Published in final edited form as:

J Mol Biol. 2013 March 25; 425(6): 977–988. doi:10.1016/j.jmb.2013.01.008.

Localized structural fluctuations promote amyloidogenic conformations in transthyretin

Kwang Hun Lim^{a,1}, H. Jane Dyson^b, Jeffery W. Kelly^{c,d}, and Peter E. Wright^{b,d,1}

^aDepartment of Chemistry, East Carolina University, Greenville, NC 27858, USA

^bDepartment of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037, USA.

^cDepartments of Chemistry and Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037, USA.

^dThe Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California 92037, USA.

Abstract

The process of transthyretin (TTR) misfolding and aggregation, including amyloid formation, appears to cause a number of degenerative diseases. During amyloid formation, the native protein undergoes a tetramer-to-folded monomer transition, followed by local unfolding of the monomer to an assembly-competent amyloidogenic intermediate. Here we use NMR relaxation dispersion to probe conformational exchange at physiological pH between native monomeric transthyretin (the F87M/L110M variant) and a small population of a transiently formed amyloidogenic intermediate. The dispersion experiments show that a majority of the residues in the β -sheet containing β -strands D, A, G and H undergo conformational fluctuations on μ s-ms time scales. Exchange broadening is greatest for residues in the outer β -strand H, which hydrogen bonds to β -strand H' of a neighboring subunit in the tetramer, but the associated structural fluctuations propagate across the entire β -sheet. Fluctuations in the other β -sheet are limited to the outer β -strand F, which packs against strand F' in the tetramer, while the B, C, and E β -strands of this sheet remain stable. The structural changes were also investigated under more forcing amyloidogenic conditions (pH 6.4–3.7), where β -strand D and regions of the D-E and E-F loops were additionally destabilized, increasing the population of the amyloidogenic intermediate and accelerating amyloid formation. Strands B, C, and E appear to maintain native-like conformations in the partially unfolded, amyloidogenic state of wild type TTR. In the case of the protective mutant T119M, the conformational fluctuations are suppressed under both physiological and mildly acidic conditions, indicating that the dynamic properties of TTR correlate well with its aggregation propensity.

Keywords

NMR; protein misfolding; relaxation dispersion; amyloid; transthyretin amyloidoses

© 2012 Elsevier Ltd. All rights reserved.

¹Corresponding Authors: Kwang Hun Lim, limk@ecu.edu, (T) 252-328-9805, (F) 252-328-6210 Peter Wright, wright@scripps.edu, (T) 858-784-9721, (F) 858-784-9822.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author contributions: K.H.L., H.J.D., and P.E.W. designed research; K.H.L. performed research; K.H.L., H.J.D., J.W.K., and P.E.W. analyzed data; and K.H.L., H.J.D., J.W.K., and P.E.W. wrote the paper.

The authors declare no conflict of interest.

Introduction

Transthyretin (TTR) is a 55 kDa tetrameric protein that binds and transports holoretinol binding protein in blood and thyroxine and holoretinol binding protein in the cerebrospinal fluid. Tetramer dissociation, monomer misfolding and self-assembly of amyloidogenic monomers into amyloid and other aggregate morphologies is known to be linked to several human degenerative diseases.^{1,2} Aggregation of wild type TTR is associated with senile systemic amyloidosis (SSA), a cardiomyopathy affecting as much as 20% of the population over age 80. Aggregation of one of more than 100 single-point mutations of TTR causes familial amyloidotic polyneuropathy (FAP) in ~ 10,000 patients, familial amyloid cardiomyopathy (FAC) in 3-4% of the African population,³ and rarely central nervous system selective amyloidosis (CNSA).⁴⁻⁶

The X-ray structure of the folded tetramer of TTR shows that each TTR subunit adopts a β -sheet sandwich structure, in which two four-stranded sheets, formed by β -strands DAGH and CBEF, respectively, are stacked on each other (Fig. 1).⁷ Two TTR monomers dimerize by formation of a hydrogen bonded anti-parallel β -sheet between the H and H' strands and close contacts, without hydrogen bonding, between the F and F' strands. The tetramer is then formed by the dimerization of the dimers through AB – GH loop interactions. TTR amyloidogenesis occurs by dissociation of tetrameric TTR into monomers that subsequently undergo a conformational transition to a partially unfolded intermediate state, which self-assembles into soluble oligomers and amyloid fibrils (Fig. 1).⁸⁻¹⁰ The majority of the pathogenic single-point mutations in TTR destabilize the tetramer or the monomer and thereby facilitate amyloid formation.^{11,12} The tetramer $\rightleftharpoons$ folded monomer $\rightleftharpoons$ misfolded monomer equilibria are strongly thermodynamically linked, which explains why both tetramer-destabilizing and monomer-destabilizing mutations can promote amyloidogenesis.¹³ A protective single-point mutant (T119M), with decreased amyloidogenicity, has also been identified.^{14,15} Structural analyses of the WT and mutant forms of tetrameric TTR reveal that the pathogenic and protective mutations induce only subtle structural changes.¹⁶ The single amino acid substitutions affect the stability of TTR rather than its structure,^{13,14} and the energetics and dynamics of the protein are implicated in the transition to the monomeric amyloidogenic intermediate.

