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Inhibiting transthyretin amyloid fibril formation via protein stabilization

(familial amyloid polyneuropathy/therapeutic strategy)

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ABSTRACT **Transthyretin (TTR) amyloid fibril formation is observed systemically in familial amyloid polyneuropathy and senile systemic amyloidosis and appears to be the causative agent in these diseases. Herein, we demonstrate conclusively that thyroxine (10.8 μM) inhibits TTR fibril formation efficiently *in vitro* and does so by stabilizing the tetramer against dissociation and the subsequent conformational changes required for amyloid fibril formation. In addition, the nonnative ligand 2,4,6-triiodophenol, which binds to TTR with slightly increased affinity also inhibits TTR fibril formation by this mechanism. Sedimentation velocity experiments were employed to show that TTR undergoes dissociation (linked to a conformational change) to form the monomeric amyloidogenic intermediate, which self-assembles into amyloid in the absence, but not in the presence of thyroxine. These results demonstrate the feasibility of using small molecules to stabilize the native fold of a potentially amyloidogenic human protein, thus preventing the conformational changes, which appear to be the common link in several human amyloid diseases. This strategy and the compounds resulting from further development should prove useful for critically evaluating the amyloid hypothesis—i.e., the putative cause-and-effect relationship between TTR amyloid deposition and the onset of familial amyloid polyneuropathy and senile systemic amyloidosis.**

Transthyretin (TTR) is present in human plasma (0.2 mg/ml; 3.63 μM, tetramer) and is composed of four identical β-sheet-rich subunits that bind and transport thyroxine (T4) and the retinol binding protein (1). In unfortunate individuals, TTR is converted into an insoluble fibrillar structure called amyloid. These fibrils putatively cause senile systemic amyloidosis (wild-type TTR composes the fibrils-late onset) and familial amyloid polyneuropathy (FAP; predominantly variant TTR composing the fibrils-earlier onset) by virtue of the amyloid's neurotoxicity and/or by physically interfering with normal organ function (2–8). A TTR amyloid fibril is ≈130Å in diameter and made up of four protofilaments, each having a twisted cross-β-helix structure (9, 10). TTR amyloid fibril formation is observed during partial acid denaturation from a conformational intermediate formed under conditions simulating a lysosome (pH 5.5 ± 0.5), which has been implicated in fibril formation *in vivo* (11, 12). TTR amyloid fibril formation can be avoided under acidic conditions by working at low TTR concentrations and low temperature (25°C) allowing identification of the quaternary, tertiary, and secondary structure of the intermediate(s) that can form amyloid (12). These studies reveal that tetrameric TTR is nonamyloidogenic; however, the dissociation of the tetramer into a monomeric intermediate having an altered, but defined, tertiary structure is capable of amyloid fibril formation and is therefore called the amyloidogenic intermediate (Fig. 1). Several of the 50 FAP-associated TTR single-site mutations still adopt a normal tetrameric structure under physiological conditions (13–16); however, these mutations significantly destabilize the tetramer (17, 18). The mutation-induced destabilization allows the amyloidogenic intermediate to be populated under lysosomal conditions (pH 5.5) in the case of the FAP associated V-30-M (most common FAP mutation) and the L-55-P TTR variants, whereas the wild-type protein remains predominantly tetrameric and nonamyloidogenic.

The TTR tetramer has an hourglass-shaped central channel where two molecules of T4 can bind at pH 7.4 (K_a values of 1×10^8 and 9.6×10^5 M^{-1}) (refs. 19 and 20; Fig. 2). However, only 10–25% of the TTR in plasma has T4 bound, because T4 binding globulin is the major T4 carrier in plasma (K_a of 6×10^9), binding ≈75% T4 present (22). Our strategy for inhibiting TTR fibril formation systemically is to design a ligand that will bind with high affinity to plasma TTR using the largely unoccupied T4 binding site. Herein, we show that ligand binding stabilizes TTR against amyloid formation, suggesting a potential new therapeutic strategy for treating TTR amyloid disease.

MATERIALS AND METHODS

Preparation of TTR, T4, and 2,4,6-Triiodophenol (TIP) Solutions. Wild-type, L-55-P, and V-30-M TTR were purified from an *Escherichia coli* expression system described previously (17). The extinction coefficient of wild-type TTR (ε_{280} = 77,600) was used for V-30-M and L-55-P TTR, as reported previously (17, 18). All studies reported in this paper use recombinant TTR, because TTR isolated from aged plasma is typically heterogeneous.

