Human-Murine Transthyretin Heterotetramers Are Kinetically Stable and Non-amyloidogenic

A LESSON IN THE GENERATION OF TRANSGENIC MODELS OF DISEASES INVOLVING OLIGOMERIC PROTEINS

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The transthyretin amyloidoses appear to be caused by rate-limiting tetramer dissociation and partial monomer unfolding of the human serum protein transthyretin, resulting in aggregation and extracellular deposition of amorphous aggregates and amyloid fibrils. Mice transgenic for few copies of amyloid-prone human transthyretin variants, including the aggregative L55P mutant, failed to develop deposits. Silencing the murine transthyretin gene in the presence of the L55P human gene resulted in enhanced tissue deposition. To test the hypothesis that the murine protein interacted with human transthyretin, preventing the dissociation and partial unfolding required for amyloidogenesis, we produced recombinant murine transthyretin and human/murine transthyretin heterotetramers and compared their structures and biophysical properties to recombinant human transthyretin. We found no significant differences between the crystal structures of murine and human homotetramers. Murine transthyretin is not amyloidogenic because the native homotetramer is kinetically stable under physiologic conditions and cannot dissociate into partially unfolded monomers, the misfolding and aggregation precursor. Heterotetramers composed of murine and human subunits are also kinetically stable. These observations explain the lack of transthyretin deposition in transgenics carrying a low copy number of human transthyretin genes. The incorporation of mouse subunits into tetramers otherwise composed of human amyloid-prone transthyretin subunits imposes kinetic stability, preventing dissociation and subsequent amyloidogenesis.

Protein misfolding and subsequent aggregation cause many human diseases, among them are the systemic amyloidoses (1–4). In these disorders the misfolded and misassembled proteins display a gain of toxic function phenotype associated with compromised organ function through a mechanism(s) that remains obscure. Although the characteristic feature of all amyloid diseases is the extracellular deposition of one protein as cross-β-sheet amyloid fibrils, additional aggregate morphologies are almost always observed in humans including amorphous aggregates, spherical aggregates, and protofibrils (5–7); this is particularly true in the case of transthyretin (TTR), one of 27 amyloidogenic proteins known to misfold and aggregate in humans (8). Wild type TTR amyloidogenesis occurs in the hearts of 10–25% of humans older than 80 years, resulting in senile systemic amyloidosis, often leading to congestive heart failure (9). More than 100 amyloidogenic mutations in TTR are responsible for the autosomal dominant disorders, familial amyloid polyneuropathy or cardiomyopathy (10), and for the rare, selective central nervous system amyloidoses (11).

TTR is synthesized and secreted by the liver, the choroid plexus of the brain and the retina (4). It circulates in cerebrospinal fluid where it is the primary transporter of thyroxine (T4) and in plasma where it acts as a backup carrier of T4 and as the primary transporter of holo retinol-binding protein. TTR may have other physiological functions, since silencing the gene by targeted disruption in mice results in behavioral phenotype that does not seem to be attributable to either thyroid dysfunction or vitamin A insufficiency (12).

TTR is a tetramer exhibiting a dimer of dimers architecture, wherein each 127-residue subunit adopts a β-sheet rich tertiary structure (13) (Fig. 1). Dissociation of the TTR tetramer into its constituent monomers is the rate-limiting step of the aggregation process in vitro and presumably in vivo (14, 15). However, dissociation is not sufficient, and the released monomer also has to partially unfold to misassemble into oligomers, soluble aggregates including protofibrils, insoluble aggregates, and amyloid fibrils in a downhill misassembly process (16).

The dimer-dimer interface bisected by the crystallographic 2-fold (x) axis (Fig. 1) and making up the T4 binding sites is the

The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S3.

The atomic coordinates and structure factors (code 2QPF) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: TTR, transthyretin; Hu-TTR, human TTR; Mu-TTR, mouse (murine) TTR; Hu/Mu-TTR, heterotetramers composed of human and mouse TTR subunits; GdnHCl, guanidinium hydrochloride; T4, thyroxine.
kinetically labile interface (17). Small molecule binding at this interface imposes kinetic stability on the tetrameric TTR and prevents aggregation and fibril formation (18, 19). This kinetic stability results from an increase in the free energy barrier associated with tetramer dissociation. Thus, the protein is effectively locked into its native tetrameric quaternary structure, because the energetic barrier is too high relative to the energy available at 37 °C (19). Covalent tethering of the subunits making up each of the T4 binding sites also precludes dissociation of the quaternary structure, providing further support that this is the kinetically labile interface (17).

In vitro experiments have demonstrated that there is a relationship between the thermodynamic and kinetic stability of TTR and its propensity to form amyloid fibrils (20, 21). Tissue culture studies have demonstrated that a conformationally heterogeneous monomer or small TTR oligomers are cytotoxic to cells of neuronal and cardiac origin, whereas large soluble aggregates and mixtures of amyloid fibrils and amorphous aggregates are not (22). In pre-symptomatic carriers of the amyloidogenic V30M TTR variant there is evidence of non-fibrillar TTR deposition with an inflammatory response in peripheral nerve biopsies, supporting the idea that non-amyloid oligomers are involved in early stages of the disease (6).