Hydrogen/deuterium (H/D) exchange NMR experiments were used to probe the conformational flexibility of the tetrameric form of TTR.^{17,18} The H/D exchange studies identified a stable core consisting of β -strands A, B, E, G and the AB loop that was destabilized in the pathogenic mutants L55P and V30M at neutral pH and under more denaturing amyloidogenic conditions (pH 4.5) for the wild type protein.¹⁹ In contrast, enhanced stability of this core was observed with the suppressor variant T119M. Wild type and pathogenic variant TTRs have been subjected to molecular dynamics simulations to obtain insights into conformational flexibility and the transition to amyloidogenic states. The amyloidogenic mechanism has been variously related to changes in conformation and dynamics in strands F, G, and H,²⁰ displacement of β -strands C and D from the β -sheet,^{21,22} destabilization of the CBEF β -sheet,²² destabilization of the DAGH β -sheet,²¹ and cooperative conformational transitions to α -sheet in both β -sheets of TTR.^{23,24}

In the present study, we utilized a monomeric variant of TTR (F87M/L110M, M-TTR)²⁵ to directly probe conformational fluctuations within the native TTR monomer that form a potentially amyloidogenic intermediate state. The crystal structure of M-TTR, which forms a tetramer at high concentration and crystallizes as such, showed that its tertiary structure is almost identical to that of wild type TTR.²⁵ In addition, amyloid formation can be induced at less acidic pH values for M-TTR (pH > 5), while a more acid pH is required for tetrameric

wild type TTR (pH < 5) in order to trigger rate-limiting tetramer dissociation. Because complications associated with the tetramer $\rightleftharpoons$ monomer equilibrium are eliminated, the monomeric variant of TTR is an ideal system to investigate structural changes between the natively folded TTR and aggregation-competent intermediate states.

To explore the conformational exchange between the native tertiary structure and intermediate states of monomeric TTR under physiological conditions (pH 7.2) we performed ^{15}N R_2 relaxation dispersion experiments on both wild type M-TTR and the monomeric form of the T119M suppressor variant (F87M/L110M/T119M). In addition, two dimensional $^1\text{H}/^{15}\text{N}$ heteronuclear single-quantum coherence (HSQC) NMR spectra were recorded for both monomers under acidic conditions to probe changes that promote amyloid formation. These combined NMR studies allowed us to map out regions exhibiting conformational fluctuations implicated in misfolding and aggregation, including amyloid formation.

Results

Conformational fluctuations at physiological pH

Amyloid formation from natively folded proteins requires local and/or global unfolding of the native state.^{1,26-29} ^{15}N R_2 relaxation dispersion experiments were recorded at two magnetic field strengths to probe spontaneous ms- μs time scale conformational fluctuations in wild type M-TTR at physiological pH (7.2). Characteristic relaxation dispersion profiles were observed for many residues and were fitted to a global two-site exchange model ($A \rightleftharpoons B$) with overall exchange rate k_{ex} and populations p_A and p_B . Representative dispersion profiles are shown in Fig. 2A and the dispersion data for all residues are shown in Supplementary Fig. S1. Residue-specific values of R_{ex} , the exchange contribution to the R_2 relaxation rate, were derived from the fits of the dispersion data. Many of the residues with large R_{ex} values are located in quaternary structure interface regions of TTR (β -strand H for the dimerization of monomers, and the AB and GH loops for the dimerization of dimers). However, we do not believe that the exchange contributions are a result of monomer-dimer or monomer-tetramer equilibria. Experiments performed at different concentrations (0.25 and 0.75 mM) of WT M-TTR gave identical relaxation dispersion profiles and the same R_{ex} value for any given residue, at each of the concentrations (Fig. 2B and Supplementary Fig. S2). If the observed dispersion was a result of oligomerization of the M-TTR, the dispersion profiles would be strongly concentration-dependent; R_{ex} would be expected to increase 2.5-fold or 8-fold over the concentration range of our experiments if the exchange process was associated with transient formation of dimers or tetramers, respectively (see legend to Supplementary Fig. S2). In addition, no changes are observed in the HSQC spectra of M-TTR over the concentration range 0.04–0.75 mM. Thus, our data show clearly that M-TTR is strictly monomeric at the concentrations used in the relaxation experiments, and the observed relaxation dispersion must therefore arise from conformational exchange within the monomer and not from transient oligomerization. Analytical ultracentrifugation experiments have confirmed that M-TTR is fully monomeric at 100 μM concentration.²⁵

^{15}N relaxation dispersion experiments were also performed for the engineered monomeric version of the suppressor variant T119M (i.e. the triple mutant F87M/L110M/T119M, denoted T119M M-TTR). Compared to wild type M-TTR, many fewer residues exhibit dispersion, and with greatly decreased R_{ex} values (Fig. 3, Supplementary Fig. S3). As for wild type M-TTR, the dispersion profiles are concentration independent over the range 0.25–0.75mM.

Many of the expected backbone amide cross peaks are absent from or are too weak to be assigned in the HSQC spectra of wild type M-TTR and, to a lesser extent, the T119M

variant. For wild type M-TTR, cross peaks are missing for residues in the D-E loop, several regions of the E-F loop, including the N-terminal turn of the helix, the beginning of β -strand F, the F-G loop, the G-H loop, and much of β -strand H. While it is possible that some cross peaks could be lost due to rapid exchange with solvent at pH 7.2, it is much more likely that the missing resonances are broadened by protein conformational exchange. Most residues with missing amide cross peaks are located in or adjacent to regions of M-TTR where ^{15}N relaxation dispersion is observed. Further, several of the cross peaks that are absent from the spectrum of wild-type M-TTR are observed for the T119M M-TTR variant, in which the exchange processes cause less severe line broadening (Fig. 3B). It is notable that ^{15}N relaxation dispersion is observed for many of these “new” cross peaks in spectra of T119M M-TTR, suggesting that the corresponding residues in wild type M-TTR experience extreme exchange broadening. The location of the missing resonances and those that we infer to be exchange broadened in wild type M-TTR are mapped to the structure in Fig. 4A.

For wild type M-TTR at pH 7.2, many residues in β -strands A, G, H, F and the A-B and E-F loops exhibit dispersion, showing that they undergo conformational fluctuations on μs -ms time scales. Indeed, dispersion is observed for most residues in the DAGH β -sheet, with the magnitude of the exchange contribution (R_{ex}) diminishing across the sheet in the H strand to D strand direction; extreme exchange broadening is observed for residues in β -strand H with only minimal broadening in β -strand D (Fig. 4A). With the exception of β -strand F and the ends of β -strand E, residues in the CBEF β -sheet exhibit little or no dispersion, implying that this β -sheet remains stably folded and undergoes minimal conformational fluctuation on the μs -ms time scale. Similar NMR relaxation dispersion experiments were performed for the monomeric form of the suppressor variant, T119M M-TTR. Residues in the same regions as wild type M-TTR exhibit conformational exchange, but the magnitude of R_{ex} is substantially decreased (Fig. 4B), consistent with the reduced aggregation propensity of the T119M TTR.¹⁵

Although many of the residues with large R_{ex} values are located in quaternary structure interface regions of tetrameric TTR (β -strands F and H for the dimerization of monomers, and the AB and GH loops for the dimerization of dimers), the exchange broadening is concentration-independent, as noted above, suggesting that it does not arise from transient oligomer formation. Rather, the results suggest that the regions of TTR that form the subunit interfaces in the tetramer are preferentially destabilized in the monomer, facilitating conformational fluctuations to a potentially amyloidogenic intermediate state.