A concentrated stock solution of T4 (Calbiochem) was prepared by dissolving T4 in 0.01 M NaOH and filtering the solution through a 0.2-μm syringe filter. The T4 solution was verified to be 99% pure by its single narrow peak in a reverse phase HPLC trace, and its identity was established by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (M^+ = 778). The concentration of the solution was obtained using its absorption at 325 nm (ε_{325} = 6185). Crude TIP purchased from Aldrich was precipitated from ethanol three times by the addition of H_2O and purified by C-4 reverse phase HPLC, and its structure was identified by GC-electron impact MS (M^+ = 472). The TIP was dried at 61°C over P_2O_5 under high vacuum and dissolved in 0.01 M NaOH to afford a 0.54 mM solution.

Amyloid Fibril Formation and Inhibition Thereof via Partial Acid Denaturation. A series of 50 mM sodium acetate-buffered 100 mM KCl solutions (1.2 ml) containing different

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Abbreviations: TTR, transthyretin; FAP, familial amyloid polyneuropathy; T4, thyroxine; TIP, 2,4,6-triiodophenol.

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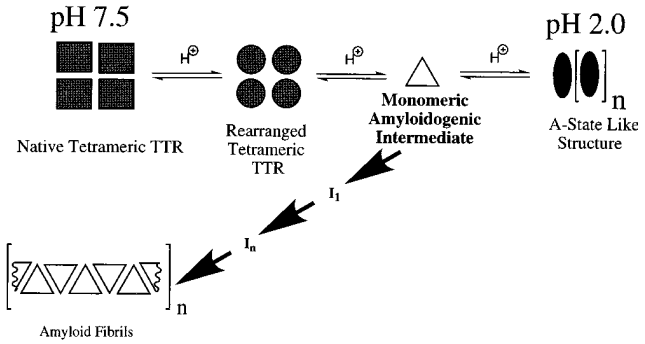


FIG. 1. Schematic representation of the acid-mediated denaturation/amyloid fibril-forming pathway of TTR.

amounts of T4 (or TIP) dissolved in 0.01 M NaOH were prepared at the desired pH in Eppendorf tubes. Wild-type TTR from a stock solution (≈5 mg of TTR per ml in 10 mM phosphate, pH 7.4/100 mM KCl/1 mM EDTA) was then added to each tube to obtain a final TTR concentration of 0.2 mg/ml. In addition, two control samples containing TTR but no inhibitor (T4 or TIP) and T4 or TIP but no TTR were also similarly evaluated. Each stationary tube was incubated at 37°C for 72 h to probe the viability of amyloid fibril formation via partial TTR denaturation. The time course of TTR fibril formation under these conditions has been reported previously (see figure 2*B* in ref. 12) and demonstrates that amyloid fibril formation plateaus after 72 h, justifying analysis of the inhibitors at 72 h. The extent of fibril formation was measured by OD at 330 nm in a standard UV cell (23, 24) and by a quantitative Congo red binding assay (12, 25) as described in detail previously (12). Analogous studies were also carried out with L-55-P and V-30-M TTR at pHs where these TTR variants make amyloid (17, 18). The integrity of the amyloid fibrils formed using the methods described above were confirmed by x-ray diffraction, as well as by light and electron microscopy using methods described previously (11, 17) .

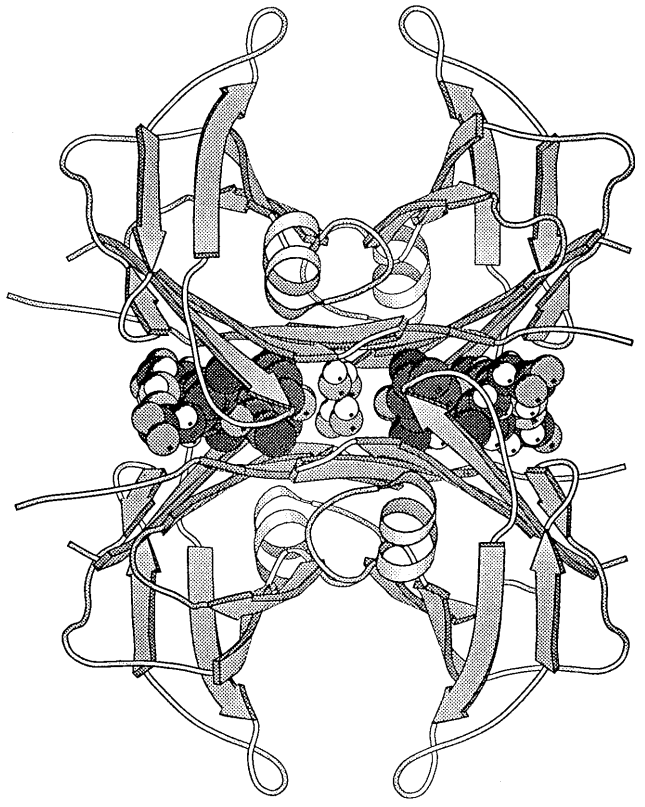


FIG. 2. Ribbon diagram of tetrameric TTR illustrating the hourglass-shaped channel that runs through the center of the protein and serves as the binding site for T4 (3,3′-diiodothyroxine shown) molecules rendered as a space-filling structure (21). The four conserved water molecules are also shown in CPK (Corey–Pauling Space Filling Models) format.

