Cys10 Mixed Disulfides Make Transthyretin More Amyloidogenic under Mildly Acidic Conditions†

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Received March 27, 2003; Revised Manuscript Received May 23, 2003

ABSTRACT: Conservative mutation of transthyretin's surface residues can predispose an individual to familial amyloidosis by dramatically changing the energetics of misfolding. Senile systemic amyloidosis (SSA), however, cannot be explained in this fashion because wild-type (WT) transthyretin (TTR) misfolds and misassembles into amyloid. Since various modifications of the SH functionality of Cys10 have been reported in humans, we sought to understand the extent to which these modifications alter the stability and amyloidosis of WT TTR as a possible explanation for SSA. Homotetrameric Cys10 TTR variants, including TTR-Cys, TTR-GSH, TTR-CysGly, and S-sulfonated TTR, were chemically synthesized starting with WT TTR. The TTR-Cys, TTR-GSH, and TTR-CysGly isoforms are more amyloidogenic than WT at the higher end of the acidic pH range (pH 4.4−5.0), and they are similarly destabilized relative to WT TTR toward urea denaturation. They exhibit rates of urea-mediated tetramer dissociation (pH 7) and MeOH-facilitated fibril formation similar to those of WT TTR. Under mildly acidic conditions (pH 4.8), the amyloidogenesis rates of the mixed disulfide TTR variants are much faster than the WT rate. S-Sulfonated TTR is less amyloidogenic and forms fibrils more slowly than WT under acidic conditions, yet it exhibits a stability and rates of tetramer dissociation similar to those of WT TTR when subjected to urea denaturation. Conversion of the Cys10 SH group to a mixed disulfide with the amino acid Cys, the CysGly peptide, or glutathione increases amyloidogenicity and the amyloidogenesis rate above pH 4.6, conditions under which TTR probably forms fibrils in humans. Hence, these modifications may play an important role in human amyloidosis.

Transthyretin (TTR)[1] is a 55 kDa homotetrameric protein present in plasma and cerebrospinal fluid that transports thyroxine and holo-retinol binding protein. TTR is the major component of the amyloid deposits that putatively cause the human amyloid diseases senile systemic amyloidosis (SSA), familial amyloidotic polyneuropathy (FAP), and familial amyloidotic cardiomyopathy (FAC). Deposition of WT TTR appears to cause SSA (*1*), whereas one of more than 80 single-site variants predominantly constitutes the fibrils in FAP or FAC (*2*, *3*).

SSA is a late onset cardiac disease characterized by extracellular fibrillar deposits in the heart, affecting as much as 25% of the population that is more than 80 years of age (*4*). These deposits and their precursors cause irreversible life-threatening organ damage and represent a significant health problem for the aging population (*5*). There is currently no effective treatment for SSA, although small molecule native state-kinetic stabilizers provide hope (*6*, *7*). While it is still not clear how and where amyloidogenesis occurs in a human, in vitro studies demonstrate that subjecting TTR to denaturing conditions leads to amyloid fibril formation through rate-limiting tetramer dissociation followed by partial monomer denaturation and misassembly (*8*−*11*).

Each subunit of the WT TTR homotetramer has a cysteine (Cys) residue at position 10. Several Cys10 isoforms of TTR have been identified in human plasma and cerebrospinal fluid by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and/or electrospray ionization mass spectrometry (ESI-MS) combined with high-performance liquid chromatography (HPLC) (*12*−*15*). These isoforms result when the Cys10 residue makes a mixed disulfide with the amino acid cysteine (TTR-Cys), the peptide glutathione (TTR-GSH), and the peptide cysteinylglycine (TTR-CysGly) or is oxidized to S-sulfonated TTR. While it is clear that single-amino acid changes in the TTR sequence, even those that are conservative, can have a significant influence on protein stability and the rates of tetramer dissociation and amyloidogenesis (*16*, *17*), the impact of Cys10 alterations on stability and amyloidosis is not so clear. The stabilities of the Cys10 isoforms of TTR have been investigated in several papers in the context of mixed tetramers composed of both WT and Cys10 modified

† This work is supported by NIH Grant DK 46335, the Skaggs Institute for Chemical Biology, and the Lita Annenberg Hazen Foundation.

