Transthyretin Mutation Leu-55-Pro Significantly Alters Tetramer Stability and Increases Amyloidogenicity†

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ABSTRACT: A recently reported variant of human transthyretin (TTR), Leu-55-Pro, implicated as the causative agent in early-onset familial amyloid polyneuropathy was expressed and characterized, and its denaturation pathway and amyloidogenicity were compared to those of wild-type transthyretin. The overlap-extension polymerase chain reaction (PCR) methodology was used to introduce the Leu-55-Pro mutation into the transthyretin DNA sequence and to construct a new expression system. The Leu-55-Pro variant of transthyretin was expressed with a leader sequence to ensure secretion into the periplasmic space of *Escherichia coli*. Transthyretin's resistance to sodium dodecyl sulfate- (SDS-) induced denaturation was utilized to measure the quaternary stability as a function of pH employing SDS–polyacrylamide gel electrophoresis (PAGE) in the presence and absence of an amyloid fibril inhibitor, Z 3-14. These studies reveal that the Leu-55-Pro TTR tetramer is significantly less stable than wild-type TTR. This is relevant because we have previously shown that the partial denaturation of transthyretin is sufficient to effect amyloid fibril formation from a denaturation intermediate which may be a structured monomer. The ability of Leu-55-Pro TTR to denature to the amyloidogenic intermediate at pHs where the wild-type protein is stable may explain the variant's propensity to form amyloid fibrils *in vitro* and *in vivo* where the wild-type protein remains stable and nonamyloidogenic. Congo red binding, polarized light microscopy, and electron microscopy confirm the characteristic structure of amyloid fibrils produced from Leu-55-Pro TTR *in vitro*. The instability of the Leu-55-Pro tetramer and the capability of this protein to form amyloid fibrils at pHs close to 5.5 suggest an explanation for the extreme pathogenicity of this variant. Several lines of evidence suggest that lysosomes may be the source of amyloid fibril formation *in vivo*. It is interesting and potentially physiologically relevant that Leu-55-Pro TTR forms amyloid fibrils at the normal operating pH of a lysosome.

Transthyretin, also known as thyroxine-binding prealbumin in older literature, is a tetrameric human plasma protein (MW 54 980) composed of identical 127-residue subunits, each having a β-sheet structure known to 1.8-Å resolution by X-ray crystallography (Blake et al., 1978, 1974). Transthyretin (TTR)[1] is encoded by a single copy gene on chromosome 18 and is secreted by hepatocytes into the blood serum where it plays a major role in the transport of thyroxine and retinol, the latter via the TTR–retinol binding protein complex (Yen et al., 1990; Jaarsveld et al., 1973; Nilsson et al., 1975; Raz et al., 1970).

Transthyretin composes the amyloid fibrils found in patients afflicted with either familial amyloid polyneuropathy (FAP) or senile systemic amyloidosis (SSA) (Benson, 1989a). Amyloid fibril formation refers to the deposition of an insoluble cross-β-sheet quaternary structure in the intracellular or extracellular space which leads to neurotoxicity and organ dysfunction. All indications are that the amyloid fibrils found in patients with systemic amyloidosis are the causative agent of the disease; however, the pathogenic mechanism remains unclear (Benson & Wallace, 1989b). Normal transthyretin is found to compose the amyloid fibrils in patients with SSA, which to some extent affects approximately 25% of the population aged more than 80 years. Conversely, one of over 20 different TTR variants carrying a single mutation is the predominant component of FAP fibrils (Benson, 1989; Benson & Wallace, 1989; Stone, 1990; Saraiva et al., 1983, 1984). The full-length TTR polypeptide is isolated as the predominant product from the amyloid fibrils in FAP patients while fragments of TTR as well as full-length polypeptide are found in SSA amyloid deposits (Westermark et al., 1990).

The majority of FAP patients are heterozygous and therefore produce one copy of wild-type TTR for every copy of amyloidogenic variant TTR. The TTR tetramers in FAP patients have a statistical distribution of wild-type and variant subunits; however, the amyloid fibrils are composed predominantly of variant TTR. Since patients are heterozygous, isolation of pure variant TTR is nearly impossible from human plasma. For this reason, we have developed an expression system for the amyloidogenic variants of TTR so that their physical properties can be compared to those of wild-type TTR without having to be concerned about the distributions of wild-type and variant subunits in the tetramers or the isolation of TTR from plasma.

Recently, members of a West Virginia kindred were reported to develop an early-onset aggressive form of FAP with rapidly progressive symptoms. All known patients afflicted with this form of amyloid disease are now deceased. The amyloid fibrils, which were found in many tissues, were composed predom-

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[1] Abbreviations: TTR, transthyretin; FAP, familial amyloid polyneuropathy; SSA, senile systemic amyloidosis; kbp, kilobase pair(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; TE buffer, 10 mM Tris–1 mM EDTA, pH 8.0; T_4, thyroxine; OD, optical density; Z 3-14, *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate.

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inantly of Leu-55-Pro TTR (Jacobson et al., 1992). Age of disease onset has been reported as the second decade, whereas other reported point mutations causing FAP, such as Val-30-Met (Saraiva et al., 1983, 1984), show onset of symptoms in the fourth to fifth decades. The reason for the more aggressive progression in patients with the Leu-55-Pro mutation is currently unknown. Insight into the mechanism may come from a consideration of the residues exchanged and their location. Position 55 lies in β-strand D on the outer edge of the TTR tetramer (Blake et al., 1978, 1974). The Leu-55-Pro mutation is a nonconservative mutation in that an imino acid residue replaces an amino acid residue. Since proline is known to be a β-strand breaker, it is conceivable that the introduction of this mutation may significantly affect the conformation of mechanism(s) TTR and/or its folding pathway so as to predispose the protein to amyloid fibril formation. We are optimistic that the characterization of the amyloidogenic variants of TTR which putatively cause FAP may prove insightful in elucidating a mechanism(s) by which normally soluble proteins are converted into insoluble amyloid fibrils *in vivo*.

