Energetic Characteristics of the New Transthyretin Variant A25T May Explain Its Atypical Central Nervous System Pathology

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SUMMARY: Transthyretin (TTR) is a tetrameric protein that must misfold to form amyloid fibrils. Misfolding includes rate-limiting tetramer dissociation, followed by fast tertiary structural changes that enable aggregation. Amyloidogenesis of wild-type (WT) TTR causes a late-onset cardiac disease called senile systemic amyloidosis. The aggregation of one of > 80 TTR variants leads to familial amyloidosis encompassing a collection of disorders characterized by peripheral neuropathy and/or cardiomyopathy. Prominent central nervous system (CNS) impairment is rare in TTR amyloidosis. Herein, we identify a new A25T TTR variant in a Japanese patient who presented with CNS amyloidosis at age 42 and peripheral neuropathy at age 44. The A25T variant is the most destabilized and fastest dissociating TTR tetramer published to date, yet, surprising, disease onset is in the fifth decade. Quantification of A25T TTR in the serum of this heterozygote reveals low levels relative to WT, suggesting that protein concentration influences disease phenotype. Another recently characterized TTR CNS variant (D18G TTR) exhibits strictly analogous characteristics, suggesting that instability coupled with low serum concentrations is the signature of CNS pathology and protects against early-onset systemic amyloidosis. The low A25T serum concentration may be explained either by impaired secretion from the liver or by increased clearance, both scenarios consistent with A25T's low kinetic and thermodynamic stability. Liver transplantation is the only known treatment for familial amyloid polyneuropathy. This is a form of gene therapy that removes the variant protein from serum preventing systemic amyloidosis. Unfortunately, the choroid plexus would have to be resected to remove A25T from the CSF—the source of the CNS TTR amyloid. Herein we demonstrate that small-molecule tetramer stabilizers represent an attractive therapeutic strategy to inhibit A25T misfolding and CNS amyloidosis. Specifically, 2-[(3,5-dichlorophenyl)amino]benzoic acid is an excellent inhibitor of A25T TTR amyloidosis in vitro. (*Lab Invest* 2003, 83:409–417).

Transthyretin (TTR) is a 55-kd homotetrameric protein composed of 127-residue β -sheet-rich subunits that is present in serum (3.6 to 7.2 μ M) and cerebral spinal fluid (CSF; 0.04 to 0.4 μ M). The function of TTR is to transport L-thyroxine (T_4) and holo-retinol-binding protein. TTR is one of > 20 nonhomologous amyloidogenic proteins that can be transformed into fibrils and other aggregates that lead to pathology in humans. These diseases do not seem to be caused by loss of function as a result of protein aggregation; instead, aggregation seems to cause neuronal/cellular dysfunction by a mechanism that is not yet clear.

Rate-limiting tetramer dissociation and tertiary structural changes within the monomer enables aggregation of wild-type (WT) TTR, leading to cardiac amyloidosis in elderly people (senile systemic amyloidosis). More than 80 mutations are known to accelerate TTR amyloidosis, exacerbating pathology by thermodynamic and/or kinetic destabilization (Connors et al, 2000; Hammarström et al, 2002; Ikeda et al, 2002; McCutchen et al, 1995). These TTR variants lead to diseases collectively referred to as familial amyloid polyneuropathy (FAP), in which aggregation putatively causes peripheral and autonomic nervous system dysfunction. Familial mutations can also lead to cardiac dysfunction, particularly in black individuals with the V122I TTR mutation (familial amyloid cardiomyopathy). Involvement of other organs such as the kidney, intestine, and eye is ordinary, but prominent central nervous system (CNS) impairment is rare in TTR amyloidosis. TTR amyloid deposition on the leptomeninges and subarachnoid vessels seems to cause CNS symptoms such as recurrent subarachnoid hemorrhage, hearing loss, ataxia, dementia, seizures, myelopathy, brain hemorrhage, and brain infarction. A

DOI: 10.1097/01.LAB.0000059937.11023.1F

Received November 13, 2002.

This study was supported by the National Institutes of Health (DK 46335), the Skaggs Institute of Chemical Biology, The Lita Annenberg Hazen Foundation, and a fellowship to Y. Sekijima from the Japan Health Science Foundation.

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few TTR variants are associated with CNS amyloidosis (Brett et al, 1999; Ellie et al, 2001; Garzuly et al, 1996; Ikeda et al, 2002; Mascalchi et al, 1999; Petersen et al, 1997; Uemichi et al, 1997; Uemichi et al, 1999); however, the requirements for a CNS selective disease as opposed to a systemic amyloid disease are unclear, and this study represents our first attempt to gain some insight.

