Comparison of Lethal and Nonlethal Transthyretin Variants and Their Relationship to Amyloid Disease†

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ABSTRACT: The role that transthyretin (TTR) mutations play in the amyloid disease familial amyloid polyneuropathy (FAP) has been probed by comparing the biophysical properties of several TTR variants as a function of pH. We have previously demonstrated that the partial acid denaturation of TTR is sufficient to effect amyloid fibril formation by self-assembly of a denaturation intermediate which appears to be monomeric. Earlier studies on the most pathogenic FAP variant known, Leu-55-Pro, revealed that this variant is much less stable toward acid denaturation than wild-type TTR, apparently explaining why this variant can form amyloid fibrils under mildly acidic conditions where wild-type TTR remains nonamyloidogenic. The hypothesis that FAP mutations destabilize the TTR tetramer in favor of a monomeric amyloidogenic intermediate under lysosomal (acidic) conditions is further supported by the data described here. We compare the acid stability and amyloidogenicity of the most prevalent FAP variant, Val-30-Met, along with the double mutant, Val-30-Met/Thr-119-Met, which serves to model the effects of these mutations in heterozygous patients where the mutations are in different subunits. In addition, we have characterized the Thr-119-Met TTR variant, which is a common nonpathogenic variant in the Portuguese population, to further investigate the role that this mutation plays in protecting individuals who also carry the Val-30-Met mutation against the classically severe FAP pathology. This biophysical study demonstrates that Val-30-Met TTR is significantly less stable toward acid denaturation and more amyloidogenic than wild-type TTR, which in turn is less stable and more amyloidogenic than Thr-119-Met TTR. Interestingly, the double mutant Val-30-Met/Thr-119-Met is very similar to wild-type TTR in terms of its stability toward acid denaturation and its amyloidogenicity. The data suggest that the Thr-119-Met mutation confers decreased amyloidogenicity by stabilizing tetrameric TTR toward acid denaturation. In addition, fluorescence studies monitoring the acid-mediated denaturation pathways of several TTR variants reveal that the majority exhibit a plateau in the relative fluorescence intensity over the amyloid-forming pH range, i.e., ca. pH 4.3−3.3. This intensity plateau suggests that the amyloidogenic intermediate(s) is (are) being observed over this pH range. The Thr-119-Met variant does not exhibit this plateau presumably because the amyloidogenic intermediate(s) do(es) not build up in concentration in this variant. The intermediate is undoubtedly forming in the Thr-119-Met variant, as it will form amyloid fibrils at high concentrations; however, the intermediate is only present at a low steady-state concentration which makes it difficult to detect.

The deposition of a normally soluble human protein into insoluble amyloid fibrils having a β-sheet quaternary structure appears to be the pathogenic determinant of amyloid disease (Benson, 1989; Benson & Wallace, 1989; Selkoe, 1990; Stone, 1990; Jacobson & Buxbaum, 1991; Sipe, 1992; Kelly & Lansbury, 1994). A comparison of the dozen known human amyloidogenic proteins reveals that there is little sequence or structural homology between these proteins, yet they can form amyloid fibrils *in vivo* which appear to have a similar structure (Kelly & Lansbury, 1994). We propose that these nonhomologous amyloidogenic proteins may be able to adopt a similar partially denatured conformation (amyloidogenic conformation) under acidic conditions (or similar conditions *in vivo*), facilitating self-assembly of the rearranged protein into amyloid fibrils. We have focused on the amyloid disease familial amyloid polyneuropathy (FAP),[1] which is an autosomal dominant disorder characterized by peripheral nerve damage and organ dysfunction. This disease is putatively caused by variant transthyretin (TTR) amyloid fibril deposition (Benson, 1989). The amyloid deposits in FAP patients are composed predominantly of 1 of over 40 single-site variants of transthyretin (Saraiva, 1995; Benson, 1989; Benson & Wallace 1989; Saraiva et al., 1983, 1984; Sipe, 1992) even though FAP patients are generally heterozygous, with approximately equal amounts of wild-type and variant TTR expressed. Typically, FAP patients exhibit symptoms around 30 years of age with death occurring within 1−2 decades; however, the age of disease

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[1] Abbreviations: TTR, transthyretin; FAP, familial amyloid polyneuropathy; SSA, senile systemic amyloidosis; Z 3-14, *N*-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography.

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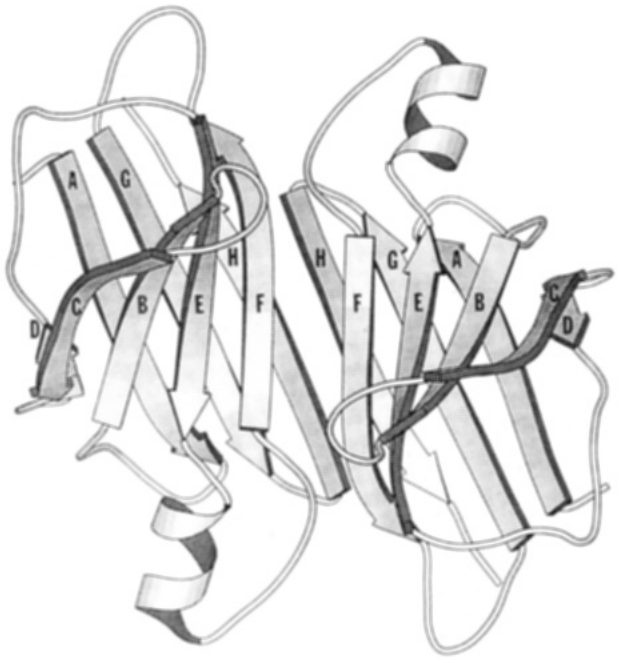

FIGURE 1: Ribbon diagram of TTR dimer generated from the coordinates of Blake et al. (1978) using the program Molscript (Kraulis, 1991).

onset, the duration of the disease, and the organ systems affected differ depending on the specific TTR variant composing the fibrils. Wild-type TTR fibrils have also been implicated as the causative agent in the geriatric amyloid disease, senile systemic amyloidosis (SSA), affecting to some extent 25% of the population over 80 years of age (Benson, 1989). The deposition of wild-type amyloid fibrils is generally benign; however, in certain individuals amyloid deposition in the heart causes significant problems. The fact that both wild-type and variant TTR can form amyloid *in vivo* suggests a common mechanism for the conversion of soluble TTR into insoluble TTR amyloid. A critical question to be answered is how the FAP mutations predispose transthyretin to amyloid fibril formation *in vivo*.