Probing Quaternary Structure Changes by Analytical Ultracentrifugation. The effect of T4 on the quaternary structure stability of wild-type TTR and single-site amyloidogenic variants thereof as a function of pH was evaluated using analytical ultracentrifugation. The quaternary structural changes were examined using sedimentation velocity and sedimentation equilibrium techniques in the presence and absence of T4 using a temperature-controlled Beckman XL-A analytical ultracentrifuge equipped with a An60Ti rotor and photoelectric scanner. Double-sector aluminum cell centerpieces and quartz windows were used in the velocity experiments at a temperature of 25 or 37°C, using 400–420 μl of sample and rotor speeds of 3000–60,000 rpm. Sedimentation of wild-type TTR was carried out at pH 4.4 (50 mM acetate/100 mM KCl). Concentrated TTR stock solutions were spun down on a desktop centrifuge for 15 min at 4°C, and all buffers were filtered through 0.2-micron filter before use. TTR samples (0.2 mg/ml) in the presence or absence of 10.8 μM T4 were prepared as described in the amyloid fibril formation section. After an incubation period of 48 h at 25°C or 72 h at 37°C, the samples were loaded into a double-sector cell and evaluated at 235 nm. TTR samples lacking T4 incubated at 37°C were not evaluated in the centrifuge due to extensive amyloid fibril formation. For boundary sedimentation analysis, the movement of the midpoint (r) of the absorbance boundary vs. time (t) was used to obtain the uncorrected sedimentation coefficient s^*, expressed in Svedberg units, from the slope of the plot of $\ln r$ vs. t/ω^2 (i.e., $d\ln r/\omega^2 dt$). The observed s^* values obtained were corrected to standard conditions by Eq. **1** using tabulated density and viscosity data (26):

$$s_{20,w} = s^* \cdot \frac{(\eta)T,b}{(\eta)w,20} \cdot \frac{(1 - \bar{v}\rho)w,20}{(1 - \bar{v}\rho)T,b} \qquad \textbf{[1]}$$

where ρ and η are the density and viscosity of the solvent at the temperature of the sedimentation velocity experiment or the viscosity of water at 20°C, and $\bar{v}$ is the partial specific volume of the protein under the conditions of the experiment. The sedimentation coefficients $s_{20,w}$ for the TTR tetramer (4.2 ± 0.2 S) was determined by sedimentation velocity experiments at pH 7, where the tetramer is the dominant species based on sedimentation equilibrium measurements.

Sedimentation equilibrium runs were performed on 130-μl samples from 3000–15,000 rpm using double-sector cells with charcoal-filled Epon centerpieces and sapphire windows. All scans were performed at 280 nm with a step size of 0.001 and 30–50 averages. Samples were allowed to equilibrate over 18–24 h, and duplicate scans 3 h apart were overlaid to determine that equilibrium had been reached. The data were analyzed by a nonlinear least square analysis (27) using the ORIGIN software provided by Beckman ($\bar{v}$ = 0.7347 based on amino acid composition). The data were fit to a single ideal species model using Eq. **2** to determine the best-fitting molecular weight.

$$A_r = \mathrm{Exp}[\ln(A_0) + (M\omega^2(1 - \bar{v}\rho)/2\mathrm{RT}) \cdot (x^2 - x_0^2)] + E, \qquad \textbf{[2]}$$

where A_r is the absorbance at radius x; A_o is the absorbance at a reference radius x_o, usually the meniscus; $\bar{v}$ is the partial specific volume of TTR (ml/mg); ρ is the density of the solvent (mg/ml); ω is the angular velocity of the rotor (radian/sec); E is the baseline error correction term; M is the gram molecular weight; R is the universal gas constant 8.314×10^7 erg/mol·K (1 erg = 0.1 μJ); and T is temperature (in K).