Thermodynamic analyses of the conformational transition

The ^{15}N relaxation dispersion profiles for both wild type and T119M M-TTR were fitted to a global two-site exchange model to estimate energetic parameters for the conformational transition (exchange rates and populations of the ground states and transient conformational substates). The population of the transient substate formed by wild type M-TTR is higher ($3.5 \pm 0.2\%$) than that formed by the protective T119M variant (population $1.0 \pm 0.1\%$). Since many more residues exhibit dispersive behavior, wild type M-TTR appears to undergo more extensive conformational fluctuations than the T119M variant. The rate of exchange, $k_{\text{ex}} (=k_f + k_b)$ is also different, $1700 \pm 50 \text{ s}^{-1}$ for wild type M-TTR and $2400 \pm 100 \text{ s}^{-1}$ for T119M M-TTR. Thus, the transient conformational substate of the T119M variant is less stable, is formed more slowly ($k_f = 24 \text{ s}^{-1}$, compared to 60 s^{-1} for wild type), and returns to the ground state more rapidly ($k_b = 2.4 \times 10^3 \text{ s}^{-1}$) than that of wild type M-TTR ($k_b = 1.6 \times 10^3 \text{ s}^{-1}$). These results indicate that the protective T119M mutation suppresses intrinsic fluctuations which, in monomeric TTR, lead to formation of a transiently populated, and potentially amyloidogenic, conformational substate.

Structural transitions at amyloidogenic pHs

Misfolding and aggregation, including amyloid formation, by TTR is accelerated under mildly acidic conditions. Tetrameric wild type TTR dissociates and forms amyloid at pH 5, with maximal aggregation rate exhibited at pH 4.4, while wild type M-TTR forms amyloid at higher pH (5.6) and aggregates very efficiently at pH greater than 5.^{8,25} In order to further investigate the conformational transitions along the amyloidogenesis pathway, 2D ¹H/¹⁵N HSQC NMR spectra of wild type M-TTR and T119M M-TTR were acquired at several acidic pH values (Fig. 5). The majority of HSQC cross peaks remain unshifted or only weakly shifted as the pH is decreased from 7.2 to 3.7, showing that the native TTR structure is maintained over this pH range. This result is consistent with X-ray crystallographic studies, which show that the native fold of the TTR tetramer is retained at pH 4.0.³⁰ Cross peaks with pH-dependent chemical shifts are mostly associated with His, Asp, and Glu residues and their immediate neighbors. At pH 4 and lower, new cross peaks appear in the center of the HSQC spectra of wild type M-TTR that indicate the onset of global TTR unfolding (Figs. S4, S5). These results are in agreement with previous biochemical studies showing that the aggregation propensity of TTR is significantly reduced under strongly acidic conditions, and that amyloid formation is strongly suppressed when the pH is lower than 4.^{8,25}

Many cross peaks in the wild type M-TTR spectrum broaden beyond detection as the pH is decreased, even at pH values where the native structure is clearly maintained (Fig. 5A). The changes in cross peak intensity were monitored as a function of pH, by normalizing to the intensity at pH 6.4 (Fig. 6A). With decreasing pH, broadening propagates across the DAGH β -sheet. Cross peaks from most residues in β -strand H are exchange broadened even at pH 7.2. At pH 5, where M-TTR forms amyloid fibrils very efficiently,²⁵ the broadening extends to β -strand D, most residues in the D-E loop, residues in the E-F helix, and residues in the A-B loop that are in contact with the helix (Fig. 7). As the pH is decreased below 5, these cross peaks become progressively broadened, and many completely disappear. Our results are in agreement with crystallographic data that show a change in the conformation of the E-F helix and E-F loop, in one subunit of the wild type TTR tetramer, when the pH is reduced from 4.6 to 4.0.³⁰ It is notable that very few residues in the CBEF β -sheet experience pH-induced exchange broadening, and the broadening that does occur begins only at pH 4, far below the onset pH for amyloid formation. Even residues in the edge β -strand F, which exhibit large R_{ex} at neutral pH, remain almost unchanged in intensity at pH 4.4, at which pH most DAGH resonances are broadened beyond detection. We conclude that partially unfolded TTR, formed by destabilization of the DAGH β -sheet under mildly acidic conditions, is the aggregation-competent state.

The pH-titration experiments were also carried out on T119M M-TTR (Fig. 5B). The HSQC spectra confirm that the native fold persists over a broad range of pH, with the unfolding transition occurring at a lower pH (pH 3.4) than for the wild type M-TTR (Fig. S5). In marked contrast to wild type M-TTR, the cross peak intensities of most residues of the monomeric T119M suppressor variant remain largely unchanged over the pH range 6.4 – 4.7, indicating a greatly reduced propensity for acid-induced conformational exchange (Fig. 6B). Cross peaks from residues in β -strand D and the D-E loop are exchange broadened at pH 3.7 and below but, overall, exchange broadening is far less extensive and occurs at much lower pHs than for wild type M-TTR (Fig. 6B). From these results it is clear that the single T119M point mutation stabilizes monomeric TTR and suppresses acid-induced conformational exchange, consistent with the decreased relaxation dispersion relative to wild type M-TTR at physiological pH. The increased stability and diminished conformational fluctuations in T119M M-TTR are accompanied by greatly decreased aggregation propensity (Fig. 8). These results suggest that the dynamic properties of the

residues exhibiting conformational exchange play an important role in TTR misfolding and amyloid formation.