Multiple attempts have been made to generate transgenic murine models of the TTR amyloidoses (5, 23–28). Successful models, i.e. those exhibiting amyloidogenesis, and most closely resembling the human disease, use the human TTR gene regulated by its own promoter with tissue specific expression. They require integration of multiple copies of the gene and production of large amounts of the human protein in the mouse. Transgenic animals expressing low concentrations of amyloid-prone mutant human TTR, including the very aggressive L55P variant (L55P TTR) do not develop amyloid deposits. Silencing the murine gene by targeted disruption in the presence of the human L55P TTR gene resulted in tissue deposition (27, 29). These observations suggest that incorporation of endogenous murine TTR subunits into heterotetramers otherwise composed of transgene-encoded human TTR increases the kinetic stability of such heterotetramers, thereby preventing dissociation, aggregation, and deposition. To test this hypothesis, we expressed, purified, and characterized the recombinant murine TTR (Mu-TTR) homotetramer as well as human/murine TTR heterotetramers (Hu/Mu-TTR) and compared their biophysical properties and aggregation propensities with those of recombinant human wild type TTR (Hu-TTR). We also crystallized Mu-TTR, determined its structure, and compared it to previously reported structures of wild type and mutant human TTRs (13, 30).

Herein, we demonstrate that incorporation of murine TTR subunits into TTR heterotetramers otherwise composed of human TTR subunits within the cellular secretory pathway imposes kinetic stability on the resulting heterotetramers, preventing their dissociation, which is the rate-limiting step in TTR misfolding, misassembly, and amyloidogenesis, processes associated with proteotoxicity (22). A similar phenomenon, referred to as interallelic transsuppression, has been observed in human carriers of both the amyloidogenic V30M TTR mutation and the naturally occurring suppressor variant T119M TTR. Compound heterozygotes carrying these mutations on distinct alleles are protected from the highly penetrant peripheral neuropathy caused by the V30M TTR mutation, because the incorporated T119M subunits kinetically stabilize the V30M/T119M TTR heterotetramers (31–33).

**EXPERIMENTAL PROCEDURES**

Recombinant TTR—Human TTR was overexpressed in *Escherichia coli* and purified as described elsewhere (22). Mouse TTR was obtained by subcloning a cDNA mouse TTR clone (ATCC 1243583) into pMMHα vector (the same vector used for human TTR expression) at the NdeI and KpnI restriction sites. The rare codon for proline in position 2 (CCC) was mutagenized to a common proline codon (CCT). The identity of the insert as that encoding mouse TTR was verified by DNA sequencing.

Mu-TTR was overexpressed in *E. coli* as above. The murine protein was precipitated from the supernatant after centrifugation (15 min at 12,000 × g, 4 °C) of the lysed cells in the 40–92% ammonium sulfate fraction (1.8–3.6 m) at 4 °C. The precipitate containing Mu-TTR was dialyzed overnight against 10 mM sodium phosphate buffer, pH 7.6, 100 mM KCl, 1 mM EDTA (phosphate buffer). An equal volume of 200 mM sodium acetate buffer, pH 4.3, 100 mM KCl, 1 mM EDTA was added to achieve a final pH of 4.4, and the mixture was incubated overnight at 37 °C to precipitate impurities. After centrifugation (30 min, 30,000 × g, 4 °C), the supernatant was purified by gel filtration (Superdex 75, GE Healthcare) eluting with phosphate buffer. The purity of the protein was >98% as determined by SDS-PAGE after Coomassie Blue staining. The yield of mouse TTR was 15 mg/liter of culture.

Monomeric mouse TTR was obtained by introducing two mutations at the dimer-dimer interface (F87M and L110M) in the Mu-TTR plasmid DNA by site-directed mutagenesis. These two mutations have been previously introduced to monomerize Hu-TTR (34). Preparation and purification of the protein was done as described for Hu-TTR (22). The identities of all the proteins were confirmed by mass spectrometry (liquid chromatography-electrospray ionization mass spectrometry).

**Crystallography, Data Collection, and Structure Solution of Mu-TTR**—Full-length Mu-TTR in 10 mM phosphate buffer, pH 7.6, 100 mM KCl, 1 mM EDTA at 10 mg/ml was crystallized by the hanging drop vapor diffusion method using 25% (w/v) polyethylene glycol 2000 monomethyl ether as precipitant dissolved in 100 mM sodium acetate, pH 4.6. Plate-like crystals grew in space group *P*3,21 with unit cell dimensions *a* = *b* = 120.51 Å, *c* = 113.19 Å. Using 20% glycerol as a cryoprotectant added to the crystallization solution, a complete dataset was collected to 2.05 Å resolution on a Rigaku FR-E X-ray generator and processed with HKL2000 (35). Six copies of chain A from the structure of Hu-TTR (Protein Data Bank code 1dvg) (13) were placed in the asymmetric unit using Phaser (36) (chains A-F in the final model). After one round of simulated annealing refinement with CNS (37), it became apparent that electron density for further copies of the monomeric subunit was present. Their position could not be determined by molecular replacement using available programs, but they were manually placed in the electron density with the program O (38), gener-
Kinetic Stabilization of Transthyretin Heterotetramers