Equilibrium Dialysis Evaluation of the Binding of T4 to TTR. Equilibrium dialysis was used to evaluate the binding stoichiometry of T4 to TTR under acidic conditions with protein and ligand concentrations identical to the concentrations used in the amyloid fibril formation and inhibition assay. The top chamber of the FT4-Nelson Dialysis Cell (Nichols Institute, San Juan Capistrano, CA), was filled with 2.5 ml of 50 mM acetate buffer containing 0.2 mg of (3.63 μM, tetramer) recombinant wild-type TTR per ml and a known amount of cold T4 with a trace amount of purified I^{125}-labeled T4. The bottom chamber was filled with 2.5 ml of acetate buffer only. After 5 days of incubation at 25°C, 200-μl aliquots from each chamber were analyzed in a UKB–Wallac (Gaithersburg, MD) Ria Gamma 1274 Counter to determine the fraction of T4 bound. The number of moles T4 bound to TTR was determined by multiplying the fraction of T4 bound by the total T4 concentration divided by the TTR concentration. A Scatchard analysis to determine the binding constant of T4 (1.5×10^{-6}–3×10^{-6} M) to TTR (0.05 mg/ml) at pH 4.4 could only be estimated, owing to the complications resulting from TTR dissociation at T4 concentrations lower than 1.5×10^{-6}M. A Scatchard analysis of the binding of T4 (1×10^{-8}–3×10^{-6} M) to TTR at pH 7.4 (0.05 mg/ml) was straightforward (20, 28).

RESULTS

The efficacy of T4 as an inhibitor of wild-type TTR fibril formation was demonstrated by incubating varying concentrations of T4 with TTR under acidic conditions, previously shown to effect fibril formation *in vitro* (11, 12). TTR fibril formation is maximal at pH 4.4 and is nearly completely inhibited by the addition of 3 eq of T4 (10.8 μM). Fibril formation was evaluated by OD measurements and by quantitative Congo red binding studies over a 72-h period (Fig. 3*A*), sufficient for TTR fibril formation to reach a maximum and plateau in the absence of inhibitor (12). As expected, the extent of fibril inhibition decreases with a decrease in added T4 (Fig. 3*A*). The inhibitory effect of T4 reaches a maximum at 10.8 μM (3 eq), where T4 binding occurs at both the low and high affinity sites in the TTR tetramer (0.2 mg/ml) as demonstrated by the binding studies (29). To be certain the inhibitory nature of T4 is not pH-specific, we evaluated TTR fibril growth inhibition at 10.8 μM T4 over the pH range of 4.2–5.0, where wild-type TTR is amyloidogenic. The inhibitory effect observed at pH 4.2 and 4.6 was nearly identical to that observed at pH 4.4 (data not shown). The effectiveness of T4 as a fibril inhibitor at pH 4.8 and 5 was not easy to evaluate, as only traces of wild-type fibrils are formed under these conditions in the absence of inhibitor (12). To be certain that T4 serves as an inhibitor of TTR fibril formation around pH 5, the L-55-P and V-30-M FAP single-site variants of TTR were studied in the absence and presence of T4. We have previously demonstrated that these amyloidogenic variants are much less stable than the wild-type protein and form the monomeric amyloidogenic intermediate, which self-assembles into amyloid at pH around 5, where the wild-type protein is largely tetrameric and resistant to fibril formation (17, 18). Fibril formation normally exhibited by the V-30-M and L-55-P TTR around pH 5 is efficiently inhibited by the addition of 10.8 μM T4 (Fig. 3 *B* and *C*). The extent of inhibition is proportional to the amount of T4 added up to 10.8 μM, at which concentration both binding sites on TTR are saturated (Fig. 3*A*). Interestingly, the other natural ligand for TTR, the retinol binding protein, does not inhibit TTR fibril formation, because retinol binding protein does not bind TTR under fibril-forming conditions (G.J.M. and J.W.K., unpublished results).

Biophysical studies carried out in this laboratory have shown that tetrameric TTR (having a sedimentation coefficient $s_{20,w} = 4.2 \pm 0.2$ S, expressed in Svedberg units) must dissociate to

