Supporting Information

Structural Characterization of Cardiac ex vivo Transthyretin Amyloid: Insight into Transthyretin Misfolding Pathway in vivo

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Materials and Methods

TTR expression and purification:

Wild type TTR (TTRwt) or monomeric variant TTR (mTTR) was transformed in to BL21 (DE3) chemically competent cells. Fifty microliter of transformed cells were spread on LB agar plate containing 100 μg/mL carbenicillin and incubated overnight at 37 ºC. Five milliliter of LB medium was inoculated with single colony from the LB agar plate and incubated at 37 ºC at 250 rpm for 4-5 hrs. A small culture (5 mL) was used to inoculate 50 mL M9 medium (supplemented with ^{13}C-glucose and ^{15}N-NH_4Cl) and grown until OD_{600} reaches 0.6. Then 50 mL M9 culture was added to 500 mL M9 medium and grown at 37 ºC. When OD_{600} reached 0.6, IPTG was added to a final concentration of 1 mM and incubated at 25 ºC with constant agitation of 250 rpm. After 12-14 hrs, the cells were harvested by centrifugation at 4 ºC and the pellet was stored at -80 ºC. To isolate TTR, pellet was resuspended in the lysis buffer (10 mM Tris buffer, 150 mM NaCl, pH 8) to sonicate and then centrifuged to collect the supernatant. Supernatant was precipitated out by adding ammonium sulfate to final concentration of 50 % and 25 % (for WT TTR and mTTR, respectively). This solution was then centrifuged at 4 ºC and saved the supernatant. The supernatant was subjected to dialysis against 20 mM tris buffer. The dialyzed solution was purified to isolate the TTR using anion exchange column followed by size exclusion chromatography using a 16/600 Superdex 16/600 column (GE Healthcare).

Preparation of in vitro TTR aggregates:

Purified monomeric variant TTR (mTTR; F87M/L110M) was filter sterilized and incubated at 0.5 mg/mL in 10 mM sodium phosphate buffer (pH 7.4) at 37 ºC with constant agitation at 250 rpm. After two days of incubation, morphology of mTTR aggregates was examined using TEM. In order to prepare mature mTTR amyloid, mTTR was incubated at 1 mg/mL in phosphate buffer at (pH 7.4, 37 ºC, 250 rpm). After three weeks of incubation, mTTR amyloid was collected by centrifugation. Amyloid sample was then washed with deionized water in order to remove any soluble TTR.

In order to obtain TTRwt oligomers TTRwt was incubated for two days at 0.2 mg/mL in 20 mM sodium acetate buffer (pH 4.4) at 4 ºC. WT TTR amyloid was prepared by incubating WT TTR at 0.2 mg/mL in 200 mM sodium acetate buffer (pH 4.4, 150 mM KCl) and incubated at 37 ºC. WT TTR amyloid was collected by centrifugation after three weeks of incubation. Amyloid sample was washed with deionized water in order to remove any soluble TTR.

Preparation of native WT TTR for solid-state NMR:

TTR native state samples were prepared by precipitating out purified TTRwt at 65-70 % ammonium sulphate at a protein concertation of 8 mg/mL. The TTR precipitate was collected by centrifuging the TTR samples at 14,000 rpm at 4 ºC for 1 hr. The precipitate was briefly rinsed with water to remove excessive ammonium sulphate.

Extraction of ex vivo TTRwt aggregates from human cardiac tissue:

Amyloid fibrils were extracted from autopsied cardiac tissue obtained on a patient with ATTRwt amyloidosis as described previously.[1] Briefly, 20 g (wet weight) of tissue was repeatedly homogenized and centrifuged in 0.15 M NaCl, 0.05 M sodium citrate in 0.01 M tris-buffered saline (pH 8.0), and deionized water. The remaining pellet was partitioned into an upper (top layer) and lower (bottom layer) portions and lyophilized separately, as were the saline, citrate, and water washes. The pooled water wash sample and top layer material were used for this study.

Fourier-transform infrared spectroscopy (FTIR) spectroscopy:

Monomeric variant TTR amyloid and ex vivo WT TTR aggregates were prepared as described above. A three microliter drop of amyloid sample was placed on the ATR crystal and was allowed to air-dry for 30 min. IR spectra for TTR amyloids were recorded using Nicolet iS50 ATR.

Transmission electron microscopy (TEM):

Five microliters of TTR sample was placed on to glow discharged formvar/carbon coated copper 400 mesh grid. The excess sample solution was blotted off with filter paper. Sample was negatively stained with 1 % uranyl acetate for 30 sec and excess stain was blotted off. Grids were air dried for five minutes and imaged under Philips CM12 Transmission Electron Microscope at 80 kV.