The final model contains residues 8–10 to the periphery of the molecule. The EFEF tetramer is in an intermediate situation. The final model contains residues 8–10 to 123–124 for each of the eight polypeptide chains and 349 solvent molecules. Data collection and refinement statistics are summarized in supplemental Table S1. Model quality is excellent for the ABCD tetramer, and we will refer to this when discussing our results. For the GHGH tetramer the quality of electron density is more than satisfactory in the vicinity of the crystallographic 2-fold axis but progressively deteriorates when moving toward the periphery of the molecule. The EFEF tetramer is in an intermediate situation. The final model contains residues 8–10 to 123–124 for each of the eight polypeptide chains and 349 solvent molecules. Data collection and refinement statistics are summarized in supplemental Table S1. Model quality is excellent as assessed with Procheck (41) with no outliers observed in the Ramachandran plot.

Aggregation and Amyloid Fibril Formation Experiments—Solutions of murine or human TTR (0.4 mg/ml) in phosphate buffer were diluted 1:1 with 200 mM buffer (100 mM KCl and 1 mM EDTA) to achieve the desired pH (sodium citrate for pH <3.2, sodium acetate for pH 3.5–5.3, and sodium phosphate buffer for pH >5.3). The protein solutions were incubated at 37 °C in cluster tubes. At the time of measurement the solutions were vortexed for 5 s and transferred to 96-well UV-transparent plates in triplicate (50 μl), and the turbidity was measured as 330 and 400 nm. The percentage of aggregation, including fibril formation, was calculated with the 100% value corresponding to the maximum signal of Hu-TTR. All measurements were made in triplicate in at least two independent experiments.

Chaotrope Stability—TTR denaturation curves were obtained by incubation of the proteins (0.1 mg/ml) in various concentrations of urea for 96 h or guanidinium hydrochloride (GndHCl) for 72 h at 25 °C in 50 mM sodium phosphate buffer, pH 7.6, 100 mM KCl, 1 mM EDTA. TTR reconstitution curves were performed by first unfolding the proteins in 6.5 M GndHCl (2 days at 25 °C). TTR was then diluted to 0.1 mg/ml in pre-designated concentrations of GndHCl in 50 mM sodium phosphate buffer, pH 7.6, 100 mM KCl, 1 mM EDTA. To prepare the reconstitution solutions in urea, TTRs (Hu-TTR or Mu-TTR) were first unfolded in 6.5 M GndHCl (Mu-TTR does not unfold in urea), the buffer was exchanged into 8 M urea, and the protein concentration was adjusted to 1 mg/ml. After buffer exchange, both TTRs were still completely unfolded, as discerned by tryptophan fluorescence (see below). Dilutions were performed to yield a final TTR concentration of 0.1 mg/ml in a range of urea concentrations (0.8–8 M) in 50 mM sodium phosphate buffer, pH 7.6, 100 mM KCl, 1 mM EDTA, and incubated for 24 h at 25 °C before recording their tertiary and quaternary structures (see below). Stocks of urea (9 M) and GndHCl (8 M) were prepared in 50 mM sodium phosphate buffer, pH 7.6, 100 mM KCl, 1 mM EDTA.

TTR Tetramer Stability Measured by Resveratrol Binding Fluorescence—Resveratrol is a stilbene that binds in the T4 binding pocket of folded tetrameric TTR (13) resulting in a sizable increase in its fluorescence quantum yield. Resveratrol fluorescence is linear with tetrameric TTR concentration for both Hu-TTR and Mu-TTR (supplemental Fig. S1). Resveratrol does not bind to or exhibit the dramatic environment-sensitive fluorescence with the double TTR mutant F87M/L110M TTR, a variant of TTR that is typically monomeric (34), and it does not shift the TTR monomer-tetramer equilibrium toward tetramer at the concentrations used in these experiments (20).

Each TTR solution (in urea or GndHCl) was vortexed for 5 s. Resveratrol (1 mM in Me2SO) was added to achieve a final resveratrol concentration of 19 μM. The samples and blank solutions (prepared in parallel) were vortexed again for 5 s and transferred into black 96-well plates. Fluorescence intensity was measured with excitation/emission 320/394 nm with a 10-nm bandwidth in a Safire 2 fluorimeter (Tecan). The concentration of urea or GndHCl at which 50% of TTR was tetrameric (Cm) was calculated with GraphPrism software (GraphPad) fitting the data to a dose-response curve with variable slope. The percentage of tetrameric protein at each denaturant concentration was calculated from the values of resveratrol binding of TTR samples that had not been denatured (100% tetramer) and those that were completely denatured (0% tetramer). All experiments were measured in duplicate in at least two independent assays.