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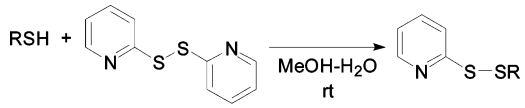
[1] Abbreviations: TTR, transthyretin; WT, wild type; SSA, senile systemic amyloidosis; FAP, familial amyloidotic polyneuropathy; FAC, familial amyloidotic cardiomyopathy; TTR-Cys, cysteine linked by a disulfide to Cys10 of transthyretin; TTR-GSH, glutathione linked by a disulfide to Cys10 of transthyretin; TTR-CysGly, cysteinylglycine linked by a disulfide to Cys10 of transthyretin; S-sulfonated TTR, TTR with the Cys10 sulfur in the side chain modified, TTR-S-SO_3^-; OD, optical density.

10.1021/bi030077a CCC: $25.00
Published on Web 07/01/2003

Scheme 1



1 R = cysteine
2 R = glutathione (GSH)
3 R = cysteinylglycine

monomers (*18*−*20*), but to date, their influence on amyloidogenesis has been debated. In this study, we have prepared homotetramers of the Cys10 isoforms of WT TTR to carefully characterize their influence on the thermodynamics and kinetics of denaturation as well as acid- and MeOH-mediated amyloidogenesis. These studies should provide insight into whether modifications of Cys10 could hasten the onset of SSA.

MATERIALS AND METHODS

Protein Preparation of WT TTR, TTR-Cys, TTR-GSH, TTR-CysGly, and S-Sulfonated TTR. Sodium tetrathionate was purchased from Aldrich; 2,2′-dithiodipyridine and glutathione were from Acros, and cysteine and cysteinylglycine were from Sigma. WT TTR was expressed and purified from an *Escherichia coli* expression system described previously (*21*). Modified TTR was prepared chemically from Cys10 WT TTR through a disulfide exchange reaction described below. All proteins were further purified by using DEAE ion exchange chromatography and gel filtration chromatography on a Superdex-75 column (Amersham Pharmacia Biotech). The micromolar concentrations mentioned in this section are for the TTR tetramer, when not specified otherwise. The pH 7.0 buffer is comprised of 10 mM phosphate, 100 mM KCl, and 1 mM EDTA. All NMR spectra were obtained on a Bruker DRX 600 MHz instrument.

S-(2-Thiopyridyl)cysteine (**1**), *S*-(2-thiopyridyl)glutathione (**2**), and *S*-(2-thiopyridyl)cysteinylglycine (**3**) were prepared from the reaction of 2,2′-dithiodipyridine (2 mmol) and the corresponding amino acid or peptide (1 mmol) in a mixure of methanol and water (1:1) at room temperature (Scheme 1). Upon completion of the reaction (monitored by TLC), most of methanol was removed in vacuo and the residue was washed with dichloromethane. The aqueous solution of **1**, **2**, or **3** was subjected to reverse phase (RP) HPLC purification, and lyophlization of the eluent gave the pure product as a white solid.

Cysteinylation of TTR (1.5 mg/mL, 108 μM monomer) was accomplished by incubating **1** (432 μM) with WT TTR in pH 7.0 buffer, affording TTR-Cys at room temperature after monitoring by LC−MS for 4 h. The glutathione mixed disulfide TTR variant and the cysteinylglycine mixed disulfide TTR variant were prepared analogously. S-Sulfonated TTR was prepared from the reaction between WT TTR (1.5 mg/mL, 108 μM monomer) and sodium tetrathionate (40 mM) in pH 7.0 buffer at room temperature, monitored by LC−MS (*22*).[2] All the prepared proteins were purified subsequently by dialysis (pH 8.0 buffer with 25 mM Tris and 1 mM EDTA), anion exchange chromatography [buffer A (pH 8.0), 25 mM Tris and 1 mM EDTA; buffer B (pH 8.0), 1 M NaCl, 25 mM Tris, and 1 mM EDTA], and gel filtration chromatography (pH 7.0 buffer). The purity of the proteins was examined by RP-HPLC and mass spectrometry (>90%). The masses of WT TTR, TTR-Cys, TTR-GSH, TTR-CysGly, and S-sulfonated TTR monomers were 13 890, 14 009, 14 195, 14 066, and 13 970 Da, respectively. The prepared proteins were stored in pH 7.0 buffer before being used.