Transthyretin is a tetrameric human plasma protein known to transport thyroxine and retinol, the latter via the intermediacy of a complex with the retinol binding protein (Yen et al., 1990; Nilsson et al., 1975; Raz et al., 1970; van Jaarsveld et al., 1973, Monaco et al., 1995). The X-ray crystal structure of wild-type TTR reveals that each monomer is folded into a β-sheet sandwich which self-associates via intermolecular β-sheet formation to form an extended dimeric β-sheet sandwich Figure 1. Two dimers self-associate in a face-to-face manner, held slightly apart by loops projecting from the edge of the sheets such that the 55 kDa tetramer afforded has a central channel where thyroxine is known to bind (Blake et al., 1978, 1974; Wojtczak et al., 1992; Hamilton et al., 1993). Despite the pathological consequences known to be associated with the most prevalent FAP variant, Val-30-Met, recent reports of the X-ray crystal structure for this variant reveal only slight perturbations in the tertiary and quaternary structures relative to wild-type TTR (Terry et al., 1993; Hamilton et al., 1992). The subtle changes between the Val-30-Met variant and the wild-type TTR structure are in the location of the Met for Val mutation, where a slight increase in the sheet-to-sheet separation within

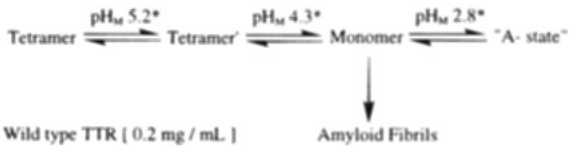

FIGURE 2: Summary of the TTR denaturation pathway which yields an amyloidogenic intermediate that can partition between two pathways: amyloid formation or further denaturation.

the monomer is reported. This result strongly suggests that the conservative FAP mutations do not significantly alter the structure of tetrameric TTR, consistent with the effects of conservative mutations on the three-dimensional structure of other proteins (Perutz & Lehmann, 1968; Hecht et al., 1983; Shortle & Lin, 1985; Alber et al., 1988; Bowie et al., 1990).

The hypothesis that a conformational change is required for transthyretin amyloid fibril formation is supported by previous studies showing that partial acid denaturation of wild-type tetrameric transthyretin is sufficient to produce a structured monomeric intermediate of TTR (amyloidogenic intermediate) which self-assembles into amyloid fibrils *in vitro* (Colon & Kelly, 1992) (Figure 2). Transthyretin acid denaturation and amyloid fibril formation are competitive processes over the pH range which supports amyloid formation, which is slightly different for each FAP variant (Colon & Kelly, 1992; McCutchen et al., 1993). The monomeric amyloidogenic intermediate appears to retain most of its native secondary and some of its native tertiary structure; however, it does structurally rearrange such that it has high propensity to self-associate. Further denaturation of the amyloidogenic intermediate, accomplished by lowering the pH even further, renders TTR incapable of amyloid formation (Figure 2). Denaturation of TTR can be studied in the absence of amyloid fibril formation employing the zwitterionic detergent Z 3-14, which inhibits amyloid fibril formation presumably by reversibly binding the amyloidogenic intermediate. Alternatively, TTR denaturation can be followed by fluorescence employing concentrations below the critical concentration, i.e., the concentration required for intermolecular self-assembly of the amyloidogenic intermediate (*vide infra*) (Andreu & Timasheff, 1986).

Previous studies involving characterization of wild-type TTR and the FAP variant Leu-55-Pro, known to cause disease symptoms as early as the second decade of life, reveal that this FAP variant is significantly less stable than wild-type TTR with regard to acid denaturation as monitored by the pH dependency of the tetramer to monomer transition (Colon & Kelly, 1992; McCutchen et al., 1993). When subjected to acid denaturation, the Leu-55-Pro TTR variant loses native quaternary structure in favor of the putative monomeric amyloid intermediate over a full pH unit higher than wild-type TTR, which is significantly more resistant to acid denaturation and amyloid fibril formation. In addition, the Leu-55-Pro variant is capable of forming amyloid fibrils under mildly acidic conditions (at lysosomal pH) and at low concentrations where wild-type TTR is stable and nonamyloidogenic. On the basis of these observations, we proposed that the amyloidogenic mutations decrease the acid stability and/or alter the denaturation pathway of TTR, thereby increasing their amyloidogenicity (Colon, 1993; McCutchen et al., 1993). To probe the generality of this observation, we have characterized three other variants of TTR in this study.

The acid stability, denaturation pathway, and amyloidogenicity of the FAP-associated Val-30-Met variant, reported to be the most prevalent FAP-causing variant in Portugal (Saraiva et al., 1983, 1984), were compared to wild-type TTR and a Val-30-Met/Thr-119-Met double mutant which serves as a model to evaluate the contributions of these mutations in heterozygous patients having these mutations in different monomeric subunits within the TTR tetramer. In addition, we evaluated the Thr-119-Met variant to further understand how this mutation protects against FAP in patients who are heterozygous for the Val-30-Met/Thr-119-Met mutation (Coelho et al., 1992). The data collected within allow us to compare the acid stabilities of several TTR tetramers. The order of acid stability from most stable to the least stable TTR variant is Thr-119-Met > Val-30-Met/Thr-119-Met > wild type > Val-30-Met ≫ Leu-55-Pro. Importantly, these data correlate with the severity of the amyloid pathology *in vivo* where the wild-type protein is less pathogenic than the Val-30-Met protein, which is less pathogenic than the Leu-55-Pro protein as gauged by the average age of disease onset. The denaturation pathways of these TTR variants were also studied by fluorescence to begin to understand the effects that these mutations have on the denaturation pathway.

MATERIALS AND METHODS

Mutagenesis to obtain the Val-30-Met/Thr-119-Met TTR variant was effected by established PCR protocols using the overlap extension methodology (MacFerrin et al., 1990; Innis et al., 1990; Ho et al., 1989). The expression systems for the single mutants, Val-30-Met and Thr-119-Met, were a kind gift from Professor Y. Sakaki, Institute of Medical Sciences, University of Tokyo, Japan. Two overlapping primers were used to introduce the Val-30-Met mutation into the Thr-119-Met expression system to produce the double mutant Val-30-Met/Thr-119-Met. The sequences of the primers are shown where the mismatch giving rise to the mutation is underlined in each primer: 5′-GCCATG CAT GTG TTC AGA-3′ and 5′-TCT GAA CAC ATG CAT GGC-3′. An expression cassette for the double mutant was produced and subcloned into the pKEN vector as previously described (McCutchen et al., 1993). Sequencing was carried out on double-stranded denatured DNA using five custom-made oligonucleotides which overlap and are complementary to the sequence of TTR. Sequencing of the PCR product, i.e., the expression cassette, before cloning into pKEN and sequencing after cloning into pKEN followed by transformation into MC 4100 *Escherichia coli* reveal a recurring host-induced mutation in the *ompA* leader sequence.[2] Wild-type, Val-30-Met, Val-30-Met/Thr-119-Met, and Thr-119-Met TTR were isolated from recombinant sources and purified (Colon & Kelly, 1992; McCutchen et al., 1993). Purification involves liberating TTR and other periplasmic proteins from the periplasmic space of *E. coli* through osmotic shock followed by a 5-fold concentration. A 60−85% ammonium sulfate cut is then subjected to DEAE ion exchange chromatography, giving >90% purity. Repeated ion exchange chromatography, either by gravity chromatography or by HPLC methods, was used to further purify TTR to >95% purity for acid denaturation experiments and to >98% purity for fluorescence experiments. The zwitterionic detergent Z 3-14 (amyloid inhibitor) was purchased from Calbiochem, and fresh solutions were made daily. All other reagents were obtained from Sigma or Fisher Scientific and were of the highest purity commercially available.