Thioflavin T (ThT) Fluorescence:

ThT stock solution was prepared by dissolving 8 mg of ThT in ten milliliters phosphate buffer (10 mM, pH 7.4). ThT working solution was prepared by diluting the stock solution in phosphate buffer (10 mM, pH 7.4) at 1:100 ratio. Fifty microliters of ex vivo WT TTR amyloid solution (5 mg/mL in deionized water) was added to 1450 microliters of ThT working solution. Fluorescence emission spectra were recorded with an excitation at wavelength of 440 nm with 1mm slit width).

SDS-PAGE:

TTR amyloid was collected by centrifuging fifty microliters of ex vivo WT TTR amyloid (5 mg/mL). The precipitates were dissolved in sixty microliters of SDS solution. The native states of WT TTR and G53A mutant were denatured in SDS sample loading buffer. All the samples were then heated at 70 ^{o}C for 10 min. Two microliters of the samples was added to each well and run on 4-12 % gradient bis tris SDS-PAGE gel using MES running buffer. Silver staining of the gel was performed according to the instructions provided in the SilverXpress™ Silver Staining Kit.

Solid-state NMR:

Solid-state NMR spectra were acquired using a Bruker Avance III HD 800 MHz NMR spectrometer equipped with a NHMFL 3.2 mm MAS probe at the National High Magnetic Field Laboratory (NHMFL) in Tallahassee, FL. Two-dimensional ^{13}C-^{13}C correlation NMR spectra were recorded using a dipolar-assisted rotational resonance (DARR) mixing scheme at a spinning frequency of 12.5 kHz. For the resonance assignment, a suite of 3D solid-state NMR experiments (NCACX, NCOCX, and CONCA) were carried out on the native state of TTR at a spinning frequency of 12.5 kHz. The DARR scheme with 80 ms mixing time was used for the ^{13}C-^{13}C correlation in the NCACX and NCACO experiments. For the one bond ^{15}N-^{13}C correlation, SPECIFIC-CP[2] was employed in the 3D experiments. The 90° pulse-lengths for ^{1}H and ^{13}C were 3.0 and 3.5 μs, respectively, and the two-pulse phase-modulated (TPPM)[3] decoupling scheme was employed with a radio-frequency field strength of 90 kHz. For the 2D DARR spectra, complex data points of 2048 × 512 and 2048 × 256 were collected for the native and amyloid states, respectively, with an acquisition delay of 2 sec, and 32 – 64 FIDs were accumulated for each t1 data point. For the 3D NMR experiments, complex datapoints of 2048 × 54 × 30 (NCACX), 2048 × 18 × 30 (NCOCX). All solid-state NMR data were processed with a squared sine window function with a sine bell shift of 3.5 using TopSpin (Bruker). Resonance assignment was carried out using Sparky (T. D. Goddard and D. G. Kneller, University of California, San Francisco).

Proteinase K digestion:

For proteinase K digestion (PK) experiments, native TTR (0.2 mg/ml), monomeric TTR amyloid (0.7 mg/ml), and cardiac extracts (7.5 mg/mL) were incubated in PBS buffer (pH 7.4) at 37 ºC in the presence of proteinase K enzyme (100 pg/μL) for 30 min and 4 hrs. The protein aggregates, particularly cardiac extracts, were highly insoluble in the PBS buffer, and thus the higher amount of aggregates was used for the PK digestion experiments. At each incubation time point, twenty microliters of incubated TTR samples were heated at 90 ºC with SDS loading buffer for 10 min. Undigested and digested protein samples were run on 4-12% gradient bis-tris SDS-PAGE gel using MES running buffer and the gel was stained with Coomassie Blue.

