A glimpse of a possible amyloidogenic intermediate of transthyretin

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Studies have indicated that partially unfolded states occur under conditions that favor amyloid formation by transthyretin (TTR), as well as other amyloidogenic proteins. In this study, we used hydrogen exchange measurements to show that there is selective destabilization of one half of the β-sandwich structure of TTR under such conditions. This provides more direct information about conformational fluctuations than previously available, and will facilitate design of future experiments to probe the intermediates critical to amyloid formation.

Amyloid fibril formation, or amyloidogenesis, is a process by which a normally soluble protein undergoes conformational changes and self-assembles into an insoluble fibril morphology *in vivo*[1,2]. This is believed to cause neurodegenerative diseases[3–6]. While the best known diseases associated with amyloidogenesis are Alzheimer's disease, light chain disease, and familial amyloid polyneuropathies (FAPs), ~20 biochemically diverse precursor proteins, including transthyretin (TTR), are known to form amyloid fibrils[7,8]. Studies have demonstrated that acidic conditions accelerate TTR amyloid fibril formation by increasing the extent of tetrameric TTR dissociating to form an assembly competent, monomeric structure with an altered tertiary structure[9,10]. We have conducted deuterium-proton (D-H) exchange experiments on the backbone amides of TTR under acidic conditions, which cause an increase in the amount of the amyloidogenic intermediate, to characterize regions of conformational change. The regions of the protein destabilized under these conditions are different and more extensive than previously thought, involving the CBEF sheet that contains most of the disease associated mutations.

TTR is found in human plasma (0.2 mg ml^{-1}) as a homotetramer of 127-residue subunits. The crystal structure of native TTR reveals that each subunit has eight β-strands, A to H (Fig. 3*a*), arranged as a β-sheet sandwich with a hydrophobic core[11]. The wild type (WT) tetramer is very stable to denaturation at neutral pH[12], yet, in certain individuals, it is converted into amyloid fibrils. WT-TTR amyloid formation is associated with the disease senile systemic amyloidosis (SSA), whereas ~70 single site variants are associated with FAPs[13,14]. While FAPs affect only one in 10^5–10^6 individuals, SSA affects 25% of the population over 80 years of age[15,16]. The accelerated amyloidogenesis in FAPs suggests that small structural perturbations can destabilize the native structure, enhancing the formation of the amyloidogenic intermediates[17,18].

The very nature of amyloids poses an intriguing problem; the 20 amyloidogenic proteins exhibit little homology in sequence or structure, yet all form amyloid fibrils of a similar overall structure[15]. One explanation for this phenomenon may be that the similarity among the amyloidogenic proteins develops not at the level of their normally folded structures, but rather the common structural features arise under partially denaturing conditions, which generate amyloidogenic intermediates[19]. Understanding the conditions that promote the accumulation of amyloidogenic intermediates, as well as their underlying common structural features, should provide further information about amyloidogenesis in general. Recent biophysical studies on TTR have identified *in vitro* conditions that stimulate amyloid formation[9,10,20] (Fig. 1).

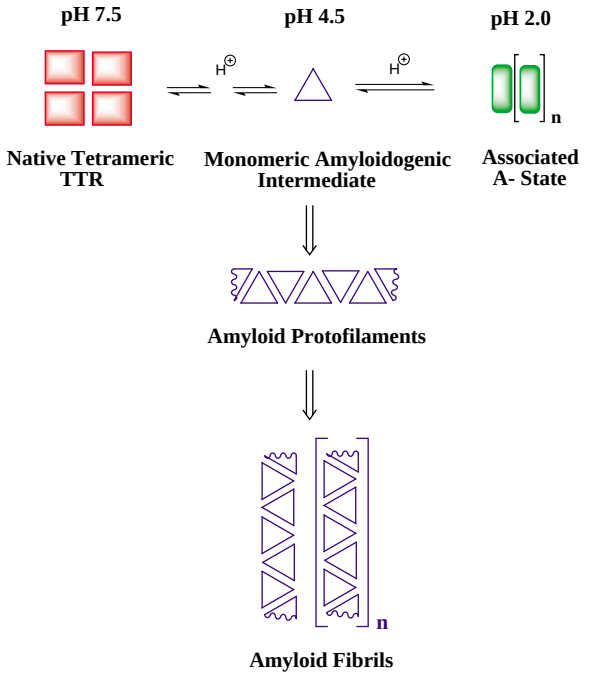

Fig. 1 The pathway of TTR denaturation and amyloid fibril formation. Tetrameric TTR dissociates into monomers with an altered structure (possibly through dimers first) at pH 4.5 that are capable of amyloid formation. Upon further acidification, TTR monomers are converted into the A-state, which is not amyloidogenic. TTR denaturation and amyloid fibril formation are competitive processes that share a common monomeric amyloidogenic intermediate.