TTR Tertiary Structure Measured by Tryptophan Fluorescence—We used the 355/335-nm emission intensity ratio (F355/F335) to follow TTR denaturation (unfolding) as reported previously (42). Exposure of the tryptophans with increasing chaotrope concentration increased the F355/F335 ratio from 0.85 to 1.4 and from 1.0 to 1.4 upon denaturation for Hu-TTR and Mu-TTR, respectively. The TTR solutions incubated in different concentrations of urea or GndHCl (both unfolding and reconstitution solutions) were vortexed for 5 s, and 100 μl of each solution were transferred into black 96-well plates. Tryptophan fluorescence was measured with excitation at 295 nm and emission at 335 and 355 nm with a 5-nm bandwidth in a Safire 2 fluorimeter. In some experiments fluorescence was measured on a Varian Cary 50 spectrofluorometer as described elsewhere (17). The concentration of urea or GndHCl at which 50% of TTR was folded (Cm) was calculated with GraphPrism software fitting the data to a dose-response curve with variable slope. The proportion of folded protein at each denaturant concentration was calculated from the fluorescence ratios of native (100% folded) or completely denatured (0% folded) TTR samples. All experiments were measured in duplicate in at least two independent assays.

Subunit Exchange Experiments—Hu-TTR and Mu-TTR were dialyzed in 25 mM Tris, pH 7.4, and stock solutions at 0.4 mg/ml were prepared. Equal volumes of Hu-TTR and Mu-TTR were mixed and incubated at 37 °C for up to 7 days and at 4 °C for 1 day. After incubation, SDS-loading buffer was added, and the samples were analyzed by SDS-PAGE without pre-boiling. The gels were stained with Coomassie Brilliant Blue.
Formation and Purification of Human/Mouse TTR Heterotetramers (Hu/Mu-TTR)—Equal amounts of Mu-TTR and Hu-TTR were denatured in 6.5 M GdnHCl for 2 days at 25 °C and then reconstituted by dilution (1:100) in 50 mM sodium phosphate buffer, pH 7.6, 100 mM KCl, 1 mM EDTA, 5% glycerol. The refolded protein was re-purified by gel filtration in phosphate buffer before analysis.

Analysis of Hu/Mu-TTR Heterotetramers—To assess whether the purified TTR tetramers resulting from denaturation/reconstitution of a solution containing equal amounts of Hu-TTR and Mu-TTR were heterotetramers (see above), we incubated 100 μl of this solution at 6 μg/ml with 100-μl equivalents of magnetic beads (Dynal protein A beads) pre-cross-linked with anti-human TTR antibody (Dako A0002) for 1 h at 4 °C following the supplier’s directions. In solution, this antibody is specific for human TTR subunits and does not bind native mouse TTR or its subunits. As controls, we incubated the beads with the same amounts of Hu-TTR, Mu-TTR, and an equimolar mixture of human and mouse TTR (heterotetramers) to demonstrate that the antibody is specific for Hu-TTR and that Hu-TTR and Mu-TTR do not exchange subunits during the experimental procedure. The TTR captured by the antibody beads was eluted with 100 μl of 100 mM triethylamine, pH 11.5. The samples were buffer exchanged in water using a Microcon filter device (3 kDa, Millipore) and adjusted to the same final volume (20 μl). SDS-loading buffer was added, and the samples were boiled for 8 min in water before loading onto 15% acrylamide/bisacrylamide Tris-HCl SDS-PAGE gels and run at 100 V. The proteins were transferred overnight to a polyvinylidene difluoride membrane at 30 V at 4 °C. The membranes were blocked for 2 h in 5% nonfat dry milk at room temperature and incubated with an anti-human TTR antibody (Dako A0002). This antibody recognizes both denatured Hu-TTR and Mu-TTR (27). Anti-rabbit IgG-AP (Sigma) was used as secondary antibody, and the membrane was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

Mouse TTR Adopts a Tetrameric Quaternary Structure—Recombinant Mu-TTR elutes from a calibrated gel filtration column at a volume consistent with a tetrameric quaternary structure (supplemental Fig S2A). An analytical ultracentrifugation sedimentation velocity analysis confirmed that Mu-TTR adopts a homogeneous structure in solution and exhibits a sedimentation coefficient of 3.8 Svedberg units, the same as that of Hu-TTR (monomeric Mu-TTR) is indistinguishable from that of Hu-TTR (34). The protein is indeed monomeric at low concentrations, as seen by its elution volume from a gel filtration column, which is very similar to the well studied monomeric human variant (34). The tryptophan fluorescence emission spectrum of F87M/L110M-Mu-TTR (0.2 mg/ml) were incubated at 37 °C over a pH range of 3.18–7.88, and aggregation was quantified after 3 days (Fig. 2A). Hu-TTR aggregated between pH 4–5, whereas Mu-TTR exhibited no turbidity over this pH range. Aggregation and fibril formation were also monitored over time at pH 4.35, the pH at which Hu-TTR exhibits its maximum rate of amyloidogenesis. Notably, Mu-TTR does not aggregate even after 46 days (1100 h) of incubation under these conditions, an observation consistent with the kinetic stability of Mu-TTR (Fig. 2B).