Recently, we diagnosed an patient who had FAP and a novel TTR mutation, threonine 25 replacing alanine (A25T). He presented with CNS amyloidosis at age 42 and peripheral nerve impairment at age 44 and exhibited very low levels of A25T TTR in the serum. Herein, we evaluate the biophysical characteristics of the A25T CNS variant revealing that the A25T TTR tetramer exhibits very low thermodynamic and kinetic stability. We show that the low serum concentration of A25T inhibits its amyloidosis, presumably explaining why this mutation does not result in an early-onset peripheral disease. We speculate that the high L-thyroxine (T_4) concentration in the choroid plexus may be able to chaperone more A25T into the CSF than is desirable, which is problematic because the concentration of T_4 in the CSF is insufficient to keep A25T TTR tetrameric, which would lead to misfolding in the leptomeninges proximal to the site of secretion, as observed. Studies within demonstrate that T_4 can stabilize the tetramer and prevent misfolding but not nearly as well as a small molecule designed for this function—providing indirect support for the T_4 chaperoning hypothesis. Importantly, we demonstrate that tetramer stabilizers represent an attractive therapeutic strategy to inhibit A25T postsecretory misfolding and CNS amyloidosis. Specifically, 2-[(3,5-dichlorophenyl)amino]benzoic acid is an excellent inhibitor of A25T TTR amyloidosis in vitro.

Results

Evaluating the pH Dependence and Rate of A25T TTR Fibril Formation Relative to WT TTR

TTR is converted into amyloid by rate-limiting pH-mediated tetramer dissociation followed by tertiary structural changes that enable self-assembly of the monomeric amyloidogenic intermediate. Unlike WT TTR, which forms fibrils over the pH range of 5.2 to 3.8, exhibiting a maximum at pH 4.4, A25T TTR exhibits maximum amyloidogenicity at approximately pH 5 and is amyloidogenic at pH as high as 6.4 (Fig. 1A). At the optimal pH for maximal fibril formation, A25T TTR forms 30% more fibrils than WT, as judged by turbidity. Whereas WT TTR requires 72 hours to reach a maximum in amyloid fibril formation (pH 4.4, 0.20 mg/ml TTR), the fibril formation of A25T TTR (pH 5.0, 0.20 mg/ml TTR) is nearly complete within 1 hour (Fig. 1B). The fibril formation rate of A25T TTR is much faster than that of other pathogenic TTR tetramers (Hammarström et al, 2002) and is similar to that of monomeric TTR (Jiang et al, 2001b), suggesting that the activation barrier for tetramer dissociation is dramatically lowered. The rate and fibril yield of A25T TTR

Figure 1.

Fibril formation as a function of pH and concentration. A, pH dependence of A25T TTR (black) and wild-type (WT) TTR (white) amyloid fibril formation quantified by turbidity at 400 nm (OD_{400}) after incubation at 37°C for 72 hours. B, Time course of A25T TTR fibril formation (filled circles) at pH 5.0 (37°C) and WT TTR fibril formation (filled triangles) at pH 4.4 (37°C) monitored by turbidity at 400 nm (OD_{400}). C, Concentration dependent time course of A25T TTR fibril formation at pH 5.0 (37°C) monitored by thioflavin T fluorescence. Open triangles represent 0.2 mg/ml A25T, filled circles represent 0.1 mg/ml A25T, and open diamonds represent 0.05 mg/ml A25T.

is highly concentration dependent (Fig. 1C). The relative yield of A25T amyloid fibrils (pH 5, 600 s) decreases from 100% at a concentration of 0.2 mg/ml to 41.1% at 0.1 mg/ml, and all of the way to 13.3% at an A25T concentration of 0.05 mg/ml, demonstrating how the low serum concentrations of A25T can protect against amyloidogenesis.

Evaluating the Quaternary and Tertiary Structure Stability of A25T TTR Chaotropic denaturation studies are used in an attempt to quantify the thermodynamic influence of the A25T mutation on TTR quaternary and tertiary structural stability. Resveratrol binds the TTR tetramer, resulting in a large increase in its fluores-

cence quantum yield, but resveratrol does not bind the monomer, allowing one to probe the tetramer-monomer equilibrium by fluorescence. Resveratrol binding can overestimate the fraction tetramer as a result of Le Chatelier's principle; however, the fraction tetramer estimated by cross-linking and resveratrol binding is similar; hence, the overestimation may not be discernible. Unlike the WT tetramer (0.1 mg/ml), which begins to dissociate when the urea concentration exceeds 3 M ($C_m = 3.3$ M urea; Fig. 2A), the A25T TTR tetramer (0.1 mg/ml) commences dissociation at 1 M urea, exhibiting complete dissociation in 2 M urea, ($C_m = 1.4$ M urea; Fig. 2A). Recently, we reported that homotetramers composed of subunits that harbor pathogenic mutations are significantly less stable than WT on the basis of urea denaturation studies (Hammarström et al, 2002). The free energy of A25T tetramer dissociation extrapolated to physiologic conditions is 31.9 kcal/mol, significantly less stable than any other TTR variant published to date. Another CNS variant D18G under investigation in the laboratory is so destabilized that it cannot tetramerize (P. Hammarström et al, unpublished data).