Monitoring pH-Induced Denaturation of TTR by SDS−PAGE. The SDS−PAGE method employed to evaluate the tetramer to monomer equilibrium takes advantage of earlier observations that the zwitterionic detergent Z 3-14, at concentrations slightly above the critical micelle concentration, inhibits amyloid fibril formation *in vitro* and facilitates refolding of acid-denatured TTR without aggregation (Colon & Kelly, 1992). Transthyretin samples, 0.2 mg/mL, were incubated at the desired pH using 50 mM phosphate- or acetate-buffered 100 mM KCl, both in the presence and in the absence of 0.075−0.1 mg/mL Z 3-14. After 40 h of incubation at 25 °C, additional Z 3-14 was added to increase the detergent concentration to 0.5 mg/mL, and the samples were immediately neutralized with 0.6 M phosphate buffer (containing 0.5 mg/mL Z 3-14) to obtain a final pH above 6.5. Neutralization-induced reconstitution of acid-denatured TTR is inhibited by concentrations of Z 3-14 ≥0.5 mg/mL, such that addition of sufficient Z 3-14 arrests the TTR quaternary structural forms present at a given pH. After neutralization, the samples were mixed with an equal volume of 4% SDS sample buffer at ambient temperature and loaded onto an SDS−PAGE gel without boiling. Under these conditions, the monomer present at equilibrium cannot undergo reconstitution to the tetramer during the neutralization step nor can the tetramer dissociate to monomer under SDS−PAGE conditions. As such, the relative amount of tetramer (which runs as dimer) and monomer present at each pH can be measured quantitatively from SDS−PAGE gels through densitometry (Figure 3). Glutaraldehyde cross-linking controls previously carried out on the wild-type TTR have also been carried out with the Leu-55-Pro and Val-30-Met variants, demonstrating the validity of this methodology for evaluating the pH_m of the variants (Colon & Kelly, 1992). In addition, preliminary analytical ultracentrifugation experiments performed on wild-typeTTR as a function of pH also validate this approach and will be reported in due course.

Monitoring Acid-Induced Denaturation/Dissociation by Fluorescence Spectroscopy. A 20 μL aliquot of a 1.5 mg/mL TTR stock solution was added to 2.98 mL of 50 mM phosphate- or acetate-buffered 100 mM KCl at the desired pH, affording a 0.01 mg/mL TTR solution for fluorescence experiments. The buffers were filtered through a 0.2 μm filter, the protein stock solution was centrifuged at 13K for 10 min, and the solution was carefully removed from the pellet for spectroscopic studies. The pH range evaluated was from pH 7.5 to 1.5, where HCl solution was used rather than a buffer for the lower pH data. TTR concentrations were determined by UV spectroscopy assuming that wild-type, Val-30-Met, Val-30-Met/Thr-119-Met, and Thr-119-Met TTR have approximately equal molar extinction coefficients (TTR's molar extinction coefficient = $7.76 \times 10^4\ M^{-1}\ cm^{-1}$) (van Jaarsveld et al., 1973). The TTR variants characterized and reported here do not involve changes in the aromatic

[2] We have characterized a recurrent mutation of Ala to Arg in the *ompA* leader sequence at position −17 (Furuya et al., 1991) which is not present in the PCR product prior to *E. coli* transformation. Since the *ompA* leader sequence is cleaved from TTR in the periplasmic space, this mutation does not affect the sequence of TTR. Recombinant expression levels of TTR variants using the pKNTR expression system (McCutchen et al., 1993) have been difficult to reproduce most likely owing to this mutation. To circumvent this problem, we freshly transform *E. coli* prior to each overexpression. Verdine has also reported loss of overexpression through mutational processes (Ezaz-Nikpay et al., 1994).

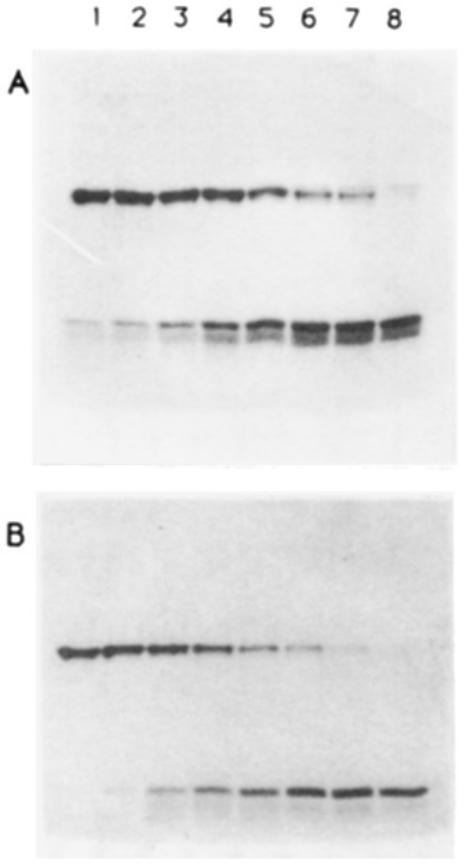

FIGURE 3: Photograph of SDS−PAGE gels from wild-type and Val-30-Met TTR acid denaturation experiments showing tetramer to monomer transitions as a function of decreasing pH. Gels were scanned by densitometry to quantify the amount of tetramer and monomer present at each pH. Samples were incubated for 40 h in the presence of 0.1 mg/mL Z 3-14, manipulated as described under Materials and Methods, and subjected to SDS−PAGE. Under these conditions, tetrameric TTR dissociates to dimer. (A) Lanes 1−8 represent wild-type TTR incubated at pH 4.25, 4.10, 3.95, 3.80, 3.65, 3.55, 3.45, and 3.00, respectively. (B) Lanes 1−8 represent Val-30-Met TTR incubated at pH 4.85, 4.65, 4.25, 4.10, 3.95, 3.80, 3.65, and 3.30, respectively.

amino acid composition, and as such, molar extinction coefficients and protein concentrations can be determined by the UV absorbance at 280 nm within ±5% (Gil & von Hippel, 1989). The 0.01 mg/mL TTR solutions were incubated at 25 °C for 40−44 h, and emission fluorescence spectra were recorded on an SLM 8000 fluorescence spectrometer at 25 °C. The conformational changes effected by acid denaturation were followed by two different fluorescence experiments. In one case, the excitation wavelength was 278 nm and emission was recorded at 334 nm to measure tyrosine to tryptophan energy transfer followed by tryptophan fluorescence. In the second case, tryptophan fluorescence was measured directly at 334 nm by excitation of tryptophan at 295 nm. A 1 cm quartz cell was used for all experiments. The excitation and emission slit widths were set at 4 nm and 8 nm, respectively. In all cases, the TTR concentrations were evaluated to minimize the inner filter effect.