Isolated WT-TTR remains stable and tetrameric from pH 7 to pH 5 and does not form amyloid. However, over the pH range of 5.0–3.9, TTR dissociates to a monomer that exhibits an altered but defined tertiary structure, as probed by fluorescence and far and near UV circular dichroism. The extent of amyloidogenesis correlates with the concentration of amyloidogenic monomer, which is maximal at pH 4.4[9,10]. Upon further acidification (< pH 3.9), the structurally defined monomers adopt alternative conformations analogous to a molten globule-like acid-denatured state (A-state), which forms low molecular weight A-state aggregates but not amyloid fibrils[21]. These observations suggest that amyloidogenesis results from interactions between specific structural elements of alternatively folded TTR monomers. Here we report the results of deuterium-proton (D-H) exchange experiments[22–24] on WT-TTR at pH 5.75 (non-amyloidogenic tetramer) and 4.5 (amyloidogenic monomer), monitored by two-dimensional NMR spectroscopy, that provide site specific information complementing previous mass spectroscopic studies[18]. The differences in the backbone amide D-H exchange rates under these two conditions reveal that one

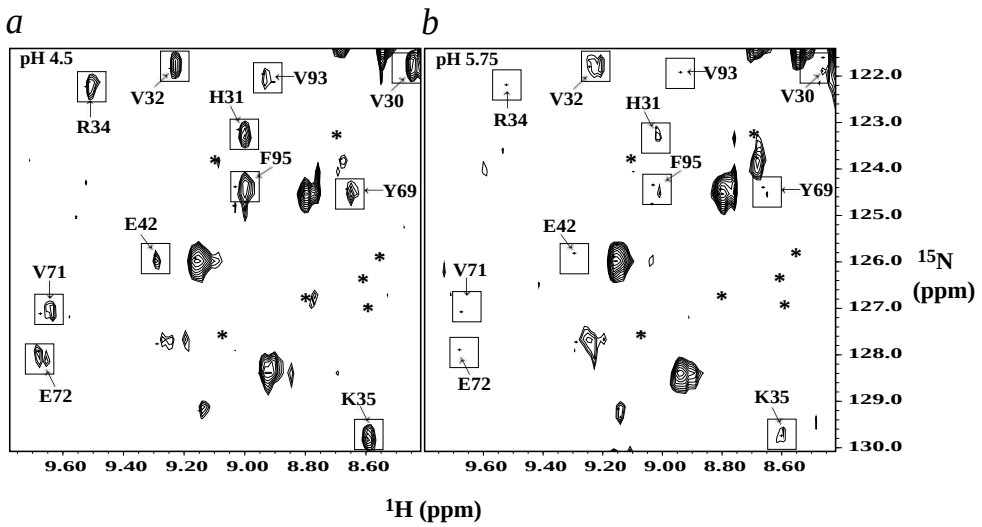

Fig. 2 D-H exchange monitored by NMR. HSQC spectra taken at pH 5.75 (40 °C) with samples re-concentrated to 10 mg ml^{-1} after 6 h incubation at 0.008 mg ml^{-1}. ***a***, D-H exchanged at pH 4.5. ***b***, D-H exchanged at pH 5.75. Boxes indicate areas of difference. Asterisks indicate sites of ^{1}H-^{15}N peaks observed for TTR with amides fully protonated.

side of the β-sheet sandwich (strands C, B, E, and F) is destabilized under conditions favoring the formation of the amyloidogenic intermediate. We suggest that this region of the protein is destabilized by the effects of pH changes and mutations, and loses its native contacts with the core β-sheet fold in the amyloidogenic intermediate.

The backbone amide ^{1}H-^{15}N resonances of 118 out of 120 nonproline residues in tetrameric WT-TTR were assigned by NMR using uniformly ^{2}H, ^{13}C and ^{15}N labeled samples[25]. In D-H exchange experiments, protected amide hydrogens are generally involved in hydrogen bonds. The crystal structure suggests that there are 59 amide hydrogen bonds within each monomer, with 44 in the β-sheet region and the helix[11] (Fig. 3*a*). At pH 5.75 (>0.2 mg ml^{-1} TTR), when TTR is predominantly tetrameric, we found that 41 amide hydrogens were completely, and 14 were partially, protected from exchange after 2 h. These amides are mainly located in the two β-sheets and serve as probes to map the changes that occur under conditions that cause an increase in the population of the amyloidogenic intermediate.

