C) where the shifts in fluorescence signals of Trp (hollow triangles), VBO (circles), and bis-ANS (diamonds) are compared. As seen, the dissociation-denaturation of T119M, as followed by the changes in the signals of these three fluorescent probes, did not change in a concerted fashion with increasing pressure, suggesting that the quaternary and tertiary structures of T119M are lost at different pressure values. Curiously the irreversibly dissociated into monomers by HHP and that the separated monomers are probably being unfolded by the combined action of pressure, urea (4 M), and low temperature (1 °C).

VBO is a fluorescent compound that only fluoresces when bound to the T4-binding site located at the interface of the TTR dimers. Fig. 2A (inset) shows the emission spectra of VBO bound to the tetramers of T119M in the presence of 4 M urea at 1 °C before compression (squares) and at increasing pressures. This experiment was performed at pH 5.5 because the fluorescence emission of free VBO is pH-dependent and considerably higher at a pH below 5.5 (not shown). Before compression, the fluorescence emission of VBO was high because of its binding to the T4-binding sites in the tetramers. As pressure increased, the tetramers dissociated, and the fluorescence emission of VBO dropped considerably (Fig. 2A). Note that after decompression the emission spectrum of VBO remained low (inset, spectrum in filled circles, which is under the spectrum in hollow squares and the isolated symbol in the left of A), indicating that after decompression tetramers do not reassemble.

Following dissociation-denaturation of T119M induced by HHP in the presence of 4 M urea at 1 °C by VBO and bis-ANS binding. A, 3 μM
Production of Heterotetramers as a Strategy to Prevent FAP

signal from VBO, which supposedly binds exclusively to the T4-binding channels, was the last one to be perturbed by HHP, suggesting that the T4-binding channels remain organized at least up to 2,000 bars. We thought that perhaps the presence of VBO would stabilize the T119M tetramers. In fact, when the center of spectral mass of the Trp emission in the absence (Fig. 2C, filled triangles) or in the presence of VBO (hollow triangles) are compared, a slight increase in stability of the tetramers with VBO is evident, suggesting that VBO protects the quaternary structure of T119M against HHP.

Bis-ANS also binds in the T4-binding channels of TTR, but lower pressure values are required to release this binding (Fig. 2C, diamonds) than with VBO. It is possible that the differences in affinity displayed by these two probes for the T4 channels would explain their different profiles of unbinding under pressure where VBO, with a higher affinity, would stabilize the tetramers most.

Because the spectra of the three different probes (tryptophan, VBO, and bis-ANS) used to evaluate the quaternary and tertiary structural changes promoted by HHP on T119M did not recover to their original position after decompression (see Fig. 2C, isolated symbols on the left), we performed size exclusion chromatography to characterize the size of the species present after pressure release (Fig. 3A). It is important to note that the sample recovered from HHP treatment still contained 4 M urea, which was washed inside the gel filtration column. Fig. 3A shows that the T119M tetramer before compression in the presence of 4 M urea eluted as a single peak at ~9 min. This elution time is compatible with the tetramer mass (56 kDa). The sample recovered after pressure release in the presence of 4 M urea eluted as a major peak at ~11 min, the same elution time of the M-TTR (arrow) that does not form tetramers (26) because of the presence of bulky residues in the dimeric interface that impede tetramerization. This result suggests that the tetramers of T119M are broken apart into monomers by the combination of HHP and 4 M urea at 1 °C; after pressure release, the monomers remain in solution (Fig. 3A).

To study the secondary structure content of the monomers formed after pressure release in the presence of 4 M urea, circular dichroism measurements were performed; the data are presented in Fig. 3B. TTR is composed mainly of β-sheets, and thus its circular dichroism spectrum has a minimum at 216 nm (continuous black line). Before compression, even in the presence of 4 M urea, T119M presents a secondary structure content similar to the one presented by the tetramers in the absence of urea (compare continuous black line with dashed black line). The presence of urea compromises the spectrum below 215 nm, making the comparison difficult in that range. As shown, after pressure release in the presence of 4 M urea, the monomers that form have low secondary structure content, suggesting that they are unfolded (dashed gray line). Thus, the presence of 4 M urea keeps the monomers apart in an unfolded conformation that impedes their reassociation into tetramers; however, upon dilution of the urea (0.4 M), the secondary structure content of the monomers immediately recovers as indicated by the continuous gray line spectrum in Fig. 3B. Taken together, these results suggest that after releasing the pressure in the presence of 4 M urea T119M remains as unfolded monomers (M(T119M, U)), which refold immediately upon urea removal (M(T119M, F)). Table 1 summarizes the spectroscopic properties of all the species thus far described.