Amyloid Fibril Formation and Electron Microscopy. Amyloid fibrils were formed from wild-type and variant TTR. Wild-type fibrils were formed under conditions previously reported (pH 4.3, 0.1 mg/mL TTR) (Colon & Kelly, 1992; McCutchen et al., 1993). Fibrils for Val-30-Met TTR were formed at pH 5.0 using a 0.02 mg/mL TTR solution incubated overnight at room temperature. The

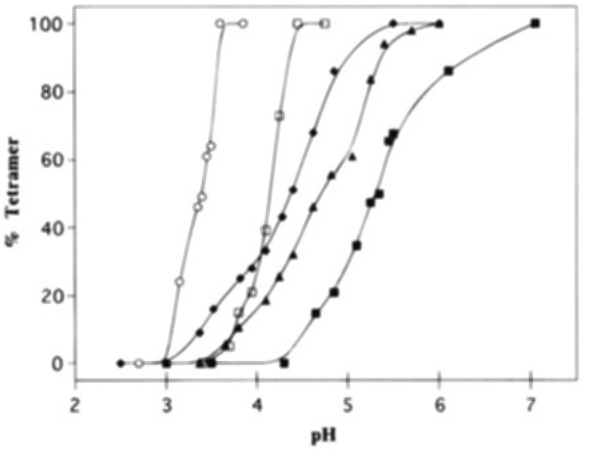

FIGURE 4: Tetramer to monomer transitions as a function of pH for Thr-119-Met (○), Val-30-Met/Thr-119-Met (□), wild type (♦), and Val-30-Met TTR (▲) as well as Leu-55-Pro TTR (■) reported previously (McCutchen et al., 1993). Samples were incubated at the designated pH for 40 h without Z 3-14 and manipulated as described under Materials and Methods. SDS−PAGE gels similar to those shown in Figure 3 were scanned by densitometry to measure the percentage of tetramer and monomer at each pH. The error in the pH_m values from these experiments is ±0.1 pH unit.

double mutant Val-30-Met/Thr-119-Met was converted into amyloid-like fibrils at pH 3.8 employing a 0.4 mg/mL TTR solution. Amyloid fibrils were formed from Thr-119-Met TTR at pH 3.8 using a 0.6 mg/mL TTR solution employing overnight incubation. Dilute suspensions of the amyloid fibrils were placed on carbon-coated copper grids, and allowed to stand for 2 min before the excess solution was removed. The grid was treated with fresh 1% uranyl acetate (pH ≈4.5) for 2 min. Excess staining solution was removed by filter paper blotting, affording negatively-contrasted TTR amyloid fibrils (Shirahama & Cohen, 1967). The fibrils were then inspected by electron microscopy using a Zeiss 10-C electron microscope as described previously (Colon & Kelly, 1992; McCutchen et al., 1993).

RESULTS

Monitoring pH-Induced Denaturation of TTR by SDS−PAGE. SDS−PAGE analysis of the quaternary structure of TTR as a function of pH shows that the tetramer to monomer transitions for wild-type and Val-30-Met TTR occur over the pH ranges of 4.4−3.0 and 4.8−3.3, respectively, in the presence of Z 3-14, indicating that Val-30-Met TTR is destabilized relative to wild-type TTR (Figure 3). We have shown that the Z 3-14 detergent stabilizes the quaternary structure of TTR by a mechanism that is under investigation (Lai and Kelly, unpublished results). The TTR quaternary structural stability analysis carried out in the absence of Z 3-14 reveals that the tetramer to monomer transition is observed over the pH range of 4.8−3.3 for wild-type TTR and 5.5−3.8 for Val-30-Met TTR, again indicating that the Val-30-Met variant is significantly destabilized relative to the wild-type protein (Figure 4). The tetramer to monomer transitions induced by acid denaturation for wild-type, Val-30-Met TTR, and other variants can be compared in Figure 4. The ranges and midpoints (pH_ms) of the tetramer to monomer transition both in the presence and in the absence of Z 3-14 are shown in Table 1. The transitions are generally shifted to a higher pH_m in the absence of Z 3-14, owing in large part to the stabilizing effect of Z 3-14 described above.

Table 1: pH Midpoints and Ranges for Tetramer to Monomer Transitions[a]

TTR	pH_m		pH ranges	
	with Z 3-14	no Z 3-14	with Z 3-14	no Z 3-14
Thr-119-Met	3.4	3.4	3.6−3.0	3.8−3.2
Val-30-Met/ Thr119-Met	3.8	4.1	4.2−3.5	4.6−3.7
wild type	3.8	4.4	4.4−3.0	4.8−3.3
Val-30-Met	4.2	4.7	4.8−3.3	5.5−3.8
Leu-55-Pro	4.4	5.4	5.7−3.9	6.1−4.0

[a] Determined by SDS−PAGE analysis (error ±0.1 pH unit).

Table 2: Fluorescence Wavelength Emission Maxima and Relative Intensities for Wild Type and Variants of TTR with Excitation at 295 nm

transthyretin protein	λ_{max} (nm), pH 7.5	relative[a] intensity	λ_{max} (nm), pH 2.0	relative[a] intensity
wild type	333	1.00	338	0.67
Val-30-Met	333	1.18	338	0.71
Thr-119-Met	335	1.37	335	0.86
Val-30-Met/Thr-119-Met	334	1.27	337	0.71

[a] Relative to a fluorescence intensity of 1 for wild-type TTR.

The observed shift in the pH_m of wild-type and Val-30-Met TTR in the absence of Z 3-14 inhibitor may also be due, in part, to amyloid fibril formation which can occur under acidic conditions in the absence of Z 3-14, but may not be detectable because the amyloid fibrils are not large enough to scatter UV light. If soluble amyloid fibrils were forming at a given pH, it would overrepresent the monomer detected at that pH by SDS−PAGE since it is known that TTR amyloid dissociates to monomer under SDS−PAGE conditions (Colon, 1993; McCutchen & Kelly, 1993). Importantly, both in the presence and in the absence of Z 3-14, the Val-30-Met tetramer is significantly destabilized with respect to acid denaturation when compared to wild-type TTR.

The tetramer to monomer transitions for the Thr-119-Met and Val-30-Met/Thr-119-Met variants of TTR occur between pH 3.6−3.0 and 4.2−3.5 in the presence of Z 3-14, and between pH 3.8−3.2 and 4.6−3.7 in the absence of Z 3-14, respectively. Only tetramer and monomer are present above and below these pH ranges. The pH_m for each variant can be found in Table 1. Both of the Thr-119-Met-containing variants are more stable toward acid denaturation than wild-type TTR (Figure 4). Interestingly, the Z 3-14 stabilizing effect against acid denaturation observed for wild-type and Val-30-Met TTR is much less pronounced for the Val-30-Met/Thr-119-Met variant and is not observed in the case of the Thr-119-Met variant. This observation is not completely understood at the moment but seems to result from the Thr-119-Met mutation which appears to be resistant to the Z 3-14 stabilization effect. Variants containing the Thr-119-Met mutation, which are less amyloidogenic as ascertained by the *in vitro* amyloid fibril formation assay, exhibit acid stability equal to or greater than wild-type TTR, indicating that the Thr-119-Met mutation is capable of stabilizing TTR toward acid denaturation.

