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Supporting Information

Impact of Deuteration on the Assembly Kinetics of Transthyretin Monitored by Native Mass Spectrometry and Implications for Amyloidoses

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Experimental Methods

1. Production and purification of HTTR, DTTR, and CNTTR

The WT-TTR expression and purification have been previously described.[1] Briefly, TTR cDNA was cloned into pETM-11 bacterial expression vector coding for an N-terminal His_6-tag and a TEV cleavage site (EMBL Protein and Purification Facility, Germany). The plasmid was transformed into *E. coli* BL21 (DE3) cells (Invitrogen, Carlsbad, U.S.A). To obtain HTTR, the cells were grown and induced in LB medium at 37°C. In the case of DTTR, the cells were adapted to perdeuterated Enfors minimal medium and grown in a fed-batch fermenter at 30°C using d_8-glycerol (99% deuterium; Euriso-top, Saint-Aubin, France) as the only carbon source.[2] For CNTTR, the cells were adapted and grown in Ross medium at 30°C with ^{13}C-glucose monohydrate and ^{15}N-ammonium chloride as the sole carbon and nitrogen sources respectively.[3] All cells were induced with IPTG during A_{600} of 0.6-0.8.

HTTR, DTTR, and CNTTR were purified in an identical manner. Cell paste was resuspended in lysis buffer (20 mM Tris pH 8, 250 mM NaCl, 3 mM imidazole) in the presence of EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). Cells were lysed by sonication and cleared cell lysate was loaded onto a Ni^{2+}-NTA column. TEV cleavage was carried out overnight at room temperature. The protein was loaded onto a Ni^{2+}-NTA column again to get rid of the cleaved His_6-tag and TEV. The final step of purification involved loading the protein through Superdex 75 Hiload 16/60 gel filtration column (GE Healthcare, Orsay, France) in 10 mM Tris pH 7.5, 50 mM NaCl, and 1 mM DTT.

While DTTR was expressed under the conditions where only deuterium atoms were present, hydrogenous solutions were used during purification and the labile deuterium atoms in the protein were allowed to exchange back to hydrogen atoms. Only non-exchangeable deuterium atoms attached to the carbon atoms of the backbone molecule stay as deuterium atoms.

2. Mass spectrometric (MS) analyses in denaturing conditions

Measurements were carried out on a electrospray time-of-flight (ESI TOF) 6210 mass spectrometer interfaced with liquid chromatography (LC) 1100 pump system (Agilent Technologies, Les Ulis, France). All used solvents were HPLC grade. Solvents A: TFA 0.03%, ACN 5%; B: ACN 95%, H_2O 5%, TFA 0.03%. Protein samples were desalted on-line on a protein cartridge (Zorbax 300SB-C8, 5 µm, 5 x 0.3 mm, Agilent Technologies) for 3 min at 100 µl/min with 100% A and eluted with 70% B at 50 µl/min. Mass spectra were acquired in the positive ion mode and in the 300-3000 *m/z* range; data acquisition and processing were done with MassHunter software (v. B.02.00, Agilent Technologies).

3. Monitoring subunit composition of intact TTR by native MS

Prior to native MS analysis,[4] proteins were buffer-exchanged into 250 mM ammonium acetate (pH 7.0) or 250 mM deuterated ammonium acetate (D-AmAc) (pD 7.4) using Amicon Ultra centrifugal filter units (Millipore). Reaction was initiated by mixing HTTR and DTTR or CNTTR in 1:1 molar ratio. A concentration of 3 µM for each protein tetramer was used for all experiments. MS analyses were carried out on a quadrupole time-of-flight mass spectrometer (Q-TOF Ultima, Waters Corporation, Manchester, U.K.). The instrument was modified for the detection of high masses.[5,6] Protein ions were generated using a nanoflow electrospray (nano-ESI) source. Nanoflow platinum-coated borosilicate electrospray capillaries were bought from Thermo Electron SAS (Courtaboeuf, France). The following instrumental parameters were used: capillary voltage = 1.2-1.3 kV, cone potential = 40 V, RF lens-1 potential = 40 V, RF lens-2 potential = 1 V, aperture-1 potential = 0 V, collision energy = 30 V, and microchannel plate (MCP) = 1900 V. All mass spectra were calibrated externally using a solution of cesium iodide (6 mg/mL in 50%

isopropanol) and were processed with the Masslynx 4.0 software (Waters Corporation, Manchester, U.K.) and with Massign software package.[7] Spectra were processed using minimal smoothing.