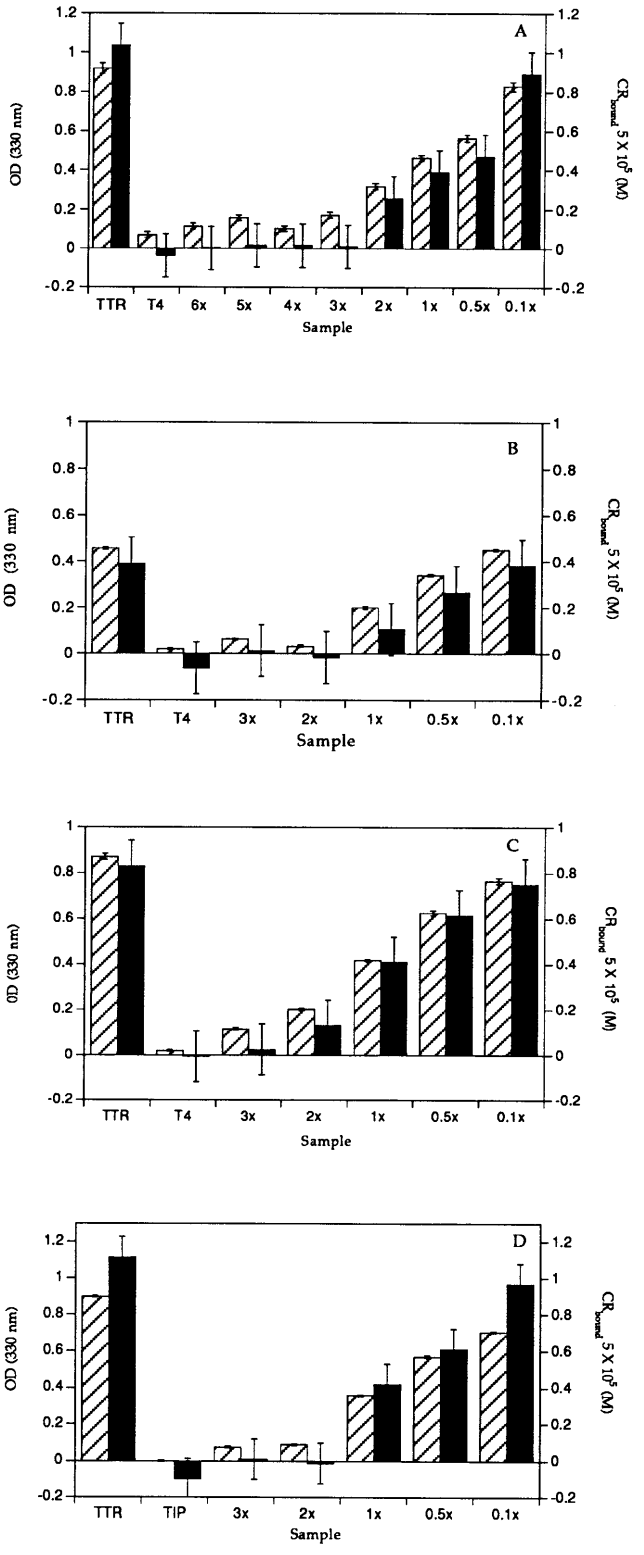
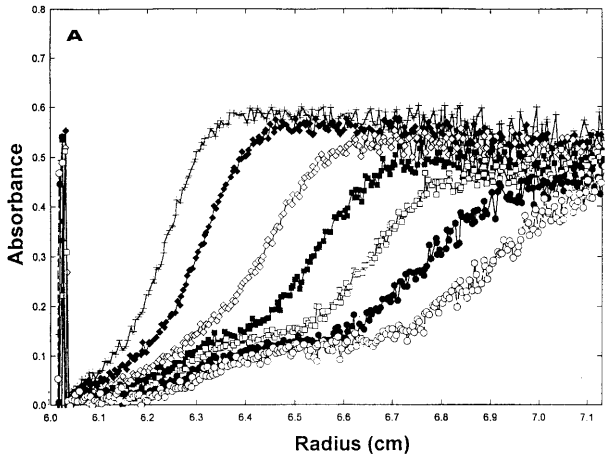


FIG. 3. Inhibition of TTR amyloid fibril formation by T4 and TIP. The extent of fibril formation in the presence and absence of inhibitor at pH 4.4 was probed at 72 h by an optical density measurement at 330 nm (hatched bars) and by a quantitative Congo red binding assay (solid bars). (*A*) Wild-type fibril formation. From left to right, fibril formation in the absence of T4 and in the presence of T4 alone (no TTR) and TTR fibril formation in the presence of the indicated equivalents of T4 (3 eq of T4 = 10.8 μM) are shown. Inhibition of (*B*) V-30-M and (*C*) L-55-P TTR by T4 at pH 5.0. (*D*) Inhibition of wild-type TTR by TIP. The labeling of the axes and the organization of the data in *B–D* is the same as that in *A*.