Amyloid Fibril Formation Assay. Solutions of TTR (7.2 μM tetramer, pH 7.0 buffer) were mixed with an equal volume of 100 mM acetate buffer (with 100 mM KCl and 1 mM EDTA) at the desired pH (3.7−6.1). The resulting solutions were incubated at 37 °C for 72 h without stirring or agitation before they were vortexed to equally distribute the fibrils, if present. The extent of fibril formation was monitored by an optical density (OD) measurement (turbidity) at 400 nm. The OD detects both fibrils and aggregates. Numerous experiments in this lab have shown that supplementary amyloid detection methods such as thioflavin T binding and Congo red binding are consistent with the OD measurement (*11*, *16*, *23*, *25*). The efficacy of small molecule inhibitors of fibril formation was also probed with these Cys10 modified TTRs. A pH 4.4 solution of TTR (3.6 μM) was incubated with known small molecule inhibitors, including *N*-(α,α,α-trifluoro-*m*-tolyl)anthranilic acid (**4**), 5-(2,4-difluorophenyl)salicyclic acid (**5**), and *N*-(3,5-dichlorophenyl)-anthranilic acid (**6**) [3.6 μM (see the Supporting Information) or 7.2 μM].

Kinetics of TTR Fibril Formation. A pH 4.4 solution of each TTR Cys10 variant (0.2 mg/mL, 3.6 μM tetramer) buffered with 50 mM acetate, 10 mM phosphate, 100 mM KCl, and 1 mM EDTA was incubated at 37 °C without stirring. One sample was utilized for each time point, which was vortexed right before analysis. The turbidity at 400 nm was measured as a function of time for up to 7 days. The rates of fibril formation at pH 4.8 were also measured in the same way. In fibril formation facilitated by MeOH addition, 107 μL of a TTR stock solution (1.5 mg/mL in pH 7.0 buffer) was added to 50% MeOH in 18 mM Tris buffer (pH 7.0) containing 100 mM KCl and 1 mM EDTA to commence the amyloidogenesis time course. The turbidity at 400 nm was continuously monitored over the course of 4000 s (8000 s for S-sulfonated TTR) with stirring.

Urea-Mediated Unfolding of TTR Measured by Tryptophan Fluorescence. The 0.1 mg/mL (1.8 μM) TTR samples were incubated in varying concentrations of urea ranging from 0 to 8 M, buffered with 50 mM phosphate, 100 mM KCl, and 1 mM EDTA (pH 7.0) at 25 °C. Equilibrium was reached in 96 h, after which tryptophan fluorescence spectra were recorded between 310 and 410 nm with excitation at 295 nm. The fluorescence intensity ratio I^{355} and I^{335} was used as a structural probe as described previously (*25*).

Urea-Mediated Dissociation of TTR Measured by Resveratrol Binding. A resveratrol (3,5,4′-trihydroxystilbene) binding protocol was used to quantify the concentration of TTR tetramer as described previously (*17*). After incubation of each TTR Cys10 variant (0.1 mg/mL, 1.8 μM) for 96 h in urea solutions (0−8 M) at 25 °C, resveratrol binding curves were recorded by measuring the fluorescence intensity

[2] Identical results were obtained in the presence or absence of sodium sulfite. Incubation of sodium sulfite with WT TTR in the absence of sodium tetrathionate also affords S-sulfonated TTR but not in quantitative yield.

at 394 nm (I^{394}) of samples each containing 18 μM resveratrol. The fraction of TTR tetramer was calculated using standard curves.

Kinetics of Tetramer Dissociation as a Function of Urea Concentration. Because TTR monomer unfolding is 5−6 orders of magnitude faster than tetramer dissociation, the tetramer dissociation step can be measured by purposefully linking the quaternary structural changes to tertiary structural changes mediated by denaturation with urea concentrations in the unfolding post-transition region (*17*). The TTR samples (0.1 mg/mL, 1.8 μM) in various urea solutions (4−6 M, pH 7.0) buffered with 50 mM phosphate, 100 mM KCl, and 1 mM EDTA were incubated at 25 °C, and their tryptophan fluorescence (I^{355}/I^{335}) ratios were recorded over a time course of 190 h.