There is a substantial amount of evidence in the literature implicating lysosomal involvement in amyloid diseases (Shirahama & Cohen, 1975; Cohen et al., 1983; Shirahama et al., 1990; Golde et al., 1992). In an effort to understand the biochemical mechanism of amyloid fibril formation, a procedure for the conversion of wild-type TTR into amyloid fibrils *in vitro* under conditions which mimic the acidic environment of a lysosome (pH ≤ 5.5) (Thoene, 1992; Winchester, 1992; Holtzman, 1989) has been developed in our lab (Colon & Kelly, 1991, 1992). We have shown that the acid-mediated partial denaturation of TTR is sufficient to achieve amyloid fibril formation *in vitro*. Both wild-type TTR and the most prevalent mutation causing FAP, the Val-30-Met variant, have been transformed into amyloid fibrils by this methodology (W. Colon and J. W. Kelly, manuscript in preparation). We have demonstrated that TTR denaturation and amyloid fibril formation are competitive processes that can now be studied individually due to the discovery of an inhibitor which prevents amyloid fibril formation and allows transthyretin to undergo reversible reconstitution from the "denatured state". Thus, in the presence of amyloid inhibitor, TTR denaturation can be studied, and in the absence of inhibitor, fibril formation can be examined. Association of a partially denatured TTR intermediate, most likely a structured monomer having native amounts of β-sheet secondary structure, is sufficient for amyloid fibril formation *in vitro*, implicating lysosomal involvement *in vivo*. We set out to examine whether the Leu-55-Pro TTR variant is predisposed to amyloid fibril formation, and if so, why. It is possible that the amyloidogenic intermediate is more stable and/or more easily populated under acidic conditions which are physiologically accessible in the case of Leu-55-Pro TTR. Alternatively, the increased amyloidogenicity exhibited by this variant may be a result of decreased stability of the TTR tetramer which could also increase the population of the amyloidogenic intermediate. The comparative studies demonstrate that the recombinant Leu-55-Pro TTR is more amyloidogenic than wild-type TTR and that the tetameric form of Leu-55-Pro TTR is less stable with respect to acid denaturation when compared to wild-type TTR.

EXPERIMENTAL PROCEDURES

Materials. Reagents for PCR were purchased from Perkin Elmer Cetus. Restriction enzymes and T4 DNA ligase were from Promega. Sequenase and sequencing reagents were from U.S. Biochemical Corp. The pHN1+ and pKEN2 vectors were a kind gift from Dr. G. Verdine, Harvard University, Cambridge, MA, and *Escherichia coli* XL1-Blue was purchased from Stratagene. PCR primers and sequencing primers were made by the Advanced DNA Technologies Laboratory, Biology Department, Texas A&M University. Polyclonal rabbit IgG antibody against human TTR was purchased from Accurate Antibodies. Other reagents were purchased from Sigma and Fisher Scientific and were of the highest purity commercially available.

Construction of TTR Expression Systems, pHNTR and pKNTR. Sakaki and co-workers have reported an expression system for TTR which is under control of the *lac* promoter (Furuya et al., 1991; Ghrayeb et al., 1984). The pINTR vector contains the TTR cDNA preceded by a sequence encoding the *ompA* leader sequence, which facilitates secretion of TTR into the periplasmic space of *E. coli* (Takagi et al., 1988). Expression levels of 5 mg/L using this expression system have been reported by Sakaki and have been reproduced by our laboratory. The pINTR expression system developed by Sakaki and co-workers was modified by flanking the TTR cDNA with *Bam*HI and *Pst*I restriction sites. This modification yields an expression cassette which facilitates easy insertion or removal of the gene containing sequence (480 bp) into the polycloning region of various expression vectors (MacFerrin et al., 1990). The advantage of this expression cassette lies in the ease of manipulating the 480-bp polynucleotide using PCR methods, which greatly simplifies mutagenesis and sequencing (Innis et al., 1990). A 1.6-kbp *Hin*dIII/*Pst*I restriction fragment containing the TTR cDNA was liberated from the original pINTR vector and purified employing a maxi DNA prep (Sambrook et al., 1989). This fragment was recovered from an agarose electrophoresis gel by running the 1.6-kbp fragment onto diethylaminoethyl (DEAE) paper and eluting the DNA from the DEAE paper with a 1 M NaCl solution in TE buffer at 55 °C. The DNA was extracted with butanol and the 1.6-kbp fragment was precipitated using EtOH. The polynucleotide was resuspended in TE buffer. The expression-cassette PCR methodology (MacFerrin et al., 1990) was used to amplify a portion of the 1.6-kbp *Hin*dIII/*Pst*I restriction fragment and to create two unique restriction sites flanking the TTR cDNA sequence in the original pINTR vector. Ten nanograms of the 1.6-kbp fragment was subjected to 25 PCR cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C on a Precision Scientific genetic thermal cycler using 10 μmol of end primers and 2.5 units of *Taq* DNA polymerase, all overlaid with mineral oil (Innis et al., 1990). Primers were designed to provide 18-bp complementarity and include the new restriction sites 5′ to the complementary region. The primers also contained a 5′ clamp (CTC) to ensure proper digestion at the newly designed restriction sites. The sequence of the primer used to incorporate the *Bam*HI site was 5′-CTC GGA TCC TAA CGA GGG CAA AAA ATG-3′, where the sequence for the restriction site is underlined. The sequence of the primer used to incorporate the *Pst*I site was 5′-CTC CTG CAG TCA TTC CTT GGG ATT GGT-3′. Amplification of the 480-bp fragment with the new restriction sites was verified by gel electrophoresis. A chloroform extraction was used to remove impurities from the PCR product. The PCR product and vectors pHN1+ and pKEN2 (Schreiber & Verdine, 1991) were double-digested with *Bam*HI and *Pst*I. The digests were mixed in a 3:1 insert to vector ratio, purified by the "glass milk" procedure described in Bio101's Geneclean kit, and resuspended in 20 μL of TE buffer. The *ompA*/TTR expression cassette was then ligated into the expression vectors

(Sambrook et al., 1989). This was followed by transformation into competent *E. coli* XL1-Blue. Ampicillin-resistant colonies were screened for the desired insert by gel electrophoresis of a restriction digest with *Bam*HI and *Pst*I. Sequencing of the clones containing the desired insert was carried out using protocols described by Kraft et al. (1988). Five custom-made oligonucleotides based on the sequence of the vector and the cDNA were used to sequence the clones. The primers were designed to have 18-bp complementarity to the gene. Approximately 200 bp could be sequenced using each primer with the next primer overlapping into the previous readable sequence. The new expression systems for TTR are referred to as pHNTR and pKNTR, and an outline of their design is shown in Figure 1.