In all subunit exchange experiments, the relative abundance of the tetramers was calculated from the intensities of the 13+ to 15+ charge states and expressed as a percentage of the total intensity of the peaks assigned to the tetrameric TTR.

4. Amyloid fibril formation

Amyloid fibril formation was performed via partial acid denaturation according to a previously described protocol.[8] The fibrillation process was initiated by incubating the sample in 100 mM sodium acetate pH 4.4 (10 mM Tris, 50 mM NaCl, 1 mM DTT) at 37°C with no stirring. Final protein concentration was set at 1 mg/ml. The extent of fibril formation was probed by fluorescence measurement of ThT binding.[9] ThT (at a final concentration of 40μM) was added to the samples before the fibrillation was initiated. ThT fluorescence was monitored using Tecan Infinite M200 PRO microplate reader (Lyon, France) in a 96-well black-wall plate up to 80 hours. Excitation of 440 nm (10 nm bandwidth) and emission of 480 nm (20 nm bandwidth) were used. The samples were subjected to shaking before every measurement. Measurements were carried out in triplicates and background subtraction was done with reaction carried out without the protein.

5. Probing the pH-dependent tetramer stability by gel electrophoresis[10]

TTR protein samples, at a final concentration of 0.2 mg/ml, were subjected to a range of buffers at pH 2.0 - 11.0, at intervals of 0.5 pH units, at 37°C for 72 hours. The buffers used were at a final concentration of 50 mM and were made up of glycine (pH 2.0 - 2.5), sodium acetate (pH 3.0 - 4.5), citric acid (pH 5), sodium citrate (pH 5.5), MES (pH 6.0 - 6.5), sodium phosphate (pH 7), Tris (pH 7.5 - 8.5), bicine (pH 9), CAPSO (pH 9.5 - 10.0), and CAPS (pH 10.5 - 11.0). The samples were then analysed on 12% Tris-tricine gel in the presence of SDS without boiling. The gels were stained with Coomassie Brilliant Blue.

6. X-ray crystallography

Crystals of H-TTR and D-TTR were grown using hanging drop-vapour diffusion in 2.15 M sodium malonate (pH 5) and 2.4 M sodium malonate (pD 5.9), respectively. Diffraction data were collected at 100K on ID23-1 beamline at ESRF, Grenoble.[11] 25% glycerol was used as cryoprotectant; the crystals were briefly soaked in the cryoprotectant before flash-freezing in liquid nitrogen and data collection. The datasets were processed with XDS[12], scaled and merged with SCALA[13], and converted to structure factors using TRUNCATE in CCP4 suite[14]. The model was refined with REFMAC5[15]. Model building was performed with COOT[16] and displayed with PYMOL[17]. The PDB structure 4PVL was used as a starting model. The models and the diffraction data have been deposited in the Protein Data Bank (http://www.rcsb.org/pdb) under accession codes 5CN3 (HTTR) and 5CNH (DTTR).

Supplementary Figures and Tables

Figure S1 The monomeric masses of A) HTTR (mass =14021.03 Da), B) DTTR (mass =14771.04 Da), and C) CNTTR (mass =14794.15 Da) were first assessed by MS in denaturing conditions to determine the level of protein labeling (summarized in Table S2).