Monitoring Acid-Induced Denaturation/Dissociation by Fluorescence Spectroscopy. The wavelengths for maximum fluorescence emission of wild type and the three variants of TTR as a function of pH were determined and are shown in Table 2. For tyrosine to tryptophan energy transfer followed by tryptophan fluorescence (excitation at 278 nm) at pH 7.5, little or no change in the wavelength of maximum fluorescence intensity is observed when wild-type TTR is compared to the three variants. At pH 2.0, wild-type TTR exhibits a red shift of 3.0 nm when compared to the pH 7.5 spectrum, while the Val-30-Met , Val-30-Met/Thr-119-Met, and Thr-119-Met variants exhibit insignificant red shifts at pH 2. Comparison of the intrinsic tryptophan fluorescence (excitation at 295 nm) data at pH 7.5 with those at pH 2.0 reveals a red shift of 5 nm for wild-type TTR, a 5 nm red shift for Val-30-Met TTR, a 3 nm red shift for the double mutant, and no observable shift for Thr-119-Met TTR at pH 2 (Table 2). In summary, the data at both excitation wavelengths suggest that the Trp residues do not become significantly solvent-exposed over this pH range as the Trp bathochromic shift is small.

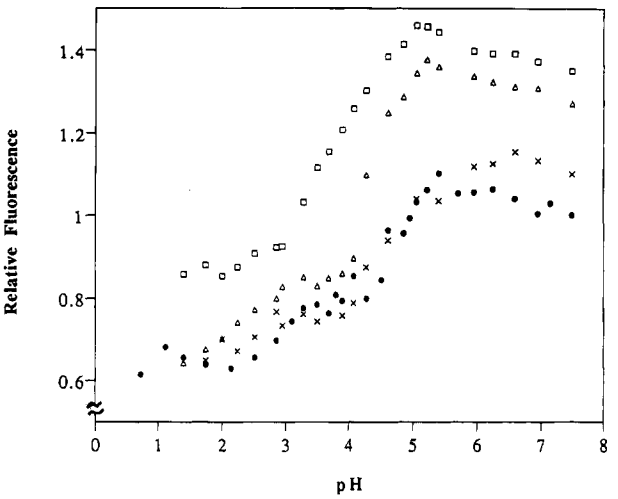

FIGURE 5: Acid denaturation of TTR and variants thereof monitored by changes in tryptophan fluorescence intensity (excitation at 295 nm). Samples were incubated at 25 °C for 40−44 h at pHs ranging from 7.5 to 1.5 in 50 mM phosphate-or acetate-buffered 100 mM KCl solution (HCl solution rather than a buffer was used for the low-pH experiments). Legend: wild type (●), Thr-119-Met (□), Val-30-Met (×), and Val-30-Met/Thr-119-Met TTR (△). The standard deviation of each fluorescence data point is <5% of the relative intensity which is an average of several trials.

The fluorescence emission data, summarized in Table 2, exhibit differences in intensity at the wavelength of maximum fluorescence. Exciting at 295 nm to directly measure the tryptophan fluorescence of the TTR solutions at pH 2.0 indicates that the fluorescence intensities of wild-type TTR, the Val-30-Met variant, and the double mutant are within ±3% of each other, while the fluorescence intensity of the Thr-119-Met variant is higher than that observed for wild-type TTR. At pH 7.5, the fluorescence intensity of Val-30-Met TTR is 18% higher than that of wild type, yet the overall tertiary structures are virtually identical (Terry et al., 1993; Hamilton et al., 1992). The increase in fluorescence intensity for the Thr-119-Met and Val-30-Met/Thr-119-Met variants is 37% and 27%, respectively, relative to wild type at pH 7.5. The tyrosine to tryptophan energy transfer followed by tryptophan fluorescence data exhibit similar features. Namely, the fluorescence intensity of the Thr-119-Met variant is significantly higher at both low and high pHs when compared with wild type TTR. In addition, the fluorescence intensity of the double mutant falls in between that of the Val-30-Met and Thr-119-Met TTR variants.

The acid denaturation curves of wild-type and the above-mentioned variants of TTR as monitored by fluorescence

are shown in Figure 5. Wild-type TTR exhibits a biphasic unfolding transition upon acid denaturation as measured by the change in tryptophan fluorescence (excitation at 295 nm). The fluorescence intensity increases slightly over the pH range 7.5−5.2, which may reflect a subtle rearrangement within the wild-type tetramer (Colon & Kelly, 1992). In the pH range of 5.2−4.2, a transition is observed which appears to represent a change in tertiary structure which may or may not be coupled to the tetramer to monomer transition. This transition accounts for approximately 50% of the total change in fluorescence intensity. A plateau region is observed from pH 4.3 to 3.3 with little fluctuation in fluorescence intensity, consistent with the formation of the amyloidogenic intermediate, and possibly other intermediates as well. The assignment of the plateau in the fluorescence spectra to the amyloidogenic intermediate(s) is supported by the observed amyloid fibril formation over this pH range at higher TTR concentrations. Over the pH range of 3.7−2.2, a second transition is observed which is thought to reflect the transition from a structured monomer to an "A-state"-like conformation (Goto et al., 1990). The A-state-like conformation is supported by its characteristic affinity for ANS, as well as TTR's native-like secondary structure and lack of tertiary structure structure at pH 2 as discerned from far- and near-UV CD spectroscopy, respectively (data not shown) (Colon, 1992; Goto & Fink, 1989; Goto et al., 1990; Baum et al., 1989; Jeng et al., 1990; Buchner et al., 1991; Fink et al., 1994).

For Val-30-Met TTR, small fluctuations in the fluorescence intensity are observed between pH 7.5 and 6.0 (Figure 5). The first transition begins at pH 6.0 and extends to pH 4.0, accounting for two-thirds of the total fluorescence change, most likely representing tertiary structural changes. A plateau region is seen from pH 4.0 to 3.0, most likely owing to the formation of the amyloidogenic intermediate(s), consistent with the observation that fibrils are formed over this pH range at higher Val-30-Met TTR concentrations. A second transition is observed from pH 3.0 extending to pH 1.5, assignable to the formation of the "A-state". A notable feature of the Val-30-Met fluorescence denaturation curve is that the wild-type and Val-30-Met proteins denature similarly (Figure 5), suggesting there are conformational changes affecting the environment of the tryptophan(s) which do not appear to be correlated with tetramer dissociation. This interpretation is supported by the SDS−PAGE experiments which indicate that the Val-30-Met TTR tetramer begins to dissociate 0.7 pH unit above the wild-type TTR tetramer at 0.2 mg/mL (the tetramer is expected to dissociate at an even higher pH at a concentration of 0.01 mg/mL, the concentration used for the fluorescence experiments). The similarity of the pH-dependent fluorescence denaturation curves for several TTR variants indicates that tertiary structural changes are responsible for the intensity changes which are not correlated explicitly with the tetramer to monomer transitions.