PCR Mutagenesis To Produce Leu-55-Pro TTR. The PCR overlap-extension method (Ho et al., 1989) was used to incorporate the point mutation in the TTR cDNA giving rise to the Leu-55-Pro variant. DNA from a maxiprep of pHNTR wild-type TTR was subjected to a *Bam*HI and *Pst*I double digest under the conditions described above. The 480-bp *ompA*/TTR fragment was purified from an agarose gel and the DNA was subjected to PCR amplification with primers designed to incorporate the T → C mutation at position 2 of codon 55. Two PCR reactions were performed under conditions previously described in the text. The first utilized the primer designed to produce the *Bam*HI restriction site in the pHNTR construct and the following primer to produce the desired mutation, 5′-GAG CCC ATG CGG CTC TCC-3′, where the mismatch giving rise to the desired mutation is underlined. The second PCR reaction utilized the primer designed to produce the *Pst*I restriction site in the pHNTR construct and the following primer to produce the desired mutation, 5′-GGA GAG CCG CAT GGG CTC-3′. DNA amplified from these two PCR reactions was then purified from an agarose gel. In the final PCR reaction, DNA from the two previous PCR reactions was used as template DNA and the primers were the original primers used to design the restriction sites for the expression cassette in the pHNTR and pKNTR constructs. This yielded the full-length *ompA*/TTR fragment with the desired mutation which was cloned into pHN1+ and pKEN2 and sequenced as described above. Recombinant Leu-55-Pro TTR was expressed from pHNTR in *E. coli* XL1-Blue at 30 °C. Six 1.5-L flasks of LB medium were inoculated with a fresh 5-mL overnight culture and expression was induced from the *tac* promoter in pHN1+ by addition of 0.1 mM IPTG after cells reached an $OD_{600} \geq 0.7$ (DeBoer et al., 1983). Cells from the 9.5-L prep were harvested 48 h after induction and were resuspended in 2.4 L of a 1.5 mM EDTA/0.03 M Tris buffer (pH 7.5) and allowed to sit at room temperature for 15 min before being centrifuged. Cells were then resuspended in 2.4 L of a 20% sucrose solution of the same buffer and stirred vigorously for 15 min at 4 °C in order to release protein from the periplasmic space. The supernatant from the osmotic shock, which contains TTR and other periplasmic proteins, was then concentrated 10-fold. TTR was precipitated in 70–85% ammonium sulfate and the pellet was dissolved in a minimal volume of 0.05 M Tris, pH 7.5. This was dialyzed against the same buffer to remove excess salt. TTR was then loaded onto a DEAE column and eluted using 800 mL of a 0–0.3 M NaCl linear gradient in 0.05 M Tris buffer, pH 7.5. This was followed by dialysis against 0.02 M phosphate buffer, pH 7.2, with 0.1 M KCl, which affords 5 mg of TTR/9.5-L prep. SDS–PAGE (Laemmli, 1970) was used to assess purity. Since expression was quite low with the Leu-55-Pro TTR variant in the pHNTR system, the expression cassette containing the sequence for

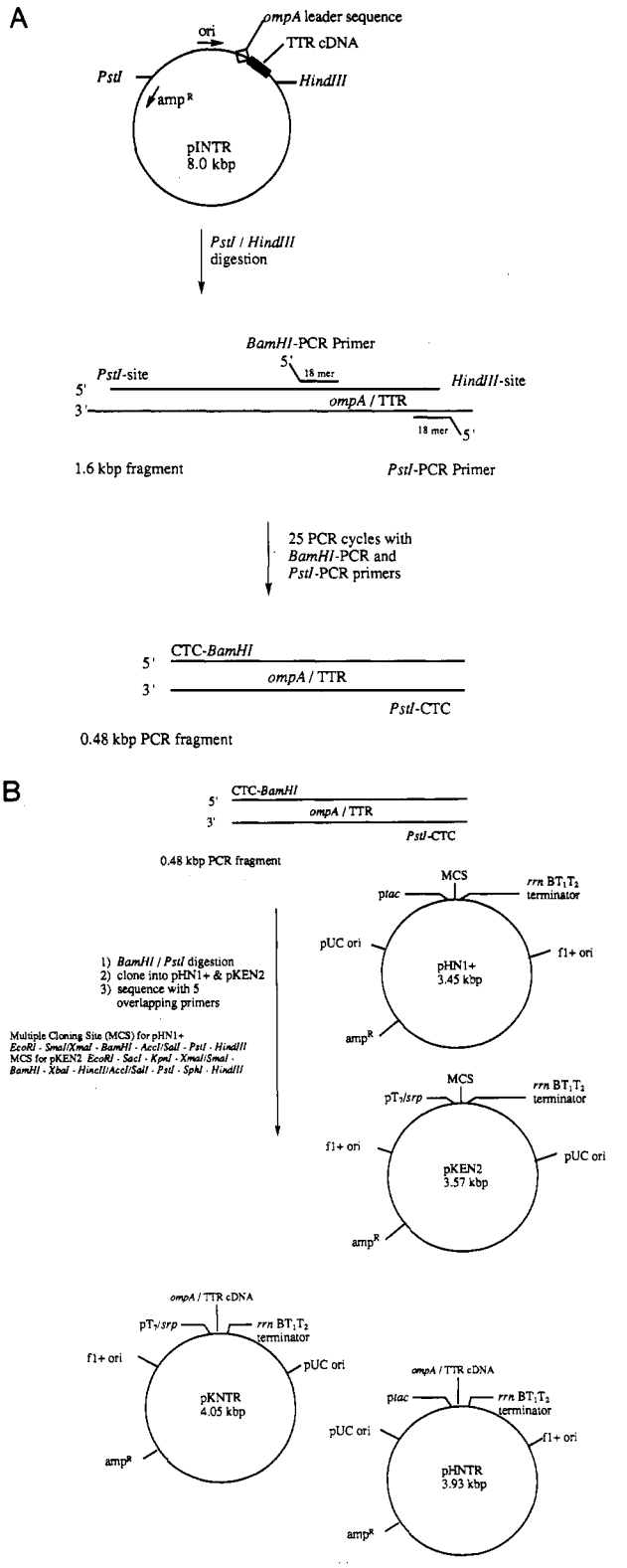

FIGURE 1: (A) Outline of PCR amplification of 480-bp *ompA*/TTR expression cassette containing the two incorporated restriction sites, the ribosome binding site, the *ompA* leader sequence, and TTR cDNA. See Experimental Procedures for sequence of primers used. (B) Outline of the design of the new TTR expression systems, pHNTR and pKNTR.