Discussion

Characterization of the transient conformational fluctuations that allow natively folded proteins to become aggregation-competent under physiological conditions is of great importance for understanding mechanisms of amyloidogenesis. The NMR experiments described herein reveal the residues and secondary structural elements within the TTR monomer that undergo transient local unfolding and promote aggregation and amyloid formation. Relaxation dispersion measurements for wild type M-TTR show that most residues located in the DAGH β -sheet undergo μ s-ms timescale conformational exchange between the native ground state structure and an intermediate state, even at physiological pH. The exchange contributions are largest for residues in β -strands H, G, and A, in that order, but Glu54 in β -strand D also exhibits weak dispersion in wild type M-TTR. Cross peaks for residues in β -strand H are missing from the HSQC spectrum or are extremely broad and weak, presumably due to exchange broadening from conformational fluctuations on ms- μ s time scales. For wild type M-TTR, residues in β -strand F, at the exposed edge of the CBEF β -sheet, also experience exchange broadening and exhibit R_2 relaxation dispersion. By contrast, residues in strands B, C, and E of the CBEF sheet show no signs of μ s-ms timescale motions in either wild type M-TTR or T119M M-TTR, either at neutral or acidic pHs (Fig. 4).

In monomeric wild type TTR, the DAGH β -sheet is clearly destabilized, with structural fluctuations propagating from β -strand H into the interior of the sheet. Within the TTR tetramer, strands H and H' in adjacent subunits align anti-parallel to form a hydrogen bonded β -sheet; destabilization of the DAGH sheet in the TTR monomer likely stems from loss of the H-H' hydrogen bonding interactions. Structural fluctuations in the other β -sheet are limited to the outer β -strand, strand F, which packs against strand F' of the neighboring subunit in the dimer but without formation of stabilizing F-F' backbone hydrogen bonds. It is highly unlikely that the conformational fluctuations in the engineered M-TTR are related to the Phe87/Met and Leu110/Met substitutions (shown in Fig. 1) introduced to disrupt the subunit interfaces of the tetramer. These mutations are conservative, lie on the surface of the monomeric subunit, and do not perturb either the tertiary structure or the stability of the subunit towards acid or urea denaturation.²⁵

The conformational fluctuations in the wild type monomer are greatly damped by introduction of the T119M suppressor mutation (Fig. 4), which increases the thermodynamic and kinetic stability of the TTR fold.²⁵ Modest exchange contributions are observed for several residues in strands F and H of T119M M-TTR, but the associated conformational fluctuations do not propagate effectively across the sheets. Importantly, and in marked contrast to wild type M-TTR, none of the residues in β -strand D exhibit relaxation dispersion in T119M M-TTR.

The 2D HSQC NMR spectra obtained at different pH values provide insights into the structural changes implicated in amyloid formation. The aggregation kinetics of TTR are highly sensitive to pH. At high concentration (15 mg/mL), the monomeric variant M-TTR forms amorphous aggregates at neutral pH following a long incubation period (more than 2 weeks) but does not spontaneously form fibrils on a biologically relevant time scale.²⁵ Aggregation under these conditions is likely limited by the relatively low population (3.5% at pH 7.2) of the transiently unfolded state. Under mildly acidic conditions (pH 4-6), the aggregation propensity and rate of fibril formation is greatly increased, suggesting that the structural transition to an amyloidogenic intermediate state is facilitated. As the pH is

decreased below 6, amide cross peaks of residues in β -strand D and in the D-E loop of wild type M-TTR, which exhibit only small exchange contributions at physiological pH, lose intensity and disappear. At pH 4, the broadening extends to residues in β -strand A, the A-B loop, and the E-F helix while residues in the CBEF β -sheet remain relatively unaffected (Fig. 6). Even residues in strand F, which undergo substantial conformational exchange at pH 7.2 (Fig. 4A), remain almost unaffected as the pH is decreased. Taken together, these results suggest that the amyloidogenic state of wild type TTR possesses a largely native-like CBEF β -sheet conformation while the DAGH β -sheet is disrupted, likely due to transient dissociation of β -strands D and H. It has been suggested previously, on the basis of biophysical measurements and limited proteolysis experiments, that the amyloidogenic intermediate is formed by transient unfolding of the C strand-loop-D strand region.^{1,8,31-33} Our NMR experiments now provide direct evidence for transient fluctuations in β -strand D, which occur to a small extent at physiological pH and become exaggerated as the pH is decreased. However, little exchange broadening is observed for residues in strand C or the C-D loop even under the most amyloidogenic conditions (pH 4.4).

Our observation that most residues in the DAGH β -sheet undergo conformational fluctuations while the BCEF β -sheet remains stable, even at acidic pH, is in apparent disagreement with amide exchange measurements on wild type TTR at pH 4.5, which suggested destabilization of the BCEF β -sheet¹⁹. The origin of this discrepancy is currently unclear but likely reflects the differing timescales of the measurements, which probe fluctuations on the millisecond (relaxation dispersion) or minutes timescale (amide exchange). It is also possible that interpretation of the amide exchange data was complicated by the presence of a substantial (25%) population of oligomers under the conditions of the experiments¹⁹. We note that conformational changes in the DAGH sheet have been predicted by molecular dynamics simulations, from which a structural transition from β -sheet to α -sheet conformation was proposed to occur during the TTR amyloid formation process.^{23,34}

The pH-titration experiments show that several regions of the protein that are relatively stable at neutral pH, including the edge strand D in the DAGH β -sheet, the D-E loop, and the E-F helix, undergo enhanced conformational fluctuations at low pH, suggesting that loss of stability in one or more of these regions is an essential trigger for effective TTR amyloid formation. This observation is consistent with previous studies: conformational changes in the E-F loop and helix were observed in the crystal structure of wild type TTR grown at pH 4.0,³⁰ and strand D is substantially destabilized in the stronger amyloidogenic mutant L55P TTR even at neutral pH.¹⁸ In addition, numerous amyloidogenic single-point mutations occur in the D-E loop (L55P, L58H, and T60A) and the E-F loop (I73V, S77F/Y, Y78F, I84S/N, E89Q/K, and A91S).⁶ Computational studies also showed that the general effect of these mutations is to increase local flexibility at the site of the mutations.^{35,36} The links between local flexibility and amyloid formation in TTR are further demonstrated by our observation that the protective T119M mutant shows consistently less dynamic behavior than WT under both physiological and acidic conditions.

