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Structural Characterization of Cardiac Ex Vivo Transthyretin Amyloid: Insight into the Transthyretin Misfolding Pathway In Vivo

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ABSTRACT: Structural characterization of misfolded protein aggregates is essential to understanding the molecular mechanism of protein aggregation associated with various protein misfolding disorders. Here, we report structural analyses of ex vivo transthyretin aggregates extracted from human cardiac tissue. Comparative structural analyses of in vitro and ex vivo transthyretin aggregates using various biophysical techniques revealed that cardiac transthyretin amyloid has structural features similar to those of in vitro transthyretin amyloid. Our solid-state nuclear magnetic resonance studies showed that in vitro amyloid contains extensive nativelike β-sheet structures, while other loop regions including helical structures are disrupted in the amyloid state. These results suggest that transthyretin undergoes a common misfolding and aggregation transition to nativelike aggregation-prone monomers that self-assemble into amyloid precipitates in vitro and in vivo.

Transthyretin (TTR) is a 55 kDa homotetrameric protein consisting of four 127-residue β-barrel subunits.[1,2] The primary role of TTR is to transport thyroid hormones and retinol binding protein in plasma and cerebrospinal fluid. TTR misfolding and aggregation are associated with numerous amyloidoses featuring cardiomyopathy and polyneuropathy.[3−6] For example, aggregation of wild type TTR (TTRwt) is implicated in senile systemic amyloidosis (SSA) that affects nearly 25% of the population over the age of 80.[7] In addition, more than 100 single-point mutations have been identified and most of these TTR variants (TTRv) can undergo misfolding and aggregation, leading to the onset of ATTR amyloidosis.

Previous extensive biochemical studies have revealed a detailed molecular mechanism of TTR misfolding and aggregation in vitro.[8−15] However, whether TTR undergoes the same misfolding and aggregation transition in vivo remains unknown. To gain insights into the misfolding and aggregation in vivo, it is essential to examine TTR aggregates formed under physiological conditions (human tissue) and compare structural features of ex vivo and in vitro TTR amyloids. In this study, we carried out comparative structural analyses of in vitro as well as ex vivo TTR amyloid extracted from human cardiac tissues by using various biophysical techniques, including Fourier transform infrared spectroscopy (FT-IR), transmission electron microscopy (TEM), and solid-state nuclear magnetic resonance (NMR).

The ex vivo TTRwt amyloid fibrils were extracted from the cardiac tissue of an SSA patient.[16] The cardiac extracts were examined with thioflavin T (ThT) fluorescence and polyacrylamide gel electrophoresis (PAGE). The enhanced ThT fluorescence confirmed the amyloid property of the cardiac extracts (Figure S1). Sodium dodecyl sulfate (SDS)−PAGE analysis also showed that TTR is the major component of the amyloid, consistent with previous structural analyses of the fibrils deposited in cardiac tissue (Figure S2).[16]

Our previous mechanistic studies of TTR misfolding in vitro revealed that nativelike aggregation-prone TTR monomers form dimers, which self-associate to form small spherical oligomers in vitro,[12] as shown in Figure 1a and Figure S3. TTR aggregates extracted from human cardiac tissue were examined by TEM (Figure 1b and Figure S4) for comparative structural analysis. Small spherical oligomers, indistinguishable from in vitro oligomers, were also observed in ex vivo cardiac TTR amyloid.

Structural features of the in vitro and ex vivo TTR amyloids were further investigated using FT-IR (Figure 2 and Figure S5). Previous FT-IR studies showed that absorption peaks arising from the stretching vibrations of main chain C═O groups (amide I) and from the N−H bending vibrations (amide II) in the peptide backbone are strongly sensitive to secondary structures and hydrogen bonding patterns in the amide backbone. Thus, FT-IR has been used to investigate structural features of amyloids formed by various aggregation-prone proteins.[17−19] In our FT-IR studies of the in vitro and ex vivo TTR amyloids, the two amyloid states exhibited almost identical absorption peaks in the IR spectra, particularly the amide I (1600−1700 cm^{-1}) and II (1500−1600 cm^{-1}) bands (Figure 2), suggesting that the two amyloids exhibit similar structural characteristics. Protease K digestion analyses of the in vitro and ex vivo aggregates are also consistent with the TEM and FT-IR results (Figure S6).