Leu-55-Pro TTR was subcloned into pKEN2 in an attempt to increase the yield. Using the same expression conditions as described for pHNTR, approximately 30 mg of Leu-55-Pro TTR/9.5-L prep was isolated using the pKNTR expression system.

Characterization of Recombinant Leu-55-Pro TTR. A Western blot analysis (Towbin et al., 1979) using polyclonal

rabbit IgG antibodies directed against TTR was performed after protein purification in order to confirm the presence of TTR. Edman N-terminal sequencing of the first 10 amino acid residues of Leu-55-Pro TTR was used to ensure that the *ompA* leader sequence was correctly processed. In addition, a fluorescence assay useful for characterizing recombinant TTR was employed to probe the integrity of the thyroxine binding site formed by the quaternary interactions between each subunit in the tetramer (Nilsson & Peterson, 1971). This assay measures the relative fluorescence of Trp using a 0.75 μM sample of TTR in the presence and absence of its native ligand thyroxine (T_4). The sample was excited at 278 nm and fluorescence intensity from 310 to 370 nm was recorded on an 8000 SLM Aminco fluorometer. T_4 was then added to afford a final concentration of 1.5 μM and the intrinsic fluorescence of TTR was measured again under identical conditions. Quenching of approximately 25% of the intrinsic fluorescence of TTR by the addition of T_4 confirms the integrity of the T_4 binding site which is found within the central channel of the TTR tetramer.

In Vitro Amyloid Fibril Studies and Characterization. Recombinant Leu-55-Pro as well as wild-type TTR were subjected to the acidic conditions previously reported to be sufficient to form amyloid fibrils *in vitro* (Colon & Kelly, 1992, 1991). The concentration of protein was lowered from 0.6 mg/mL used in previous experiments to 0.2 mg/mL due to the difficulty encountered in working with concentrated Leu-55-Pro TTR. The Leu-55-Pro TTR variant has a high tendency to self-associate when kept in stock concentrations greater than 2 mg/mL, at pH 7.5. The fibril formation assays were carried out at 37 °C with slow stirring and were performed at various pHs using either 0.1 M sodium acetate or sodium phosphate buffer in the presence of 0.1 M KCl. Light scattering at 330 nm was used to monitor amyloid fibril formation as a function of time using a Milton Roy 3000 diode array spectrophotometer. Light scattering measures the total mass of the amyloid fibrils formed (Andreu & Timasheff, 1986; Mulkerrin & Wetzel, 1989). TTR amyloid fibrils were known to bind congo red, which results in a 5–15-nm red shift in the UV absorbance spectrum of congo red (Glenner et al., 1974). To probe whether the aggregated protein observed in the assay described above was amyloid, a congo red binding assay was employed in each case. Approximately 7 μmol of suspected TTR amyloid fibrils was added to a 10 μM solution of congo red. The absorbance spectra were recorded from 300 to 700 nm looking for a red shift in the congo red absorbance maximum ca. 490 nm. For those samples exhibiting congo red binding, the amyloid fibril–congo red complex was viewed under a light microscope with a polarized light source in search of the expected green birefringence known to be exhibited by the amyloid–congo red complex (Glenner et al., 1974; Klunk et al., 1989; Puchtler et al., 1962). A Zeiss C-10 electron microscope was used after preliminary characterization to examine the structure of the amyloid fibrils, which, when negatively contrasted with uranyl acetate, should have a diameter of approximately 70 Å, be >1000 Å in length, and appear laterally associated with a macromolecular twist.

Determination of the Relative Stability of Quaternary Structure of Leu-55-Pro TTR by SDS–PAGE. Leu-55-Pro TTR was subjected to incubation at a variety of acidic pHs in the presence and absence of the micellar detergent Z 3-14 (Tandon & Horowitz, 1986, 1987), which inhibits amyloid fibril formation. We have previously shown that Z 3-14 makes it possible to study the denaturation pathway without competitive amyloid fibril formation (Colon & Kelly, 1991). The concentration of TTR employed for the experiments in the presence of detergent was 0.2 mg/mL while the Z 3-14 concentration was 0.1 mg/mL. The detergent not only prevents aggregation but more importantly allows TTR to undergo reversible denaturation and reconstitution. The concentration of TTR for those experiments performed in the absence of Z 3-14 was also 0.2 mg/mL. After TTR was incubated at the desired pH for 40 h at 25 °C, the protein solutions were quenched to prevent TTR from undergoing reconstitution by raising the detergent concentration to 0.5 mg/mL at the same time the pH was increased to pH 7.0 by addition of 150 μL of 0.6 M phosphate buffer, pH 7.5. Concentrations of Z 3-14 $\geq$ 0.5 mg/mL have been previously demonstrated to prevent reassociation of denaturation intermediate(s) which are present at the time of the pH jump (Colon & Kelly, 1991). SDS–PAGE is utilized to evaluate the quaternary species present under the specified conditions. That the experiment gives an accurate description of the quaternary structural changes that are occurring has been verified by glutaraldehyde cross-linking experiments performed as described previously (Colon & Kelly, 1992). The tetramer to monomer transition was evaluated by SDS–PAGE using samples of wild-type and Leu-55-Pro TTR subjected to the incubation/pH jump conditions described above. Samples were mixed with SDS–PAGE sample buffer, loaded, and run on an SDS–polyacrylamide gel which was subsequently scanned by densitometry to quantitate the relative amounts of tetramer and monomer present. The samples were not boiled prior to loading. The tetramer is not resistant to SDS and has been shown to run as the dimer on an SDS–PAGE gel; hence a dimer band is observed and has been shown to represent the amount of tetramer actually present. Gluteraldehyde cross-linking of the samples has revealed that the amount of dimer that is present is negligible under the conditions used here; therefore, we can say with confidence that what is actually observed is a tetramer to monomer transition.

RESULTS

pKNTR Construct for Leu-55-Pro TTR Variant. The PCR fragment for the Leu-55-Pro variant was successfully cloned into pKEN2 as seen by gel electrophoresis of a *Bam*HI and *Pst*I digest of DNA isolated from an ampicillin- and tetracycline-resistant colony. Sequencing was carried out directly from the above-mentioned clone rather than the PCR product itself using denatured double-stranded DNA. DNA sequencing verified that the desired mutation was introduced and no other mutations were incurred during the PCR amplification procedure, which has been estimated to incorporate an incorrect nucleotide every 9000 nucleotides (Tindall & Kunkel, 1988).

