Cys-10 Mixed Disulfide Modifications Exacerbate Transthyretin Familial Variant Amyloidogenicity: A Likely Explanation for Variable Clinical Expression of Amyloidosis and the Lack of Pathology in C10S/V30M Transgenic Mice?†

Qinghai Zhang and Jeffery W. Kelly*

Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road BCC265, La Jolla, California 92037

Received February 28, 2005; Revised Manuscript Received May 7, 2005

ABSTRACT: The marked variation in clinical expression and age of familial amyloid disease onset is not well understood. One possibility is that metabolite modification(s) of a disease-associated mutant protein can change the energetics and propensity for misfolding, influencing the disease course. Each subunit of the transthyretin (TTR) tetramer has a single Cys residue that can exist in the SH form or as a mixed disulfide with the amino acid Cys or the peptide glutathione or fragments of the latter. The stability and amyloidogenicity of the clinically most important TTR variants (V30M and V122I) in their SH oxidation state were compared with those of their mixed disulfide adducts. All the Cys-10 mixed disulfide conjugates exhibited substantially decreased protein stability (urea, pH 7) and a higher rate and extent of amyloidogenesis (slightly acidic conditions). We also investigated the amyloidogenicity and stability of a C10S/V30M TTR double mutant which lacks the ability to make mixed disulfides, but retains the disease-associated V30M mutation. Unlike V30M TTR, this double mutant is nonamyloidogenic in transgenic mice. Our in vitro data reveal that the C10S/V30M and V30M TTR homotetramers have identical amyloidogenicity and stability, implying that Cys-10 mixed disulfide formation enhances amyloidogenesis in V30M transgenic mice. Given the high proportion of TTR subunits having mixed disulfide modifications in human plasma (∼50%), and the data within demonstrating their increased amyloidogenicity, we submit that disulfide metabolite modifications have the potential to influence the course of amyloidoses, including TTR amyloidoses caused by mutations.

Specific mutations appear to cause early-onset protein misfolding diseases, including Alzheimer's, Parkinson's, and the familial amyloidoses (*1*, *2*). A single amino acid change within the sequence of a protein, even a conservative surface mutation, can dramatically change folding energetics so as to predispose an individual to misfolding, misassembly, and familial amyloidosis (*3*−*5*). It is also true that covalent protein modifications by abnormal metabolites (*6*, *7*), by spontaneous side chain alterations (*8*), or by more conventional posttranslational modifications (including phosphorylation or glycosylation) can alter folding energetics. However the role of normal and abnormal covalent modifications in protein misfolding diseases is often overlooked because it is difficult to demonstrate linkage to pathology (*6*, *7*, *9*).

Transthyretin (TTR)[1] is a 55 kDa tetrameric plasma protein that carries thyroxine and holo-retinol binding protein in the blood and cerebrospinal fluid. Under certain conditions, TTR undergoes rate-limiting tetramer dissociation, and subsequent partial monomer unfolding enabling misassembly into aggregates of varying morphology including fibrillar cross β-sheet quaternary structures known as amyloid (*10*−*14*). The aggregation of wild-type (WT) TTR is thought to cause senile systemic amyloidosis (*15*), while the misfolding and misassembly of one of over 100 point mutations of TTR is genetically and biochemically linked to familial amyloid polyneuropathy (FAP), familial amyloid cardiomyopathy (FAC), and central nervous system selective amyloidosis (CNSA) (*16*, *17*). V30M is the most prevalent FAP mutation in Japan, Sweden, and Portugal (*16*), whereas V122I is the most common variant associated with FAC in African-Americans (*17*); these two TTR variants will be the focus of this study.

Transthyretin is covalently modified both in healthy humans and in amyloidosis patients. Cys-10, the sole cysteine residue in each TTR subunit, is generally the site of modification (*18*−*21*). Of all possible Cys-10 modifications, mixed disulfide formation with the amino acid cysteine (cysteinylation) is the predominant modification, found on up to 50% of TTR subunits in plasma (*18*−*21*). Less prevalent modifications at Cys-10 include oxidation, *S*-sulfonation, glutathionylation, cysteinylglycylation, and homocysteinylation (*22*, *23*).

S-Sulfonation of Cys-10 is known to stabilize TTR inhibiting its amyloidogenesis (*24*, *25*). In contrast, we have

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

* To whom correspondence should be addressed. Phone: (858) 784-9880. Fax: (858) 784-9899. E-mail: jkelly@scripps.edu.

[1] Abbreviations: TTR, transthyretin; WT, wild-type; FAP, familial amyloid polyneuropathy; FAC, familial amyloid cardiomyopathy; V30M-Cys, cysteine linked by a disulfide to Cys-10 of Val30Met-transthyretin; V30M-GSH, glutathione linked by a disulfide to Cys-10 of Val30Met-transthyretin; V122I-Cys, cysteine linked by a disulfide to Cys-10 of Val122Ile-transthyretin; V122I-GSH, glutathione linked by a disulfide to Cys-10 of Val122Ile-transthyretin.

10.1021/bi050378f CCC: $30.25
Published on Web 06/04/2005

recently shown that mixed disulfide formation between the Cys-10 residue in WT TTR and cysteine or glutathione, or a fragment of the latter (cysteinylglycine), dramatically destabilizes TTR which accelerates TTR amyloidogenesis in vitro under mildly acidic conditions, where the unmodified form of the protein is natively folded and nonamyloidogenic (*26*). Deleterious mixed disulfide bond formation with the normal metabolite cysteine may explain the genesis of the sporadic TTR amyloid disease, senile systemic amyloidosis. Herein, we explore whether the incomplete clinical expression of familial TTR amyloidosis could also be explained by the extent of and nature of metabolite modifications (*26*−*31*).