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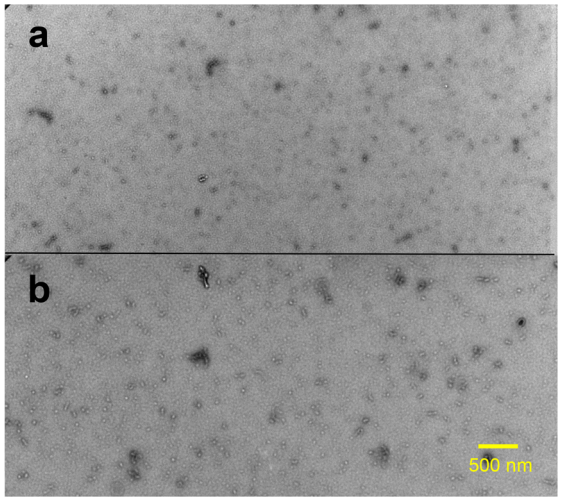

Figure 1. TEM images of (a) in vitro and (b) ex vivo TTR amyloid. The in vitro TTR amyloid was obtained by incubating recombinantly generated monomeric TTR (mTTR; F87M/L110M) at a protein concentration of 0.5 mg/mL in PBS buffer (pH 7.4) at 37 °C for 2 days. The ex vivo TTR amyloid was extracted from human cardiac tissue (TTRwt).

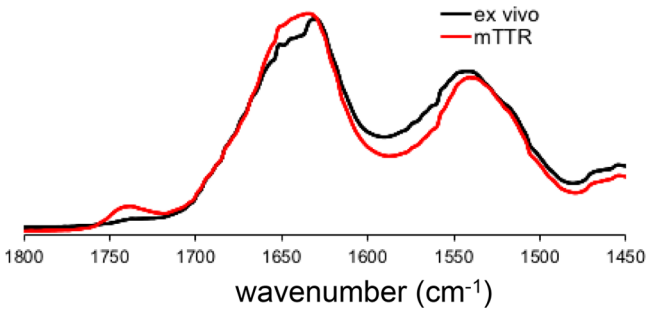

Figure 2. Amide I and II bands of the FT-IR spectra for in vitro mTTR (red) and ex vivo (black) TTR amyloid.

Our combined structural studies indicate that in vitro mTTR amyloid formed at pH 7.4 has structural properties similar to those of ex vivo cardiac TTR amyloid. However, previous mechanistic studies, including our structural studies, have utilized mildly acidic conditions (pH 4−5) to induce amyloid formation of TTRwt.[12,15,20,21] Thus, structural characteristics of the mTTR and TTRwt amyloids formed at pH 7.4 and 4.4, respectively, were compared by using solid-state NMR (Figure S7). The ^{13}C chemical shifts of backbone (Cα) and aliphatic side chains are highly sensitive to local environments such as secondary structures and ϕ and ψ dihedral angles. Thus, the two-dimensional (2D) $^{13}C-^{13}C$ correlation NMR experiment is an ideal tool for comparative structural studies of biological molecules. In the overlaid 2D $^{13}C-^{13}C$ correlation NMR spectra, NMR cross-peaks between the backbone (50−65 ppm) and side chain carbons (10−70 ppm) and between side chain carbons (10−50 ppm) overlap well. The nearly identical 2D spectra for the two TTR amyloids clearly indicate that the TTR amyloid states have similar molecular conformations. These solid-state NMR results suggest that TTR has similar misfolding and aggregation pathways at both pH 7.4 and 4.4, and the mechanistic studies of TTR misfolding and aggregation at pH 4.4 may also provide valuable insight into in vivo TTR misfolding and aggregation.

Previous structural studies showed that TTR amyloid formed at pH 4.4 had extensive nativelike β-sheet structures.[20,22−27] In this study, more detailed solid-state NMR experiments were carried out to clearly identify the nativelike β-structured regions and other regions that undergo a misfolding transition. The 2D $^{13}C-^{13}C$ correlation NMR spectra were acquired and compared for the native (red) and amyloid (black) states of TTR (Figure 3). The NMR spectra

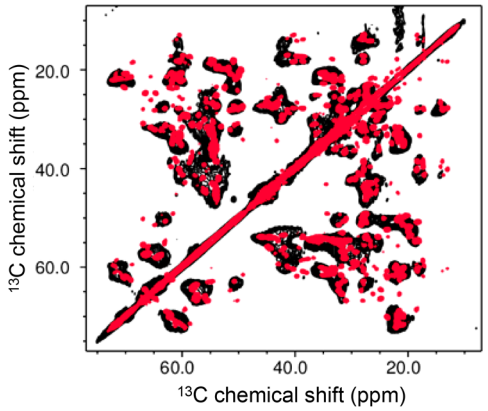

Figure 3. Overlaid two-dimensional $^{13}C-^{13}C$ correlation solid-state NMR spectra for the native (red) and amyloid (black) states of TTRwt formed at pH 4.4 obtained at a proton frequency of 800 MHz. A dipolar-assist rotational resonance (DARR) with a 20 ms mixing time was used for the mixing scheme.

for the two states overlap well, suggesting that the amyloid state contains extensive nativelike structures. It is also notable that numerous cross-peaks from native TTR disappear in the amyloid state, indicative of structural changes during misfolding and aggregation.

To identify the residues that undergo structural changes, three-dimensional solid-state NMR experiments (NCACX, NCOCX, and CANCO)[28,29] were performed for the sequential resonance assignment of the native TTR (Figure S8). The chemical shift changes in the 2D DARR spectra were mapped into the crystal structure of native TTR (Figure 4). The residues with cross-peaks that disappeared in the amyloid spectrum are colored green, and the overlapped regions are colored yellow. The cross-peaks in the boundary in Figure 3 and Figure S9 (green asterisks) are indicated in red, and the loops in purple could not be assigned presumably due to the high flexibility of the loop regions in the native state.