monomer ($s_{20,w}$ = 1.6 ± 0.2 S) and undergo a conformational change to self-assemble into amyloid (11, 12, 17, 18, 30). In the absence of T4, TTR exists as a mixture of quaternary species at pH 4.4 (25°C), including the monomeric amyloidogenic intermediate as discerned from the velocity profile of wild-type TTR (Fig. 4*A*). Analysis of the two separate boundaries using the second moment method gave an average *s* value of 4.8 S for the fast-moving species and 1.75 S for the slow-moving boundary. Since this simple analysis cannot determine the number of components in each boundary, the "dcdt" method for calculating the apparent sedimentation coefficient distribution, $g^*(s)_t$, for sedimenting samples was used (31). This analysis identifies multiple sedimenting species with s_{25} values of (1.5 S, 4.3 S, 5.4 S, and 6.3 S; Fig. 4*B*), corresponding to the amyloidogenic monomeric intermediate, the acid-rearranged tetramer, and two higher-order aggregated species, respectively. The higher-order assemblies may be on the pathway to amyloid but have not yet been identified. Wild-type TTR and, to a greater extent, V-30-M and L-55-P TTR exhibit significant aggregation in the absence of T4, indicating their instability toward acid denaturation and subsequent self-assembly, complicating the sedimentation analysis.



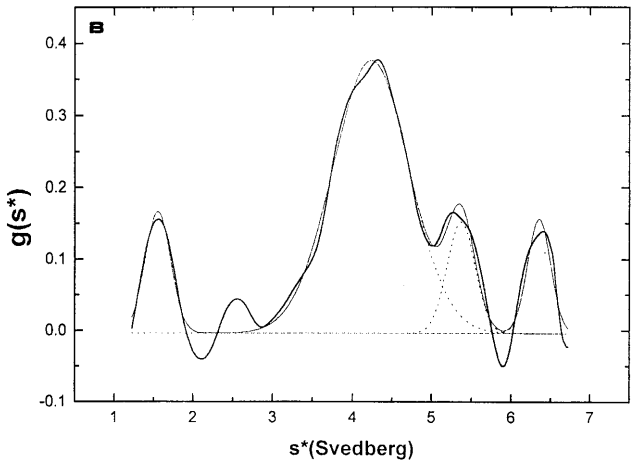
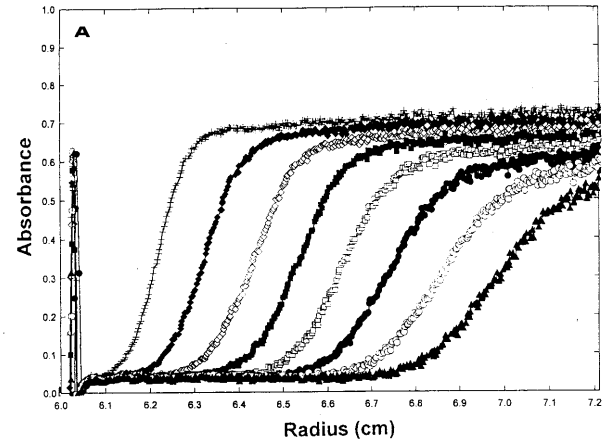


FIG. 4. (*A*) Sedimentation velocity profile of wild-type TTR in the absence of T4 at pH 4.4. Solute distribution recorded at 235 nm (60,000 rpm). Scans for analysis were recorded every 3 min; for simplicity, only scans from every 24 min are shown. (*B*) Apparent sedimentation coefficient distribution ($g^*(s)_t$) as a function of sedimentation coefficient for wild-type TTR (0.2 mg/ml) in the absence of T4, indicating the formation of multiple species during the partial acid denaturation of TTR.



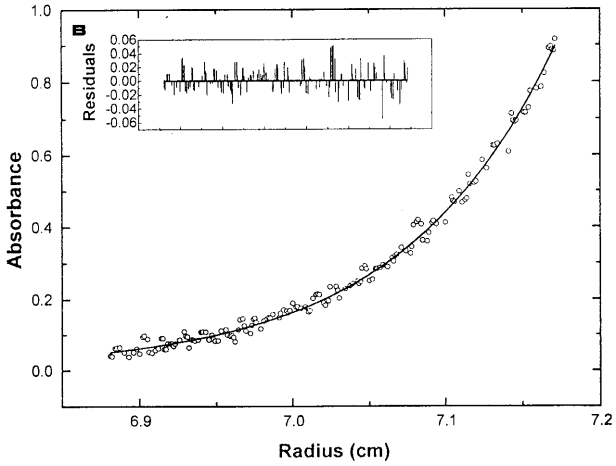


FIG. 5. (*A*) Sedimentation velocity profile of wild-type TTR in the presence of T4 at pH 4.4. Solute distribution recorded at 235 nm (60,000 rpm). Scans for analysis were recorded every 3 min; for simplicity, only scans from every 15 min are shown. (*B*) Sedimentation equilibrium analysis of wild-type TTR at pH 4.4 in the presence of 10.8 μM. The solid line drawn through the data was obtained by fitting the absorbance vs. radial position *r* to Eq. **2** for a homogeneous tetrameric species. The residual difference between the experimental data and the fitted data for each point is shown in the *Inset*.