RESULTS

Thiol Conjugation Influences TTR Amyloid Fibril Formation. TTR can be converted to amyloid fibrils by acid-mediated partial denaturation. Acidification initiates rate-limiting tetramer dissociation and transforms the β-sheet sandwich into a partially unfolded monomer, which is able to misassemble into amyloid fibrils (*8*−*11*). The highest yield of amyloid fibril formation for the Cys10 conjugated TTRs occurs over the pH range of 4.2−4.8 (72 h). The pH dependency and extent of deposition of the majority of the variants were similar to those of WT TTR over the pH range of 4.2−4.6, with the exception of S-sulfonated TTR which is significantly less amyloidogenic (Figure 1a). Most importantly, the pH range for amyloidogenesis is shifted higher for TTR-Cys, TTR-GSH, and TTR-CysGly compared to that of WT TTR. This difference is dramatic at pH 4.8 and 5.0 where WT TTR amyloidogenicity is low in contrast to the high aggregation tendency of TTR-Cys, TTR-GSH, and TTR-CysGly. The enhanced amyloidogenicity of the Cys10 disulfide variants at higher pH is likely important as these pHs are readily accessible in human physiology. S-Sulfonated TTR is less amyloidogenic over the whole pH range than WT TTR. If the misfolding reaction is allowed to proceed for extended periods (168 h at pH 4.4), it is still clear that S-sulfonated TTR is less amyloidogenic than WT TTR (see below).

Previous studies demonstrate that high-affinity small molecule binding to the native state of WT TTR and its variants can substantially inhibit amyloid fibril formation because of thermodynamic and kinetic implications (*7*). Binding of at least 1 equiv and ideally 2 equiv of inhibitor to the two thyroxine binding sites within TTR stabilizes the native state of the TTR·I and TTR·I_2 complexes sufficiently that the activation barrier for tetramer dissociation (the rate-limiting step of fibril formation) becomes insurmountable under physiological and most denaturing conditions. The previously identified native state stabilizers **4**−**6** are also very good inhibitors against fibril formation for all the Cys10 TTR variants. The extent of fibril formation is reduced to <50% of that observed with the Cys10 variants of TTR when 1 equiv (3.6 μM) of inhibitor was added relative to TTR concentration (Figure 1S, Supporting Information), whereas increasing the binding stoichiometry by increasing the inhibitor concentration (7.2 μM) reduced the extent of fibril formation to less than 15% (Figure 1b).

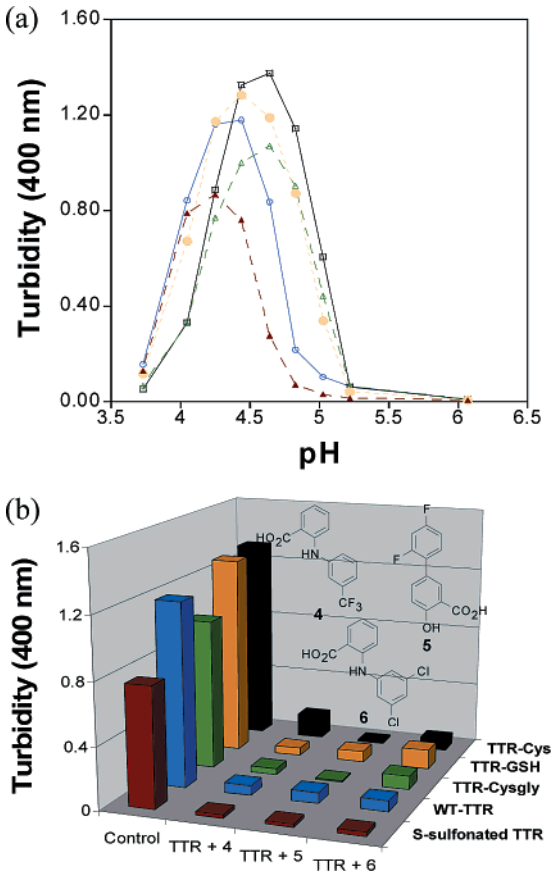


FIGURE 1: (a) pH dependence of WT TTR (blue, ○), TTR-Cys (black, □), TTR-GSH (yellow, ●), TTR-CysGly (green, △), and S-sulfonated TTR (red, ▲) aggregation including amyloid fibril formation (37 °C for 72 h) measured by turbidity at 400 nm. (b) Extent of acid-mediated TTR (3.6 μM) fibril formation (pH 4.4) in the presence of established TTR amyloid inhibitors **4**−**6** (7.2 μM).