The Reassociation of the Separated Monomers of T119M into Tetramers Is a Slow Process—To investigate the kinetics of the retetramerization of the separated monomers of T119M, a concentrated solution of T119M (20 μM) was subjected to 3,000 bars for 2 h in the presence of 4 M urea to produce monomers. Then after pressure was removed, this solution was diluted 10 times at pH 7.0 at 25 °C, and aliquots of this solution were withdrawn and injected into a gel filtration column (Fig. 4A). Immediately after urea dilution, there were only monomers, which eluted at ~11 min (gray line). Over time, this species disappeared, giving rise to tetramers (peak at ~9 min). The complete reassociation of T119M monomers into tetramers was very slow, taking several hours (Fig. 4A, inset), and even 8 h after the urea dilution there were still 40% monomers remaining. The
The monomer of T119M produced by HHP treatment were diluted with 25 mM Tris-HCl, 100 mM KCl, pH 7.0, resulting in a solution with 0.2 μM monomers and 0.2 M urea. This solution was compressed in steps at 25 °C, and the center of spectral mass of Trp emission was collected in each pressure value ( ). The wt engineered monomer of TTR was diluted at the same conditions and subjected to HHP ( ). From these data and Equation 3, a plot of ln(α/1−α) versus pressure was constructed, and the folding thermodynamic parameters for the monomers were calculated ( and Table 1). The isolated symbols on the left represent the center of mass achieved after returning the sample to atmospheric pressure.


table 1

| Parameter | T119M (native or refolded tetramer) | M-TTR (unfolded monomer under pressure) | M-TTR (unfolded monomer after pressure) | M-TTR (folded monomer after urea removal) |
|-----------|-------------------------------------|-----------------------------------------|----------------------------------------|------------------------------------------|
| Trp emission (nm) | 341 | 351 | 349 | 343 |
| VBO binding | - | - | - | - |
| Bis-ANS binding | ++ | - | - | - |
| Secondary structure | + (β-sheet) | NMa | - (residual) | + (β-sheet) |

*a Not measured; - , no binding or unstructured; + , moderate binding or structured; ++ , strong binding.*

| Parameter | T119M (native or refolded tetramer) | M-TTR (unfolded monomer under pressure) | M-TTR (unfolded monomer after pressure) | M-TTR (folded monomer after urea removal) |
|-----------|-------------------------------------|-----------------------------------------|----------------------------------------|------------------------------------------|
| Trp emission (nm) | 341 | 351 | 349 | 343 |
| VBO binding | - | - | - | - |
| Bis-ANS binding | ++ | - | - | - |
| Secondary structure | + (β-sheet) | NMa | - (residual) | + (β-sheet) |

*a Not measured; - , no binding or unstructured; + , moderate binding or structured; ++ , strong binding.*

FIGURE 5. The monomers of the wt and T119M present a similar thermodynamic stability during HHP. The monomers of T119M produced by HHP treatment were diluted with 25 mM Tris-HCl, 100 mM KCl, pH 7.0, resulting in a solution with 0.2 μM monomers and 0.2 M urea. This solution was compressed in steps at 25 °C, and the center of spectral mass of Trp emission was collected in each pressure value ( ). The wt engineered monomer of TTR was diluted at the same conditions and subjected to HHP ( ). From these data and Equation 3, a plot of ln(α/1−α) versus pressure was constructed, and the folding thermodynamic parameters for the monomers were calculated ( and Table 1). The isolated symbols on the left represent the center of mass achieved after returning the sample to atmospheric pressure.