The data of Takaota et al. suggest to us that modifications of the Cys-10 residue of TTR are important in amyloidogenesis in vivo, in the context of a V30M transgenic murine model of FAP (*32*). A comparison of transgenic mice expressing human WT, V30M, or the C10S/V30M TTR gene reveals amyloidosis only in the V30M mice after 21−24 months. The basis for the difference in amyloid propensity between the V30M and the Cys-free C10S/V30M mice was not explained. On the basis of our previous results (*26*), we envision that modification of Cys-10 by mixed disulfides could be an important determinant for amyloidogenesis, potentially explaining why the V30M mice, but not the C10S/V30M mice, exhibit amyloidogenesis. Herein, we report data strongly supporting this hypothesis. In particular, we show that the formation of a mixed disulfide between Cys-10 of variant TTR and either cysteine or glutathione results in hyperamyloidogenic derivatives of V30M and V122I TTR. These data suggest that the combination of a destabilizing mutation and mixed disulfide formation may be required to observe amyloid pathology, potentially explaining the incomplete clinical expression of the familial amyloid diseases.

MATERIALS AND METHODS

Expression and Purification of V30M, V122I, and C10S/V30M TTR. The genes encoding the TTR mutant proteins V30M, V122I, and C10S/V30M were cloned into pmmHα plasmids utilizing the QuickChange protocol (Stratagene, La Jolla, CA). Expression and purification of the homotetrameric proteins was performed as previously described (*33*). Briefly, respective plasmids were transformed into chemically competent Epicurian Gold *Escherichia coli* that were grown at 37 °C. Cells were harvested by centrifugation and lysed by sonication (3 × 3 min, 4 °C). The lysate was collected and treated with two ammonium sulfate salt cuts of 0−50% and 50−90% (w/v), the latter leading to TTR precipitation. The precipitate was dissolved in pH 8.0 solution buffered with 25 mM Tris-HCl (1 mM EDTA), and then dialyzed overnight (10 000 MW cutoff membrane). Transthyretin was initially purified using a Source Q anion exchange column (Pharmacia, Piscataway, NJ) utilizing a NaCl gradient of 200 to 450 mM at pH 8.0 (buffered with 25 mM Tris-HCl and 1 mM EDTA). The protein was further purified using a Superdex 75 gel filtration column (Pharmacia) eluting with a pH 7.0 solution buffered with 10 mM sodium phosphate (100 mM KCl and 1 mM EDTA). LC−MS analysis was performed to confirm the purity (>95%) and expected mass of the protein (monomer: V30M, MW = 13 921 Da; V122I, MW = 13 904 Da; C10S/V30M, MW = 13 905 Da). The purified proteins were stored in pH 7.0 buffer (10 mM sodium phosphate, 100 mM KCl and 1 mM EDTA) at 4 °C until experiments were performed.

Cysteinylation and Glutathionylation of V30M and V122I through Cys-10. Cysteinylated or glutathionylated V30M (V30M-Cys or V30M-GSH) and V122I (V122I-Cys or V122I-GSH) homotetramers were individually prepared using the reaction of *S*-(2-thiopyridyl)cysteine or *S*-(2-thiopyridyl)glutathione and the respective proteins as described previously (*26*). The TTR conjugates were subsequently purified by dialysis to remove low molecular weight organic molecules, and then by gel filtration using pH 7.0 buffer (10 mM sodium phosphate, 100 mM KCl, and 1 mM EDTA). The purity of the conjugates is above 95% based on HPLC integration. The masses of the V30M-Cys, V30M-GSH, V122I-Cys, and V122I-GSH subunits are 14 040, 14 227, 14 022, and 14 209 Da, respectively.

Amyloid Fibril Formation of TTR and Its Mixed Disulfides at Various pHs. A TTR stock solution (0.4 mg/mL; 7.2 $\mu M_{tetramer}$ in pH 7.0 buffer) was mixed with an equal volume of 100 mM acetate buffer at the desired pH (3.7−5.8), containing 100 mM KCl and 1 mM EDTA, to initiate amyloidogenesis. Phosphate buffer (100 mM sodium phosphate, 100 mM KCl, and 1 mM EDTA) was used in amyloidogenesis reactions having a final pH of ≥6. The resulting solutions were incubated at 37 °C for 72 h without agitation. Each sample was vortexed for 5 s just before measurement to equally suspend the precipitate (if any). The extent of amyloid formation was monitored by an optical density (OD) measurement (turbidity) at 400 nm on an HP 845x UV−visible spectrometer. That turbidity is a faithful metric of TTR amyloid fibril formation has been demonstrated previously by comparison with thioflavin T fluorescence (*14*, *34*−*37*).

Rates of TTR Amyloid Fibril Formation under Mildly Acidic Conditions. To compare the kinetics of amyloid fibril formation of the disease-associated variants in their SH form to their respective Cys-10 mixed disulfides, a pH was selected from the upper end of the pH range where TTR amyloid fibril formation was observed by turbidity (pH 5.8 for V30M series and pH 5.1 for V122I series). TTR variants and their mixed disulfides (0.2 mg/mL; 3.6 $\mu M_{tetramer}$) were incubated in acetate buffer at the desired pH as described above (37 °C) without stirring. The turbidity at 400 nm was measured as a function of time over a 7 day time course employing individual samples for each time point. The samples were vortexed just before each turbidity measurement. A comparison of the rates of amyloid fibril formation of V30M and C10S/V30M was performed at pH 5.1, close to the pH maximum for fibril formation.