Kinetics of Fibril Formation. The rates of fibril formation were studied with all the Cys10 TTR variants at pH 4.4 and 4.8 (37 °C) by utilizing turbidity measurements (400 nm) over a time course of 168 h which detects both fibrils and aggregates as they scatter light (Figure 2a,b). Each time course is a single exponential consistent with previous results demonstrating that tetramer dissociation is rate-limiting for fibril formation (*17*). The fibril formation rates of TTR-Cys, TTR-GSH, and TTR-CysGly are quite similar to that of WT at pH 4.4, unlike the situation with S-sulfonated TTR which forms amyloid more slowly (~2-fold) (Figure 2a). In contrast to the similar amyloidogenesis rates of WT, TTR-CysGly, TTR-GSH, and TTR-Cys at pH 4.4, the disulfide modified variants form amyloid much faster than WT TTR at pH 4.8 (Figure 2b), which could be very important from the perspective of amyloidogenesis under physiological accessible conditions. The amyloidogenesis rate of S-sulfonated TTR is also slower than that of WT TTR at pH 4.8.

MeOH addition likely lowers the dielectric constant and changes the water activity of the aqueous medium solvating TTR, also leading to partial denaturation and amyloid fibril formation (*24*). The rates of TTR amyloidogenesis mediated by methanol [50% (v/v)] treatment are dramatically faster than those mediated by acid denaturation (Figure 2c). The rank ordering of the fastest to the slowest Cys10 analogues is similar to the data at pH 4.4.