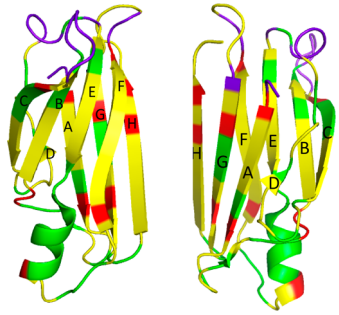

Figure 4. Crystal structure of the monomeric form of native TTR with colors for the residues that are absent (green). The cross-peaks in the boundary (Figure S9) are colored red. The crystal structure was drawn in two different views.

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The comparative solid-state NMR structural analyses of the native and amyloid states of TTR indicate that the loop regions (AB, CD, EF, and GH loops) and EF helix undergo structural changes or become disordered during amyloid formation. The AB and GH loops are located in the tetrameric interfacial regions.[30] Thus, the dissociation of the tetramers into monomers may lead to the changes in local environments of the loop regions, resulting in the chemical shift changes. In addition, side chain interactions between strands C and B and the DE loop appear to be disrupted in the amyloid state (Figure S10a). It is also interesting to note that the chemical shifts of the residues in A108 in strand G are affected during misfolding and aggregation (Figure 4 and Figure S10b). Residue A108 is involved in the formation of the binding pocket in native tetrameric TTR. The dissociation of the native tetramer into aggregation-prone monomers is the initial step in the aggregation process, and thus, the disruption of the binding pocket may result in the chemical shift changes of the residues in strand G.

On the other hand, a majority of cross-peaks overlap well in the overlaid spectra. Most of the cross-peaks come from the residues in the two β-sheets (CBEF and DAGH), suggesting that the overall nativelike β-sheet structures appear to remain unchanged in the amyloid state (Figure 4). These results are consistent with our previous structural studies using selective $^{13}CO/^{13}C\alpha$ labeling schemes that revealed the two β-sheet structures are maintained in the amyloid state of TTR.[20]

Understanding the molecular mechanism of protein misfolding and aggregation is essential for developing therapeutic approaches. Previous mechanistic studies of TTR misfolding and aggregation have provided detailed insights into the molecular basis for misfolding and aggregation in vitro. Under the amyloidogenic condition at mildly acidic pH values of 4−5, tetrameric native TTR is dissociated into aggregation-prone monomers that self-assemble into small oligomers and subsequently amyloid precipitates.[11,21] Comparative structural analyses of ex vivo and in vitro TTR amyloids are essential for investigating whether a similar TTR misfolding and aggregation process takes place in vivo. Previous studies showed that TTR forms a heterogeneous mixture of amyloids, including thick and long filaments as well as less-ordered aggregates in vivo depending on the age of onset and mutations.[31,32] Very recently, a cryo-EM structure of TTR fibrils extracted from ATTRV30M cardiac tissues was reported.[33] The structural studies revealed that TTRV30M adopts distinctive non-native conformations in the ex vivo filaments, suggesting that the TTR variant protein becomes unfolded during amyloid formation. The filamentous aggregates were, however, not observed in the cardiac extracts from a patient with ATTRwt amyloidosis used in this study. Moreover, the ex vivo cardiac TTRwt amyloid resembles in vitro TTR amyloid with extensive nativelike β-structures. These observations suggest that TTR misfolding and aggregation may take place through multiple pathways in vivo depending on the disease phenotypes and mutations.

In summary, we report solid-state NMR studies that suggest in vitro TTR amyloid formed at pH 4.4 contains extensive nativelike β-sheet structures, while most of the native loop structures are changed in the amyloid state, consistent with previous structural studies.[20,22,23,34] In addition, the in vitro TTR amyloid has structural characteristics similar to those of ex vivo cardiac TTR amyloid, suggesting that the TTR misfolding and aggregation observed under mildly acidic conditions may occur in vivo, as well. Structural characterization of ex vivo TTR variant amyloids associated with distinct disease phenotypes will be of great importance for understanding tissue-selective deposition of TTR amyloid, which is currently underway in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.0c00091.

ThT fluorescence emission spectrum of ex vivo TTRwt amyloid, SDS−PAGE of cardiac extracts, TEM images of in vitro and ex vivo TTR amyloids, FT-IR spectra of in vitro mTTR and ex vivo TTR amyloids, overlaid 2D DARR for mTTR and TTRwt amyloids, three-dimensional strip plots for the sequential assignments, crystal structure of the monomeric form of native TTR showing side chain interactions, and overlaid 2D DARR for the native and amyloid states of TTRwt (PDF)

Accession Codes

Transthyretin, UniProtKB entry P02766.

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Notes

The authors declare no competing financial interest.

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