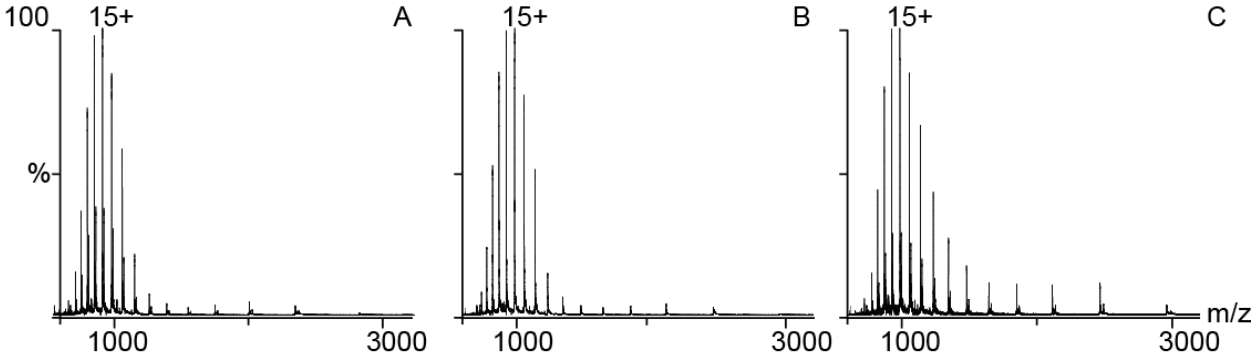

Figure S2 Intact tetrameric masses of A) HTTR (mass = 56121 ± 4 Da), B) DTTR (mass = 59089 ± 8 Da), and C) CNTTR (mass = 59175 ± 6 Da) (4H, 4D, and 4CN respectively) were analyzed in native conditions and their experimental masses were determined (Table S3). In both cases the mass difference between the unlabeled and labeled proteins is ~3 kDa. The *m/z* signals of 4H, 4D, and 4CN did not overlap.

The tetrameric masses of D) HTTR (mass = 56594 ± 11 Da) and E) DTTR (mass = 59589 ± 8 Da) were also assessed under native conditions in D-ammonium acetate. Figure S2D and S2E were essentially duplication of Figure S2A and S2B respectively, apart from the fact that the solvent used was D_2O based instead of H_2O based. A key difference between the samples in normal solvent and in D-AmAc was mass shift toward higher *m/z*. This mass shift can be attributed to the binding of D_2O and D-AmAc ions to the macromolecules.[18]

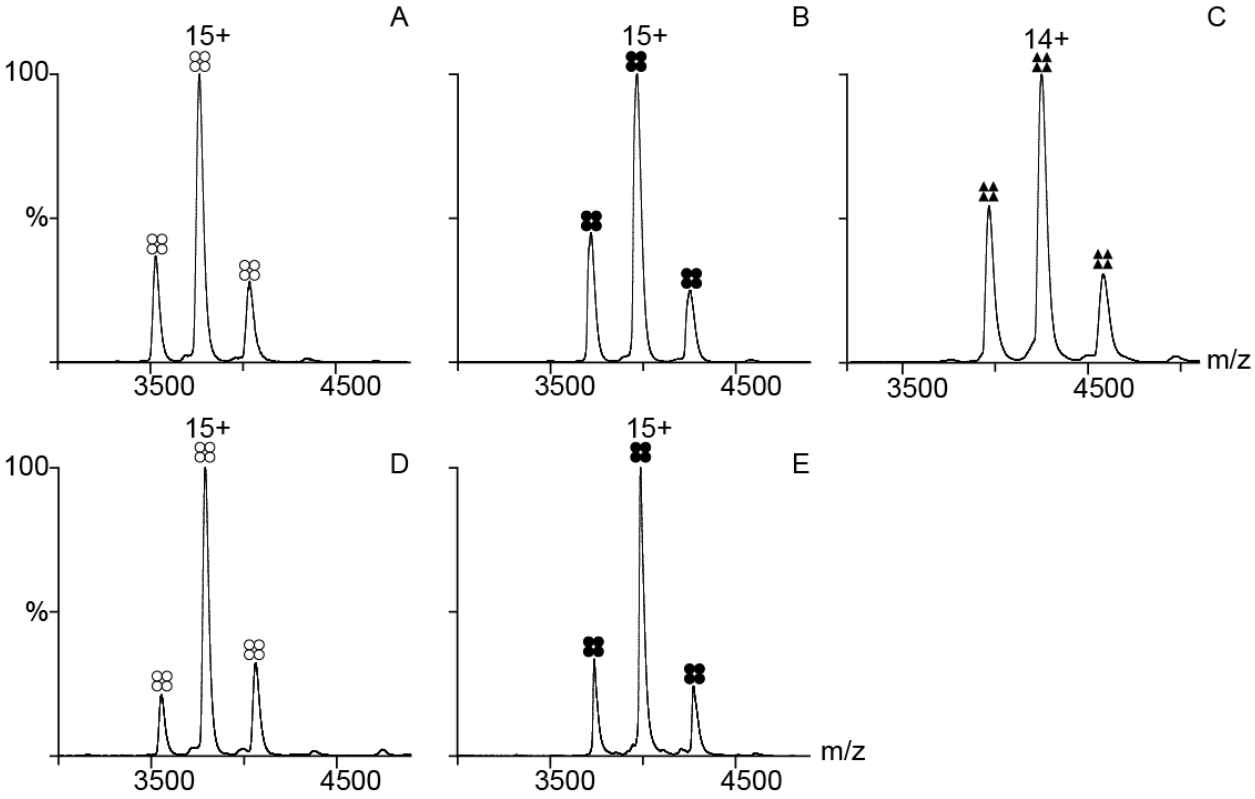

Figure S3 Possible TTR tetramer hybrids formed from mixing pure HTTR and pure DTTR samples (A) and from mixing pure HTTR and pure CNTTR samples (B).