We prepared a monomeric variant of Mu-TTR by introducing two mutations at the dimer-dimer interface (F87M and L110M) that block tetramerization by steric hindrance, as previously demonstrated in the case of Hu-TTR (34). The protein is indeed monomeric at low concentrations, as seen by its elution volume from a gel filtration column, which is very similar to the well studied monomeric human variant (34). The tryptophan fluorescence emission spectrum of F87M/L110M-Mu-TTR (monomeric Mu-TTR) is indistinguishable from that of Mu-TTR homotetramer, indicating that the environment of the tryptophan residues in the two proteins is identical. In Hu-
TTR and presumably in Mu-TTR, one of the two Trp residues is quenched by a nearby Tyr, whereas the other is directly excited and is also excited by Tyr to Trp FRET; therefore, the Trp residues are very sensitive reporters of native TTR tertiary structure. Then, the identical tryptophan spectra found for Mu-TTR and monomeric Mu-TTR suggests that the tertiary structures of the two proteins are likely to be the same, as are the engineered monomeric human TTR (F87M/L110M-TTR) and its corresponding tetramer, for which the two crystal structures have been solved (13, 34). Notably, monomeric Mu-TTR forms fibrils at pH 4.35 (Fig. 2), demonstrating that once the dissociation of the tetramer is achieved (by mutation in this case), the mouse TTR tertiary structure can be altered to render it amyloidogenic under conditions where Hu-TTR aggregates.

**The Mouse TTR Tetramer Exhibits Much Greater Kinetic Stability Than the Human TTR Tetramer**—The stability of the Mu-TTR homotetramer was compared with that of the Hu-TTR homotetramer utilizing both urea and GndHCl denaturation curves. Because the quaternary and tertiary structural changes occurring within TTR appear to be linked (20) (see below), we evaluated the quaternary structural changes using resveratrol binding to the tetramer, which is accompanied by a substantial increase in the fluorescence quantum yield (see “Experimental Procedures”). To discern whether equilibrium had been reached, we recorded denaturation curves starting with native tetramers and reconstitution curves starting with unfolded TTR. In experiments starting with native TTR, the tetramer was exposed to different concentrations of denaturant for 3–4 days, after which the fraction of tetrameric protein was measured employing resveratrol fluorescence. In experiments in which equilibrium was approached from the unfolded protein, TTR in the presence of high denaturant concentrations was transferred to predetermined lower concentrations of denaturant (keeping the final TTR concentration constant). The reconstitution samples were incubated for 1 day, after which the fraction of tetrameric protein in solution was measured by resveratrol fluorescence.

In Fig. 3, panels A and B, we display the urea denaturation and reconstitution curves of Hu-TTR (triangles) and Mu-TTR (circles). For Hu-TTR the denaturation/reconstitution curves were essentially superimposable and exhibited reversible transitions that reached equilibrium within the incubation periods described above, indicated by the same C_m of 3.1 M (Table 1). In contrast, Mu-TTR exhibited hysteresis when comparing the urea denaturation curve (C_m = 6.1 M) to the
urea reconstitution curve ($C_m = 2.1 \text{ M}$; Table 1), demonstrating that the denaturation samples had not yet reached thermodynamic equilibrium at the time of measurement (96 h), explaining the lack of inflection usually displayed by fast cooperative transitions. The kinetically stable trans-suppressor variant T119M TTR exhibits behavior similar to that of Mu-TTR (20). A time course demonstrates that the reassembly process takes less than 200 s (data not shown). Analysis of the reconstitution reaction as a function of urea concentration by SDS gel after glutaraldehyde cross-linking showed that the reassembly transitions for Hu-TTR and Mu-TTR exhibit $C_m$ values in the range of 3.0–3.2 and 2.0–2.25 M, respectively, in excellent agreement with the resveratrol-based $C_m$ values (supplemental Fig. S3). That Mu-TTR does not reach equilibrium in these studies suggests that it is kinetically more stable than Hu-TTR, which reaches equilibrium under the same conditions.

We studied the tertiary structural changes of Mu-TTR and Hu-TTR upon incubation with chaotropes, utilizing both the denaturation and reconstitution paradigms to further probe whether equilibrium had been reached. In Fig. 3, panels C and D, we display the urea denaturation and reconstitution curves for Hu-TTR (triangles) and Mu-TTR (circles) as ascertained by tryptophan fluorescence (i.e. monitoring tertiary structural changes). Hu-TTR exhibits identical denaturation and reconstitution curves, with a $C_m$ of 3.3 M (Table 1). In contrast, the denaturation/reconstitution curves for Mu-TTR exhibit hysteresis, indicating that the Mu-TTR denaturation solutions had not reached thermodynamic equilibrium, consistent with the hypothesis that Mu-TTR is kinetically much more stable than Hu-TTR.