The monomer of T119M exhibits thermodynamic stability similar to that of the wt monomer—Because we found a condition where folded monomers of the T119M exist (short times after decompression upon urea dilution), its pressure-induced unfolding at 25 °C was followed by Trp fluorescence emission and compared with that of M-TTR (Fig. 5). As seen in Fig. 5, the unfolding curves for M-TTR and the T119M monomers are very similar, suggesting equivalent thermodynamic stabilities. In both cases, the unfolding process was fully reversible as seen by the complete recovery of the center of spectral mass of Trp after decompression (hollow symbols on the left). The thermodynamic parameters (ΔGo and ΔV) for the folding process of these two monomeric proteins were calculated from the curves in Fig. 5 and Equation 3 (Fig. 5, inset, and Table 2).
It is possible to conclude from the analysis of the change in free energy of folding that the T119M monomer is as stable as the TTR (ΔG = −2.14 versus −2.47 kcal/mol, respectively), which suggests that the difference in stability that is observed between the two tetramers, wt and T119M, resides in the inter-subunit contacts (quaternary structure) and not in the intrasubunit contacts (tertiary structure). In addition, the volume changes upon folding for the T119M and wt monomers were equal to 50 and 65 ml/mol, respectively (Table 2), values which are similar to those calculated for other monomeric proteins (35, 36). The difference between the volume changes, although small, seems to be significant. It is possible that the mutation at position 119 creates a more compact monomer with lower internal void volumes. Additionally the change in volume calculated for the dissociation of the wt tetramer by NMR (37) at pH 7.5 is equal to 212 ml/mol, a value that is ∼4-fold higher than the one obtained for the unfolding of the wt monomer.

Production of Heterotetramers: The Incorporation of T119M Monomers into Amyloidogenic Tetramers Reduces Its Amyloidogenicity—With an elegant approach, Kelly and co-workers (29) have been able to isolate T119M/V30M hybrid tetramers by co-expressing V30M and a tagged version of T119M in the same bacterial cell. The tagged protein has a short sequence of anionic residues incorporated into its N terminus. With this tag, it is possible to isolate by ion exchange chromatography the hybrid tetramers, which have incorporated none, one, two, three, or four tagged T119M subunits. By studying these mixed tetramers, it was clear that the incorporation of only two subunits of T119M in a V30M tetramer was enough to almost abrogate its amyloidogenic potential.

Here we took advantage of the folded T119M monomers, which we were able to isolate after HHP treatment upon urea dilution, and prepared heterotetramers by mixing these monomers with tetramers composed of V30M, A25T, and L55P, variants involved in FAP or central nervous system amyloidosis. We emphasize that the monomers that we isolate with a cycle of compression-decompression are native and have no tag added to its sequence.

Initially to force subunit exchange, we took advantage of HHP, which dissociates V30M tetramers in the absence of urea (18). Thus, 8 μM T119M monomers was mixed with 1 μM V30M tetramers at pH 7.0; this mixture was subjected to 3,000 bars for 60 min at 25 °C to allow for the complete dissociation of V30M tetramers. Then pressure was released, and tetramers with mixed subunits were randomly formed. This solution was kept for 24 h at 4 °C to allow for complete heterotetramer formation. Then it was injected into a gel filtration column to confirm the presence of tetramers (not shown). The peak corresponding to these mixed tetramers was collected, concentrated, and used in the next step of the experiment where aggregation was probed, now at pH 5.0. Of course, this sample was a heterogeneous population of tetramers composed of zero, one, two, three, and four subunits of T119M. We subjected these heterotetramers to HHP using a protocol previously described by our group (17). Briefly we have shown that after a cycle of compression-decompression at pH 5.0 and 37 °C, TTR (wt and variants) aggregates to form amyloid fibrils in less than 30 min. We postulated that HHP induces a “sick fold” in the protein, rendering it amyloidogenic.

As seen in Fig. 6A, there was a progressive increase in the amount of light scattering corresponding to fibril formation after decomposition of a 3 μM solution of pure V30M, which had been compressed for 60 min at pH 5.0 (continuous black line). At the same concentration (3 μM), the mixed tetramers of T119M and V30M subunits did not show any sign of aggregation after decompression (gray line), confirming the incorporation of T119M subunits into these tetramers and the abolishment of their ability to aggregate. As a control, the same experiment was performed by incubating 1 μM V30M with 2 μM T119M tetramers (dashed line). There was some aggregation after decompression probably as a result of the 1 μM V30M that was present in the solution. Thus, from these data we can conclude that the monomers of T119M that are produced after a pressure treatment can be incorporated into V30M tetramers with the aid of HHP and that this incorporation inhibits amyloidogenesis.