Our D-H exchange experiments commenced with concentrated perdeuterated TTR solutions, which were diluted into H_2O at pH 4.5 or pH 5.75. Dilution to 0.008 mg ml^{-1} shifts the equilibrium from tetramer to monomer while inhibiting irreversible assembly into amyloid. After different incubation periods, both samples were adjusted to pH 5.75, to reform the tetramer, allowing the protein to be concentrated for NMR detection of the ^{1}H-^{15}N resonances. Starting with deuterated TTR (^{2}H-^{15}N), any signals reflect the exchange-in of protons from H_2O. SDS-PAGE analysis, analytical ultracentrifugation and gel-filtration studies of TTR at 0.005–0.01 mg ml^{-1} in the buffers used showed that about 75% of TTR is monomeric at pH 4.5, while 25% is monomeric at pH 5.75, consistent with previous results[10]. D-H exchange data collected at pH 5.75, with the protein in a non-amyloidogenic state, gave very similar exchange patterns at both high (10 mg ml^{-1}) and low (0.008 mg ml^{-1}) concentrations of TTR. These data demonstrate that even if TTR dissociates to the monomeric state at low concentration its structure remains native like at pH 5.75, consistent with previous observations[10]. Differences in the D–H exchange patterns at pH 4.5 and 5.75 should therefore reflect structural changes that occur under amyloidogenic conditions.

This comparison unambiguously revealed increased exchange rates for the backbone amides of Ser 23, Val 30, His 31, Val 32, Phe 33, Arg 34, Lys 35, Glu 42, Tyr 69, Val 71, Glu 72, Val 93, and Phe 95 (Fig. 2 and 3*b*) at the lower pH. Note that intrinsic D-H exchange rates are pH dependent, following V-shaped log (rate) versus pH curves, with minima between pH 3 and 4 (ref. 26). Accordingly, intrinsic D-H exchange rates are >10 times faster at pH 5.75 than at pH 4.5. In addition, the exchange rates of the more stable amides from both sheets, including those in strands A, B, E, G, and Glu 42 from strand C, are very similar to one another at high protein concentration (10 mg ml^{-1}) and pH 5.75. Consequently, the increased exchange rates at pH 4.5, observed for the aforementioned 13 residues, must be caused by disruption of hydrogen bonds. All of the amides deprotected at pH 4.5, except Ser 23, are involved in interstrand hydrogen bonds in the tetramer (pH 5.75) and are located within the C, B, E, and F strands (CBEF sheet) of the β-sandwich (Fig. 3).

This study identifies the residues that undergo increased conformational fluctuations under amyloidogenic conditions, and leads to unexpected conclusions. Previous thinking about structural changes in the amyloidogenic intermediate, based on low resolution spectroscopic data, suggested that TTR amyloidogenesis begins with dissociation of tetramer followed by separation of β-strands C and D from the core of the monomer[9,10,14,19,27]. Our study reveals that all hydrogen bonds connecting β-strands B and C are destabilized, while those connecting β-strands D and A remain partially intact. Interestingly, the amides on β-strand A remain protected but those defining β-strand D exchange, indicating that there is local labilization. The inherent instability of this region is indicated by the fact that β-strand C (Ala 45, Gly 47) and β-strand D (Leu 55) amides undergo relatively rapid exchange even at pH 5.75. However, under native conditions, β-strands C and D remain connected to β-strands A and B through hydrogen bonds donated by the latter strands and one donated by β-strand C (Glu 45).

In addition to disruptions of hydrogen bonds connecting β-strands B and C at pH 4.5, we observed similar effects on hydrogen bonds connecting β-strands B to E, and E to F. Such disruptions are likely concurrent, suggesting a cooperative disruption of half of the β-sandwich, the sheet composed of the B, C, E, and F strands. Although this region is destabilized, the exchange rates of the residues do not increase enough to equal those of fully exposed residues; the protection factors decrease dramatically, but the most highly populated structure probably still contains native-like hydrogen bonds. Under the acidic con-