To further demonstrate the kinetic stability of Mu-TTR, we followed the time courses of homotetramer disassembly (by resveratrol binding) and β-sheet unfolding (by tryptophan fluorescence) of human and mouse TTR in 6 M urea over a period of 19 days. Hu-TTR (triangles) exhibits disassembly and unfolding that is complete within 5 days, a time at which Mu-TTR (circles) is still ~60% tetrameric (Fig. 4). Mu-TTR continued to disassemble and unfold over the course of a total of 19 days, confirming the observations in the denaturation experiments (Fig. 3) that the Mu-TTR solutions had not reached equilibrium because of the kinetic stability of the homotetramer. The data are also consistent with our perception at the outset of these studies (20) that the processes of disassembly and unfolding are likely linked in both Mu-TTR and Hu-TTR; as soon as the TTR tetramers disassemble into monomers, they unfold.

Thus, the limiting step in the denaturation process, as in fibril formation, is the dissociation of the tetramer.

Mouse and Human TTR Do Not Exchange Subunits under Physiologic Conditions—Human TTR tetramers slowly exchange subunits under physiologic conditions, pH 7.4, reaching equilibrium after 1 and 7 days of incubation, at 4 and at 37 °C, respectively (33, 44). The faster exchange rate observed at 4 °C compared with that at 37 °C may reflect a significant contribution from the hydrophobic effect to tetramer stability (44). The kinetically stable T119M TTR tetramer does not exchange subunits with wild type TTR because it does not dissociate to any measurable extent on this time scale (32); therefore, if Mu-TTR is also kinetically stable under physiologic conditions, it should not exchange subunits with Hu-TTR tetramers. Equimolar amounts of homotetrameric Mu-TTR and homotetrameric Hu-TTR (0.2 mg/ml, physiologic concentrations) were co-incubated at 37 °C, pH 7.4, under the same conditions where a mixture of wild type human homotetramers and disease-associated variant human TTR homotetramers exhibit subunit exchange. After incubation, SDS-loading buffer was added, and the samples were analyzed by SDS gel electrophoresis without pre-boiling. Under these partially denaturing conditions in which the proteins migrate not only as a function of their mass but also according to their charge and shape, Hu-TTR and Mu-TTR can be clearly distinguished (Fig. 5); whereas

|                  | Disassembly | Reassembly | Unfolding | Refolding |
|------------------|-------------|------------|-----------|-----------|
|                  | $C_m$       | $m$        | $C_m$     | $m$       |
| Urea             |             |            |           |           |
| Hu-TTR           | 3.1 ± 0.04  | −2.10      | 3.1 ± 0.01| −2.42     |
| Mu-TTR           | 6.1 ± 0.2   | −0.55      | 2.1 ± 0.02| −2.05     |
| GndHCl           |             |            |           |           |
| Hu-TTR           | 4.9 ± 0.06  | −1.07      | 1.3 ± 0.01| −7.82     |
| Mu-TTR           | 4.7 ± 0.04  | −1.21      | 0.7 ± 0.02| −2.72     |

FIGURE 4. Kinetics of tetramer disassembly (closed symbols) and unfolding (open symbols) in 6 M urea for Hu-TTR (triangles) and Mu-TTR (circles). For both Hu-TTR and Mu-TTR the loss of quaternary structure (disassembly) and the loss of tertiary structure (unfolding) are tightly linked. Mu-TTR is kinetically stabilized compared with Hu-TTR as seen by its significantly slower dissociation/unfolding kinetics. Tetramer disassembly was measured by resveratrol binding fluorescence; unfolding was measured by tryptophan fluorescence.
Hu-TTR appears to migrate as a dimer, Mu-TTR is probably migrating as a tetramer. The lane comprising pre-made Hu/Mu-TTR heterotetramers (produced by 6.5 M GndHCl unfolding/reconstitution of equimolar amounts of Hu-TTR and Mu-TTR; see below) shows several discrete bands presumably corresponding to a statistical distribution of tetramers having from 0 to 4 murine subunits. More experiments are necessary to demonstrate unequivocally the size of the unboiled TTR in SDS-PAGE. For the purpose of the experiment however, the important fact is that Hu/Mu-TTR heterotetramers migrate as several (more than two) species. Mu-TTR does not exchange subunits with Hu-TTR over time, since only two discrete bands, one corresponding to Mu-TTR and another corresponding to Hu-TTR, are observed in the gel. The lack of exchange between Hu-TTR and Mu-TTR was also observed when the two proteins were incubated at 4 °C for 24 h (data not shown).

Denaturation/Reconstitution of 1:1 Mixtures of Human and Mouse TTR Results in the Formation of Heterotetramers with a Statistical Distribution of Subunits—In mice transgenic for the highly amyloidogenic human variant L55P TTR that also express murine TTR, the proteins circulate in plasma as heterotetramers (tetramers composed of L55P TTR and Mu-TTR subunits) (27) because of heterotetramer formation in the cellular secretory pathway. To investigate the influence of mouse TTR subunit incorporation into the Hu-TTR tetramer, we prepared mixed Hu/Mu-TTR heterotetramers and determined their amyloidogenicity and stability in urea.

Equal amounts of Hu-TTR and Mu-TTR were incubated for 2 days in 6.5 M GndHCl, conditions at which both human and murine TTR are denatured, as shown by their tryptophan fluorescence spectra. The denatured proteins were then subjected to reconstitution and purification as described under “Experimental Procedures.” Gel filtration chromatography revealed a single species with the same elution volume as that of human and mouse TTR homotetramers, indicating that the refolded product was tetrameric TTR.