A25T tertiary structural stability can be ascertained by Trp fluorescence monitored urea denaturation

Figure 2.

Protein denaturation as a function of urea. A, Evaluation of the quaternary and tertiary structure stability of A25T (unbroken lines) and WT (broken lines) TTR as a function of urea concentration. Open symbols represent tetramer dissociation curves for A25T (circles) and WT (triangles) measured by resveratrol binding after 96 hours of incubation. Filled symbols represent tertiary structure unfolding for A25T (circles) and WT (triangles) measured by intrinsic tryptophan fluorescence. B, The logarithm of the rate of tetramer dissociation ($\ln k_{diss}$; k_{diss} in h^{-1}) for A25T (filled circles) and WT TTR (filled triangles) as a function of urea concentration is plotted (employing urea concentrations that will not support tertiary structure refolding). The $\ln k_{diss}$ versus urea concentration plot is linear allowing extrapolation to physiological conditions.

curves. The free energy of monomer unfolding of A25T is 2.80 kcal/mol ($C_m = 2$ M urea), which indicates that the A25T tertiary structure is less stable than that of WT TTR ($C_m = 3.4$ M urea; Fig. 2A). The tertiary structural instability exhibited by A25T is similar to the other CNS variant that we have recently characterized (D18G; 2.40 kcal/mol) (P Hammarström et al, unpublished data). The C_m s of A25T unfolding are independent of TTR concentration (0.01 to 0.1 mg/ml), demonstrating that the tetramer $\leftrightarrow$ folded monomer and folded monomer $\leftrightarrow$ unfolded monomer equilibria are unlinked for this variant. In contrast, tetramer dissociation and monomer unfolding of WT TTR are clearly linked, as evidenced by the nearly coincident curves for resveratrol binding and tryptophan fluorescence (Fig. 2A). Furthermore, the C_m for unfolding of WT TTR is dependent on TTR concentration, providing additional evidence that the quaternary and tertiary structural equilibria are linked, even at 0.01 mg/ml (A. Hurshman et al, unpublished data). Other variants studied thus far (V30M, L55P, and V122I) also exhibit linked equilibria (Hammarström et al, 2002); hence, the free energy of tetramer dissociation and monomer unfolding cannot be calculated by the methods described here for A25T. Nevertheless, although a direct comparison of the free energy is not possible, it is clear from the urea denaturation curves that the A25T mutation significantly destabilizes both the TTR quaternary and tertiary structure compared with WT and the other FAP variants.

A25T Homotetramer Dissociation Kinetics

The rate of tetramer dissociation can be ascertained by linking the slow dissociation step to rapid tertiary structural changes by a method described previously (Hammarström et al, 2002; Jiang et al, 2001a). Tertiary structural changes are easy to monitor by fluorescence in urea concentrations exceeding those required for subunit unfolding, rendering the process irreversible. The logarithm of the dissociation rate constant ($\ln k_{diss}$) varies linearly with urea concentration (Fig. 2B), allowing extrapolations to physiological conditions to be made. The k_{diss} of A25T TTR extrapolated to 0M urea was calculated to be 19.8 hours^{-1} , which equates to a half-life ($t_{1/2}$) of only 2.1 minutes, 1200-fold faster than that of WT TTR and 126-fold faster than L55P tetramer dissociation (Hammarström et al, 2002), the mutation associated with the most aggressive systemic amyloidosis.

Quantification of A25T and WT TTR in the Serum and CSF of a Patient with Heterozygous FAP

The serum and CSF from a heterozygous patient with A25T TTR was analyzed by ELISA and immunoprecipitation followed by liquid chromatography/mass spectrometry (LC/MS). The total serum protein and albumin concentrations were normal (76.0 mg/ml and 45.0 mg/ml, respectively); however, the total TTR concentration was low (0.0702 mg/ml; normal, 0.2000