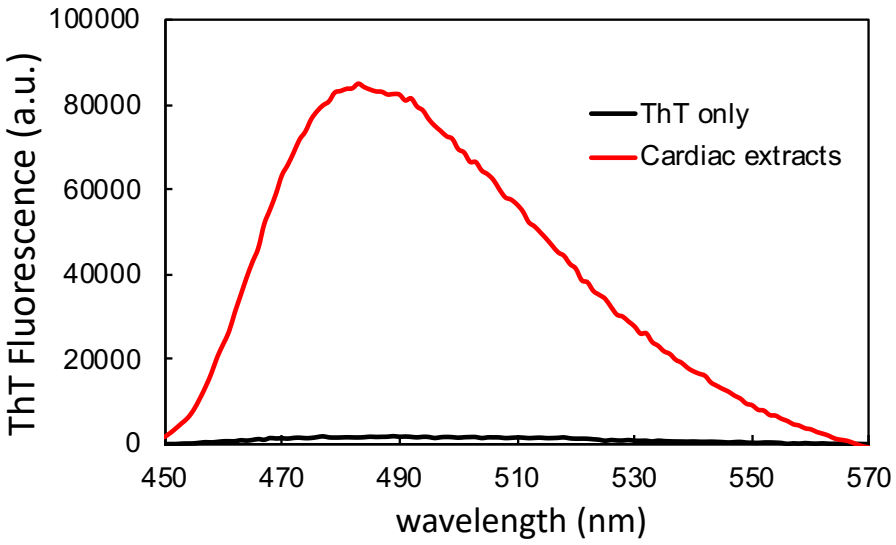

Figure S1. ThT fluorescence emission spectrum of ex vivo WT TTR amyloid (red) recorded with an excitation wavelength of 440 nm.

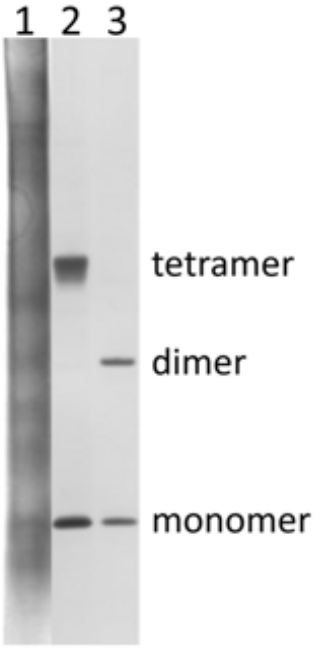

Figure S2. SDS-PAGE analyses of cardiac extracts, and in vitro WT TTR and G53A TTR. Cardiac extracts were dissolved in 8M urea (lanes 1), whereas in vitro WT TTR (lane 2) and G53A TTR (lane 3) were denatured in SDS. Protein bands were then silver stained.

The SDS-PAGE of the cardiac extracts (lane 1) is consistent with previous analyses of the ex vivo TTR aggregates[1]. Monomeric and dimeric forms of TTR as well as a series of TTR fragments were observed in previous analyses using mass spectrometry, which are also observed in our SDS-PAGE analyses. The previous analyses also revealed that the two bands between the monomer and dimer correspond to disulfide-linked dimers consisting of full-length monomer and truncated monomer.