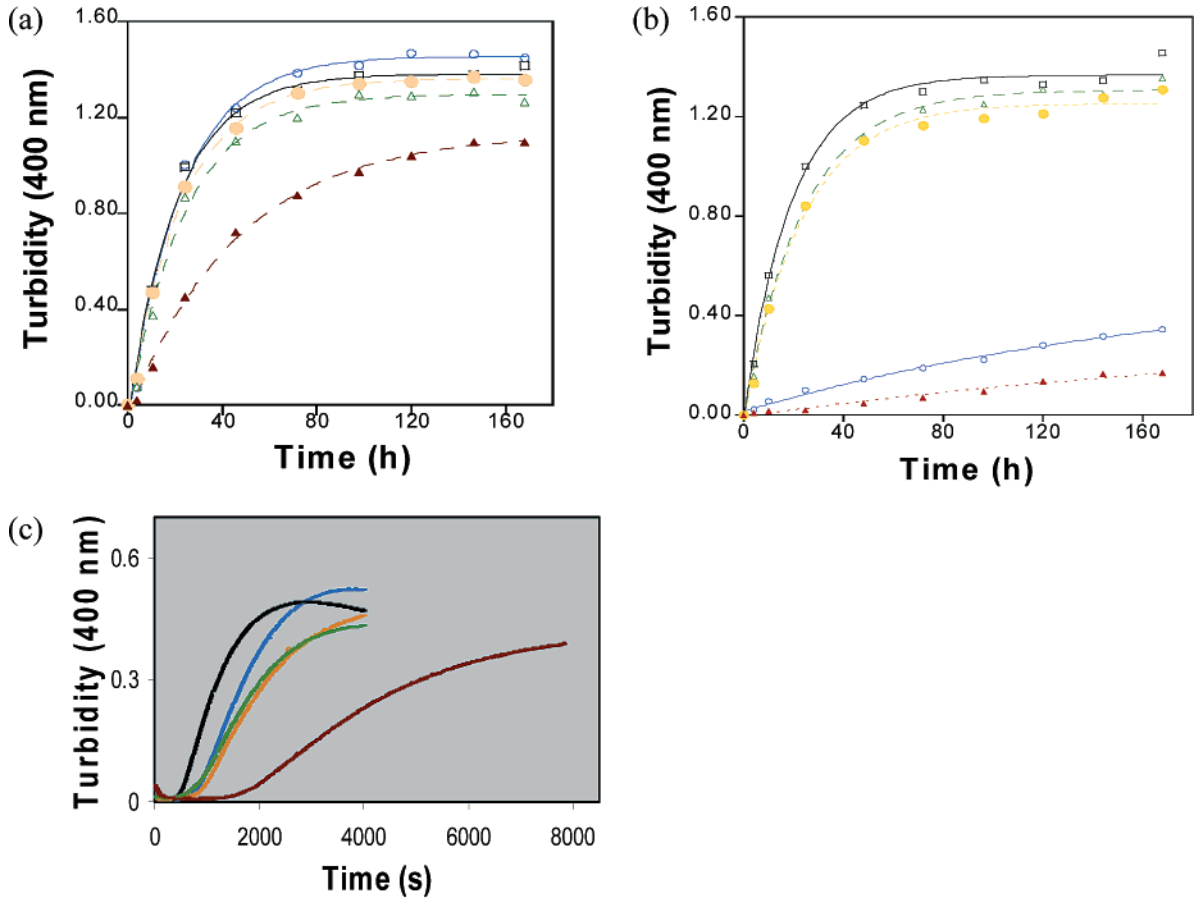


FIGURE 2: Rates of TTR amyloid fibril formation followed by turbidity at 400 nm (same color scheme as in Figure 1). (a) Fibril formation of WT TTR, TTR-Cys, TTR-GSH, TTR-CysGly, and S-sulfonated TTR initiated and mediated by a pH jump to 4.4. (b) Fibril formation rates of TTR and its variants at pH 4.8. (c) Rates of TTR fibril formation initiated and mediated by MeOH-induced denaturation.

The Cys10 TTR Disulfide Analogues Are Less Stable than WT and S-Sulfonated TTR. As in amyloidogenesis, the urea denaturation of TTR requires tetramer dissociation before monomer unfolding (*11*, *16*, *25*). Direct TTR tetramer denaturation in urea is not possible. The small molecule resveratrol increases its fluorescent quantum yield when it binds to the TTR tetramer, but does not bind to the monomer; hence, the fraction of tetramer of each TTR isoform as a function of denaturant can be estimated from calibration curves for resveratrol fluorescence as a function of tetramer concentration (Figure 2S, Supporting Information) (*17*). Tryptophan fluorescence as a function of urea concentration is used to follow the TTR tertiary structural changes, which can only occur after subunit dissociation.

In all cases, the quaternary structural transitions are nearly coincident with the unfolding curves mediated by increasing urea concentrations, suggesting that the quaternary and tertiary structural transitions are linked for all the Cys10 TTR variants tested herein (cf. panels a and b of Figure 3). This linkage makes it very difficult to extract $\Delta G_{tetramer}$ and $\Delta G_{unfolding}$. Nonetheless, qualitative information about protein stability can still be obtained by comparing denaturant concentrations required to reach 50% unfolding (C_m) and the slopes of the denaturation curves, the so-called *m* values. On the basis of C_m values, the tetramer stability of the mixed disulfide Cys10 modified TTRs was decreased for all the variants except for the S-sulfonated TTR homotetramer which exhibited a stability very similar to that of WT-TTR (Figure 3).

Urea-Mediated Tetramer Dissociation Rates of the Cys10 Variants Are Similar. TTR monomer unfolding detected by tryptophan fluorescene is approximately 500 000 times faster in urea than tetramer dissociation; therefore, it is possible to monitor the rate of dissociation by linking this process to tertiary structural changes (*17*). As depicted in Figure 4, the dissociation rate of each homotetrameric Cys10 variant is similar to the WT rate at pH 7. Rates were measured at several urea concentrations, in all cases exceeding a concentration that can support refolding to make this process irreversible (5.5 M urea data are depicted in Figure 4; time courses in 4.0, 4.5, 5.0, and 6.0 M urea are displayed in Figure 3S, Supporting Information). It is interesting that the S-sulfonated TTR homotetramer does not exhibit the substantially slowed tetramer dissociation kinetics in urea at pH 7, relative to those of WT TTR, unlike the situation under acidic denaturing conditions where this variant dissociates slowly, demonstrating that this variant behaves differently in acid and urea. This could be explained by the extra negative charge on S-sulfonated TTR because of its pK_a (<2). The extra charge would influence pH denaturation, which causes denaturation by perturbing the pK_a of the ionizable groups in the vicinity of Cys10, more than urea denaturation which increases the solubility of hydrophobic side chains.