Probing TTR Stability by Urea Denaturation. Tryptophan fluorescence shifts to longer wavelength and increases in intensity when TTR dissociates and unfolds in urea; hence it is used to monitor the protein stability, specifically the linked tetramer to monomer and monomer to unfolded monomer equilibria (*4*, *38*). Urea denaturation studies were carried out by incubating 0.1 mg/mL (1.8 $\mu M_{tetramer}$) TTR samples in urea concentrations ranging from 0 to 8 M, buffered with 50 mM phosphate, 100 mM KCl, 1mM EDTA (pH 7.0 at 25 °C). Tryptophan fluorescence spectra between 310 and 410 nm (excitation at 295 nm) were recorded to examine the tertiary structural changes after 96 h of incubation when equilibrium was reached. The fluorescence ratio

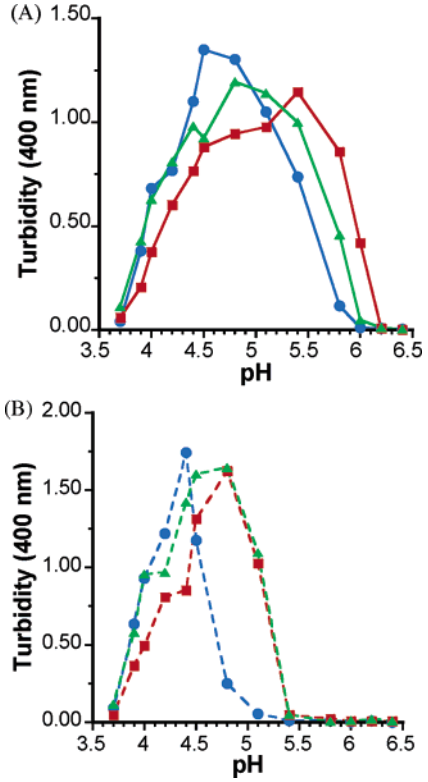

FIGURE 1: Acid-induced TTR aggregation, including amyloid fibril formation, over a pH range of 3.7−6.4 at 37 °C (72 h) without agitation was assessed for the thiol forms of the familial TTR variants, as well as for the mixed disulfide conjugates by turbidity at 400 nm. (A) pH-dependent aggregation of V30M (blue, ●) and its mixed disulfide variant homotetramers V30M-Cys (red, ■) and V30M-GSH (green, ▲). (B) pH-dependent aggregation of V122I (blue, ●) and its mixed disulfide variants V122I-Cys (red, ■) and V122I-GSH (green, ▲). The lines connecting data points facilitate comparisons.

at 355 and 335 nm was used as a structural probe as described previously (*38*). The midpoint from the urea denaturation curves (C_m), evaluated as described previously, was utilized to compare relative stabilities (*39*, *40*).

RESULTS

Familial Amyloid Variant Homotetramers with Cys-10 Mixed Disulfides Exhibit Elevated Amyloidogenicity at Mildly Acidic pH. Although the exact mechanism of TTR amyloidogenesis in humans remains unclear, it appears to require tetramer dissociation and partial monomer unfolding, affording misassembly competent intermediates (*10*−*14*). The rate of tetramer dissociation is hastened under acidic conditions, making the process of amyloidogenesis easily observable on a laboratory time scale; hence amyloid fibril formation was assessed over an acidic pH range by measuring the turbidity of solutions of V30M and V122I, as well as their Cys-10 mixed disulfide conjugates after incubation for 72 h. The Cys-10 mixed disulfide variants exhibit dramatically different pH profiles of aggregation in comparison to the unmodified (SH) proteins, the former being much more amyloidogenic near neutral pH (Figure 1). While the SH form of the V30M tetramer forms slightly more amyloid at pH 4.5, the mixed disulfide homotetrameric V30M adducts are much more amyloidogenic at pH 5.8 (Figure 1A). The enhanced endpoint amyloidogenicity at higher pH where the SH form of the protein is nonamyloidogenic is even more clear when the V122I SH form of the homotetramer is compared with its mixed disulfides (Figure 1B). For the WT data published previously, as well as the V30M and V122I data presented here, it is clear that mixed disulfide formation leads to increased amyloidogenicity under mildly acidic conditions for all TTR sequences examined thus far. Mixed disulfide formation does not appear to modify the morphology of the TTR aggregates produced, which are indistinguishable from the laterally associated fibrils resulting from the SH forms of V122I and V30M.