to 0.4000 mg/ml). The CSF was bloody and exhibited a high total protein level of 2.97 mg/ml and a high TTR concentration of 0.0242 mg/ml (normal, 0.0020 to 0.0200 mg/ml), possibly as a result of asymptomatic subarachnoid hemorrhage. LC/MS was used to quantify the A25T:WT TTR ratio in serum and CSF. First, we examined the serum and CSF of a patient with heterozygous V30M FAP as a control. The LC/MS spectra of DTT-treated immunoprecipitated TTR from a patient with heterozygous V30M FAP showed equally intense major ion peaks of molecular mass (m/z) $13,762 \pm 1$ and $13,794 \pm 1$ (Fig. 3A), consistent with the $[M + H]^+$ ion of the WT and V30M TTR monomer, respectively. The ratio of WT and V30M TTR ion count peak areas in serum was approximately 1:1, consistent with previous matrix-assisted laser desorption ionization/time-of-flight (MALDI/TOF) mass studies (Sekijima et al, 2001; Tachibana et al, 1999) and radioimmunoassay results (Nakazato et al, 1984). The LC/MS method applied to V30M heterozygotes CSF also showed a 1:1 ratio of V30M to WT ion counts (Fig. 3A). Applying the same methods to determine the ratio of A25T TTR relative to WT in the heterozygote described within reveals that the serum level of A25T TTR was very low. In fact, no A25T TTR was detected in two of three examinations of serum TTR. In one attempt A25T TTR (molecular mass 13,792 m/z) was detected, but the peak area of A25T TTR was < 25% of that of WT TTR (Fig. 3B). In the CSF, the peak area of A25T TTR was 19% of that of WT TTR (Fig. 3B); however, this result did not reflect pure CSF because of a possible subarachnoid hemorrhage (contamination with blood).

Figure 3.

Liquid chromatography/mass spectrometry (LC/MS) of immunoprecipitated TTR in heterozygotes. A, Analysis of WT and V30M TTR in the serum (left column) and CSF (right column) of a heterozygous V30M FAP patient (upper panels). Peak 1 represents the WT TTR monomer ion counts (m/z 13,762), and peak 2 indicates the V30M TTR monomer ion counts (m/z 13,794). B, WT and A25T TTR analysis for the Japanese FAP patient described within. Peak 1 represents the WT TTR monomer ion counts (m/z 13,762), and peak 3 indicates the A25T TTR monomer ion counts (m/z 13,792). Note that the CSF is contaminated by blood (see text).

Figure 4.

Subunit exchange between FT_2 -WT and A25T homotetramers ($4^\circ C$). A, Chromatographic trace from analytical anion exchange after 0 hours of exchange. Peak 1 is composed of (A25T)4, peak 2 is composed of (A25T)3(FT_2 -WT)1, peak 3 is composed of (A25T)2(FT_2 -WT)2, peak 4 is composed of (A25T)1(FT_2 -WT)3, and peak 5 is composed of (FT_2 -WT)4. B, Analytical anion exchange chromatographic trace after 24 hours of exchange. Organization is the same as that in (A).

Figure 5.

Small molecule inhibition of A25T and WT TTR amyloid fibril formation. A, Inhibition of A25T (black bars; 3.6 μM , pH 5.0) and WT TTR (white bars; 3.6 μM , pH 4.4) amyloid fibril formation by T_4 and (B) 2-[(3,5-dichlorophenyl)amino] benzoic acid at pH 5 (A25T) or pH 4.4 (WT) as a function of inhibitor concentration as evaluated by turbidity at 400nm (OD_{400}).

Can A25T and WT TTR Form Hybrid Tetramers

Mixing equimolar amounts (3.6 μM) of A25T and FT₂-WT TTR homotetramers at 4° C reveals that a statistical distribution of tetramers composed of WT and A25T subunits can form by subunit exchange within 24 hours as discerned by ion exchange chromatography (Fig. 4). The other CNS TTR variant (D18G) under evaluation by this laboratory is unable to form significant amount of hybrid TTR tetramers with WT TTR by a subunit exchange mechanism (P. Hammarström et al, unpublished data).

Inhibition of A25T TTR Fibril Formation by T₄ and 2-[(3,5-dichlorophenyl)amino]benzoic acid

The efficacy of T₄ as an inhibitor of TTR fibril formation was demonstrated by incubating varying concentrations of T₄ with TTR (3.6 μM) under acidic conditions. WT TTR fibril formation was nearly completely inhibited by the addition of 2 eq of T₄ (7.2 μM). In contrast, A25T TTR fibril formation was incompletely inhibited, even when 10 eq of T₄ (36 μM) was used relative to A25T TTR concentration (Fig. 5A). 2-[(3,5-dichlorophenyl)amino]benzoic acid is one of the best TTR amyloid fibril formation inhibitors discovered to date by our laboratory. This small molecule completely inhibited both WT and A25T TTR fibril formation at a concentration of 7.2 μM (Fig. 5B).