Expression and Characterization of Leu-55-Pro. The IPTG-induced expression level of Leu-55-Pro TTR variant was 30 mg/9.5 L of growth medium using the pKNTR expression system. We found that addition of IPTG directly after inoculation inhibited cell growth and gave very low levels of TTR expression. The expression of TTR was found to be greatest when cells were induced after cells reached an OD_{600} > 0.7. The purification scheme described under Experimental Procedures gave greater than 90% purity after passage through the DEAE ion-exchange column as seen by SDS–PAGE. Purified Leu-55-Pro TTR was characterized and compared to wild-type TTR. A Western blot analysis confirmed the immunoreactivity of recombinant Leu-55-Pro TTR as shown by the fact that the protein cross-reacted with polyclonal rabbit IgG TTR antibody. Also, N-terminal sequencing revealed that the first 10 residues of Leu-55-Pro TTR were present

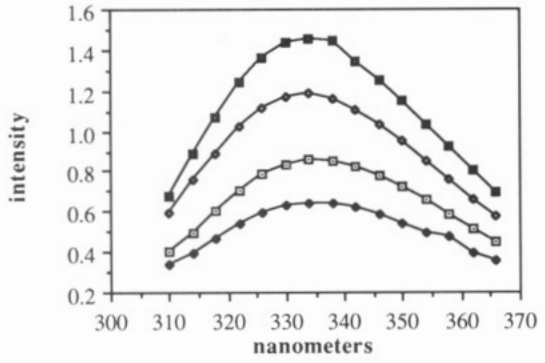

FIGURE 2: Intrinsic Trp fluorescence of Leu-55-Pro TTR and observed quenching upon binding of T_4 when excited at 278 nm and scanned from 310 to 370 nm. TTR (0.75 μM) and T_4 (1.5 μM) were used. Percent quenching was determined from values at 335 nm where maximum fluorescence and quenching are observed. Open squares represent intrinsic fluorescence of wild-type TTR. Solid diamonds represent wild-type TTR fluorescence after T_4 quenching. Solid squares represent intrinsic fluorescence of Leu-55-Pro TTR. Open diamonds represent Leu-55-Pro TTR fluorescence after T_4 quenching.

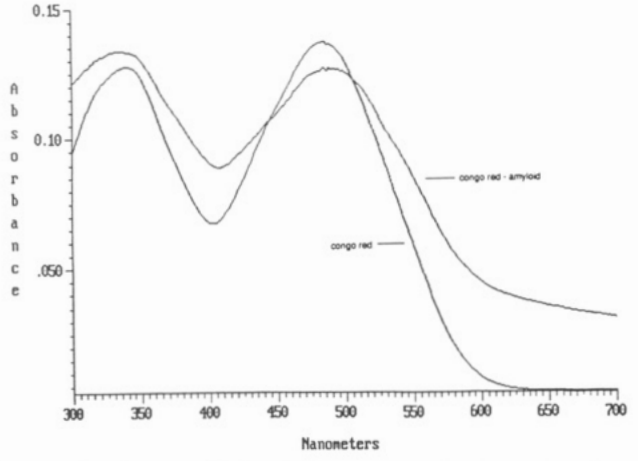

FIGURE 3: Congo red absorbance spectra showing red shift in aborbance when congo red is bound to amyloid fibrils.

and that the *ompA* leader sequence was properly processed after TTR was secreted into the periplasmic space of *E. coli*. The T_4 fluorescence assay confirmed the integrity of the quaternary structure of Leu-55-Pro insofar as TTR exhibited the expected quenching of the Trp fluorescence upon binding of T_4. Approximately 25% of the Trp fluorescence is quenched when T_4 is added to wild-type TTR. Leu-55-Pro TTR exhibited an increase in the overall intrinsic fluorescence when compared to wild-type TTR. The Leu-55-Pro TTR fluorescence is 170% of that observed for wild-type TTR. The relative quenching observed upon addition of T_4 to Leu-55-Pro TTR was 18%, slightly less than that observed for wild-type TTR (Figure 2). The fluorescence data suggest that the quaternary and/or tertiary structure of Leu-55-Pro TTR is altered; however, it is still tetrameric as determined by glutaraldehyde cross-linking studies.

In Vitro Amyloid Fibril Formation. We have previously demonstrated that the partial acid-mediated denaturation of wild-type TTR affords amyloid fibrils at pHs between 4.5 and 3.3. Amyloid fibril formation for Leu-55-Pro TTR has been shown to be significant at pHs between 6.1 and 3.6. That these aggregates are amyloid has been demonstrated by a congo red binding assay in which a red shift was observed in the congo red absorbance spectra (Figure 3). Polarized microscopy reveals green birefringence of the amyloid fibrils when stained with congo red, and electron microscopy shows

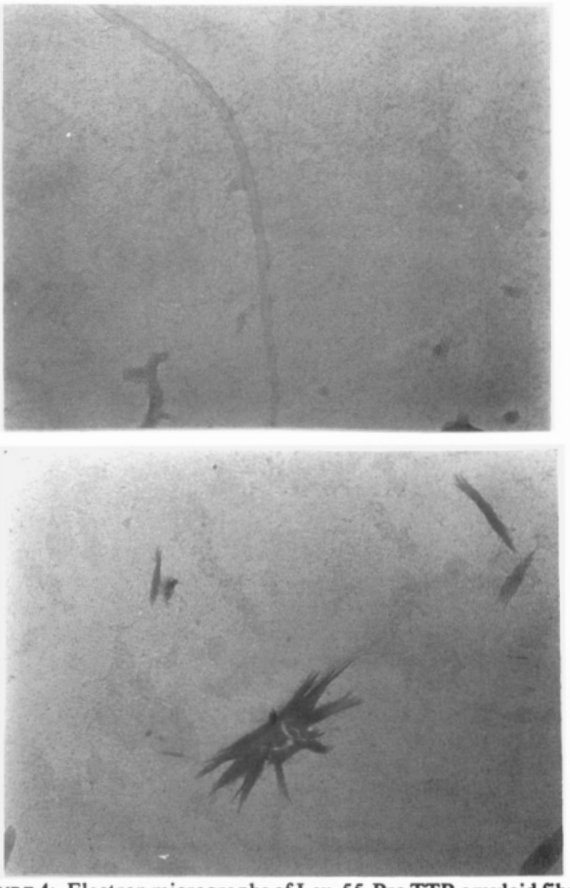

FIGURE 4: Electron micrographs of Leu-55-Pro TTR amyloid fibrils formed at pH 5.5 at 0.2 mg/mL. The scale for the single fibril is 1 mm = 125 Å. The scale for the laterally associated fibrils is 1 mm = 400 Å.