Previous CD experiments showed that further acidification of TTR below pH 4 induces global unfolding and reduces the amyloidogenic property significantly.⁸ Our NMR results clearly demonstrate two successive conformational transitions. Under mildly acidic conditions, M-TTR fluctuates between the native folded state and a locally unstructured state with strong aggregation propensity. At pH < 4, the HSQC spectra show that the protein begins to unfold more globally (Fig. S4) and would therefore be less amyloidogenic.

The NMR experiments described herein provide new insights into the molecular basis of TTR monomer misfolding and amyloid formation. At physiological pH, the intersubunit

regions (the H and F strands, and the AB and GH loops) and β -strands A and G in the DAGH β -sheet undergo conformational fluctuations that lead to exchange broadening of amide resonances. Exchange broadening is particularly pronounced at the C-terminus of β -strand H and in the adjacent β -strand, strand G, with which it forms hydrogen bonds. Although further NMR experiments will be required to determine the detailed nature of the structural changes associated with the exchange process, we note that fluctuations that transiently disrupt the secondary structure near the C-terminus of β -strand H would likely expose an aggregation-prone region of β -strand G which, as a peptide fragment, is competent to form amyloid fibrils.³⁷ Interestingly, recent relaxation dispersion experiments on a mutant of the Fyn SH3 domain have led to a strikingly similar observation; fluctuations at the C-terminus expose the aggregation-prone N-terminal β -strand and lead to formation of a small population of an aggregation competent intermediate.³⁸ In the amyloidogenic state of wild type M-TTR populated under mildly acidic conditions, where fibril formation is much more efficient, the structural fluctuations in the DAGH β -sheet extend to β -strand D and the D-E and E-F loops and appear to result in transient local unfolding that accelerates the rate of aggregation. Transient unfolding of β -strand D would expose β -strand A, which as an isolated peptide is highly aggregation-prone and spontaneously forms fibrils³⁹. The amyloidogenic intermediate formed by M-TTR at acidic pH retains elements of native-like structure, most notably in the CBEF β -sheet, consistent with some models of TTR amyloid fibrils proposed on the basis of biophysical measurements.^{33,40}

Materials and Methods

Protein Purification and Sample Preparation

¹⁵N-labeled and ¹⁵N, ¹³C-labeled wild type M-TTR and the M-T119M variant were expressed and purified from an *Escherichia coli* expression system as described previously.⁴¹

NMR Spectroscopy

NMR spectra were recorded using Bruker 500-, 600-, and 800-MHz spectrometers. Assignments for T119M M-TTR were made using standard multidimensional triple resonance NMR experiments.⁴² Many resonances are broadened in spectra of wild type M-TTR and triple resonance spectra were of poor quality. Assignments were therefore transferred, as far as possible, from T119M M-TTR and verified and extended using a 3D ¹⁵N-edited NOESY-HSQC spectrum.

R₂ Relaxation Dispersion Experiments

Relaxation dispersion data were acquired for M-TTR and T119M M-TTR samples (0.25-0.75mM) in phosphate buffer (10mM, pH 7.2) at 15°C. ¹⁵N R₂ relaxation rates were measured at proton Larmor frequencies of 500 and 800 MHz using relaxation-compensated CPMG (Carr-Purcell-Meiboom-Gill) pulse sequences implemented in a constant-time manner.^{43,44} A total relaxation time of 40 ms was used for all of the dispersion experiments. The R₂ relaxation dispersion data at the two field strengths were fitted with a two-site exchange model using the in-house computer program GLOVE with the following equations for the two state conformational exchange:⁴⁵

$$R_2^{eff}(1/\tau_{CP}) = R_2^0 + \frac{1}{2} \left[k_{ex} - \frac{1}{\tau_{CP}} \cosh^{-1} [D_+ \cosh(\eta_+) - D_- \cosh(\eta_-)] \right]$$

where,

$$\begin{aligned}
 D_{\pm} &= \frac{1}{2} \left[\pm 1 + \frac{\psi + 2\Delta\omega^2}{(\psi^2 + \zeta^2)^{1/2}} \right] \\
 \eta_{\pm} &= \frac{\tau_{CP}}{2} \left[\pm \psi + (\psi^2 + \zeta^2)^{1/2} \right]^{1/2} \\
 \psi &= (R_A - R_B - p_A k_{ex} + p_B k_{ex})^2 - \Delta\omega^2 + 4p_A p_B k_{ex}^2 \\
 \zeta &= 2\Delta\omega (R_A - R_B - p_A k_{ex} + p_B k_{ex})
 \end{aligned}$$

where $\Psi = k_{ex}^2 - \Delta\omega^2$, $\zeta = -2\Delta\omega k_{ex}(p_A - p_B)$, τ_{cp} is the time between successive 180° pulses in the CPMG pulse train, R_2^0 is the R_2 relaxation rate in the absence of conformational exchange, p_A and p_B are the populations of the ground- and excited-state conformations, respectively ($p_A + p_B = 1$), $\Delta\omega$ is the chemical shift difference between the two states, and it is assumed that $R_A = R_B$. Rate constants for the ground-to-excited state (k_f) and excited-to-ground state (k_b) transitions can be determined by $p_B \cdot k_{ex}$ and $p_A \cdot k_{ex}$ respectively. The dispersion data for all of the residues were fit with global k_{ex} and $p_A p_B$ values while allowing $\Delta\omega$ values to change for each residue. Uncertainties were estimated using Monte Carlo simulations.