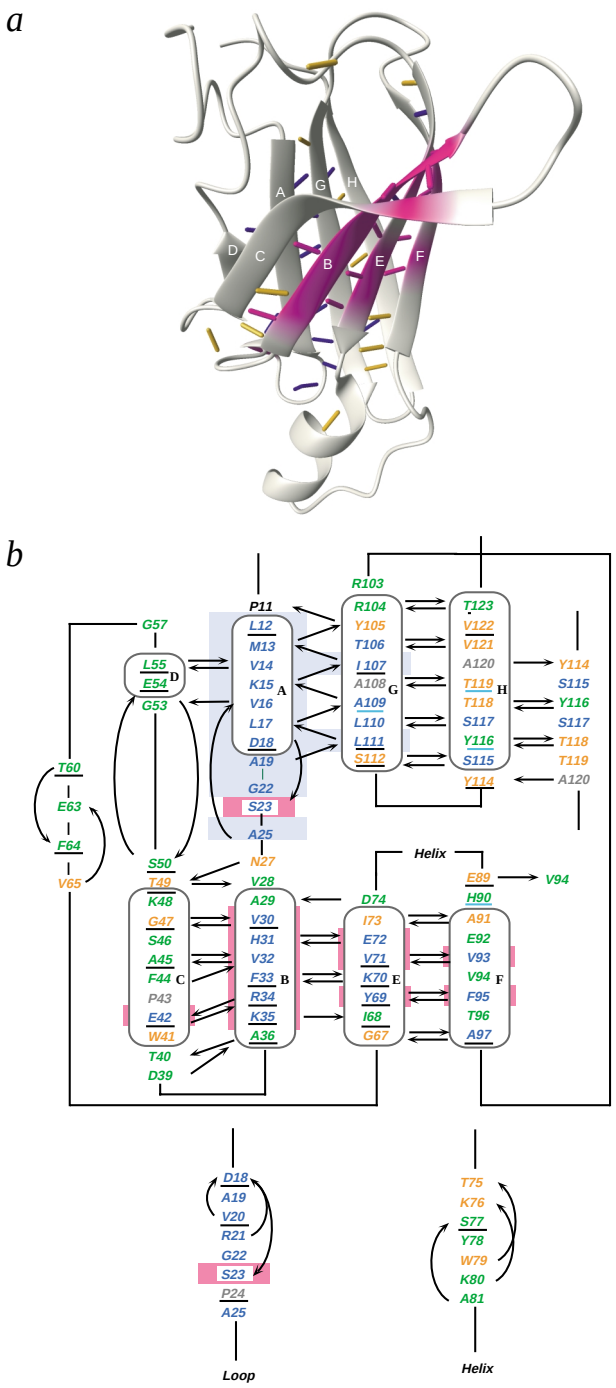

Fig. 3 The low pH conformational changes in TTR. ***a***, Ribbon diagram representation of a TTR monomer in the native state (prepared using MOLMOL[30]) with β-strands labeled A through H[11]. Regions highlighted in magenta contain amide hydrogens more labile at pH 4.5 than at pH 5.75. Color coded bars indicate amide hydrogens protected at pH 5.75 and 10 mg ml^{-1} TTR that were used as probes for intermediate states of TTR: orange, NHs partially exchanged after 72 h at pH 5.75 and 10 mg ml^{-1} TTR; blue, NHs protected for more than 500 h at the same conditions; magenta, same as blue, but NHs do exchange at pH 4.5 and 0.008 mg ml^{-1} TTR. ***b***, Schematic diagram of TTR consisting of one TTR monomer and the H strand of a neighboring monomer. Boxes indicate native state β-strands labeled A through H. Arrows indicate backbone hydrogen bonds pointing from donor to the acceptor residue. Only residues in secondary structure elements or protected at pH 5.75 are shown. The residue labels are color coded according to the exchange behavior at pH 5.75 and 20 mg ml^{-1} TTR. Green, NHs are completely exchanged after 2 h; orange, NHs are partially exchanged after 72 h; blue, NHs are protected for more than 500 h; black, either Pro or the unassigned Ala 120. The ^{1}H-^{15}N of Ala 108, which was assigned although it is very broad and not useful as a D–H exchange probe, is also labeled in black. Residues underlined with black bars have pathogenic variants, and residues underlined with cyan bars have nonpathogenic variants. Magenta colored bars highlight residues of TTR that are deprotected under amyloidogenic conditions (pH 4.5) and correspond to the magenta hydrogen bonds in (*a*). The area shaded in blue indicates residues that remain protected under all conditions tested.

ditions, many more fluctuations occur to states in which the C, B, E, and F strands are unfolded than to the totally unfolded state. The fact that amyloidogenicity increases under conditions that give rise to such an intermediate state, but drops again under conditions favoring full unfolding, argues that a partially unfolded intermediate is important for generation of amyloid.