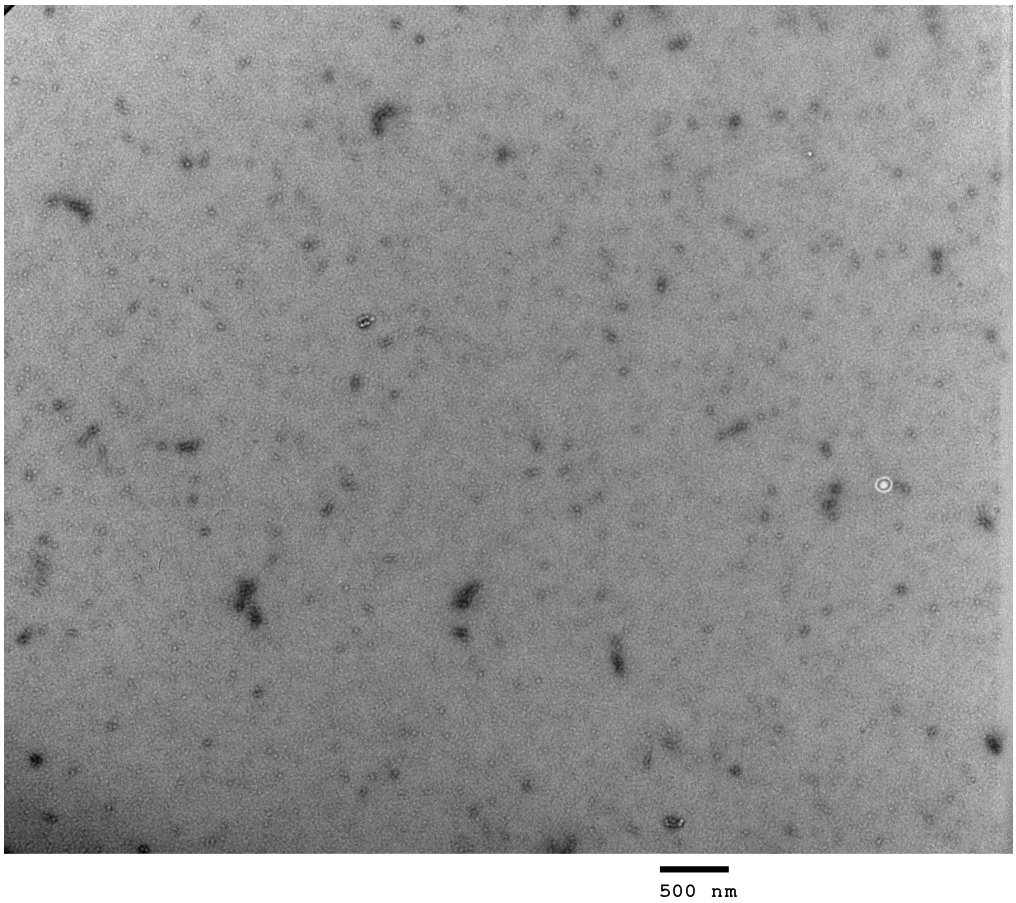

Figure S3. TEM image of WT TTR (0.2 mg/ml) incubated in sodium acetate buffer (pH 4.4) for two days at 4 ºC. The small spherical oligomers were observed for both WT TTR and monomeric TTR (mTTR) at pH 4.4 and 7.4, respectively.

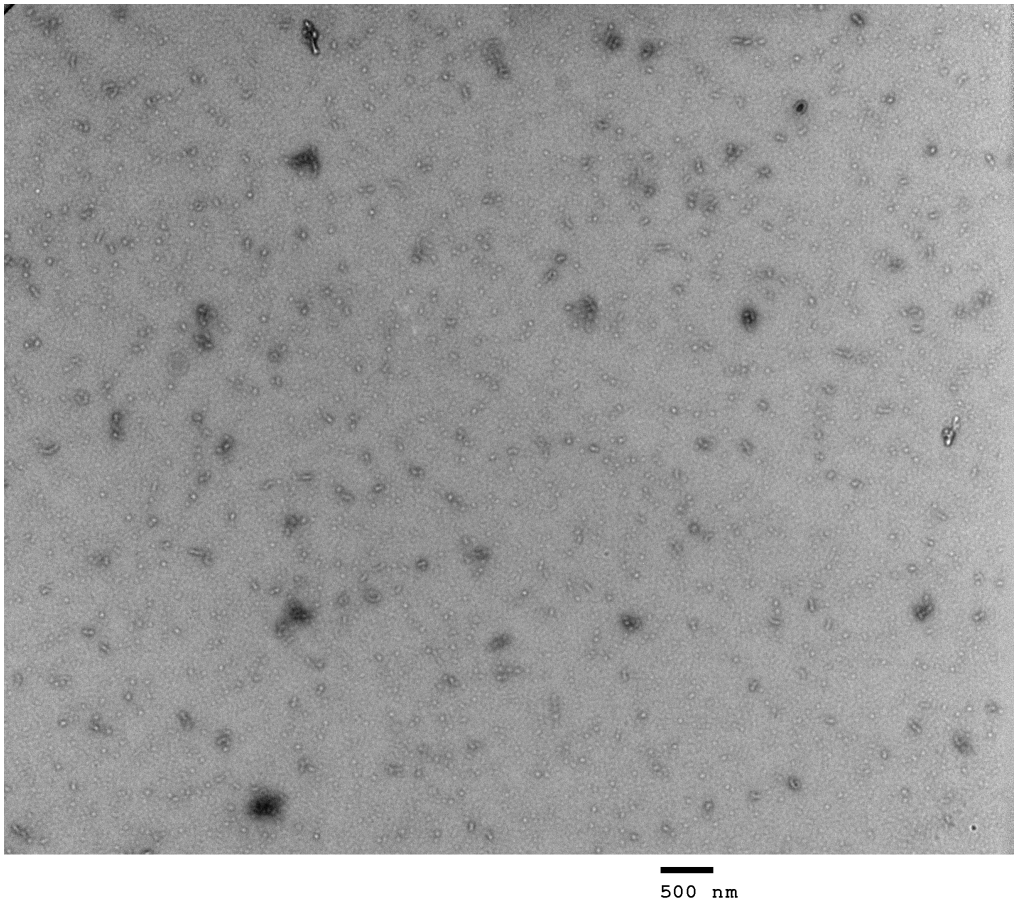

Figure S4. TEM image of cardiac tissue extracts of WT TTR isolated from an SSA patient.