HSQC pH-titrations

2D ^1H - ^{15}N HSQC spectra were recorded at 15°C over a range of pH values in 20mM phosphate (pH >5.5) or 50mM acetate buffers (pH <5.0). At low pH, M-TTR forms amyloid within several hours at 37°C (Fig. 8). However, the aggregation rate of TTR decreases substantially at lower temperatures⁷; at 15 μM concentration, TTR does not form amyloid at pH 4.4 and 25°C. The pH-titrations were therefore performed at low temperature (15°C) and low concentration (15 – 70 μM), under which conditions the NMR samples remained soluble for more than 12 hours.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Gerard Kroon for assistance with NMR experiments, Steve Bourgault for measurement of TTR amyloid formation kinetics, and Maria Martinez-Yamout and Euvel Manlapaz for technical support. This work was supported by the National Institutes of Health Grant DK34909 (P.E.W.), GM57374 (H.J.D.), the Skaggs Institute of Chemical Biology (P.E.W. and J.W.K.), and the ECU research development award (K.H.L.).

Abbreviations

TTR	transthyretin
HSQC	heteronuclear single-quantum coherence
WT	wild type
SSA	senile systemic amyloidosis
FAP	familial amyloidotic polyneuropathy
FAC	familial amyloid cardiomyopathy
CNSA	central nervous system selective amyloidosis (CNSA)
M-TTR	monomeric TTR

NOE	nuclear Overhauser effect
H/D exchange	hydrogen/deuterium exchange

Reference List

1. Kelly JW. Alternative conformations of amyloidogenic proteins govern their behavior. *Curr. Opin. Struct. Biol.* 1996; 6:11–17. [PubMed: 8696966]
2. Benson MD, Uemichi T. Transthyretin amyloidosis. *Amyloid.* 1996; 3:44–56.
3. Jacobson DR, Pastore RD, Yaghoubian R, Kane I, Gallo G, Buck FS, Buxbaum JN. Variant-sequence transthyretin (isoleucine 122) in late-onset cardiac amyloidosis in black Americans. *N. Engl. J. Med.* 1997; 336:466–473. [PubMed: 9017939]
4. Westermark P, Sletten K, Johansson B, Cornwell GG III. Fibril in senile systemic amyloidosis is derived from normal transthyretin. *Proc. Natl. Acad. Sci. USA.* 1990; 87:2843–2845. [PubMed: 2320592]
5. Saraiva MJ. Transthyretin mutations in health and disease. *Hum. Mutat.* 1995; 5:191–196. [PubMed: 7599630]
6. Connors LH, Lim A, Prokaveva T, Roskens VA, Costello CE. Tabulation of human transthyretin (TTR) variants, 2003. *Amyloid.* 2003; 10:160–184. [PubMed: 14640030]
7. Blake CC, Geisow MJ, Oatley SJ, Rerat B, Rerat C. Structure of prealbumin: secondary, tertiary and quaternary interactions determined by Fourier refinement at 1.8 Å. *J. Mol. Biol.* 1978; 121:339–356. [PubMed: 671542]
8. Lai Z, Colon W, Kelly JW. The acid-mediated denaturation pathway of transthyretin yields a conformational intermediate that can self-assemble into amyloid. *Biochemistry.* 1996; 35:6470–6482. [PubMed: 8639594]
9. Lashuel HA, Lai Z, Kelly JW. Characterization of the transthyretin acid denaturation pathways by analytical ultracentrifugation: implications for wild-type, V30M, and L55P amyloid fibril formation. *Biochemistry.* 1998; 37:17851–17864. [PubMed: 9922152]
10. Quintas A, Vaz DC, Cardoso I, Saraiva MJ, Brito RM. Tetramer dissociation and monomer partial unfolding precedes protofibril formation in amyloidogenic transthyretin variants. *J. Biol. Chem.* 2001; 276:27207–27213. [PubMed: 11306576]
11. Sekijima Y, Wiseman RL, Matteson J, Hammarström P, Miller SR, Sawkar AR, Balch WE, Kelly JW. The biological and chemical basis for tissue-selective amyloid disease. *Cell.* 2005; 121:73–85. [PubMed: 15820680]
12. Hammarström P, Jiang X, Hurshman AR, Powers ET, Kelly JW. Sequence-dependent denaturation energetics: A major determinant in amyloid disease diversity. *Proc. Natl. Acad. Sci. USA.* 2002; 99(Suppl 4):16427–16432. [PubMed: 12351683]
13. Hurshman Babbes AR, Powers ET, Kelly JW. Quantification of the thermodynamically linked quaternary and tertiary structural stabilities of transthyretin and its disease-associated variants: the relationship between stability and amyloidosis. *Biochemistry.* 2008; 47:6969–6984. [PubMed: 18537267]
14. Hammarström P, Wiseman RL, Powers ET, Kelly JW. Prevention of transthyretin amyloid disease by changing protein misfolding energetics. *Science.* 2003; 299:713–716. [PubMed: 12560553]
15. Hammarström P, Schneider F, Kelly JW. Trans-suppression of misfolding in an amyloid disease. *Science.* 2001; 293:2459–2462. [PubMed: 11577236]
16. Hornberg A, Eneqvist T, Olofsson A, Lundgren E, Sauer-Eriksson AE. A comparative analysis of 23 structures of the amyloidogenic protein transthyretin. *J. Mol. Biol.* 2000; 302:649–669. [PubMed: 10986125]
17. Liu K, Cho HS, Hoyt DW, Nguyen TN, Olds P, Kelly JW, Wemmer DE. Deuterium-proton exchange on the native wild-type transthyretin tetramer identifies the stable core of the individual subunits and indicates mobility at the subunit interface. *J. Mol. Biol.* 2000; 303:555–565. [PubMed: 11054291]