Next we investigated whether subunit exchange could take place spontaneously, without the aid of HHP, and render the heterotetramers less amyloidogenic. Several aggressive amyloidogenic variants of TTR, including V30M, L55P, and A25T (1 μM), were incubated initially in the presence of T119M monomers (8 μM) or T119M tetramers (2 μM) at 4 °C for 24 h at pH 7.0. It was expected that, under these conditions, subunit exchange would take place to produce mixed tetramers. Then the pH was dropped to 4.4, and the samples were kept at 37 °C for 72 h, a condition that favors aggregation. Turbidity at 400 nm and Congo red binding assays were used to measure the extent of fibril formation. As a positive control (100% aggregation in Fig. 6B), the mentioned tetramers were incubated alone at pH 4.4 for 72 h. Fig. 6B shows the results of these experiments. It is evident that the extent of aggregation was significantly decreased in all samples that were previously incubated with T119M monomers, confirming that subunit exchange had occurred and provided protection against aggregation. The variants that were previously incubated with T119M tetramers (T119M(T)) during the exchange phase showed around 90% aggregation, confirming that there was no subunit exchange when tetramers of T119M were used because of their high stability.

The Production of Heterotetramers in the Human Plasma: Is It Possible to Use the T119M Monomers Produced after HHP as a Strategy to Combat FAP?—Once the production of heterotetramers of T119M subunits combined with V30M, L55P, or A25T proved to be feasible in buffer and very effective in preventing fibrillogenesis, we wondered whether this feat could be accomplished in a more physiological environment, such as the blood plasma. In this case, it was necessary to label the variants of TTR with a fluorescent probe to track them in a complex
solution. Acrylodan, which binds covalently to cysteine residues, was chosen. TTR has only one cysteine at position 10. Fig. 7A (as well as C and D, insets) shows native gels illuminated with UV light, which acrylodan absorbs. In Fig. 7A, lane 0 it is possible to see the band corresponding to the soluble A25T_Acryl which was added to the plasma. Because the concentration of albumin in the plasma is very high, it is also possible to detect its presence under UV illumination (all lanes and lane 1, which shows the plasma before the addition of A25T_Acryl).

When the pH of the plasma containing 3.5 μM TTR variants labeled with acrylodan was decreased to 4.4 by adding a known amount of HCl, the proteins aggregated and were seen in the top of the gels after incubating at 25 °C for 72 h (Fig. 7, A (A25T), C (L55P), and D (V30M), lanes 2). The acrylodan-labeled aggregates were also detected by gel filtration chromatography (not shown). These results show that it is possible to follow the aggregation of acrylodan-labeled TTR in a complex mixture like the human plasma.

Because we were able to successfully follow the aggregation of acrylodan-labeled TTR in the plasma, the next step was to investigate whether the aggregation of heterotetramers would be inhibited in the plasma as it was inhibited in buffer (Fig. 6).
Production of Heterotetramers as a Strategy to Prevent FAP

To answer that question, 3.5 \( \mu M \) TTR\(_{Acryl} \) ((A25T (Fig. 7, A and B), L55P (Fig. 7C), and V30M (Fig. 7D)) were initially mixed with 28 \( \mu M \) T119M monomers in the plasma. These plasma solutions were kept at 4 °C for 24 h to allow for subunit exchange (production of heterotetramers). Then the pH was decreased to 4.4 to induce the aggregation of TTR. After 72 h at 25 °C, native gels were run to measure the amount of remaining tetramers; the results are presented in lane 3 of the gels in Fig. 7. As controls, 3.5 \( \mu M \) TTR\(_{Acryl} \) was subjected to these treatments in the presence of 7 \( \mu M \) T119M tetramers (lanes 4) or wt tetramers (lane 5). In the gels as well as in the bar plots where the extent of remaining tetramers was quantified by densitometry (Fig. 7, B, C, and D), it is evident that only around 10–15% of tetramers remained in the positive control samples (homotetramers, bars 2). On the other hand, there was about 35–45% of tetramers remaining in the samples that incorporated T119M subunits in all TTR cases (bars 3), suggesting that the incorporation of T119M monomers to form heterotetramers can take place in a complex milieu like the human plasma and render the tetramers less amyloidogenic. When the exchange phase was performed with T119M tetramers (lanes 4 and bars 4) or wt tetramers (lanes 5 and bars 5), aggregation was not inhibited; the amount of tetramer remaining remained as low as the control. The monomers of T119M, when incubated in buffer at pH 7 or in the plasma, did not show any sign of aggregation (not shown). Taken together, these results have an important implication because it is possible to envision a therapy based on adding T119M monomers to plasma of FAP patients as an alternative strategy to treat this disease.