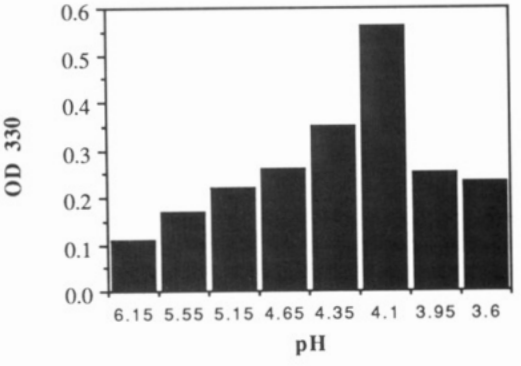

FIGURE 5: Bar graph representing overall amyloid fibril formation as a function of total increase in OD at 330 nm observed after samples were incubated for 1 h under described conditions.

fibrillar structures with the correct dimensions and the expected macromolecular twist (Figure 4). In addition, a pH-dependent study of amyloid fibril formation revealed that Leu-55-Pro TTR forms amyloid fibrils under less acidic conditions and at lower protein concentrations when compared to wild-type TTR. Clearly this variant is much more amyloidogenic than wild-type TTR under these conditions. In fact, wild-type TTR shows negligible amyloid fibril formation at the concentrations used to study Leu-55-Pro TTR fibril formation (0.2 mg/mL), irrespective of pH. Figure 5 illustrates the relative amount of amyloid fibril formation for Leu-55-Pro TTR as a function of pH after 1 h. Aggregation was monitored by a light scattering time course and the final OD at 330 nm after 1 h of incubation is reported. Maximum fibril formation in this experiment was seen at pH 4.1. Importantly, the Leu-55-Pro TTR variant can form amyloid fibrils to a significant extent at pH 5.5, which is the accepted operating pH of a

normal lysosome (Thoene, 1992; Winchester, 1992; Holtzman, 1989).

Tetramer to Monomer Transition as a Function of pH. The stability of the Leu-55-Pro TTR tetramer as a function of pH was probed taking advantage of the amyloid fibril inhibitor Z 3-14. The Leu-55-Pro variant exhibits a tetramer to monomer transition centered at pH 4.35 in the presence of Z 3-14. This is significantly higher than the pH_m observed for wild-type TTR, which in the presence of Z 3-14 has been shown to be centered at pH 3.8. In the absence of Z 3-14, the transition is centered at pH 5.35 for Leu-55-Pro TTR and at pH 4.4 for wild-type TTR, which indicates that in addition to inhibiting fibril formation, the detergent also appears to stabilize the tetrameric form of TTR. It should be stressed that some amyloid fibrils do form in the absence of Z 3-14 perturbing the equilibrium between monomer and tetramer. Stabilization of TTRs quaternary structure by Z 3-14 apparently results from the SO_3^- functionality interacting with the cationic sites in the protein. It is well-known that the sulfate anion generally stabilizes the folded structure of proteins and it is interesting that 0.5 M sodium sulfate stabilizes the quaternary structure of TTR to the same degree that Z 3-14 does (Z. Lai and J. W. Kelly, unpublished observation). The data obtained from the SDS–polyacrylamide gels through densitometry to determine the relative amounts of tetramer and monomer at the designated pHs are summarized in Figure 6. Detailed studies on the wild-type protein have shown that the pH_m of the tetramer to monomer transition determined by near-UV CD is similar to the pH_m determined by the SDS–PAGE analysis, further verifying the validity of the SDS–PAGE approach.

DISCUSSION

Since the majority of the transthyretin mutations associated with FAP are not easily available via isolation from plasma of heterozygous patients, we have constructed an expression cassette for TTR which facilitates rapid site-directed mutagenesis using PCR methodology and facilitates TTR expression from readily available expression vectors. The size of the expression cassette and the ease with which mutations can be introduced makes this new construct much more convenient than the original TTR construct (Ho et al., 1989). We found that cloning the 480-bp *ompA*/TTR expression cassette into the pKEN2 vector engineered by Verdine and co-workers gave higher expression levels for Leu-55-Pro TTR than did other commercially available vectors. This is presumably due to the better control of the inducible *lac*-regulated overexpression phagemid, pKEN2. The strong control in this vector proves to be of importance because *E. coli* did not grow well, nor did cells express TTR when they were induced directly after inoculation. Another factor contributing to the appeal of the pKEN2 vector is the strong *rrn* BT_1T_2 ρ-independent transcriptional terminator which prevents runoff transcription of the vector (Brosius et al., 1981). In addition, the ribosome binding site used in this construct is close to the consensus Shine–Dalgarno sequence with optimum spacing between the Shine–Dalgarno sequence and the initiation codon (Gold & Stormo, 1990). We encountered severe reproducibility problems with expression of Leu-55-Pro TTR using the pHNTR expression system which is under control of the *tac* promoter (DeBoer et al., 1983). Expression levels of recombinant protein using the pHNTR system varied between 5 and 60 mg/9.5-L prep, and for this reason, we opted to use the pKNTR expression system.

The concept that a single amino acid substitution in a protein can give rise to an altered folding/unfolding pathway leading