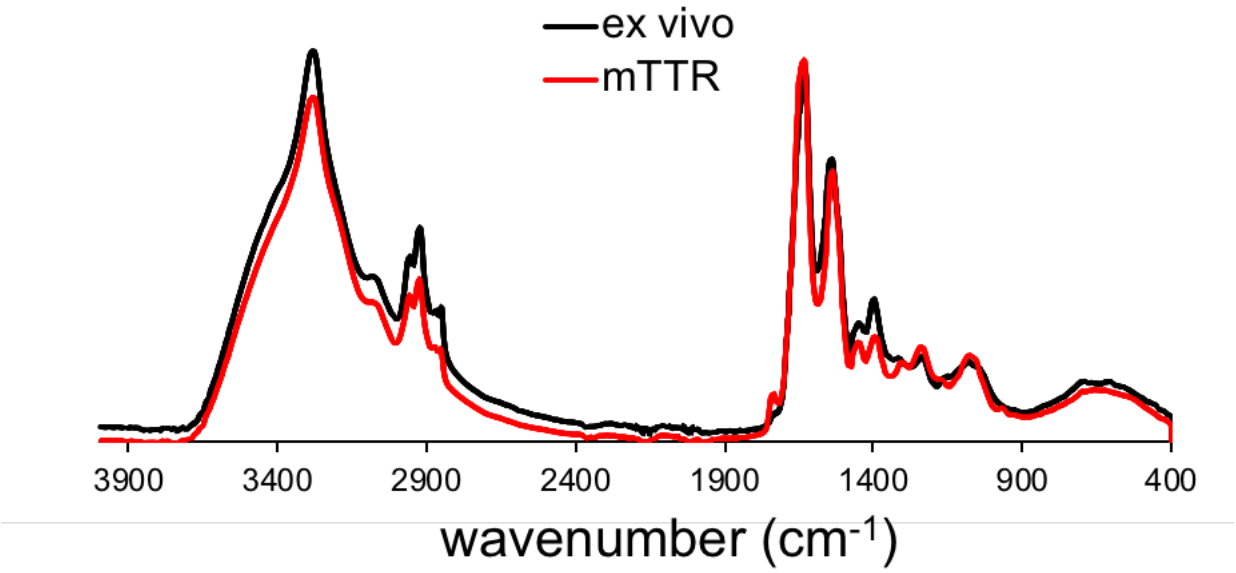

Figure S5. FTIR spectra of in vitro mTTR (red) and ex vivo TTR (black) amyloids.

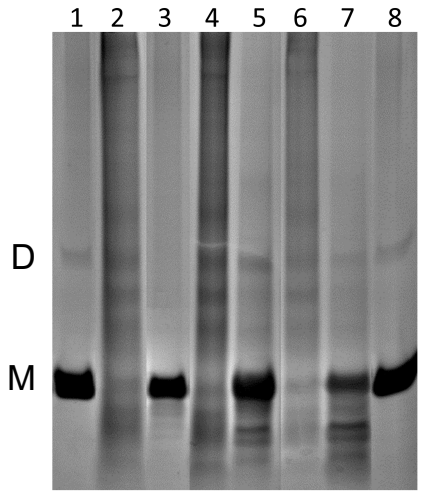

Figure S6. Proteinase K (PK) digestion assay of the native state of WT TTR, mTTR aggregates, and cardiac extracts. The proteins samples were treated with Proteinase K enzyme (200 pg/μL) at 37 ºC for 30 min and 4 hrs. Monomeric and dimeric TTR were indicated as M and D, respectively. Due to the insoluble nature of the aggregates, particularly cardiac extracts, the higher amount of aggregates was used for the PK digestion experiments and SDS-PAGE analyses. The bands for the ex vivo aggregates are weaker due to the higher insolubility of the aggregates.

1: WT native TTR (0.2 mg/ml) with no digestion
2: Cardiac extracts (7.5 mg/ml) with no digestion
3: mTTR aggregates (0.2 mg/ml) with no digestion
4: Cardiac extracts (7.5 mg/ml) treated with PK for 30 min
5: mTTR aggregates (0.7 mg/ml) treated with PK for 30 min
6: Cardiac extracts (7.5 mg/ml) treated with PK for 4 hrs
7: mTTR aggregates (0.7 mg/ml) treated with PK for 4 hrs
8: WT native TTR (0.2 mg/ml) treated with PK for 4 hrs

Previous detailed analyses using mass spectrometry revealed that the cardiac extracts consist of not only full-length monomeric and dimeric forms of TTR, but also a series of C-terminal fragments with N-termini ranging from residues 46 to 55.[1] The broad band under monomeric TTR in our SDS-PAGE (undigested cardiac extracts, lane 2) may come from the various TTR fragments. During the incubation period, the small fragments might be more easily digested away by PK enzymes, and thus additional clear bands, presumably from the digestion of full-length TTR, appear under the monomer band in lane 4 and 6. The small fragment bands are almost identical to those from mTTR aggregates in lane 5 and 7, suggesting that the ex vivo and in vitro TTR aggregates have similar structural features consistent with our TEM and FT-IR structural analyses.