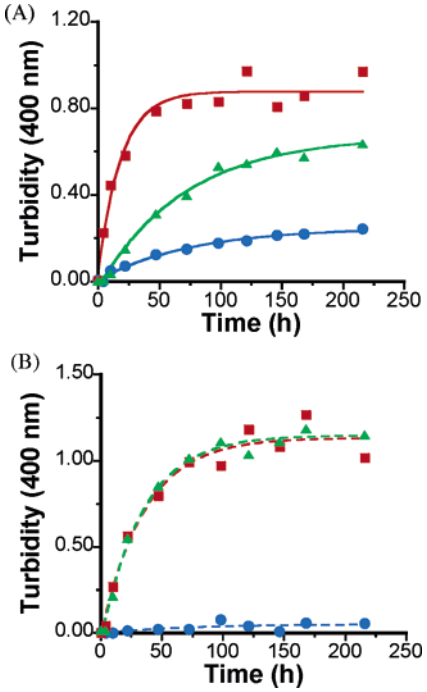

FIGURE 2: Comparison of the rates of amyloid formation of familial TTR variants in their Cys-10 SH form and their Cys-10 mixed disulfide form at slightly acidic pH. Aggregation was assessed by turbidity at 400 nm (same color and symbol scheme as in Figure 1). (A) Amyloidogenesis of V30M, V30M-Cys, and V30M-GSH was evaluated at pH 5.8, a pH at which all these sequences form amyloid on a convenient laboratory time scale. (B) Amyloid formation of V122I, V122I-Cys, and V122I-GSH was studied at 5.1, the highest pH at which all three sequences form amyloid on a convenient laboratory time scale.

The rate of amyloid fibril formation was also compared among V30M, V122I, and their respective Cys-10 mixed disulfides (Figure 2). The time course of V30M amyloidogenesis at pH 5.8 reveals that the V30M-Cys mixed disulfide has the lowest tetramer dissociation barrier and therefore the fastest rate of amyloidogenesis, followed by the glutathione mixed disulfide adduct which still is much faster than V30M-SH (Figure 2A). The kinetics of V122I amyloidogenesis (pH 5.1) follow the same trend in that tetramer dissociation and amyloidogenesis is sluggish for V122I-SH, whereas it is much faster for the mixed cysteine and glutathione disulfides, the latter exhibiting very similar time courses (Figure 2B).

The Cys-10 Mixed Disulfide Modification Renders TTR Less Stable. Tetramer dissociation has to occur to achieve urea-mediated TTR denaturation because urea is capable of denaturing the TTR monomer, but not the tetramer (*38*). TTR monomer unfolding, detected by tryptophan fluorescence changes described above, is about 500 000 times faster in urea than is tetramer dissociation; hence the monomer does not generally accumulate as a kinetic intermediate. Therefore,

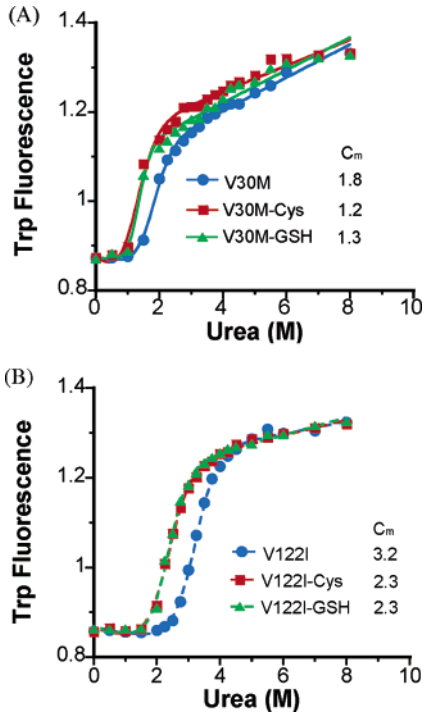

FIGURE 3: Comparison of the thermodynamic stability of reduced (SH form) familial TTR variants to the stability of Cys-10 mixed disulfide variants employing urea denaturation curves (pH 7, Figure 1 color and symbol scheme applies). Tryptophan fluorescence (I^{355}/I^{335}, λ_{ex} = 295 nm) was monitored as a function of urea concentration (0−8 M) to probe the linked quaternary and tertiary structural transitions. (A) Urea denaturation curves of V30M, V30M-Cys, and V30M-GSH. (B) Urea denaturation curves of V122I, V122I-Cys, and V122I-GSH. Tabulated C_m values were determined as described previously (*39*, *40*).

urea denaturation curves report on the tetramer to folded monomer and folded monomer to unfolded monomer linked equilibria, without being affected by monomer unfolding kinetics (*4*). Urea denaturation was used to interrogate the stability of the reduced SH form and mixed disulfide conjugates of V30M and V122I TTR by comparing urea denaturation midpoints (C_m), determined as described previously (*39*, *40*). All of the known pathogenic TTR variants exhibit decreased protein stability relative to WT TTR, including the V30M and V122I variants studied herein (*3*, *4*, *41*). It is clear that the mixed disulfide conjugates of V30M (Figure 3A) and V122I (Figure 3B) are substantially less stable than the SH form of the homotetramers. In fact, the mixed disulfide V30M TTR homotetramers (C_m = 1.2 for V30M-Cys, and C_m = 1.3 for V30M-GSH) are among the least stable TTR proteins characterized to date (*3*).