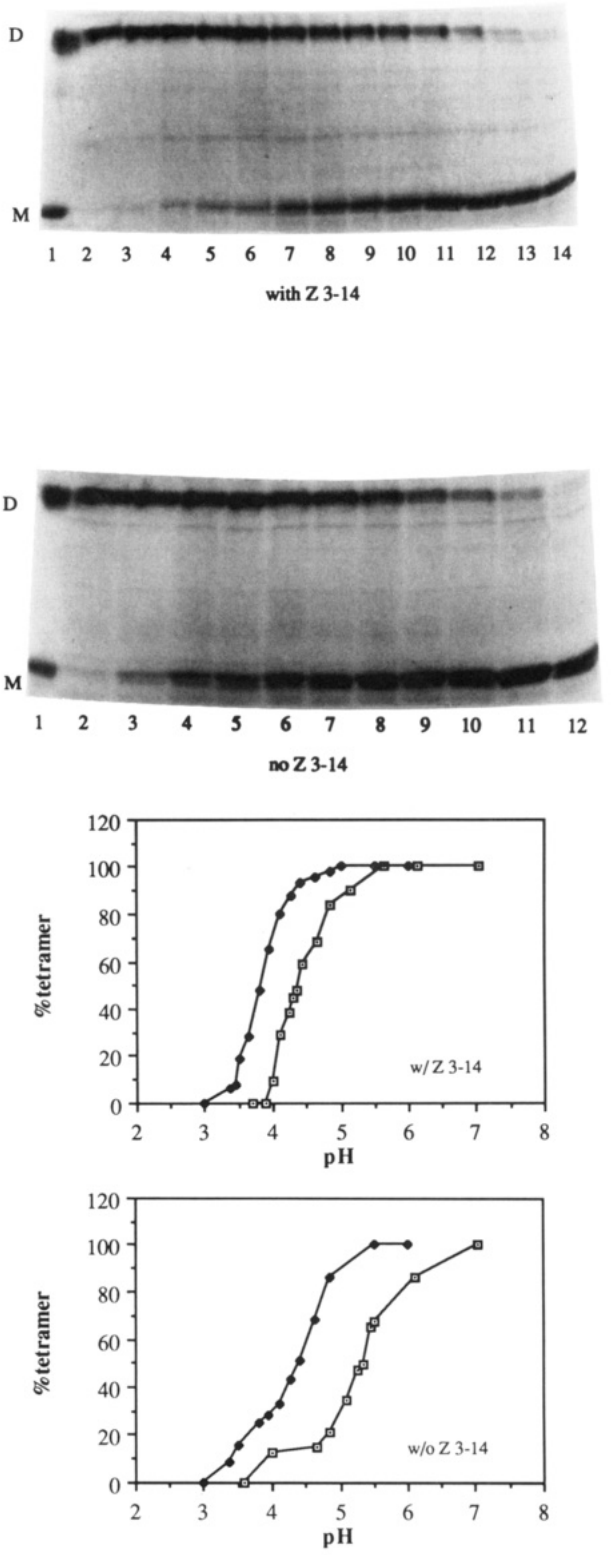

FIGURE 6: (Top two panels) SDS–PAGE showing tetramer to monomer transition for Leu-55-Pro TTR in the presence and absence of Z 3-14, where the amount of protein running as dimer is taken to represent the total amount of tetramer. For SDS–PAGE with Z 3-14 (top panel) lanes 2–14 represent samples incubated at pHs 7.05, 6.15, 5.65, 5.15, 4.85, 4.65, 4.45, 4.35, 4.3, 4.25, 4.1, 4.0, and 3.9. For SDS–PAGE without Z 3-14 (second panel) lanes 2–12 represent samples incubated at pHs 7.05, 6.1, 5.5, 5.45, 5.35, 5.25, 5.1, 4.85, 4.65, 4.0, and 3.9. Lane 1 in both cases is double-loaded with unboiled and boiled TTR standards running as dimer (D) and monomer (M), respectively. (Bottom two panels) Graphs representing data obtained from densitometry readings of gel data from the top two panels showing a tetramer to monomer transition for Leu-55-Pro TTR (open squares) centered at pH 4.35 with Z 3-14 present and at pH 5.35 in the absence of Z 3-14. For comparison, wild-type TTR (solid diamonds) is also shown with a transition centered at pH 3.8 with Z 3-14 and at pH 4.4 without Z 3-14.

to self-association has been well substantiated in the literature (Brems et al., 1988; Seckler et al., 1989; Yu & King, 1984; King & Yu, 1986). Jaenicke and King have described single amino acid substitutions in the P22 tailspike protein which dramatically influence the folding pathway (Seckler et al., 1989; Yu & King, 1984; King & Yu, 1986). Temperature-sensitive mutations have been identified which render the P22 tailspike protein capable of aggregation at nonpermissive temperatures. The thermolabile folding intermediate identified in the wild-type folding pathway is destabilized in the temperature-sensitive mutations and there is an increase in the competing off-pathway process leading to aggregation. Also, Brems has shown that a single mutation in bovine growth hormone leads to slower refolding and an increase in aggregation due to stabilization of a folding intermediate (Brems et al., 1988). With these results as precedent, it is conceivable that single amino acid substitutions such as those associated with FAP could dramatically affect the reconstitution/denaturation pathway of TTR and therefore be responsible for the onset of amyloid fibril deposition by either stabilizing the amyloidogenic intermediate and/or destabilizing the native quaternary structure of TTR.

The experiments described within demonstrate that although the tetramer of Leu-55-Pro TTR can form, it is significantly less stable toward acid-mediated denaturation when compared with wild-type TTR. The increased Trp fluorescence intensity in Leu-55-Pro TTR combined with the decreased ability of T_4 to quench the intrinsic Trp fluorescence suggests that the tertiary and/or quaternary structure is different than that of the wild-type protein. This is not surprising and most likely results from the nonconservative Leu to Pro mutation. The overall increase in fluorescence intensity for the variant may be explained by a shift in the environment surrounding either one or both of the Trp residues in each monomer or a conformational change which masks an intrinsic quenching factor. The decreased quenching of the Trp fluorescence upon T_4 binding in the case of Leu-55-Pro TTR could be a result of various scenarios which cannot be distinguished until high-resolution structural data becomes available.

The resistance of TTR to SDS-induced denaturation proves useful for comparing the stabilities of wild-type and Leu-55-Pro TTR, employing SDS–PAGE to determine the extent of quaternary structural change as a function of pH. These studies reveal that the wild-type tetramer is significantly more stable toward acid denaturation than is the Leu-55-Pro tetramer. In the presence of the Z 3-14 amyloid inhibitor, the tetramer to monomer transition is centered at pH 3.8 for wild-type TTR and at pH 4.35 for Leu-55-Pro TTR. In the absence of inhibitor, the transition for wild-type TTR is centered at pH 4.4, whereas that for Leu-55-Pro TTR is centered at pH 5.35. We have recently determined that the stabilization of the transthyretin tetramer by Z 3-14 is most likely the result of the SO_3^- functionality in Z 3-14 interacting with positively charged TTR. Analogous shifts in stability have resulted from the addition of 0.5 M sodium sulfate to TTR solutions. That sodium sulfate has no inhibitory effect on amyloid fibril formation suggests that Z 3-14's tetramer-stabilizing and amyloid inhibitory functions are separable. To ensure that the SDS–PAGE method faithfully reports on the quaternary structural changes occurring in acidic solution, the TTR samples were cross-linked after incubation but before subjecting the Leu-55-Pro samples to SDS–PAGE analysis. The pH_ms determined for cross-linked and non-cross-linked Leu-55-Pro samples were identical, as was the case for the wild-type TTR, confirming the validity of the method for measuring quaternary structural changes (Colon & Kelly, 1992). Furthermore, a comparison of wild-type TTR pH_ms by near-UV CD and SDS–PAGE reveals pH_ms that are similar.