On the other hand, the small TTR fragments were not observed in the native state of WT TTR in lane 8, indicating that the native state TTR is more ordered and protected than the TTR aggregates.

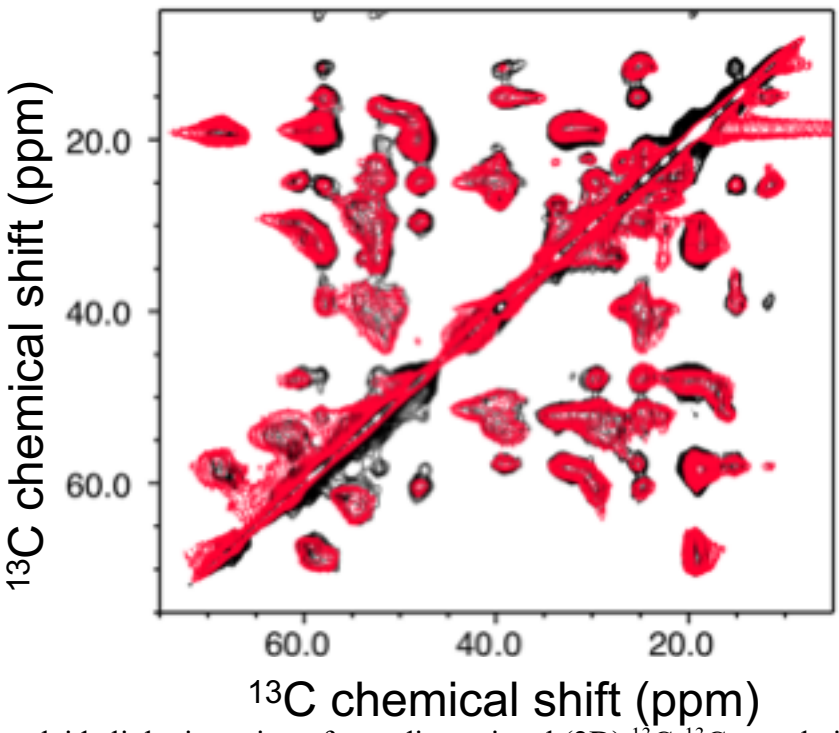

Figure S7. Overlaid aliphatic region of two-dimensional (2D) ^{13}C-^{13}C correlation solid-state NMR spectra for mTTR (red) and WT TTR (black) amyloids.