The C10S/V30M TTR Double Mutant Carrying the FAP Mutation but Lacking Cys-10 Exhibits Amyloidogenicity and Protein Stability Identical to That of V30M. Mice transgenic for the human C10S/V30M double mutant TTR do not present with amyloid disease, whereas mice transgenic for human V30M develop amyloidosis (*32*). A possible explanation is that the V30M mice produce amyloid because their subunits are subject to Cys-10 mixed disulfide modifications. Another plausible explanation is that the V30M and C10S/V30M proteins differ in stability and amyloidogenicity. Therefore, it is important to establish their biophysical properties using purified homotetramers in vitro. The pH-dependent amyloidogenicity of V30M and C10S/V30M

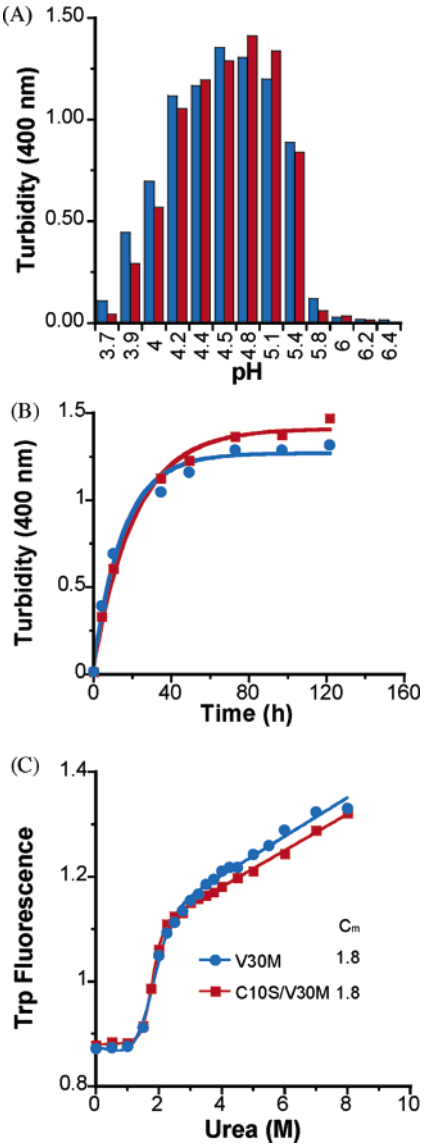

FIGURE 4: Comparison of the amyloidogenicity and thermodynamic stability of reduced (SH form) V30M (blue, ●) and C10S/V30M (red, ■) starting from homotetramers. (A) Endpoint analysis of pH-dependent aggregation of V30M and C10S/V30M at 37 °C (72 h) without agitation, assessed by turbidity at 400 nm. (B) Time course of V30M and C10S/V30M aggregation at pH 5.1 followed by turbidity (400 nm). (C) Urea unfolding curves of V30M and C10S/V30M probed by tryptophan fluorescence as described in Figure 3 (pH 7, 0−8 M urea). Tabulated C_m values were determined as described previously (*39*, *40*).

double mutant is identical taking into account the ±5% error (Figure 4A). In addition, their rates of fibril formation at pH 5.1 (Figure 4B) and their stability based on urea denaturation curves (Figure 4C) are also identical. Thus, from the perspective of biophysical experiments, the V30M homotetramer and the C10S/V30M homotetramer have identical stability and amyloidogenicity, making it likely that mixed disulfide formation plays a role in amyloidogenicity in the V30M transgenic mice.

DISCUSSION

The TTR proteins utilized in this in vitro study are purified homotetramers, whereas the tetramers found in the majority of heterozygous familial patients are composed of a statistical distribution of disease-associated and WT subunits (*36*). We

have previously and consistently demonstrated that one can observe the extremes of behavior using tetramers composed of a single subunit, and as a consequence studying homotetramers is very useful (*3*, *42*, *43*). The comparison of different homotetrameric TTR proteins herein illustrates unambiguously that mixed disulfide modification at Cys-10 affects TTR stability and amyloidogenicity.

We have shown that Cys-10 mixed disulfide formation shifts the amyloidogenic pH range toward neutral pH and increases the rate of amyloidogenesis at the upper end of the pH range for all the TTR sequences studied to date, including the familial amyloid disease variants V30M and V122I TTR evaluated herein, and the WT homotetramer studied previously (*26*). Cys-10 mixed disulfide formation decreases protein stability substantially at pH 7, beyond that conferred by the FAP and FAC mutations, making amyloidogenesis more feasible under physiological conditions, albeit on a time scale not practical for laboratory experiments. That the TTR tetramer dissociation barrier is also lowered at pH 7 by mixed disulfide formation can be inferred by the increased rate of amyloidogenesis observed herein under slightly acidic conditions.

We have shown previously that the Cys10Ala TTR homotetramer exhibits stability and amyloidogenicity comparable to those of the WT TTR homotetramer having a Cys residue at position 10 in the reduced (SH) state (*44*). Hence, we expected that the FAP variant V30M and the double mutant C10S/V30M would be similar in stability and amyloidogenicity. In fact, our experiments indicate that these two homotetramers have identical stability and amyloidogenicity. These data indicate that stability differences between these TTR proteins in mice do not explain why V30M mice present with amyloidogenesis whereas C10S/V30M mice remain amyloid free. In contrast, the data within fully support the hypothesis that the ability of the Cys-10 residues within the V30M homotetramer to make mixed disulfides enhances amyloidogenicity in humans and rodents. The elevated levels of Cys-10 mixed disulfides and related conjugates in symptomatic FAP patients relative to controls reported by Suhr et al. further support the idea that the risk for amyloidogenesis increases with covalent metabolite (mixed disulfide) modification (*45*, *46*).