18. Liu K, Kelly JW, Wemmer DE. Native state hydrogen exchange study of suppressor and pathogenic variants of transthyretin. *J. Mol. Biol.* 2002; 320:821–832. [PubMed: 12095258]
19. Liu K, Cho HS, Lashuel HA, Kelly JW, Wemmer DE. A glimpse of a possible amyloidogenic intermediate of transthyretin. *Nat. Struct. Biol.* 2000; 7:754–757. [PubMed: 10966644]
20. Yang M, Lei M, Brüschweiler R, Huo S. Initial conformational changes of human transthyretin under partially denaturing conditions. *Biophys. J.* 2005; 89:433–443. [PubMed: 15821170]
21. Yang M, Yordanov B, Levy Y, Brüschweiler R, Huo S. The sequence-dependent unfolding pathway plays a critical role in the amyloidogenicity of transthyretin. *Biochemistry.* 2006; 45:11992–12002. [PubMed: 17002298]
22. Rodrigues JR, Simões CJV, Silva CG, Brito RMM. Potentially amyloidogenic conformational intermediates populate the unfolding landscape of transthyretin: Insights from molecular dynamics simulations. *Protein Sci.* 2010; 19:202–219. [PubMed: 19937650]
23. Armen RS, Alonso DOV, Daggett V. Anatomy of an Amyloidogenic Intermediate: Conversion of β -Sheet to α -Sheet Structure in Transthyretin at Acidic pH. *Structure.* 2004; 12:1847–1863. [PubMed: 15458633]
24. Steward RE, Armen RS, Daggett V. Different disease-causing mutations in transthyretin trigger the same conformational conversion. *Protein Engineering Design and Selection.* 2008; 21:187–195.
25. Jiang X, Smith CS, Petrassi HM, Hammarström P, White JT, Sacchettini JC, Kelly JW. An engineered transthyretin monomer that is nonamyloidogenic, unless it is partially denatured. *Biochemistry.* 2001; 40:11442–11452. [PubMed: 11560492]
26. Dobson CM. Protein folding and misfolding. *Nature.* 2003; 426:884–890. [PubMed: 14685248]
27. Jahn TR, Radford SE. The Yin and Yang of protein folding. *FEBS J.* 2005; 272:5962–5970. [PubMed: 16302961]
28. Chiti F, Dobson CM. Amyloid formation by globular proteins under native conditions. *Nat Chem Biol.* 2009; 5:15–22. [PubMed: 19088715]
29. Colon W, Kelly JW. Partial denaturation of transthyretin is sufficient for amyloid fibril formation in vitro. *Biochemistry.* 1992; 31:8654–8660. [PubMed: 1390650]
30. Palaninathan SK, Mohamedmohaideen NN, Snee WC, Kelly JW, Sacchettini JC. Structural insight into pH-induced conformational changes within the native human transthyretin tetramer. *J. Mol. Biol.* 2008; 382:1157–1167. [PubMed: 18662699]
31. Kelly JW, Colon W, Lai Z, Lashuel HA, McCulloch J, McCutchen SL, Miroy GJ, Peterson SA. Transthyretin quaternary and tertiary structural changes facilitate misassembly into amyloid. *Adv. Protein Chem.* 1997; 50:161–181. [PubMed: 9338081]
32. Nettleton EJ, Sunde M, Lai Z, Kelly JW, Dobson CM, Robinson CV. Protein subunit interactions and structural integrity of amyloidogenic transthyretins: evidence from electrospray mass spectrometry. *J. Mol. Biol.* 1998; 281:553–564. [PubMed: 9698569]
33. Serag AA, Altenbach C, Gingery M, Hubbell WL, Yeates TO. Arrangement of subunits and ordering of β -strands in an amyloid sheet. *Nat. Struct. Biol.* 2002; 9:734–739. [PubMed: 12219081]
34. Yang M, Lei M, Yordanov B, Huo S. Peptide plane can flip in two opposite directions: implication in amyloid formation of transthyretin. *J. Phys. Chem. B.* 2006; 110:5829–5833. [PubMed: 16553385]
35. Cendron L, Trovato A, Seno F, Folli C, Alfieri B, Zanotti G, Berni R. Amyloidogenic potential of transthyretin variants: insights from structural and computational analyses. *J. Biol. Chem.* 2009; 284:25832–25841. [PubMed: 19602727]
36. Yang M, Lei M, Huo S. Why is Leu55-->Pro55 transthyretin variant the most amyloidogenic: insights from molecular dynamics simulations of transthyretin monomers. *Protein Sci.* 2003; 12:1222–1231. [PubMed: 12761393]
37. Tartaglia GG, Pawar AP, Campioni S, Dobson CM, Chiti F, Vendruscolo M. Prediction of Aggregation-Prone Regions in Structured Proteins. *J. Mol. Biol.* 2008; 380:425–436. [PubMed: 18514226]
38. Neudecker P, Robustelli P, Cavalli A, Walsh P, Lundström P, Zarrine-Afsar A, Sharpe S, Vendruscolo M, Kay LE. Structure of an Intermediate State in Protein Folding and Aggregation. *Science.* 2012; 336:362–366. [PubMed: 22517863]

39. Jarvis JA, Kirkpatrick A, Craik DJ. ¹H NMR analysis of fibril-forming peptide fragments of transthyretin. *Int. J. Pept. Protein Res.* 1994; 44:388–398. [PubMed: 7875942]
40. Olofsson A, Ippel JH, Wijmenga SS, Lundgren E, Ohman A. Probing solvent accessibility of transthyretin amyloid by solution NMR spectroscopy. *J. Biol. Chem.* 2004; 279:5699–5707. [PubMed: 14604984]
41. Lashuel HA, Wurth C, Woo L, Kelly JW. The most pathogenic transthyretin variant, L55P, forms amyloid fibrils under acidic conditions and protofilaments under physiological conditions. *Biochemistry.* 1999; 38:13560–13573. [PubMed: 10521263]
42. Cavanagh, J.; Fairbrother, WJ.; Palmer, AG., III; Rance, M.; Skelton, NJ. *Protein NMR Spectroscopy: Principles and Practice.* Elsevier Academic Press; Burlington MA: 2007.
43. Loria JP, Rance M, Palmer AG. A relaxation-compensated Carr-Purcell-Meiboom-Gill sequence for characterizing chemical exchange by NMR spectroscopy. *J. Am. Chem. Soc.* 1999; 121:2331–2332.
44. McElheny D, Schnell JR, Lansing JC, Dyson HJ, Wright PE. Defining the role of active-site loop fluctuations in dihydrofolate reductase catalysis. *Proc. Natl. Acad. Sci. USA.* 2005; 102:5032–5037. [PubMed: 15795383]
45. Millet O, Loria JP, Kroenke CD, Pons M, Palmer AG. The static magnetic field dependence of chemical exchange line broadening defines the NMR chemical shift timescale. *J. Am. Chem. Soc.* 2000; 122:2867–2877.
46. Hamilton JA, Steinrauf LK, Braden BC, Liepnieks J, Benson MD, Holmgren G, Sandgren O, Steen L. The x-ray crystal structure refinements of normal human transthyretin and the amyloidogenic Val-30-->Met variant to 1.7-Å resolution. *J. Biol. Chem.* 1993; 268:2416–2424. [PubMed: 8428915]
47. Koradi R, Billeter M, Wüthrich K. MOLMOL: A program for display and analysis of macromolecular structures. *J. Mol. Graphics.* 1996; 14:51–55.