DISCUSSION

All of the TTR FAP variants characterized to date exhibit decreased tetramer stabilities, enabling tetramer dissociation and partial monomer misfolding (required for amyloidogen-

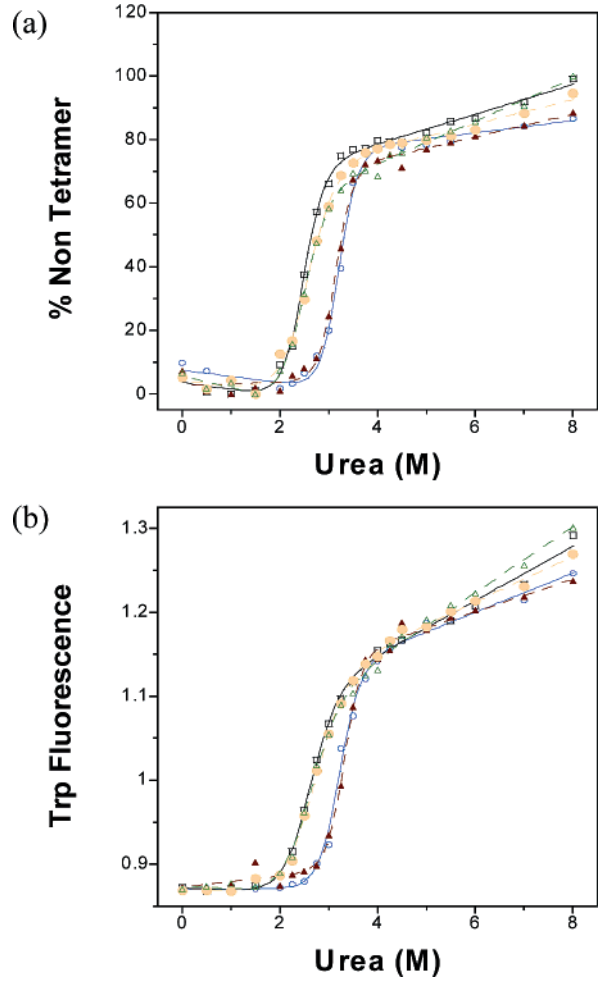


FIGURE 3: Stability of TTR as a function of urea concentration (pH 7; same color scheme as in Figure 1). (a) Tetramer dissociation measured by resveratrol fluorescence. Resveratrol binding to the tetramer but not the monomer results in a dramatic fluorescence quantum yield increase, allowing the concentration of the folded tetramer to be followed. (b) Monomer unfolding detected by tryptophan fluorescence, first requiring tetramer disssociation.

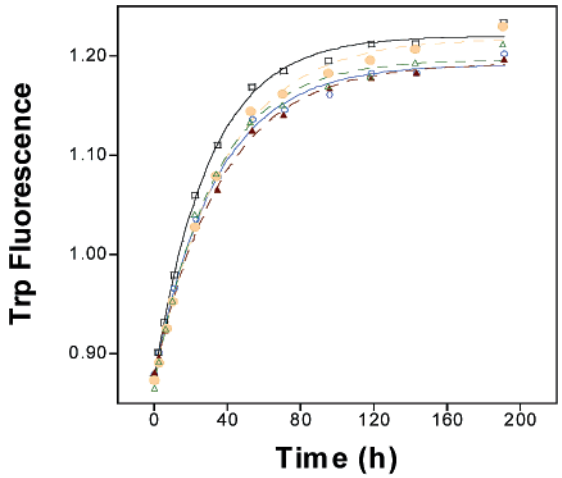


FIGURE 4: TTR tetramer dissociation rates of Cys10 modified TTR homotetramers in comparison to WT TTR measured by tryptophan fluorescence (5.5 M urea at pH 7; same color scheme as in Figure 1).

esis) under conditions in which the wild-type protein would be tetrameric and nonamyloidogenic. The hypothesis to be tested in these studies focuses on whether modifications of Cys10 are sufficiently destabilizing to facilitate WT amyloidogenesis leading to the onset of SSA. The most prevalent modification of TTR in human serum is the formation of a mixed disulfide between TTR Cys10 and the amino acid cysteine. The second most prevalent mixed disulfide is with cysteinylglycine; minor forms include a mixed disulfide with glutathione and S-sulfonated TTR (*12*−*15*).