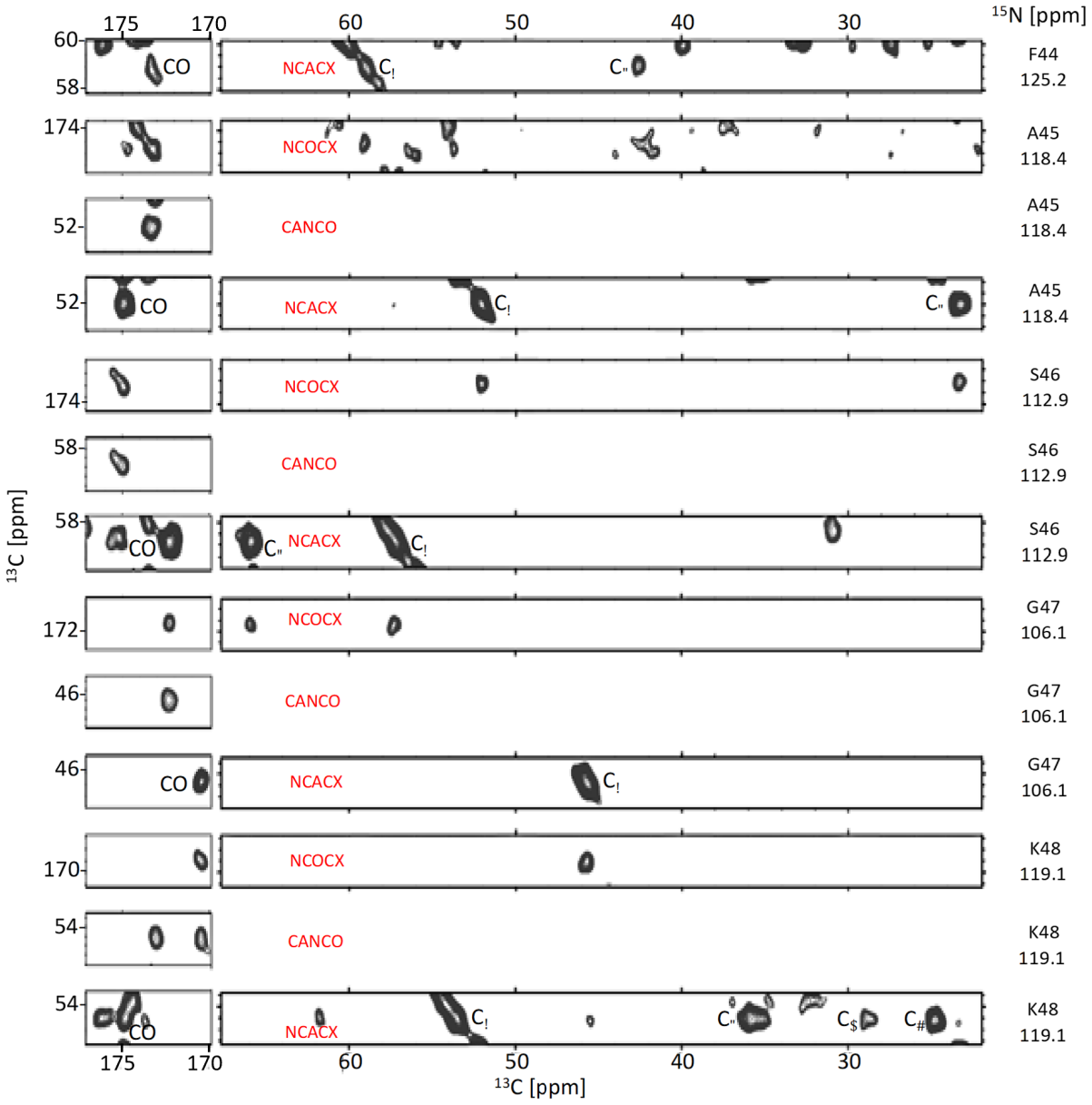

Figure S8. Strip plots for the 3D solid-state NMR spectra (NCACX, NCOCX, and CANCO) for the sequential resonance assignment of the native TTR.