The fluorescence intensity of the Thr-119-Met variant increases slightly when the pH is decreased from pH 7.5 to 5.2, analogous to what is observed with the wild-type fluorescence denaturation curve. Unlike wild-type TTR, the Thr-119-Met TTR variant exhibits a more cooperative unfolding transition as monitored by fluorescence, indicating that the mutation not only stabilizes the TTR tetramer but also changes the folding pathway such that the plateau indicative of the formation of the amyloidogenic intermediate is not observed. This does not mean that the amyloidogenic intermediate is not formed, but it does suggest that the amyloidogenic intermediate is destabilized by the Thr-119-Met mutation, explaining its apparently low steady-state concentration. The more cooperative structural transition from tetramer to monomer (A-state) observed over the pH range of 5.2−3.0 by fluorescence, when considered in combination with the SDS−PAGE quaternary structural analysis, indicates that the Thr-119-Met mutation not only increases tetramer stability toward acid denaturation but also alters the denaturation pathway so as to destabilize the amyloidogenic intermediate.

The Val-30-Met/Thr-119-Met TTR denaturation curve shows characteristics similar to the wild-type fluorescence denaturation curve (Figure 5). There is a slight increase in fluorescence intensity over the pH range of 7.5−5.2. The first structural transition is observed between pH 5.2 and 4.0, accounting for two-thirds of the total fluorescence intensity change. A plateau region exists from pH 4.0 to 3.0, followed by a second transition below pH 3.0 which leads to the "A-state". The Thr-119-Met mutation seems to stabilize the Val-30-Met/Thr-119-Met tetramer such that this variant is less amyloidogenic than the Val-30-Met variant as a function of pH, but the Thr-119-Met variant does not prevent formation of the amyloidogenic intermediate, nor does it appear to significantly alter the tertiary structural changes occurring as a function of decreasing pH in the double mutant.

The denaturation curves derived from tyrosine to tryptophan energy transfer followed by tryptophan fluorescence are similar to the curves derived from the direct tryptophan fluorescence, i.e., Figure 5. The only notable difference appears between pH 4.0 and 3.0, where both the Val-30-Met and Val-30-Met/Thr-119-Met variants exhibit a slight decrease in the fluorescence intensity in the energy transfer fluorescence experiment (excitation at 278 nm, data not shown). Experiments in progress outlined in the Discussion should guide us in the proper interpretation of the fluorescence data.

Amyloid Fibril Formation and Electron Microscopy. Electron microscopy of the Val-30-Met amyloid fibrils produced at pH 5.0 (Figure 6) demonstrates that they are very similar to fibrils isolated from FAP patients, having the appropriate dimensions of approximately 70 Å in diameter by >1000 Å in length with a laterally associated twist (Puchtler et al., 1962; Glenner et al., 1974; Klunk et al., 1989). The Val-30-Met amyloid fibrils created *in vitro* appear to be more stable than the wild-type or Thr-119-Met amyloid fibrils. We were able to clearly see Val-30-Met amyloid fibrils on the carbon-coated copper grid several weeks after the sample was prepared. In contrast, amyloid fibrils derived from wild-type TTR were only stable for a few hours after the uranyl acetate-stained samples were prepared. Although amyloid fibril formation was suggested for the Val-30-Met/Thr-119-Met variant as discerned by congo red binding and a small but discernible red shift in the absorbance spectra of congo red when it is bound to amyloid fibrils (McCutchen & Kelly, 1993; Glenner et al., 1974), these presumed amyloid fibrils were never clearly visualized by electron microscopy, suggesting that amyloid fibrils for the double mutant are even less stable than wild-type fibrils under these conditions or that their structure is

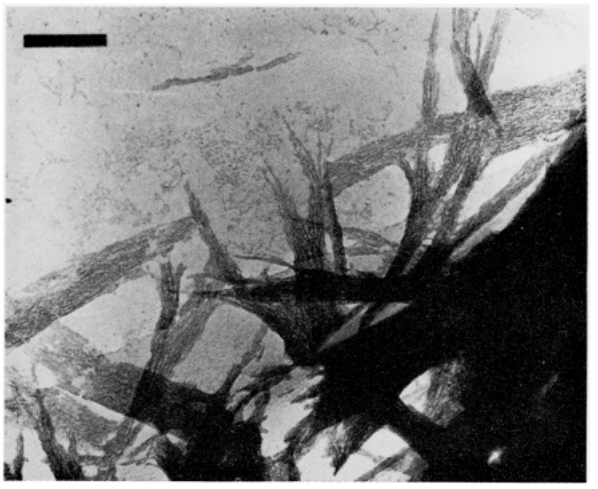

FIGURE 6: Photograph of negatively-contrasted (uranyl acetate) Val-30-Met TTR amyloid fibrils, viewed under a Zeiss 10-C electron microscope, showing lateral association of fibrils. Val-30-Met amyloid fibrils were formed at 0.02 mg/mL in 50 mM acetate-buffered 100 mM KCl at pH 5.0 and incubated at room temperature overnight. Magnification = 63250× (bar = 1000 Å).

not consistent with amyloid. Interestingly, it was possible to visualize amyloid fibrils of Thr-119-Met TTR by electron microscopy; however, the concentration required to form these fibrils was high, 0.6 mg/mL TTR. At this concentration, the other variants reported here form massive amounts of amyloid fibrils such that electron microscopy cannot be used to inspect the structure of the aggregates due to overloading of the carbon-coated copper grids. The high concentration of Thr-119-Met TTR required for amyloid fibril formation reflects the low amyloidogenicity of this variant and the relative instability of the amyloidogenic intermediate as indicated by the absence of a fluorescence plateau in the denaturation curve of the Thr-119-Met variant.

DISCUSSION

It is known that the amyloid deposits of heterozygous FAP patients are composed predominantly of variant TTR subunits, implying that FAP mutations confer increased amyloidogenicity on the protein. The goal of this study was to understand how these mutations increase the amyloidogenicity of TTR. We have expressed and purified Val-30-Met, Val-30-Met/Thr-119-Met, and Thr-119-Met tetrameric variants of TTR using the *E. coli* expression system developed previously in an effort to compare both their acid stabilities and their denaturation pathways (McCutchen et al., 1993).