Discussion

TTR amyloid fibril formation requires tetramer dissociation followed by a rapid conformational change in the monomer, allowing the TTR to misassemble into oligomers, protofilaments, and filaments as well as amorphous aggregates (Colon and Kelly, 1992; Kelly, 1996, 1997; Lai et al, 1996). The soluble intermediates preceding or formed in parallel with amyloid seem to be the entities responsible for pathology (Sousa et al, 2001). Biophysical data presented above on A25T reveals that this mutation destabilizes the quaternary structure of TTR (Fig. 2A). Moreover, the A25T homotetramer dissociates faster than any tetramer studied to date, which is significant because tetramer dissociation is known to be rate limiting for amyloid fibril formation (Figs. 1B and 2B). The ability of the A25T homotetramer to dissociate and misfold at pHs as high as 6.4 is a significant risk factor, as this pH can be achieved in several intra- and extracellular environments (Fig. 1A). The A25T TTR homotetramer dissociates 126-fold faster and is more destabilized than the L55P TTR homotetramer in the test tube, yet the age of disease onset for A25T is 42 years of age, whereas the age of onset for the severe systemic amyloidosis caused by the L55P mutation is in the second decade; (Jacobson et al, 1992; Yamamoto et al, 1994). We know that thermodynamic and kinetic stability considerations as well as the pH dependence of fibril formation correlate with disease severity (Hammarström et al, 2002; McCutchen et al, 1993, 1995). On the basis of these considerations, A25T TTR should result in an aggressive systemic amyloid dis-

ease with early onset, but this is not case. Interestingly, the patient with the A25T mutation exhibited a low A25T serum TTR concentration (< 25% of WT), suggesting that this factor protected the patient from an early-onset systemic disease. This is not surprising because self-assembly of the amyloidogenic intermediate is a concentration-dependent process (J.T. White et al, unpublished data). In fact, the rate of fibril formation and the total yield of fibrils diminished dramatically when the concentration of A25T TTR was reduced (Fig. 1C).

There are at least two possible explanations for the low serum A25T TTR concentration. One is decreased secretion of variant TTR from liver, and the other is rapid degradation of A25T TTR postsecretion. We propose that the low serum level of A25T TTR detected in this patient is a result of degradation by the quality control machinery of the cells that compose the liver. L55P TTR may represent the lowest stability tetramer amenable to efficient secretion, explaining why L55P carriers exhibit an early-onset systemic disease. This hypothesis will be tested by future studies. We speculate that the A25T variant leads to a CNS disease because of the high T₄ concentration in the choroid plexus, which could chaperone enough A25T TTR out of the choroid plexus to facilitate amyloidosis (Dickson et al, 1987; Fan et al, 1999; Sawkar et al, 2002; Schreiber et al, 1990). T₄ binding is known to stabilize the quaternary structure (Miroy et al, 1996; Fig. 5A) and render D18G tetrameric by a tetramer stabilization mechanism at high concentrations. However, the T₄ concentration in the CSF is not sufficient to stabilize A25T TTR (owing to the K_d of T₄) after secretion enabling TTR to dissociate and misfold into amyloid fibrils in the CSF. This hypothesis is consistent with the observation that A25T TTR deposits in the leptomeninges and subarachnoid space—regions facing the CSF. It is also possible that A25T prefers CNS deposition because of some unique feature of that environment, such as binding of the monomer to the leptomeningeal membrane. Other TTR variants seem to have a predilection toward tissue-selective deposition (Connors et al, 2000; Ikeda et al, 2002; Jacobson et al, 1996; Jenne et al, 1996; Ranlov et al, 1992).

Eight of > 80 TTR disease-associated variants are reported to have CNS involvement at an early stage of pathology (Brett et al, 1999; Ellie et al, 2001; Garzuly et al, 1996; Ikeda et al, 2002; Mascalchi et al, 1999; Petersen et al, 1997; Uemichi et al, 1997, 1999) (Table 1). These TTR variants share common features, including a fifth decade age of disease onset, low serum variant TTR levels, and, in the two cases examined thus far, extreme mutation induced protein destabilization. TTR serum levels of several CNS-associated variants including D18G (P Hammarström et al, unpublished data), Δ V122 (Uemichi et al, 1997), Y114C (Ando et al, 1999), and A25T are very low relative to WT levels, unlike the situation in FAP heterozygotes that do not exhibit CNS involvement. The most destabilized TTR variant characterized to date (D18G) results in prominent CNS amyloidosis with benign low-