It is conceivable that during reconstitution human TTR subunits could have greater affinity for human TTR subunits and mouse TTR subunits more affinity for other mouse TTR subunits. If that were the case, the refolded tetramers would be Mu-TTR and Hu-TTR homotetramers. To determine whether the refolded tetramers were composed of human and mouse subunits (i.e. heterotetramers), we incubated the protein with magnetic beads to which an anti-human TTR specific antibody had been cross-linked to capture only the TTR that had incorporated human subunits. The adsorbed TTR was then eluted from the beads and analyzed by SDS-PAGE. The Western blot of the gel was developed with anti-TTR antibody that recognizes Hu-TTR and Mu-TTR (Fig. 6A). In SDS gels under standard conditions (boiled samples), both Hu-TTR and Mu-TTR run mainly as monomers; however, Mu-TTR migrates faster during reconstitution.
than Hu-TTR, and the two proteins can be easily distinguished. The control lanes (Hu-TTR and Mu-TTR homotetramers) demonstrate that the antibody used for immunoadsorption is specific for Hu-TTR subunits because a TTR band is seen in the Hu-TTR lane but not in the Mu-TTR lane. The Hu-TTR + Mu-TTR control lane (mixed homotetramers) demonstrates that the human and mouse tetramers did not exchange subunits during the process of immunoadsorption and analysis because only a Hu-TTR band is seen in that lane. The Hu/Mu-TTR lane clearly shows that the GdnHCl denatured/reconstituted proteins are heterotetramers comprised of human and mouse subunits. This observation is consistent with the statistical distribution of tetramers detected after analysis of Hu/Mu-TTR by SDS-PAGE without pre-boiling (Fig. 5).

Human/Mouse TTR Heterotetramers Are More Stable Than Human TTR Homotetramers under Acidic Conditions—Hu/Mu-TTR heterotetramers produced using the GdnHCl denaturation/reconstitution method were subjected to acid-mediated fibril formation at pH 4.35 (pH of maximum fibril formation for Hu-TTR) as detailed under “Experimental Procedures” (Fig. 6B). The turbidity of the Hu/Mu-TTR heterotetramers (23%) is less than that of Hu-TTR (100%) and more than that of Mu-TTR (3%) at the same concentration, indicating that the incorporation of mouse TTR subunits into the human protein stabilizes the resulting heterotetramer. Moreover, the turbidity of Hu/Mu-TTR heterotetramers is significantly lower than that of an equimolar mixture of Hu-TTR and Mu-TTR (homotetramers, 41%), consistent with the unfolded/refolded TTR being composed of human and murine subunits.

**DISCUSSION**

Although the highly amyloidogenic human L55P TTR variant is responsible for aggressive amyloidosis with fatal cardiomyopathy, nephropathy, and autonomic neuropathy in young adults (45), L55P TTR transgenic mouse models with one or two copies of the L55P TTR transgene failed to show deposition (5, 29). However, when the L55P TTR animals were crossed with mice in which the Mu-TTR gene was silenced (29), the L55P TTR transgenic mice displayed amyloidosis with fatal cardiomyopathy (45). The primary structures of Hu-TTR and Mu-TTR are 80% identical (Fig. 1A), and both are homotetramers (supplemental Fig. S2). Despite their similarity, heterotetrameric Mu-TTR does not form aggregates or amyloid fibrils under the same conditions in which Hu-TTR is amyloidogenic (Fig. 2).

A comparison of the crystal structures of 23 TTR variants, including wild type, amyloidogenic, and non-amyloidogenic mutants, shows no significant differences that can account for their diverse stabilities (46). Similarly, the crystal structure of Mu-TTR is essentially the same as that of Hu-TTR (Fig. 1B); this finding excludes the possibility of an alternate fold, such as that seen in other highly similar proteins (47), as an explanation for its stability.

The stability of TTR was evaluated by subjecting homo- and heterotetramers to differing concentrations of denaturants (urea or GdnHCl) and measuring the amount of tetrameric protein (by resveratrol fluorescence) and folded protein (by tryptophan fluorescence) in each of the solutions. One of the hallmarks of a kinetically stable protein is resistance to denaturation since a kinetic barrier prevents it from sampling the unfolded state. Such proteins do not reach equilibrium in a chaotrope denaturation experiment on any reasonable laboratory timescale, we thus carried out both denaturation and reconstitution experiments to study whether equilibrium was reached in each of these paradigms. Refolding and reassembly processes occur in less than 200 s, whereas tetramer dissociation can be fast or very slow depending on the sequence being studied. The Mu-TTR Cm values of refolding and reassembly are lower than those of Hu-TTR under all the experimental conditions tested (Table 1), indicating that Mu-TTR is less stable than Hu-TTR. These results predict that Mu-TTR would be more amyloidogenic than Hu-TTR. However, our data demonstrate that this is not the case because Mu-TTR is kinetically stabilized compared with Hu-TTR (Figs. 3 and 4), and it does not dissociate and denature under conditions in which Hu-TTR is completely disassembled and unfolded. This interpretation is supported by the fact that under physiologic conditions the mouse and human proteins do not exchange subunits (Fig. 5). This last observation also implies that the heterotetrameric Hu/Mu-TTR found in the transgenic animals must be folded and assembled intracellularly before secretion (27), because monomeric mouse TTR subunits are not present in the serum; thus, they cannot form heterotetramers with the slowly dissociating Hu-TTR.