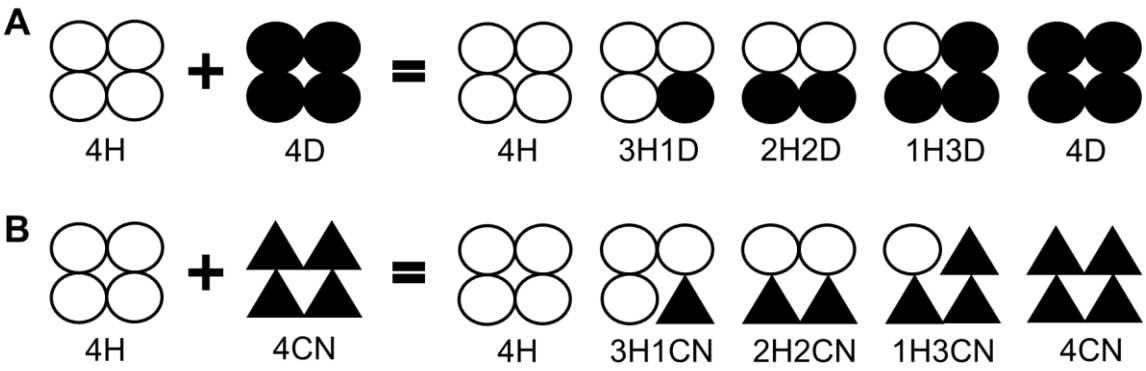

Figure S4 Unlabeled and labeled TTR were mixed and the exchange of subunits was monitored by native MS. A) 16 hours (i.e., 968 minutes) after mixing HTTR and DTTR in equimolar ratio, peaks matched the masses of 4H, 2H2D, 1H3D, and 4D TTR tetramers. B) TTR spectrum 16 hours (i.e., 968 minutes) after mixing HTTR and CNTTR. 4H, 2H2CN, 1H3CN, and 4CN TTR tetramers were detected. C) 4H and 4D were mixed in D-ammonium acetate and monitored by native MS. 23 hours (i.e., 1371 minutes) after mixing the samples, 4H, 2H2CN, 1H3CN, and 4CN TTR tetramers were observed.

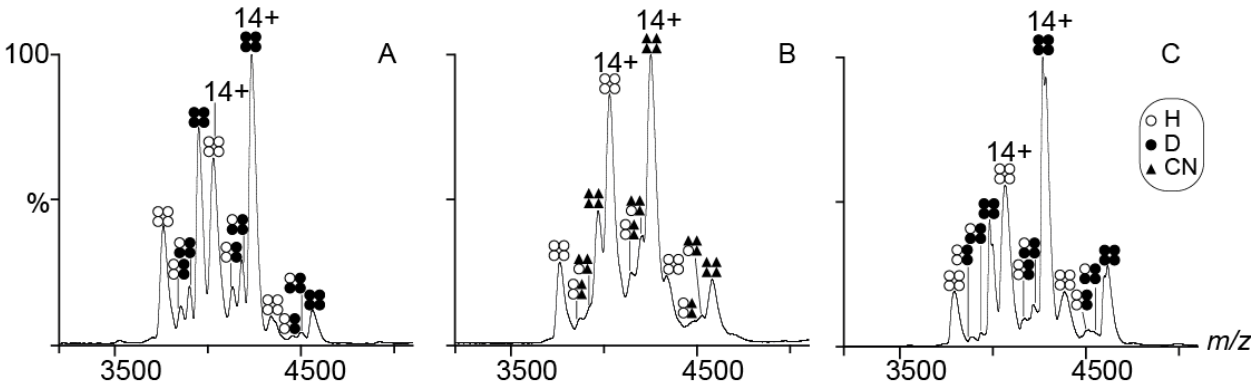

Table S1 Data collection and refinement statistics of H-TTR and D-TTR X-ray crystal structures