Table 1. TTR Variants Associated with CNS Amyloidosis and Serum Levels

Mutations/Ethnic background	Age of Onset	CNS Amyloidosis	Systemic Amyloidosis	Serum Variant TTR Level	Protein Stability
D18G/Hungarian	36, 37, 53, 51 (mean age: 44.3)	+++	+	very low (undetectable)	very low (monomer)
V30G/German	30, 43, 56, 35, 44, 47 (mean age: 42.5)	+++	+	n.d.	n.e.
F64S/Italian	28, <20, <20	+++	++	n.d.	n.e.
A36P/Italian	37, 37, 40 (mean age: 38.0)	+++	++	n.d.	n.e.
L12P/English	38	+++	++	n.d.	n.e.
G53E/French	37, 42 (mean age: 39.5)	+++	+++	n.d.	n.e.
ΔV122/Ecuadorian	57	++ ^a	+++	Very low	n.e.
Y114C/Japanese	39, 34, 31, 34 (mean age: 34.5)	++	+++	Very low	n.e.
A25T/Japanese	42	++	++	Very low (<25% of WT)	(unstable tetramer)

CNS, central nervous system.

+++ , severe clinical symptoms resulting in death; ++ , moderate clinical symptoms; + , slight clinical symptoms or subclinical amyloid deposition; n.d., not described; n.e., not examined.

^a The relationship between CNS symptoms and amyloidosis was not confirmed.

level systemic deposition, suggesting that extreme destabilization prevents systemic amyloidosis. The stability of A25T, which is between that of L55P and D18G, leads to a disease in which CNS symptoms and systemic amyloidosis symptoms appear at nearly the same time.

FAP was considered until recently to be an incurable disease; however, liver transplantation developed 10 years ago proves to be an effective therapeutic strategy for replacing the variant TTR gene in the liver by the WT gene-reversing systemic amyloidosis and halting progression of FAP pathology (Holmgren et al, 1993). Variant TTR in the serum disappears several days after liver transplantation; however, CSF levels of variant TTR do not significantly decrease even after long-term postsurgical observation (Adams et al, 2000; Ando et al, 1995). Hence, this approach will not correct CNS selective amyloidosis. More worrisome is that amyloid deposition on subarachnoid vessels and leptomeninges was found in a patient with V30M FAP (Ushiyama et al, 1991). Although CNS involvement in V30M FAP is not generally considered to be clinically significant, three reports question this generally held perception (Herrick et al, 1996; Horta et al, 1964; Sakashita et al, 2001). Therefore, CNS amyloidosis could be a future problem for the > 500 patients who have received V30M transplantation (Familial Amyloidotic Polyneuropathy World Transplant Register, www.fapwtr.org/). The need to elucidate the precise mechanism of TTR-related CNS amyloidosis and to develop effective therapeutic strategies is compelling. Herein, we demonstrate that tetramer stabilizers such as 2-[(3,5-dichlorophenyl)amino]benzoic acid inhibit A25T amyloidosis in vitro (Fig. 5B) providing hope that compounds of this type that cross the blood-brain barrier could be useful in CNS-selective or -specific TTR amyloid diseases.

Materials and Methods

Patient

The patient is a 45-year-old Japanese man. At age 42, he first began to notice vertigo and nausea. Two years later, he developed a gait disturbance. Dysesthesia of the right hand and hearing loss appeared at age 45. On examination, he had right sensorineural hearing loss, superficial and deep sensory disturbance of extremities, generalized areflexia, and ataxic gait. Motor nerve conduction velocities were decreased in both upper and lower extremities. Magnetic resonance imaging disclosed marked linear gadolinium enhancement over the surface of the brain and spinal cord. Sural nerve biopsy showed no abnormal findings such as amyloid deposition; however, biopsied leptomeninges disclosed heavy deposits of amyloid, which reacted with an anti-TTR antibody. We performed a genetic diagnosis for TTR-related amyloidosis with informed consent. Direct DNA sequencing of TTR exon 2 showed a single base transversion at the first nucleotide of codon 25 (GCC to ACC), indicating replacement of alanine by threonine in the protein (A25T). No other mutations were found in the coding region of the TTR gene.

Protein Expression and Purification

Recombinant A25T TTR was expressed in BL21/DE3 Epicurean gold *Escherichia Coli* (Stratagene, La Jolla, California) transformed with the pmmHα plasmid, containing A25T TTR and ampicillin resistant genes. Expression and purification were performed as described previously (Lashuel et al, 1999).