One possible explanation for the increased amyloidogenicity exhibited by the FAP variants is that the folded TTR structure is perturbed by these mutations. However, this seems unlikely since most of the FAP mutations are conservative internal substitutions or conservative surface mutations. It is known from structural studies on other proteins that those mutations usually do not affect the overall structure adopted by a protein (Perutz & Lehmann, 1968; Hecht et al., 1983; Shortle & Lin, 1985; Alber et al., 1988; Bowie et al., 1990; Baldwin et al., 1993) In fact, the X-ray crystal structure of the tetrameric FAP variant, Val-30-Met, confirms the expectation that the conservative Met for Val mutation only slightly perturbs the structure of TTR (Terry et al., 1993; Hamilton et al., 1992). This finding strongly suggests that FAP mutations do not operate through an effect on the folded structure. Furthermore, earlier work from our laboratory has shown that TTR must undergo a conformational change in order for it to self-assemble into amyloid fibrils. It is logical then to propose that the FAP mutations in TTR operate by affecting the protein denaturation pathway by making the conformational changes that lead to amyloid fibril formation more facile. In other words, the amyloidogenic mutations are thought to affect the denaturation pathway facilitating the formation of the amyloidogenic intermediate. Several other examples are known whereby a single site mutation can affect the folding pathway/denaturation pathway of a protein dramatically, predisposing it to abnormal aggregation facilitated by the self-association of a denaturation intermediate (Seckler et al., 1989; King & Yu, 1986; Yu & King, 1984; Buchner et al., 1991; Arakawa et al., 1987; London et al., 1974; Brems, 1988; Turkewitz et al., 1988; Teschke & King, 1993; Jaenicke, 1987; Zettlmeissl et al., 1979; Hurle et al., 1994; Wetzel, 1994).

The data outlined here, and our earlier results on Leu-55-Pro TTR, reveal that the FAP variants we have studied to date have the ability to self-assemble into amyloid fibrils under mildly acidic denaturing conditions and at low concentrations where the wild-type protein is nonamyloidogenic. Our *in vitro* methodology for making amyloid fibrils has been developed on the basis of literature reports suggesting the importance of lysosomal involvement in amyloid disease (Smetana, 1927; Glenner et al., 1971; Shirahama & Cohen, 1975; Cohen et al., 1983; Shirahama et al., 1990; Westermark et al., 1990). As a result, the conditions are meant to simulate the acidic environment of the lysosome *in vivo*. Most of the reports invoking lysosomal involvement in amyloidogenesis stress the importance of proteolysis within the lysosome in amyloid fibril formation. However, in the case of TTR, it is likely that the acidic partially denaturing environment of the lysosome (pH 5.5 $\pm$ 0.5) is the most important factor for TTR amyloid fibril formation, since proteolysis does not appear to be required in TTR amyloid fibril formation (Colon & Kelly, 1992; McCutchen et al., 1993; McCutchen & Kelly, 1993). We envision that the lysosomal pH is sufficient to populate the amyloidogenic intermediate which can partition between two pathways. The normal pathway includes acid-mediated denaturation followed by proteolysis to afford peptides and amino acids. Alternatively, the amyloidogenic intermediate can self-assemble into protease-resistant amyloid fibrils at a rate that competes with the proteolysis rate. The lysosome would presumably fill with amyloid, resulting in the exocytosis of the lysosomal contents to the extracellular space.

Previous studies on the most pathogenic FAP variant, Leu-55-Pro TTR, demonstrate that the tetramer is significantly destabilized (pH_m 5.4) under acidic denaturation conditions when compared to the wild-type tetramer (pH_m 4.4). The low pH_m of the wild-type protein would ensure that the concentration of the amyloidogenic intermediate would be low in the lysosome (pH 5.5). This is consistent with the relatively low amyloidogenicity of wild-type TTR, seemingly explaining why wild-type amyloid deposition is not problematic before age 80. Alternatively, patients with the Leu-55-Pro mutation present with FAP in the second decade of life, consistent with the idea that this variant can readily form the monomeric amyloidogenic intermediate in relatively high concentrations in a normal lysosome, leading to an increased rate of fibril formation and an early disease onset. A similar

evaluation of the most prevalent FAP mutation, Val-30-Met, reveals that its acid stability falls in between those of wild type and Leu-55-Pro as does its amyloidogenicity *in vivo*.

The most prevalent FAP mutation, Val-30-Met TTR, destabilizes the tetrameric protein toward acid denaturation as clearly demonstrated by its elevated pH_m when compared to wild-type TTR (Figure 4). The destabilization of the Val-30-Met tetramer toward acid denaturation (pH_m 4.7) enables it to form the amyloidogenic intermediate under lysosomal acidic conditions (Thoene, 1992; Winchester, 1992; Holtzman, 1989) where wild-type TTR (pH_m 4.4) is more stable. The fluorescence denaturation curve reported here for the Val-30-Met variant is biphasic, suggesting the presence of at least one intermediate. A plateau in the fluorescence intensity across the amyloid-forming pH range appears to indicate the presence of an amyloidogenic intermediate(s) and possibly other conformational intermediates. The fluorescence-based acid denaturation curves for the wild-type tetramer and the Val-30-Met tetramer are very similar, indicating that the pH-dependent tertiary structural changes exhibited by these proteins dominate their fluorescence intensity changes. SDS−PAGE analysis appears to be a more reliable method for assessing the quaternary structural changes, which do not appear to be the predominant contributor to the fluorescence curves, consistent with the dogma that fluorescence studies are not very sensitive to quaternary structural changes. It is not clear what the relative contributions of the two Trp residues are to the fluorescence denaturation curve; however, it is clear that the environment of the contributing Trp's does not change markedly in terms of solvent exposure upon denaturation as evidenced by the small bathochromic shift exhibited by the fluorescence spectra at lower pH (Table 2). Ongoing studies with variants of TTR containing only a single Trp should allow us to better delineate the relative contributions of Trp 41 and Trp 79.

A recent report suggests that the pathogenic effects of the most prevalent FAP variant, Val-30-Met TTR, can be repressed by a second mutation, Thr-119-Met, in another subunit (Coelho et al., 1992). The Val-30-Met/Thr-119-Met heterozygous mutations were detected in a patient who presented with the first clinical manifestations of FAP and through a skin biopsy was initially diagnosed with Val-30-Met amyloid disease. Two decades post diagnosis, however, the patient had still not developed the classical symptoms of FAP. DNA studies to recheck the initial report of the Val-30-Met mutation revealed the presence of a second mutation, Thr-119-Met, hence this patient has one copy of the Val-30-Met gene and one copy of the Thr-119-Met TTR gene. The Thr-119-Met TTR mutation is benign and highly prevalent in the Portuguese population; and quite interestingly, when combined with the Val-30-Met mutation, it appears to suppress the symptoms of FAP (Coelho et al., 1992; Scrimshaw et al., 1992; Setsuko et al., 1992).

Studies on the nonpathogenic Thr-119-Met mutation, which is found at the dimer interface, show that this mutation stabilizes the tetramer significantly with regard to acid denaturation ($pH_m = 3.4$). The stability of the Thr-119-Met tetramer makes it unlikely that the amyloidogenic intermediate would form to a significant extent under mildly acidic lysosomal conditions, which appears to explain why this mutation is nonpathogenic. Interestingly, the Thr-119-Met variant exhibits a strikingly different fluorescence denaturation curve when compared with the other TTR tetramers studied here. The Thr-119-Met denaturation appears to be more cooperative and clearly lacks the plateau region, suggesting that the concentration of the amyloidogenic intermediate is quite low. This result indicates that the amyloidogenic intermediate is destabilized relative to the monomeric "A state".