	HTTR	DTTR
PDB code	5CN3	5CNH
Beamline	ID23-1, ESRF	ID23-1, ESRF
Wavelength (Å)	0.972	0.972
Space group	$P\ 2_1\ 2_1\ 2$	$P\ 2_1\ 2_1\ 2$
Unit cell length (Å) / angle (°)	42.75 85.49 64.54 90.00 90.00 90.00	42.64 85.79 64.16 90.00 90.00 90.00
Resolution, Å	42.74 – 1.30	38.18 – 1.42
Completeness, %	99.6 (98.1)	99.5 (98.6)
Multiplicity	4.2	4.3
Total reflections	249764 (35178)	193286 (27612)
Unique reflections	58869 (8349)	45102 (6427)
Mean I / sigma (I)	15.8 (1.4)	15.6 (1.0)
Rmerge	0.032 (0.940)	0.043 (1.392)
Rpim (±)	0.017 (0.519)	0.023 (0.732)
Wilson B-factor (Å²)	19.64	21.58
Reflections used in refinement	55840	42792
Reflections used for R-free value	2981	2268
RMSD of bond lengths (Å) / angles (°)	0.020 / 1.985	0.020 / 1.777
R / R-free value	0.153 / 0.180	0.152 / 0.198
Average B-factor (Å^2)	26.95	29.13

*Statistics for the highest-resolution shell are shown in parentheses.

Table S2 Mass spectrometric analysis of HTTR, DTTR and CNTTR in denaturing conditions

	Calculated mass (Da)	Measured mass (Da)	% of labeling
HTTR	14019.15 [(a)]	14021.03	-
DTTR	14772.15 [(b)]	14771.04	99.85 [(d)]
CNTTR	14809.15 [(c)]	14794.15	98.10 [(e)]

[(a)] The calculated mass corresponds to the sum of mass of all 130 amino acids in the protein sequence of wild type TTR.
[(b)] The calculated mass is expressed as the calculated mass of HTTR in which all 753 non-exchangeable hydrogen atoms are replaced by deuterium (adds 753 mass units).
[(c)] The calculated mass is expressed as the calculated mass of HTTR in which all 627 [^{12}C]-atoms are replaced by [^{13}C]-atoms (adds 627 mass units) and all 163 [^{14}N]-atoms are replaced by [^{15}N]-atoms (adds 163 mass units).
[(d)] The % of labeling is expressed as [(measured mass of DTTR – calculated mass of HTTR) / (calculated mass of DTTR – calculated mass of HTTR)]
[(e)] The % of labeling is expressed as [(measured mass of CNTTR – calculated mass of HTTR) / (calculated mass of CNTTR – calculated mass of HTTR)]

Table S3 Mass spectrometric analysis of HTTR, DTTR, and CNTTR in native conditions

	Mass in H-ammonium acetate (Da)	Mass in D-ammonium acetate (Da)
Homotetramers		
4H	56121 ± 4	56594 ± 11
4D	59089 ± 8	59589 ± 8
4CN	59175 ± 6	-
Heterotetramers		
2H2D	57603 ± 4	58124 ± 2
1H3D	58338 ± 5	58871 ± 3
2H2CN	57644 ± 5	-
1H3CN	58414 ± 2	-

Table S4 Half-life ($t_{1/2}$) of each species of TTR tetramer hybrids during the subunit exchange

	Relative abundance (%)		Half-life ($t_{1/2}$)	
	Start (y1)	End (y2)	mins	hours
4D	50.68	30.45	1160	**19.33**
4CN	49.26	33.04	3439	**57.32**
(D) 4D	55.25	34.55	3915	**65.25**

* Half-life is defined by the amount of time required for the relative abundance of each tetramer hybrid to reach mid-point between its initial and end value [i.e. |y1 – y2| / 2].

* 4D represents the relative abundance of TTR tetramers with four D-labeled subunits in subunit exchange between equimolar mixtures of HTTR and DTTR in H_2O-based solvent.

* 4CN represents the relative abundance of TTR tetramers with four [^{13}C, ^{15}N]-labeled subunits in subunit exchange between equimolar mixtures of HTTR and CNTTR in H_2O-based solvent.

* (D) 4D represents the relative abundance of TTR tetramers with four D-labeled subunits in subunit exchange between equimolar mixtures of HTTR and DTTR in D_2O-based solvent.

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