We have previously demonstrated that the rate of fibril formation can be predicted from tetramer dissociation rates (29). Assuming that the mechanisms of dissociation are the same for both human and murine TTR, the fact that Mu-TTR fibril formation is much slower than that of Hu-TTR (in reality it was not observed, Fig. 2) indicates that its tetramer dissociation is also much slower, consistent with kinetic stabilization. Mono-
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meric Mu-TTR (F87M/L110M-Mu-TTR) is amyloidogenic under the same conditions as the Hu-TTR homotetramer aggregates (Fig. 2C) because in this case the tetramer cannot form as a consequence of steric hindrance. In practical terms, kinetic stabilization means that the energetic barrier for tetramer disassembly into monomers is so high that the process does not happen on a biologically relevant time scale. This finding explains why mice do not spontaneously develop transthyretin amyloidosis, although they are susceptible to other systemic amyloidoses derived from the endogenous proteins serum amyloid A and apoaII (48).

Our data also show that the experimental incorporation of mouse TTR subunits into Hu-TTR (by the GndHCl unfolding/reconstitution method) kinetically stabilizes the resulting mixture of heterotetramers, as seen by its decreased amyloidogenic potential with respect to Hu-TTR and its resistance to urea denaturation (Fig. 6). Unboiled samples of SDS-loaded Mu-TTR and Hu/Mu-TTR heterotetramers seem to behave as tetramers in SDS-PAGE, whereas Hu-TTR migrates as a dimer (Fig. 5), further demonstrating their increased stability. These results explain why it is difficult to obtain transgenic murine models of the human TTR amyloidoses; the presence of endogenous Mu-TTR results in heterotetramer formation in the secretory pathway, preventing Hu-TTR fibrillogenesis and the appearance of the disease phenotype because the mixed tetramers cannot dissociate. The human TTR transgenics with appropriate tissue-specific TTR expression that have been shown to develop deposition in the presence of endogenous Mu-TTR have a high number of transgene copies (30 or more per allele), which results in a sufficiently high expression level of human TTR homotetramers to allow the formation of enough misfolded monomer to aggregate (5, 49). Although these animals are useful models for the study of TTR pathology, they are difficult to utilize for therapeutic studies, particularly for testing compounds that impose kinetic stability by binding to the tetramer because they require very high drug concentrations (often unobtainable) to bind to all the circulating TTR, which may exceed the toxic threshold. Our data suggest that the most useful transgenic model should have its endogenous Mu-TTR silenced and an intermediate level of a misfolding-prone human TTR homotetramer.

Mice having one or two copies of the human L55P TTR gene in the presence of the intact mouse TTR gene make mixed human/mouse TTR heterotetramers in the secretory pathway. These transgenics fail to show aggregation or amyloid fibril formation (5, 29) because of kinetic stabilization imposed by Mu-TTR subunit incorporation into the human TTR protein, as demonstrated herein. Although these lines were considered familial amyloid polyneuropathy disease model failures as a consequence of this work, the failure to make amyloid can now be interpreted to provide strong support for the amyloid hypothesis, the idea that TTR has to dissociate and partially denature to form amyloid and nonfibrillar aggregates, associated with proteotoxicity in humans.

Transgenic mouse models of human disease have been very useful for the analysis of disease pathogenesis and the development of therapeutics. Many of these models display overexpression of the protein responsible for the disease. Our studies show that in at least one instance the biophysical properties of the murine host protein can interfere with disease development. In the case of TTR the same phenomenon appears to prevail in a rat transgenic for the human amyloidogenic variant V30M TTR in which the phenotype was relatively mild (50). Such a model would benefit from the silencing of the endogenous rat TTR, which like Mu-TTR is likely very stable (Fold Rx).

In other systems interaction of human and mouse amyloid β peptide have been reported in mouse models of Alzheimer disease (51); such an interaction produces aggregates and fibers that are more stable than those consisting of human-only peptide. Similarly, a variant of superoxide dismutase 1 related to familial amyotrophic lateral sclerosis, expressed in transgenic mice, forms heterodimers with the endogenous murine protein. These heterodimers might have different biophysical properties than the human-only or mouse-only homodimers (52).

In summary, the complications created by incorporation of the murine subunit(s) into an oligomeric protein also composed of human subunits stresses the importance of careful design of transgenic models for all diseases in which the protein of interest adopts a quaternary structure. Structural similarities between the human and endogenous proteins might result in the formation of mixed heteroproteins, which can impair or poison the observation and understanding of a desired phenotype.

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