DISCUSSION

It has been observed that the allele carrying the T119M mutation alleviates the aggressiveness of the V30M mutation in Portuguese families that accumulate these two mutations (8, 9). In vitro studies performed with this non-pathogenic variant under aggregating conditions also point to its non-amyloidogenicity because no aggregation has been observed thus far. It has been postulated that aggregation of TTR into amyloid fibrils depends on the formation of a monomeric, amyloidogenic species that is present at acidic pH (38, 39). Because T119M tetramers are highly stable even when incubated under acidic condition for long times, the T119M amyloidogenic intermediate is not present, rendering this tetramer non-amyloidogenic. These observations suggest that the T119M mutation seems to protect against FAP disease by impeding tetramer dissociation. Thus, it has been reasoned that the incorporation of T119M subunits into tetramers would render them more stable and less amyloidogenic.

Some studies have already shown that heterotetramers that incorporate T119M subunits have a diminished extent of aggregation in accordance with what has been observed in heterozygous individuals who do not have FAP symptoms. These studies were performed with a tagged version of T119M, an ingenious strategy to precisely map the minimum number of T119M subunits to be incorporated into the heterotetramers to render them less amyloidogenic or even non-amyloidogenic (29). However, in these studies there was no attempt to express stable, homogeneous T119M monomers as would be required for therapy. Recently Reixach et al. (40) showed that the very stable tetramer of mouse TTR did not exchange subunits with human TTR even when both proteins were incubated for 24 h at 4 °C, a condition that favors subunit exchange. Heterotetramers were only observed after diluting a solution containing equal amounts of mouse and human TTR denatured in 6.5 M guanidinium hydrochloride for 2 days. Thus, our study complements what has been described previously because here we are proposing a protocol that can produce stable monomers of T119M, and these monomers have proved to be effective in preventing the aggregation of several TTR variants even in a complex environment such as the human plasma, mimicking what would be envisioned for therapeutic purposes against FAP disease. Up to now, because of the high thermodynamic stability of the T119M tetramers, it has not been possible to isolate T119M monomers either for experimental study or as a possible therapy for FAP disease.

Thus, the HHP-protocol presented here represents a possible strategy to be explored to produce monomers of T119M to be administered to FAP patients. Our results are schematically summarized in Fig. 8. Under pressure, in the presence of sub-denaturing concentrations of urea, such as 4 M, T119M tetramers undergo dissociation and unfolding (step 1). When pressure and urea are removed, the unfolded monomer immediately refolds into its native state (step 2); retetramerization is, as expected, concentration-dependent and slower especially in the plasma (step 3). When subunits of the variant tetramers of TTR are allowed to exchange even in a complex solution like the human plasma, they produce heterotetramers (step 4) that are less amyloidogenic (step 5) than variant homotetramers (step 6). This protection against aggregation was observed even at a molar ratio of 1:2 tetramers:T119M monomers. It is possible that by increasing the amount of T119M monomers aggre-
Production of Heterotetramers as a Strategy to Prevent FAP

The study is an engineered version of the protein that has two bulky mutations that impede tetramerization (F87M/L110M) (26). Jiang et al. (26) calculated the thermodynamic parameters for the urea-induced denaturation of M-TTR. The AG° of folding for the M-TTR was found to be −5.5 kcal/mol, twice that obtained in the present study where HHP was the perturbing tool (ΔG° = −2.5 kcal/mol). This discrepancy probably arises from the fact that HHP is a gentle tool for denaturing proteins; in general, partially folded states are present under pressure. The similarity between the thermodynamic stability of the T119M monomer with that of the wt monomer is consistent with the resolved x-ray atomic structure of the T119M tetramer where only a few new H-bonds can be seen within each monomer (9).

Currently the only therapy available for FAP is liver transplantation, which imposes risks for the patients and does not clear the amyloid deposits that are already present in the patients (41, 42). Thus, new strategies are clearly required. Several non-steroidal anti-inflammatory compounds and molecules derived from them have been shown to be effective in inhibiting aggregation of TTR in vitro (43, 44). These molecules are not currently available to be administered to FAP patients, however. New therapies are necessary to treat amyloidogenic diseases in general and FAP in particular. Of course, additional experiments are necessary before our strategy can be used in the clinic, but the results described here point to its potential use as a treatment.

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