The accumulation of Cys-10 mixed disulfides and related conjugates in symptomatic patients appears to be explained by an increase in oxidative stress, which has been suggested to be a risk factor in many amyloidoses including those involving TTR (*47*, *48*). The ratio of reduced and oxidized glutathione is a sensitive indicator of oxidative stress in vivo (*49*). Interestingly, glutathione and its thiol-containing fragments including cysteine and cysteinylglycine are all known to conjugate with TTR, implying that these modifications may originate from glutathione (*50*). Increasing oxidative stress in rats by administration of paraquat resulted in an increase of TTR amyloid deposition, relative to untreated rats, but no data on the redox status of Cys-10 were provided (*51*). Another case illustrating the importance of oxidative stress as a risk factor for amyloidosis comes from an autopsy report on an FAP patient with mutations in both TTR (V30M) and extracellular superoxide dismutase (Arg213Gly). This individual developed peripheral neuropathy at the early age of 25, revealing massive amyloid deposition at autopsy 13 years later (*52*).

A common phenomenon in familial TTR amyloidosis is incomplete clinical expression. For example, individuals that have the same disease-associated mutation, even individuals within a family, often exhibit very different disease courses or none at all (*30*, *31*). The penetrance of the common FAP type I V30M mutation can be as low as 2% in Sweden or as high as 80% in Portugal (*28*, *29*). These observations are difficult to explain on the basis of genetics alone, although we cannot rule out genetic modifiers not yet identified (*53*). We previously published evidence that metabolite modification could initiate protein misfolding in sporadic Alzheimer's disease and in the sporadic TTR amyloid disease, senile systemic amyloidosis (*6*, *26*), and provide compelling data within that these covalent modifications could also increase the risk of familial amyloid disease.

The results presented herein suggest that metabolite modification increases the misfolding propensity, possibly contributing to the amyloidosis observed within a subset of individuals having a given disease-associated TTR mutation. Altering Cys-10 with a protective modification such as sulfonation (*24*−*26*) or lowering oxidative stress in plasma may be useful therapeutic strategies to slow or prevent TTR amyloidosis.

ACKNOWLEDGMENT

We are grateful to R. Luke Wiseman and Maria T. Dendle for providing V30M, V122I, and C10S/V122I for this work. We thank Dr. Daryl Bosco for acquiring electron microscopy images, and Dr. Joel N. Buxbaum, Dr. Evan T. Powers, and Dr. Wim D'Haeze for valuable discussions.

SUPPORTING INFORMATION AVAILABLE

TTR amyloid sample preparation details for electron microscopy analysis of the aggregation products of V30M and V122I homotetramers and their mixed disulfide variants. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Forloni, G., Terreni, L., Bertani, I., Fogliarino, S., Invernizzi, R., Assini, A., Ribizzi, G., Negro, A., Calabrese, E., Volonté, M. A., Mariani, C., Franceschi, M., Tabaton, M., and Bertoli, A. (2002) Protein misfolding in Alzheimer's and Parkinson's disease: genetics and molecular mechanisms, *Neurobiol. Aging 23*, 957−976.
2. Buxbaum, J. N., and Tagoe, C. E. (2000) The genetics of the amyloidoses, *Annu. Rev. Med. 51*, 543−569.
3. Yoshiki, S., Wiseman, R. L., Matteson, J., Hammarström, P., Miller, S. R., Balch, W. E., and Kelly, J. W. (2005) Biological and chemical basis for tissue-selective amyloid disease, *Cell 121*, 73−85.
4. Hammarström, P., Jiang, X., Hurshman, A. R., Powers, E. T., and Kelly, J. W. (2002) Sequence-dependent denaturation energetics: A major determinant in amyloid disease diversity, *Proc. Natl. Acad. Sci. U.S.A. 99*, 16427−16432.
5. Kelly, J. W. (1996) Alternative conformations of amyloidogenic proteins govern their behavior, *Curr. Opin. Struct. Biol. 6*, 11−7.
6. Zhang, Q., Powers, E. T., Nieva, J., Huff, M. E., Dendle, M. A., Bieschke, J., Glabe, C. G., Eschenmoser, A., Wentworth, P., Jr., Lerner, R. A., and Kelly, J. W. (2004) Metabolite-initiated protein misfolding may trigger Alzheimer's disease, *Proc. Natl. Acad. Sci. U.S.A. 101*, 4752−4757.
7. Brownlee, M. (1995) Advanced protein glycosylation in diabetes and aging, *Annu. Rev. Med. 46*, 223−234.