Fibril Formation Assay

A TTR stock solution [0.4 mg/ml in 10 mM sodium phosphate, 100 mM KCl, 1 mM EDTA, 1 mM DTT (pH 7)] was diluted 1:1 with 100 mM acidification buffer containing 100 mM KCl, 1 mM EDTA, and 1 mM DTT. Citrate buffer was used when a final pH of ≤ 3.4 was desired; acetate buffer was used when the pH range under evaluation was 3.8 to 5.8, and phosphate buffer was used for evaluating amyloidogenesis from pH 6.2 to 7.0. The influence of T₄ and 2-[(3,5-dichlorophenyl)amino]benzoic acid was evaluated by adding them to a 0.4 mg/ml TTR solution, which was incubated for 2 hours (37° C) before the pH was dropped to 4.4 (WT TTR) or 5.0 (A25T TTR) via a 1:1 dilution with acetate buffer. All fibril formation samples were incubated at 37° C for 72 hours without stirring after acidification. The extent of fibril formation was probed by turbidity measurements at 400 nm on an HP 845× UV-visible spectrometer equipped with a Peltier temperature-controlled cell holder. Single-time point samples (72 hours) were vortexed immediately before the measurement to suspend the precipitate equally.

Fibril Formation Kinetics

Solutions of TTR (0.4 mg/ml) were mixed with an equal volume of 100 mM acetate buffer (with 100 mM KCl, 1 mM EDTA, and 1 mM DTT) to yield a final pH of 4.4 for WT TTR and 5.0 for A25T TTR. The samples were incubated at 37° C with stirring. The turbidity at 400 nm was continuously monitored over the course of 3600 seconds. Fibril formation kinetics of A25T TTR were also monitored by thioflavin T (ThT) fluorescence on an AVIV ATF-105 fluorometer (AVIV Instruments, Lakewood, NJ) with 440 nm excitation and 485 emission (J.T. White et al, unpublished data). For A25T TTR concentration-dependent studies of amyloidogenicity, 1 ml of 100 mM sodium acetate buffer [with 100 mM KCl, 1 mM EDTA, and 1 mM DTT (pH 4.8)] was added to a 10 × 10 mm cuvette equipped with circular stirbar along with 20 μ l of 2 mM ThT in 10 mM sodium phosphate, 100 mM KCl (pH 7.6). One milliliter of A25T TTR (0.4 mg/ml or 0.2 mg/ml or 0.1 mg/ml) in 10 mM sodium phosphate buffer [with 100 mM KCl, 1 mM EDTA, and 1 mM DTT (pH 7)] was added to the cuvette using a 1-ml titration syringe to start the time course. The final TTR concentration was half the initial concentration (0.2 mg/ml or 0.1 mg/ml or 0.05 mg/ml), and the final pH was 5.0. The samples were incubated at 37° C with stirring, and fluorescence intensity at 485 nm was continuously monitored over the course of 1800 seconds.

Urea-Mediated TTR Tetramer Dissociation Measured by Resveratrol Binding

Resveratrol displays a large increase in its fluorescence quantum yield and a blue shift upon binding to tetrameric TTR, but does not bind to the TTR monomer (X Jiang et al, unpublished data). Resveratrol binding standard curves were recorded to quantify the

concentration of WT and A25T TTR tetramer (0 to 0.12 mg/ml; 0 to 2.18 μ M_{tetramer}) utilizing 18 μ M resveratrol. The fluorescence intensity at 394 nm (I^{394}) was plotted versus the concentration of TTR tetramer, exhibiting a linear fit as expected. Resveratrol fluorescence was recorded from 350 to 550 nm following excitation at 320 nm. Samples containing TTR (0.01 to 0.1 mg/ml; 0.18 to 1.8 μ M_{tetramer}) were incubated (25° C) in various concentrations of urea buffered with 50 mM phosphate (pH 7.0; 100 mM KCl, 1 mM EDTA, 1 mM DTT) to evaluate tetramer stability. Urea solutions were checked by refractive index to verify the concentrations prepared by weight. After incubation (96 hours) 3.6 μ l of resveratrol from 2.5 mM stock solution (in DMSO) was added to a 500 μ l protein sample just prior to the measurement yielding a final concentration of 18 μ M resveratrol (Hammarström et al, 2001).

Urea Mediated Monomer Unfolding Measured by Tryptophan Fluorescence

Samples containing TTR (0.01 to 0.1 mg/ml) were incubated (25° C) in various concentrations of urea in 50 mM phosphate buffer (pH 7.0; 100 mM KCl, 1 mM EDTA, 1 mM DTT) for 96 hours. Urea concentrations were verified as above. All TTR Trp fluorescence spectra were recorded over the range of 310 to 410 nm using an ATF 105 Aviv spectrofluorometer equipped with a thermostated cell holder using excitation at 295 nm. Native wild-type TTR exhibits a fluorescence maximum at 337 to 338 nm, whereas the unfolded protein shows maximum emission between 355 and 358 nm. Each subunit of TTR has two tryptophans (residues 41 and 79). We used the 355 nm:335 nm emission intensity ratio (F₃₅₅/F₃₃₅) to follow denaturation by the exposure of the tryptophans as a function of chaotrope concentrations. The ratio increased from 0.85 to 1.4 upon denaturation.