Several studies have been carried out with Cys10 modified TTR samples derived from human subjects (*18*−*20*). These samples are heterogeneous with regard to the post-translational modifications at Cys10; in other words, the tetramers are comprised of modified and wild-type subunits. Using these samples, opposite conclusions have been drawn with regard to whether S-sulfonation of Cys10 protects or predisposes individuals to amyloidosis (*18*, *20*). Atland and colleagues have utilized an electrophoretic method to evaluate these heterogeneous samples and have concluded that the mixed disulfide forms of TTR are more amyloidogenic than reduced TTR (*19*). Furthermore, they conclude that S-sulfonated TTR produced by sulfite treatment reduces TTR amyloidogenicity substantially (*20*).

In this study, we have employed transthyretin tetramers comprised of subunits that are identically modified. Homotetramers of WT TTR, TTR-Cys, TTR-CysGly, TTR-GSH, and S-sulfonated TTR were subjected to acid- and MeOH-mediated amyloidosis conditions and urea denaturation in an effort to understand the relative amyloidogenicity and stability of the variants. The mixed disulfide forms of TTR, particularly TTR-Cys, are more amyloidogenic than WT TTR, substantially so under mildly acidic conditions (pH 4.8 and 5.0). Significantly, the rates of acid-mediated fibril formation of TTR-Cys, TTR-CysGly, and TTR-GSH are substantially faster at the higher end of the acidic pH range (pH 4.8 vs pH 4.4) relative to that of WT TTR, implying a lowered energetic barrier for fibril formation at physiological accessible conditions for these Cys10 mixed disulfide variants. S-Sulfonated TTR is less amyloidogenic than WT TTR over the entire pH range associated with amyloidogenicity. Its aggregation is also slower than that of WT under acidic conditions as well as amyloidogenesis mediated by MeOH addition. The urea denaturation curves reveal that mixed disulfide formation destabilizes the tetramer as discerned from linked tetramer dissociation and monomer unfolding curves in urea. S-Sulfonated TTR and WT TTR exhibit very similar stabilities in urea, unlike the modest difference observed under acidic conditions.

It is interesting that the most prevalent modification, cysteinylation of TTR, renders TTR substantially more amyloidogenic than WT TTR at pH 5, which may be a risk factor for the onset of SSA. It is important to learn more about the genomic and environmental variables that lead to this modification. It is potentially relevant that FAP patient serum shows an increased level of Cys10 conjugation, indicating a vulnerability to amyloidosis (*26*−*27*). It is likely that oxidative stress would increase the population of TTR subunits modified by mixed disulfide formation. Oxidative stress is thought to be important in several amyloid diseases (*28*−*30*).

It is reassuring to see that the small molecule native state stabilizers that prevent amyloidosis by increasing the dissociative activation energy are nearly equally effective for all the TTR variants.

In summary, mixed disulfide formation with Cys10 of TTR may be a risk factor for SSA. Statistically relevant modification data on TTR and the fibrils in SSA patients and age-matched controls will be required to judge the relevance of these in vitro studies.

ACKNOWLEDGMENT

We thank Prof. Evan T. Powers for helpful discussions.

SUPPORTING INFORMATION AVAILABLE

^{1}H and ^{13}C NMR spectra and characterization data of compounds **1**−**3**. Figure 1S depicts the extent of acid-mediated TTR fibril formation (pH 4.4) in the presence of small molecules (3.6 μM). Standard curves for resveratrol binding (18 μM) as a function of TTR concentration are depicted in Figure 2S. Tetramer dissociation rates of TTR in varying post-transition urea concentrations are shown in Figure 3S. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI030077A