8. Nilsson, M. R., Driscoll, M., and Raleigh, D. P. (2002) Low levels of asparagine deamidation can have a dramatic effect on aggregation of amyloidogenic peptides: implications for the study of amyloid formation, *Protein Sci. 11*, 342−349.
9. Drewes, G. (2004) MARKing tau for tangles and toxicity, *Trends Biochem. Sci. 29*, 548−555.
10. Kelly, J. W. (1998) The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways, *Curr. Opin. Struct. Biol. 8*, 101−106.
11. Colon, W., and Kelly, J. W. (1992) Partial denaturation of transthyretin is sufficient for amyloid fibril formation in vitro, *Biochemistry 31*, 8654−8660.
12. Lai, Z., Colon, W., and Kelly, J. W. (1996) The acid-mediated denaturation pathway of transthyretin yields a conformational intermediate that can self-assemble into amyloid, *Biochemistry 35*, 6470−6482.
13. Liu, K., Cho, H. S., Lashuel, H. A., Kelly, J. W., and Wemmer, D. E. (2000) A glimpse of a possible amyloidogenic intermediate of transthyretin, *Nat. Struct. Biol. 7*, 754−757.
14. Jiang, X., Smith, C. S., Petrassi, H. M., Hammarström, P., White, J. T., Sacchettini, J. C., and Kelly, J. W. (2001) An engineered transthyretin monomer that is nonamyloidogenic, unless it is partially denatured, *Biochemistry 40*, 11442−11452.
15. Westermark, P., Sletten, K., Johansson, B., and Cornwell, G. G., 3rd. (1990) Fibril in senile systemic amyloidosis is derived from normal transthyretin, *Proc. Natl. Acad. Sci. U.S.A. 87*, 2843−2845.
16. Saraiva, M. J. (2001) Transthyretin mutations in hyperthyroxinemia and amyloid diseases, *Hum. Mutat. 17*, 493−503.
17. Jacobson, D. R., Pastore, R. D., Yaghoubian, R., Kane, I., Gallo, G., Buck, F. S., and Buxbaum, J. N. (1997) Variant-sequence transthyretin (isoleucine 122) in late-onset cardiac amyloidosis in black Americans, *N. Engl. J. Med. 336*, 466−473.
18. Ando, Y., Ohlsson, P.-I., Suhr, O., Nyhlin, N., Yamashita, T., Holmgren, G., Danielsson, Å., Sandgren, O., Uchino, M., and Ando, M. (1996) A new simple and rapid screening method for variant transthyretin-related amyloidosis, *Biochem. Biophys. Res. Commun. 228*, 480−483.
19. Ando, Y., Suhr, O., Yamashita, T., Ohlsson, P.-I., Holmgren, G., Obayashi, K., Terazaki, H., Mambule, C., Uchino, M., and Ando, M. (1997) Detection of different forms of variant transthyretin (Met30) in cerebrospinal fluid, *Neurosci. Lett. 238*, 123−126.
20. Kishikawa, M., Nakanishi, T., Miyazaki, A., Shimizu, A., Nakazato, M., Kangawa, K., and Matsuo, H. (1996) Simple detection of abnormal serum transthyretin from patients with familial amyloidotic polyneuropathy by high-performance liquid chromatography/electrospray ionization mass spectrometry using material precipitated with specific antiserum, *J. Mass Spectrom. 31*, 112−114.
21. Bergquist, J., Andersen, O., and Westman, A. (2000) Rapid method to characterize mutations in transthyretin in cerebrospinal fluid from familial amyloidotic polyneuropathy patients by use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Clin. Chem. 46*, 1293−1300.
22. Gales, L., Cardoso, I., Fayard, B., Quintanilha, A., Saraiva, M. J., and Damas, A. M. (2003) X-ray absorption spectroscopy reveals a substantial increase of sulfur oxidation in transthyretin (TTR) upon fibrillization, *J. Biol. Chem. 278*, 11654−11660.
23. Lim, A., Sengupta, S., McComb, M. E., Théberge, R., Wilson, W. G., Costello, C. E., and Jacobsen, D. W. (2003) In vitro and in vivo interactions of homocysteine with human plasma transthyretin, *J. Biol. Chem. 278*, 49707−49713.
24. Altland, K., and Winter, P. (1999) Potential treatment of transthyretin-type amyloidoses by sulfite, *Neurogenetics 2*, 183−188.
25. Altland, K., Winter, P., Saraiva, M. J. M., and Suhr, O. (2004) Sulfite and base for the treatment of familial amyloidotic polyneuropathy: two additive approaches to stabilize the conformation of human amyloidogenic transthyretin, *Neurogenetics 5*, 61−67.
26. Zhang, Q., and Kelly, J. W. (2003) Cys10 mixed disulfides make transthyretin more amyloidogenic under mildly acidic conditions, *Biochemistry 42*, 8756−8761.
27. Coelho, T. (1996) Familial amyloid polyneuropathy: new developments in genetics and treatment, *Curr. Opin. Neurol. 9*, 355−359.
28. Holmgren, G., Costa, P. M., Andersson, C., Asplund, K., Steen, L., Beckman, L., Nylander, P. O., Teixeira, A., Saraiva, M. J., and Costa, P. P. (1994) Geographical distribution of TTR met30 carriers in northern Sweden: discrepancy between carrier frequency and prevalence rate, *J. Med. Genet. 31*, 351−354.
29. Sousa, A., Coelho, T., Barros, J., and Sequeiros, J. (1995) Genetic epidemiology of familial amyloidotic polyneuropathy (FAP)-type I in Povoa do Varzim and Vila do Conde (north of Portugal), *Am. J. Med. Genet. 60*, 512−521.
30. Holmgren, G., Wikstrom, L., Lundgren, H. E., and Suhr, O. B. (2004) Discordant penetrance of the trait for familial amyloidotic polyneuropathy in two pairs of monozygotic twins, *J. Intern. Med. 256*, 453−456.
31. Holmgren, G., Ando, Y., Wikstrom, L., Rydh, A., and Suhr, O. (1997) Discordant symptoms in monozygotic twins with familial amyloidotic polyneuropathy (FAP) (TTR Met 30), *Amyloid 4*, 178−180.
32. Takaoka, Y., Ohta, M., Miyakawa, K., Nakamura, O., Suzuki, M., Takahashi, K., Yamamura, K., and Sakaki, Y. (2004) Cysteine 10 is a key residue in amyloidogenesis of human transthyretin Val30Met, *Am. J. Pathol. 164*, 337−345.
33. Lashuel, H. A., Wurth, C., Woo, L., and Kelly, J. W. (1999) The most pathogenic transthyretin variant, L55P, forms amyloid fibrils under acidic conditions and protofilaments under physiological conditions, *Biochemistry 38*, 13560−13573.
34. White, J. T., and Kelly, J. W. (2001) Support for the multigenic hypothesis of amyloidosis: the binding stoichiometry of retinol-binding protein, vitamin A, and thyroid hormone influences transthyretin amyloidogenicity in vitro, *Proc. Natl. Acad. Sci. U.S.A. 98*, 13019−13024.
35. Jiang, X., Buxbaum, J. N., and Kelly, J. W. (2001) The V122I cardiomyopathy variant of transthyretin increases the velocity of rate-limiting tetramer dissociation, resulting in accelerated amyloidosis, *Proc. Natl. Acad. Sci. U.S.A. 98*, 14943−14948.
36. Schneider, F., Hammarström, P., and Kelly, J. (2001) Transthyretin slowly exchanges subunits under physiological conditions: A convenient chromatographic method to study subunit exchange in oligomeric proteins, *Protein Sci. 10*, 1606−1613.
37. Hurshman, A. R., White, J. T., Powers, E. T., and Kelly, J. W. (2004) Transthyretin aggregation under partially denaturing conditions is a downhill polymerization, *Biochemistry 43*, 7365−7381.
38. Hammarström, P., Jiang, X., Deechongkit, S., and Kelly, J. (2001) Anion shielding of electrostatic repulsions in transthyretin modulates stability and amyloidosis: Insight into the chaotrope unfolding dichotomy, *Biochemistry 40*, 11453−11459.
39. Foss, T. R., Kelker, M. S., Wiseman, R. L., Wilson, I. A., and Kelly, J. W. (2005) Kinetic Stabilization of the native state by protein engineering: implications for inhibition of transthyretin amyloidogenesis, *J. Mol. Biol. 347*, 841−854.
40. Fersht, A. (1999) *Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding*, Vol. 1, W. H. Freeman and Company, New York.
41. McCutchen, S. L., Lai, Z., Miroy, G. J., Kelly, J. W., and Colon, W. (1995) Comparison of lethal and nonlethal transthyretin variants and their relationship to amyloid disease, *Biochemistry 34*, 13527−13536.
42. Hammarström, P., Wiseman, R., Powers, E., and Kelly, J. (2003) Prevention of transthyretin amyloid disease by changing protein misfolding energetics, *Science 299*, 713−716.
43. Hammarström, P., Schneider, F., and Kelly, J. W. (2001) Trans-suppression of misfolding in an amyloid disease, *Science 293*, 2459−2462.
44. McCutchen, S. L., and Kelly, J. W. (1993) Intermolecular disulfide linkages are not required for transthyretin amyloid fibril formation in vitro, *Biochem. Biophys. Res. Commun. 197*, 415−421.
45. Suhr, O. B., Ando, Y., Ohlsson, P.-I., Olofsson, A., Andersson, K., Lundgren, E., Ando, M., and Holmgren, G. (1998) Investigation into thiol conjugation of transthyretin in hereditary transthyretin amyloidosis, *Eur. J. Clin. Invest. 28*, 687−692.
46. Suhr, O. B., Svendsen, I. H., Ohlsson, P.-I., Lendoire, J., Trigo, P., Tashima, K., Ranløv, P. J., and Ando, Y. (1999) Impact of age and amyloidosis on thiol conjugation of transthyretin in hereditary transthyretin amyloidosis, *Amyloid 6*, 187−191.
47. Ando, Y., Nyhlin, N., Suhr, O., Holmgren, G., Uchida, K., el Sahly, M., Yamashita, T., Terasaki, H., Nakamura, M., Uchino, M., and Ando, M. (1997) Oxidative stress is found in amyloid deposits in systemic amyloidosis, *Biochem. Biophys. Res. Commun. 232*, 497−502.
48. Pratico, D. (2002) Oxidative imbalance and lipid peroxidation in Alzheimer's disease, *Drug Dev. Res. 56*, 446−451.
49. Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) Oxidized redox state of glutathione in the endoplasmic reticulum, *Science 257*, 1496−1502.