In the presence of 10.8 μM T4, wild-type TTR exclusively sediments as a single symmetrical band at pH 4.4 with an *s* value close to that of the tetramer under physiological conditions (Fig. 5*A*). The V-30-M and L-55-P TTR variants with bound T4 also show the movement of a single boundary across the cell with identical *s* values (4.8 S) at 25°C corresponding to tetrameric TTR. The second moment analysis of the sedimentation data in the presence of 10.8 μM T4 gave the following *s* values at 25°C, 37°C, and corrected to 20°C in water for wild-type, V-30-M, and L-55-P TTR, respectively: $s_{25,buffer}$ = 4.9 S, 4.8 S, and 4.8 S; $s_{37,buffer}$ = 5.8 S, 5.9 S, and 6.2S; $s_{20,w}$ = 4.2 ± 0.2 S, 4.1 ± 0.1 S, and 4.3 ± 0.2 S, demonstrating the presence of tetrameric TTR at pH 4.4.

From sedimentation equilibrium analysis of wild-type TTR at pH 4.4 (0.2 mg/ml) in the presence of T4 (10.8 μM), no heterogeneity could be detected upon linearization of the sedimentation equilibria. The fit of the exponential to a single species gave an average molecular weight of 52,720 g/mol, which corresponds to the molecular weight of the tetramer (54,980 from amino acid composition; Fig. 5*B*). These data, along with the binding data presented below, demonstrate that 2 mol of T4 bind to and stabilize the normal quaternary structure of TTR, thus preventing denaturation-mediated

fibril formation. Attempts to carry out equilibrium ultracentrifugation experiments on V-30-M and L-55-P in the presence of T4 failed due to their instability in the centrifuge for long periods of time.

T4 binding to wild-type TTR was evaluated at pH 4.4 to be sure that the binding constant for T4 did not change significantly at lower pH. At physiological TTR concentration (0.2 mg/ml), it is clear that two equivalents of T4 are bound to TTR at 10.8 μM T4, which is also the case for T4 binding to TTR at pH 7.4. A Scatchard analysis cannot be rigorously accomplished at pH 4.4 because of TTR dissociation (0.05 mg/ml) or dissociation and amyloid formation (0.2 mg/ml) at low T4 concentrations, which complicates the evaluation of the binding curves. A fit of a portion of the T4 binding data to TTR at pH 4.4 over the T4 concentration range ($1.5–3 \times 10^{-6}$ M), where TTR is largely tetrameric yields an apparent binding constant of 6.4×10^5 M^{-1} for binding to both the low and high affinity sites. A Scatchard analysis of the binding of T4 to TTR at pH 7.4 yields the expected values ($K_a1 = 2.3 \times 10^7 \pm 6 \times 10^6$ M^{-1}; $K_a2 = 4.8 \times 10^5 \pm 2 \times 10^5$ M^{-1}), corroborating the authenticity of the recombinant protein and the validity of the methods used at pH 4.4.

To verify the generality of this small molecule approach to inhibit TTR fibril formation, we studied the commercially available nonnatural ligand TIP, which is reported to bind TTR with 3-fold higher affinity relative to T4 (32). This simple phenol also efficiently inhibits TTR fibril formation and appears to be very similar to T4 in terms of its efficacy (Fig. 3*D*). These results have motivated our lab to design and synthesize high affinity small molecule inhibitors of TTR fibril formation using a structure-based design approach.

DISCUSSION

The deposition of a normally soluble protein into cross-β amyloid fibrils is the hallmark of human amyloid disease (6, 33, 34). There is ample evidence to show that conformational changes are sufficient for the conversion of a number of normally soluble human proteins into amyloid fibrils, including the immunoglobulin light chains, lysozyme, and TTR, and variants thereof (11, 12, 34–36). Fibril formation is believed to be intimately involved in the pathological mechanism of human amyloid disease based on the demonstrated neurotoxicity of amyloid fibrils produced *in vitro*, the observation of lower levels of amyloid in age-matched controls relative to Alzheimer disease patients, and the correlation of improved health with the clearance of amyloid in FAP patients, where liver transplantation is used to replace mutant TTR with wild-type TTR (37). The amyloid hypothesis has been difficult to prove, and, as a result, the pharmaceutical industry has been reluctant to make a large commitment in the amyloid disease area until the pathogenic mechanism becomes more clearly established (6, 34).