Calculation of the Thermodynamic Stability of TTR

The dissociation constant of the tetramer (K_{diss}), the free energy of tetramer dissociation (ΔG_{diss}), and the free energy of monomer unfolding (ΔG_U) were calculated using formulas: $K_{diss} = 256c^3(1-\alpha)^4\alpha^{-1}$, $\Delta G_{diss} = -RT\ln K_{diss}$, $\Delta G_U = -RT\ln(F_U/1 - F_U)$, where c is the concentration of tetramer, α is the fraction tetramer and F_U is the fraction of unfolding.

Rate of Tetramer Dissociation Measured by Tryptophan Fluorescence

The evaluation of tetramer dissociation rates was carried out using TTR (0.1 mg/ml; 50 mM phosphate buffer, 100 mM KCl, pH 7, 25° C) samples in 3.5 to 6.0 M urea (A25T TTR) or 4.5 to 9.0 M urea (WT TTR), concentrations in the post-transition region for tertiary structural changes. The Trp fluorescence emission ratio (F₃₅₅/F₃₃₅) as a function of time (25° C) was measured to evaluate the tetramer dissociation rate by linking it to tertiary structural changes, as described in detail previously (Hammarström et al, 2002; Jiang et al, 2001a). The kinetic data fit well to a single expo-

nential function: $I^{355/335} = I^{355/335}_N + A(1 - e^{-k_{\text{diss}} t})$; where $I^{355/335}_N$ is the native protein fluorescence intensity ratio (355/335 nm), A is the amplitude difference, k_{diss} is the tetramer dissociation rate constant, and t is time in hours.

Evaluating the Ability of A25T TTR to Exchange Subunits with WT TTR

Equal concentrations (3.6 μM) of two different TTR tetramers, FT₂-WT and A25T, were mixed and incubated at 4° C for 24 hours. The extent of hybrid tetramer formation was evaluated by analytical ion exchange chromatography as described previously (Hammarström et al, 2001; Schneider et al, 2001).

ELISA Quantification of TTR Concentration in Human Serum and CSF

ELISA quantification of TTR concentration was performed as described previously (Purkey et al, 2001). Briefly, 100- μl samples of recombinant TTR of known concentration and serum samples (diluted 4000 $\times$) or CSF samples (diluted 200 $\times$) were applied to an Immulon-4HBX microtiter plate (Thermal Labsystems, Helsinki, Finland) coated with antihuman prealbumin (TTR) antibody (DiaSorin, Stillwater, Minnesota) and incubated at 4° C for 24 hours. The Ab-bound TTR was then allowed to react with alkaline phosphatase-conjugated goat antihuman prealbumin (TTR) antibody (EY Laboratories). Bound alkaline phosphatase activities were assayed by color development using the ELISA substrate solution from the GIBCO/BRL ELISA amplification system (Life Technologies).

Immunoprecipitation and LC/MS Analysis of Serum and CSF TTR

A 1.5-ml Eppendorf tube was filled with 100 μl of serum or 500 μl of CSF from the patient. A 1:1 gel/TSA (10 mM Tris HCl, pH 8.0/140 mM NaCl/0.025% NaN_3) slurry (10 μl) of quenched Sepharose (Purkey et al, 2001) was added to the solution and incubated on a rocker at 18 rpm and 4° C for 1 hour. After centrifugation (16,000 $\times g$), 100 μl of a 1:1 gel/TSA slurry of the anti-TTR antibody-conjugated Sepharose was added to the supernatant, and the tube was rocked at 4° C for 20 minutes. The samples were centrifuged (16,000 $\times g$), and the supernatant was removed. The gel was washed with 1 ml of TSA/0.05% saponin (Fisher Scientific) (3 $\times$, 10 minutes each) at 4° C and additionally with 1 ml of TSA (2 $\times$, 10 minutes each) at 4° C. The samples were centrifuged (16,000 $\times g$), the final wash was removed, and 155 μl of 100 mM triethylamine (pH 11.5) was added to elute the TTR from the antibodies. After gentle agitation at 4° C for 30 minutes, the sample was centrifuged (16,000 $\times g$) and 145 μl of the supernatant containing TTR was removed. The supernatant was treated with 14.5 μl of 1 M DTT at 100° C for 15 minutes. Forty microliters of each sample was injected on to a Hewlett-Packard 1100-MSD mass-spectrometer, running a linear gradient of acetonitrile (0% to 100%) in water. Both the water and the acetonitrile phases are acidified with TFA (0.05%).

Acknowledgments

We thank E. Powers and A. Hurshman for help with evaluating thermodynamic parameters, J.T. White for help in the fibril formation assay, R.L. Wiseman for help in the subunit exchange assay, and D. French for help with ELISA quantification.

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