50. Pettersson, T. M., Carlstrom, A., Ehrenberg, A., and Jornvall, H. (1989) Transthyretin microheterogeneity and thyroxine binding are influenced by non-amino acid components and glutathione constituents, *Biochem. Biophys. Res. Commun. 158*, 341−347.
51. Tajiri, T., Ando, Y., Hata, K., Kamide, K., Hashimoto, M., Nakamura, M., Terazaki, H., Yamashita, T., Kai, H., Haraoka, K., Imasato, A., Takechi, K., Nakagawa, K., Okabe, H., and Ishizaki, T. (2002) Amyloid formation in rat transthyretin: effect of oxidative stress, *Clin. Chim. Acta 323*, 129−137.
52. Sakashita, N., Ando, Y., Marklund, S. L., Nilsson, P., Tashima, K., Yamashita, T., and Takahashi, K. (1998) Familial amyloidotic polyneuropathy type I with extracellular superoxide dismutase mutation: a case report, *Hum. Pathol. 29*, 1169−1172.
53. Soares, M. L., Coelho, T., Sousa, A., Batalov, S., Conceiçãdo, I., Sales-Luis, M. L., Ritchie, M. D., Williams, S. M., Nievergelt, C. M., Schork, N. J., Saraiva, M. J., and Buxbaum, J. N. (2005) Susceptibility and modifier genes in Portuguese transthyretin V30M amyloid polyneuropathy: complexity in a single-gene disease, *Hum. Mol. Genet. 14*, 543−553.

BI050378F