The Thr-119-Met mutation has also been shown to render the heterozygous tetramer Val-30-Met/Thr-119-Met TTR less amyloidogenic *in vivo* despite the pathogenic effects of the single Val-30-Met mutation in FAP patients (Coelho et al., 1992; Scrimshaw et al., 1992; Setsuko et al., 1992). We have also shown that Val-30-Met/Thr-119-Met TTR is less amyloidogenic *in vitro* than wild-type TTR owing to its acid stability (pH_m 4.1), which likely explains the altered pathogenic behavior of the mixed Val-30-Met/Thr-119-Met tetramer *in vivo*. Thus, the Thr-119-Met subunit present in combination with the Val-30-Met FAP subunit appears to render the protein less amyloidogenic by increasing the stability of the tetramer toward acidic denaturation.

In accordance with the amyloidogenic characteristics of the TTR variants *in vivo*, the Val-30-Met variant is capable of forming amyloid fibrils *in vitro* at a higher pH and at a lower concentration (pH 5.0, 0.02 mg/mL) than wild-type TTR (pH 4.3, 0.1 mg/mL). In addition, electron microscopy indicates that Val-30-Met TTR forms amyloid fibrils which are significantly more stable than wild-type amyloid fibrils. The *in vitro* amyloidogenicity of Val-30-Met TTR correlates with the enhanced amyloidogenicity exhibited by Val-30-Met TTR *in vivo*. The *in vitro* studies also show that the Thr-119-Met mutation increases tetramer stability and decreases amyloidogenicity, consistent with the observation that the Thr-119-Met mutation protects against amyloid disease *in vivo* in Val-30-Met/Thr-119-Met heterozygous individuals. At present, conditions have not been found to convert the Val-30-Met/Thr-119-Met variant into amyloid fibrils *in vitro*. The Thr-119-Met variant is capable of amyloid fibril formation, albeit at much higher TTR concentrations (pH 3.8, 0.6 mg/mL) than are required for the visualization of amyloid fibrils formed from any of the other TTR variants reported here. Even at 0.6 mg/mL, only very small quantities of Thr-119-Met TTR amyloid fibrils are observed by electron microscopy, while other variants of TTR form massive quantities of amyloid fibrils under these conditions as discerned by light scattering and electron microscopy. This result is consistent with the fluorescence results which suggest that the Thr-119-Met amyloidogenic intermediate is destabilized relative to the other conformations accessible to the protein.

This investigation corroborates an earlier interpretation that TTR instability toward acid denaturation is correlated with amyloidogenicity both *in vitro* and *in vivo* (Colon & Kelly, 1992; McCutchen et al., 1993). The results presented within further demonstrate that the acid instability correlates well with the pathogenicity associated with these variants as evaluated by their amyloidogenicity *in vitro* and by the age of onset of amyloid disease *in vivo*, which is most likely a result of the extent of amyloid fibril deposition (Table 3). The order of the acid stability of the TTR variants which have been characterized to date, from most stable to least stable, is Thr-119-Met > Val-30-Met/Thr-119-Met > wild type > Val-30-Met ≫ Leu-55-Pro (McCutchen et al., 1993). This correlates with the severity of the amyloid disease associated with each variant *in vivo*; i.e., wild-type TTR is

Table 3: Correlation between Increasing pH_m *in Vitro* and Decreasing Age of Amyloid Disease Onset *in Vivo*[a]

TTR variant	pH_m[b]	age of disease onset
Thr-119-Met	3.4	none reported
Wild Type	4.4	> 80 (SSA)
Val-30-Met	4.7	30 (FAP)
Leu-55-Pro	5.4	teens to early 20's (FAP)

[a] (McCutchen et al. (1993); Coelho et al. (1992); Scrimshaw et al. (1992); Setsuko et al. (1992); Jacobson & Buxbaum (1991); Saraiva et al. (1983, 1984). [b] pH_m values determined without Z 3-14 present (error ± 0.1 pH units).

less pathogenic than the Val-30-Met variant which is less pathogenic than the Leu-55-Pro variant (Table 3) (Saraiva et al., 1983, 1984; Jacobson & Buxbaum, 1991). As the correlation would predict, the single Thr-119-Met mutation, which confers increased stability upon the TTR tetramer, has not been associated with amyloid disease, although it is a common TTR variant in Portugal. More importantly, the Thr-119-Met mutation appears to suppress the pathogenic effects of the FAP mutation, Val-30-Met, in the case of the heterozygous Val-30-Met/Thr-119-Met patient. Similar mutations which suppress the influence of another aggregation-causing mutation have been characterized previously by King and co-workers (Mitraki et al., 1993).

The correlation between acid instability of TTR *in vitro* and amyloidogenicity *in vivo* is striking. The results of this comparative study strongly support a mechanism for amyloid fibril formation which involves partial denaturation of tetrameric TTR to a putatively structurally rearranged monomeric intermediate capable of self-assembling into amyloid fibrils. Lysosomal acidic conditions appear to be sufficient to dissociate the tetramers of the Leu-55-Pro and Val-30-Met FAP variants into a monomeric intermediate which can form amyloid fibrils (McCutchen et al., 1993); however, these conditions do not appear to be sufficient to significantly dissociate wild-type, Val-30-Met/Thr-119-Met, or Thr-119-Met TTR. Whether partial acid denaturation resulting in tetramer dissociation and amyloid fibril formation is accomplished under acidic conditions *in vivo* remains to be demonstrated. Nonetheless, these results, when combined with previous data, clearly indicate a correlation between TTR acid instability and amyloidogenicity, and the importance of denaturation intermediates in amyloid fibril formation.

CONCLUDING REMARKS

Transthyretin amyloid fibril formation and denaturation are competitive processes. As a result, the acid stability of the TTR tetramer is a critical factor in amyloidogenicity because dissociation/denaturation of the tetramer affords an amyloidogenic intermediate which is capable of self-assembling into amyloid fibrils. The order of acid stability of the TTR variants studied to date, from most stable to least stable, is Thr-119-Met > Val-30-Met/Thr-119-Met > wild type > Val-30-Met ≫ Leu-55-Pro. These data correlate with the severity of the amyloid pathology where the wild-type protein is less pathogenic than the Val-30-Met protein, which is less pathogenic than the Leu-55-Pro protein. Results from the characterization of the nonpathogenic variant, Thr-119-Met, and the double mutant, Val-30-Met/Thr-119-Met, suggest that the Thr-119-Met mutation confers increased acid stability to the TTR tetramer and suppresses the known effect of the FAP-associated mutation, Val-30-Met. It is also interesting that the fluorescence-monitored denaturation curve for the Thr-119-Met variant does not indicate a significant concentration of the amyloidogenic intermediate, implying that the amyloidogenic intermediate is destabilized and is present at very low steady-state concentration. This is not the case for the other TTR variants evaluated where the amyloidogenic intermediate(s) is (are) readily detected by fluorescence over the amyloid-forming pH range.

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