At the present time, there is no general strategy for treating human amyloid disease. The results presented within are important because the strategy of using ligand binding to stabilize the native protein conformation may prove useful for other monomeric and oligomeric amyloidogenic proteins, which adopt well-defined physiological conformations. Moreover, medicinal chemists are very good at making high affinity ligands for proteins and at understanding structure–activity relationships using the principles of structure-based drug design (38, 39). Last, and perhaps most importantly, the approach demonstrated within produces molecules that may allow scientists to probe the pathogenic mechanisms of certain amyloid diseases in animal models and ultimately in humans (40). With molecules that inhibit TTR amyloid formation, one could ask and answer a very important question: Does the inhibition of amyloid fibril formation inhibit the onset of amyloid disease?

The results presented within demonstrate that T4 and TIP are effective inhibitors of TTR amyloid fibril formation *in vitro* at an inhibitor concentration of 10 μM. It is important to point out that the physiological concentration of TTR is 0.2 mg/ml (3.63 μM, tetramer), and, therefore, even if the binding constant of the inhibitor were 10^{10} M^{-1}, the mechanism of inhibition still requires a 7.26 μM inhibitor concentration in plasma to occupy both sites in TTR. The sedimentation velocity experiments of wild-type TTR and variants thereof showed that in the absence of T4 at acidic pH values, TTR forms amyloid and exists in multiple quaternary forms in solution, including the monomeric amyloidogenic intermediate. However, in the presence of three equivalents of T4, wild-type and variant TTR are stabilized in a tetrameric nonamyloid form at acidic pH values, as discerned by sedimentation velocity and equilibrium ultracentrifuge data. However, T4 does not prevent fibril formation systemically in humans by binding to TTR, because the majority of T4 (>75%) is bound to T4 binding globulin, which has a higher affinity (6×10^9 M^{-1}) for T4 than TTR. Contrary to the case in plasma, TTR is the major T4 carrier in the cerebral spinal fluid, perhaps explaining why TTR fibril formation is generally not observed in the brain [ref. 41; occasionally TTR fibrils are observed in the blood vessels of the choroid plexus, which is the site of TTR synthesis in the cerebrospinal fluid (42)]. Based on the *in vitro* results presented, a synthetic T4 mimic that is neither an agonist nor antagonist (does not bind to the thyroid receptor) and that has higher affinity for TTR than T4 binding globulin may prove to be an effective TTR amyloid fibril inhibitor systemically *in vivo*. This strategy should prove to be general for inhibiting fibril formation in the case of the wild-type TTR, which putatively causes senile systemic amyloidosis, and for the vast majority of the TTR variants associated with FAP, since in the majority of the cases the T4 binding is unaffected. There are a few reported mutations out of the 50 examples of FAP-associated TTR variants that appear to be incapable of T4 binding due to altered T4 binding sites, implying that these particular cases may not be susceptible to small molecule inhibition (43). However, it may be possible based on structure–activity relationships to design a molecule that will bind to all 50 FAP variants with higher affinity than TIP, but that remains to be seen. Recent demonstrations that amyloid can be cleared *in vivo* suggest that there may be a delicate balance between fibril formation and fibril clearance (44). Even if a given compound were only moderately effective as an inhibitor of TTR fibril formation, it still may prove effective as a therapeutic agent, because it may only be necessary to slightly shift the balance between synthesis and degradation of amyloid to avoid the onset of amyloid disease (34). All indications are that humans can tolerate low levels of amyloid deposition, but not high levels.

The strategy outlined here where a ligand is used to stabilize the normal structure of a protein against deleterious conformational changes should prove useful for preventing fibril formation in other amyloid diseases where the precursor protein is structurally well-defined and undergoes denaturation before self-assembly into amyloid but may not easily be applied in Alzheimer disease, where the β-peptide exhibits conformational heterogeneity in normal individuals.

CONCLUSION

A common mechanistic hypothesis appears to be operating in several, but perhaps not all amyloid diseases, where a normally folded protein undergoing a conformational change yields an amyloidogenic intermediate that self-assembles into amyloid fibrils (34). We demonstrate a potentially useful therapeutic strategy for TTR-based amyloid diseases where the amyloidogenic protein of interest is stabilized against partial denaturation by ligand binding to the native state, making the

conformational change affording the amyloidogenic intermediate difficult to achieve, thus preventing fibril formation.

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