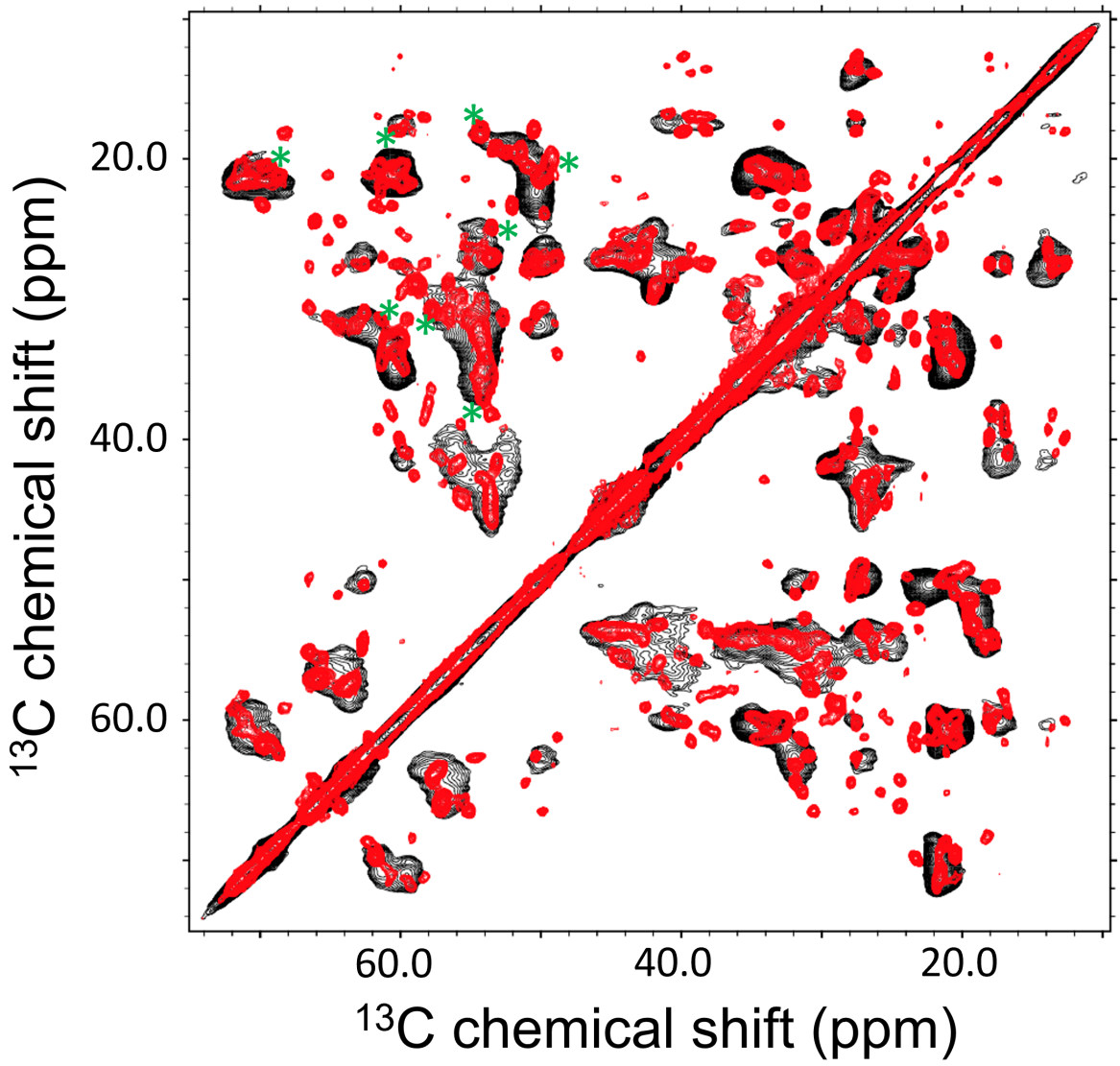

Figure S9. Overlaid two-dimensional ^{13}C-^{13}C correlation solid-state NMR spectra for the native (red) and amyloid (black) states of WT TTR formed at pH 4.4 obtained at a proton frequency of 800 MHz. Cross-peaks in the boundary are indicated by *.

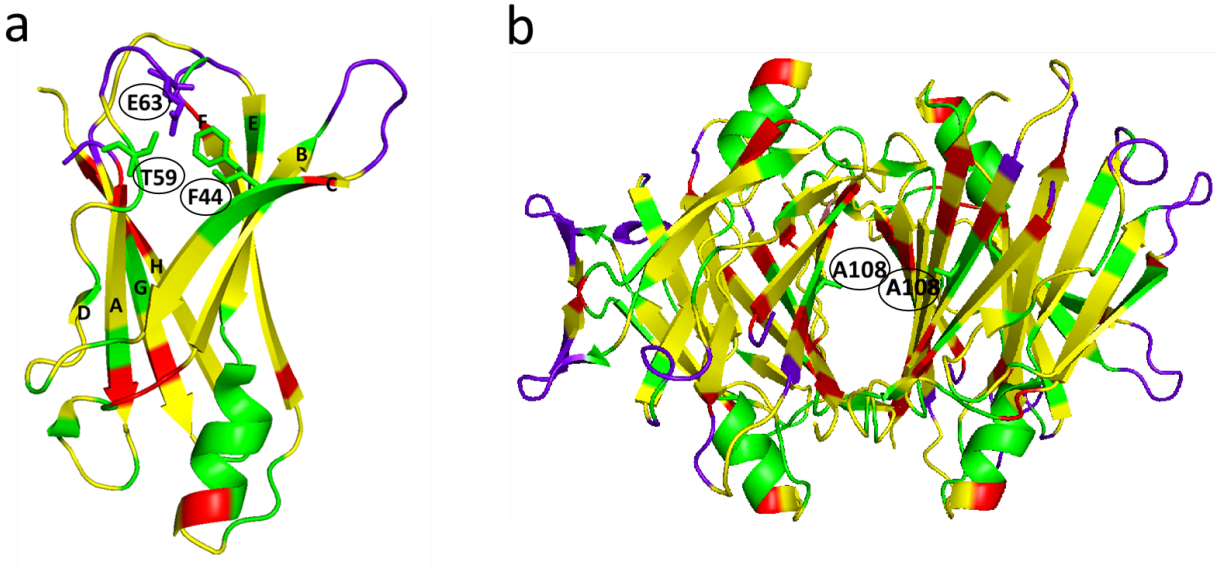

Figure S10. (a) Crystal structure of monomeric form of native TTR that shows sidechain interactions in strands C and B, and DE loop. (b) Crystal structure of tetrameric form of native TTR with residue A108 in the binding pocket.

References

1. Kingsbury, J. S.; Theberge, R.; Karbassi, J. A.; Lim, A.; Costello, C. E.; Connors, L. H. Detailed structural analysis of amyloidogenic wild-type transthyretin using a novel purification strategy and mass spectrometry. *Anal. Chem.* **2007***, 79*, 1990-1998.

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