Such a partially unfolded state could facilitate the formation of intermolecular interactions that are essential for amyloidogenesis. Interestingly, there are pathogenic variants at many residue positions that are protected by hydrogen bonds in the native fold for the wild type sequence (pH 5.75), but become deprotected under amyloidogenic conditions (pH 4.5). Importantly, there are more pathogenic mutations within β-strands of the CBEF sheet than on the other β-sheet of the sandwich (DAGH), which is the location of most of the nonpathogenic variants (Fig. 3*b*). It is presently impossible to dissect the effects of mutations on stability. In most proteins, mutations generally result in a decrease in global stability. However, it seems likely that in partially unfolded states, such as the intermediate, the destabilization will be more localized to the region around the site of the mutation. The distribution of TTR variants thus also suggests that destabilizing β-strands of the CBEF sheet is crucial for TTR amyloidogenesis[14,16]. Future hydrogen exchange measurements on pathogenic variants can help clarify such a relationship by detecting changes in localized destabilization.

At both pH 4.5 and pH 5.75, residues 12–25, as well as Ile 107 and Leu 111, remain protected against D–H exchange. This indicates that β-strand A is anchored to β-strand G through well protected hydrogen bonds and maps out the core of TTR that remains unaltered even under amyloidogenic conditions (Fig. 3). Amyloidogenic conditions thus appear to stimulate changes in only part of the molecule, rather than causing global unfolding[10].

The D–H exchange under amyloidogenic conditions has been studied at the whole protein level by mass spectroscopy[18]. That study established that there are ~60 amides in the whole WT-TTR protected against exchange, but could not identify the residues involved. This number is close to the number of amides detected in our NMR experiments. It was also found that the number of amides protected dropped to ~20 in the V30M variant, which is more amyloidogenic. The reduction in the number of protected amides between WT-TTR and the V30M variant is greater than that between WT-TTR at neutral and low pH. Intrinsic differences in the techniques, site specific for NMR but molecule averaged for mass spectroscopy, make quantitative comparisons of partial exchange difficult.

Our data demonstrate that the pH dependent changes that occur under conditions giving rise to the monomeric amyloidogenic intermediate are best characterized as a destabilization and increase in mobility of the CBEF β-sheet. The wide distribution of sites in the protein and variety of pathogenic variants suggest that their enhanced amyloidogenesis is not due to local structural perturbations, but rather results from destabilization of the tertiary fold. Furthermore, the preponderance of FAP associated variants on one side of the β-sheet sandwich, coupled with the D-H exchange data at pH 4.5 suggests that conformational

changes involve β-strands CBEF and possibly cause displacement of the CBEF sheet from the core of the β-sandwich. The disruption of these four strands would expose hydrogen bond donors and acceptors as well as hydrophobic patches that enable self-assembly of the amyloidogenic intermediate, ultimately causing formation of amyloid fibrils[28,29].

Methods

Per-deuteration of TTR amide groups. ^{2}H and ^{15}N labeled TTR was produced in *Escherichia coli* in D_2O and $^{15}NH_4Cl$ defined media, and purified as described[17]. The lyophilized TTR powder was dissolved in D_2O solution at 0.25 mg ml^{-1} and adjusted to pH 2.0 with DCl. At pH 2.0, TTR is a random coil that allows complete exchange of its amide hydrogens with the solvent[20]. TTR was exchanged for over 12 h at room temperature to allow complete deuteration. The protein was then diluted four-fold with D_2O, and neutralized to pH 6.0 with NaOD to initiate refolding of TTR. Dilution was necessary to reduce protein aggregation during refolding. Renatured TTR was centrifuged to remove precipitate, and was then used in hydrogen exchange experiments.

Deuterium-proton exchange at pH 4.5. ^{2}H-^{15}N doubly labeled TTR stock solution (0.25 mg ml^{-1}) was diluted into H_2O containing 5 mM KH_2PO_4 to a concentration of 0.008 mg ml^{-1}, and the pH adjusted with HCl to 4.5. The solution was kept at 37 °C for different periods of time. Concentrated buffer was then introduced to bring TTR back to the native state. The final solution was at pH 5.75 (50 mM phosphate and 100 mM NaCl). The TTR solution was concentrated to 0.4 mg ml^{-1} within 2 h by a MiniKros® Sampler System (MICROGON). Before NMR studies, further concentration to 10 mg ml^{-1} was carried out using Amicon Centriprep and Centricon filters.

Deuterium–proton exchange at pH 5.75. The same procedure was performed as above, except that TTR was kept at pH 5.75 throughout the entire process.

SDS-PAGE analysis. SDS-PAGE was performed with a gradient gel (10–20%) and silver stained. Tetrameric TTR in SDS loading buffer without boiling produces a 30 kDa band (dimer) while monomeric TTR runs as a 14 kDa band.

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