- We report relaxation dispersion measurements of monomeric transthyretin (TTR)
- Monomeric TTR shows μ s-ms timescale fluctuations at neutral pH
- The fluctuations are localized to one of the two TTR β -sheets
- Enhanced amyloidogenic conditions at lower pH show increased fluctuation
- These conformational fluctuations are abolished in the protective mutant T119M

Fig. 1.

Proposed process of misfolding and amyloid formation of TTR. The monomer structure shows the designation of β -strands A-H and the positions of the F87 and T110 residues (green), which are both changed in the monomeric variant M-TTR to Met. The position of the additional mutation at T119, which is changed in the stabilized mutant T119M M-TTR is shown in black. The coordinates are of the native tetramer 1TTA.⁴⁶ The monomer structure shown in pink and blue was derived from the tetramer 1TTA. Figure prepared using MOLMOL.⁴⁷

Fig. 2.

Representative ^{15}N R_2 dispersion data of M-TTR (0.4 mM) at pH 7.2 and 15°C. A. R_2 dispersion curves at static magnetic fields of 18.8T (800 MHz ^1H frequency) are shown in red and 11.7T (500 MHz ^1H frequency) are shown in black. Lines represent global fits to the data for all residues; the full data set is shown in Fig. S1. B. Representative curves showing relaxation dispersion at 18.8T for WT M-TTR at concentrations of 0.25 mM (orange) and 0.75 mM (green); results for all residues are shown in Fig. S2.

Fig. 3.

A. Representative ^{15}N R_2 dispersion data of T119M M-TTR (0.4 mM) at pH 7.2 and 15°C. R_2 dispersion curves at static magnetic fields of 18.8T (800 MHz ^1H frequency) are shown in red and 11.7T (500 MHz ^1H frequency) are shown in black. Lines represent global fits to the data for all residues; the full data set is shown in Fig. S3. B. Contribution of the conformational exchange (R_{ex}) to the transverse relaxation rate for WT M-TTR (blue square) and T119M M-TTR (red circle). Vertical blue arrows denote residues for which R_{ex} could be quantitated for observable cross peaks in T119M M-TTR, but where the corresponding cross peaks in WT M-TTR were invisible or broadened to such an extent that relaxation dispersion measurements could not be made. Black diamonds at the x-axis denote proline residues and those for which no cross peaks were observed for either WT or T119M M-TTR. Secondary structure is mapped onto each plot, color-coded according to the β -sheets shown in the inset structure.

Fig. 4.

Residues showing evidence for slow-timescale dynamics, mapped onto the M-TTR X-ray structure (1GKO²⁵). A. WT M-TTR. B. T119M M-TTR. Residues for which $R_{ex} > 20 \text{ s}^{-1}$ are shown in red, residues with $10 \text{ s}^{-1} < R_{ex} < 20 \text{ s}^{-1}$ are shown in orange, and residues with $3 \text{ s}^{-1} < R_{ex} < 10 \text{ s}^{-1}$ in green. Purple denotes residues for which the amide cross peak is absent or excessively broadened in WT M-TTR but which could be observed in spectra of T119M M-TTR. Black indicates the position of prolines and of residues whose amide cross peaks are missing from the spectra of both wild type and T119M M-TTR.

Fig. 5. Region of the HSQC spectra of M-TTR and T119M M-TTR showing changes as a function of pH. A. M-TTR. B. T119M M-TTR. Spectra shown are at pH 7.2 (black), 6.4 (red), 5.7 (green, A), 5.5 (green, B), 5.0 (blue) and 4.7 (yellow).

Fig. 6.

Changes in cross peak intensity with decreasing pH. A. M-TTR. B. T119M M-TTR. Cross peak intensities are plotted as the ratio of peak intensity at the indicated pH relative to the intensity of the corresponding cross peak at pH 6.4. Secondary structure is mapped onto each plot, color-coded according to the β -sheets shown in the inset structure.

Fig. 7.

Mapping of pH-dependent cross peak intensity changes to M-TTR structure. A. Red indicates residues in wild type M-TTR with cross peaks that lose >70% intensity in the pH 5.0 HSQC spectrum relative to the spectrum at pH 7.2. Purple indicates the location of residues that are exchange broadened in the pH 7.2 HSQC spectrum or which exhibit relaxation dispersion with $R_{ex} > 15 \text{ s}^{-1}$. Black indicates the position of prolines and of residues whose amide cross peaks are missing from the spectra of both wild type and T119M M-TTR. B. Location of residues that lose >70% cross peak intensity in the pH 5.0 HSQC spectrum T119M M-TTR (relative to the pH 7.2 spectrum). Color code is the same as panel A.

Fig. 8. Aggregation kinetics of WT and T119M M-TTR at 37°C and pH 4.4 measured by recording optical density at 400 nm as a function of incubation time. Samples were at 15 or 30 μ M concentration in 0.2mM acetate buffer containing 100 mM KCl and 0.1 mM EDTA.