The decreased stability of the Leu-55-Pro tetramer is potentially important in the pathology of FAP because the tetramer must dissociate to what appears to be a structured monomer in order to self-associate into amyloid fibrils (W. Colon, Z. Lai, and J. W. Kelly, manuscript in preparation). Interestingly, similar unpublished studies from our laboratory on another FAP-causing TTR variant (Val-30-Met) reveal that this variant is also less stable than the wild-type protein and its stability falls between that of wild-type and Leu-55-Pro TTR (pH_m of 4.2 with Z 3-14 and pH_m of 4.7 without Z 3-14) (W. Colon and J. W. Kelly, manuscript in preparation). Collectively, this data demonstrates that wild-type TTR is more stable than Val-30-Met TTR, which is more stable than Leu-55-Pro TTR. Interestingly, the instability of each TTR tetramer correlates with the severity of the pathogenicity of each TTR sequence.

We have previously proposed that amyloid fibril formation could occur in the lysosome by an off-pathway process which competes with normal protein turnover. Normal protein turnover mediated by acid denaturation followed by proteolysis affords amino acids and small peptides. Alternatively, TTR could undergo acid-mediated partial denaturation leading to a conformational intermediate which is capable of self-association at a rate that is faster than the rate at which it is proteolytically processed, affording amyloid fibrils which are known to be protease-resistant. It is conceivable that these amyloid fibrils could then be exported out of the cell to locations where amyloid fibrils have been observed *in vivo*. The known facts regarding TTR amyloidosis can be explained by a mechanism of this type. Interestingly, Leu-55-Pro TTR appears to be capable of forming amyloid fibrils at a significant rate at the pH at which a normal lysosome operates. This may explain the much earlier onset of Leu-55-Pro FAP.

The correlation between the increasing pH at which the tetramer to monomer transition occurs and the increasing pathogenicity of TTR amyloid disease further supports the lysosomal hypothesis wherein a structured putatively monomeric form of TTR self-associates into amyloid fibrils. We have demonstrated that the FAP-causing TTR variant homotetramers Val-30-Met and Leu-55-Pro are able to self-associate into amyloid fibrils at pHs as high as 5.0 and 6.15, respectively. Both of these pHs are easily obtained within a lysosome and are significantly higher than the pHs at which wild-type TTR is capable of amyloid fibril formation (pH 3.3–4.5). The *in vitro* experiments demonstrate that Leu-55-Pro TTR is the least stable tetramer and as such can dissociate to the amyloidogenic intermediate, which readily self-associates into amyloid fibrils at pHs accessible within a normal lysosome. At lysosomal pHs, the equilibrium between tetramer and monomer lies further toward the amyloidogenic intermediate as the acid stability of TTR tetramer decreases from wild-type to Val-30-Met to Leu-55-Pro TTR. Shifting the equilibrium toward the amyloidogenic intermediate increases the rate of amyloid fibril formation in the order of wild-type $\ll$ Val-30-Met $<$ Leu-55-Pro. The amyloidogenicity both *in vitro* and *in vivo* correlates with the acid stability of the TTR tetramer.

Amyloid fibrils formed from Leu-55-Pro and wild-type TTR under the acidic conditions described within run as monomeric TTR when loaded onto a denaturing SDS–PAGE gel. As described previously, tetrameric TTR is resistant to SDS and will run as a dimer when an unboiled sample is loaded onto

an SDS–PAGE gel. Since monomer is the only species visible when fibrils are subjected to SDS–PAGE, this experiment suggests that the amyloid fibrils are composed of monomeric subunits of TTR which are noncovalently associated. Alternatively, we cannot rule out the possibility that a nonnative dimer or tetramer which is not SDS-resistant is the amyloidogenic precursor. However, these studies suggest that the stability of the TTR tetramer is directly related to amyloidogenicity and that an amyloidogenic monomer leads to amyloid fibril formation.

CONCLUDING REMARKS

The results presented here demonstrate that the mutation of Leu to Pro at position 55 in TTR gives rise to a variant form of TTR which behaves dramatically differently than wild-type TTR with respect to the pH at which a tetramer to monomer transition is observed as well as pHs and concentrations at which amyloid fibril formation is observed. The Leu-55-Pro pH_m in the absence of Z 3-14 is 5.35, approximately a full pH unit higher than that of wild-type TTR (pH_m 4.4 in the absence of Z 3-14). The acid stability of the tetramer appears to be a critical factor in amyloidogenicity because denaturation of the tetramer affords an amyloidogenic intermediate which is capable of self-association into amyloid fibrils. The acid stability of the TTR tetramer appears to determine the concentration of the amyloidogenic intermediate present at a given pH, which in turn governs the rate of amyloid fibril formation. The Leu-55-Pro TTR variant has been found in FAP patients as amyloid depsoits 2–3 decades prior to other known pathogenic variants of TTR and approximately 6 decades prior to amyloid deposition derived form wild-type TTR observed in SSA patients. It would follow from these facts that the Leu-55-Pro variant is able to adopt the amyloidogenic conformation necessary for amyloid fibril formation under physiological conditions with greater ease than other FAP variants and wild-type TTR. The Leu-55-Pro mutation dramatically affects TTR denaturation and appears to stabilize the amyloidogenic conformation at the expense of the quaternary structure at pHs where wild-type TTR is tetrameric. The lowered stability of the Leu-55-Pro TTR tetramer toward acidic denaturation has been verified by SDS–PAGE studies described within. The increased overall fluorescence of the Leu-55-Pro TTR variant suggests that there is a conformational change in the tetramer in which either one or both of the Trp residues found in each monomer of TTR is in an altered environment. The reduced quenching of intrinsic Trp fluorescence upon addition of T_4 suggests that this mutation may alter the quaternary structure of TTR. The fact that monomeric Leu-55-Pro TTR is present at pHs around 5.5 confirms the altered stability of TTR. In addition, the ability to form amyloid fibrils from Leu-55-Pro TTR at the normal operating pH of a lysosome further implicates lysosomal involvement in amyloid fibril formation *in vivo*. Taken together, these *in vitro* studies reveal significant differences between wild-type and Leu-55-Pro TTR and, most importantly, begin to shed light on why this recently discovered TTR variant exhibits such aggressive and extreme pathogenicity.

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