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Fluorotryptophan incorporation modulates the structure and stability of transthyretin in a site-specific manner

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Abstract

Abnormal deposition of aggregated wild-type (WT) human transthyretin (TTR) and its pathogenic variants is responsible for cardiomyopathy and neuropathy related to TTR amyloidosis. The tryptophan (Trp) fluorescence measurements typically used to study structural changes of TTR do not yield site-specific information on the two Trp residues per TTR protomer. To obtain such information, tryptophan labeled with fluorine at the 5 and 6 positions (5FW and 6FW) was incorporated into TTR. Fluorescence of 5FW and 6FW-labeled WT-TTR (WT-5FW and WT-6FW) and a single-Trp mutant W41Y showed that the photophysics of incorporated fluoro-Trp is consistent with site-specific solvation of the indole ring of W41 and W79. ^{19}F -NMR showed that solvent accessibility depends on both the location of the Trp and the position of the fluorine substituent in the indole ring. Unexpectedly, differences were observed in the rates of aggregation, with WT-6FW aggregating more rapidly than WT-5FW or WT TTR. Real-time ^{19}F -NMR urea unfolding experiments revealed that WT-5FW is kinetically more stable than WT-6FW, consistent with the aggregation assay. In addition, structural perturbations of residues distant from either Trp site are more extensive in WT-6FW. Notably, residues in the dimer interfaces are perturbed by 6FW at residue 79; pathogenic mutations in these regions are associated with reduced tetramer stability and amyloidogenesis. The differences in behavior that arise from the replacement of a fluorine at the 5-position of a tryptophan with one at the adjacent 6-position emphasizes the delicate balance of stability in the TTR tetramer.

Graphical Abstract

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Supporting Information

Three tables containing validation of protein constructs by mass spectrometry, longitudinal relaxation times and translational diffusion coefficients for TTR samples, together with 7 figures showing size exclusion profiles, UV absorbance, fluorescence and circular dichroism spectra for labeled TTR, a comparison of ^{19}F NMR spectra of labeled TTR in the presence and absence of internal reference, a series of ^1H - ^{15}N HSQC spectra overlaid to show differences arising from mutagenesis and fluorine labeling, and a comparison of the aggregation of mutant and fluorine labeled TTR.

Introduction

Human amyloid diseases are directly associated with misfolding and aberrant aggregation of certain proteins.¹ One of these aggregation-prone proteins, transthyretin (TTR), is a 55-kDa homotetramer present in plasma and cerebrospinal fluid.² The normal function of TTR is to transport thyroxine and retinol-binding protein/retinol complex in the blood.^{3, 4}

Amyloidogenic deposits of wild-type transthyretin (WT-TTR) in the heart cause senile systemic amyloidosis, which affects as much as 25% of the population over age 80.⁵ In addition, formation of mis-assembled aggregates of more than 100 autosomal dominant pathogenic TTR mutants results in familial amyloid cardiomyopathy and familial amyloid polyneuropathy.⁶ The V122I mutation, for example, leads to cardiac amyloidosis in 3–4% of African Americans.⁷ Because of the high prevalence and the causative connection to TTR amyloidosis, it is clear that understanding the molecular basis of TTR amyloidosis is of great importance.

Native TTR is a tetramer (formed as a dimer of dimers) in which each subunit consists of a β -sandwich fold formed from a pair of four-stranded β -sheets (DAGH and CBEF, Fig. 1A, B) that enclose a hydrophobic core. Extensive inter-protomer interactions contribute to the stabilization of the native tetrameric TTR architecture.⁸ To initiate amyloidogenic aggregation, the native TTR tetramer must first dissociate to form a folded and structured monomer,^{9–11} which undergoes conformational fluctuations¹² and samples a partially unfolded state to self-assemble into fibrils in a downhill polymerization process.¹³ Importantly, the aggregation propensities of some well-studied pathogenic mutants correlate well with their tetramer dissociation rates as probed by the intrinsic tryptophan (Trp) fluorescence changes as a function of urea concentrations.¹⁴ These mechanistic observations point to tetramer dissociation as the rate-limiting step;^{13, 15} the energetics of the multi-step dissociation-unfolding-aggregation equilibrium is indeed encoded in the sequences of TTR variants. However, the molecular basis of the tetramer destabilization in pathogenic TTR mutants remains poorly understood.

There are two native Trp residues in WT-TTR: W41 is located at the beginning of the C strand and W79 is within the sole helix, between the E and F strands (the EF helix, Fig. 1B). Their well-separated locations provide a useful means to probe site-dependent structural information regarding TTR. In a routine Trp fluorescence measurement of TTR, however,

such information is lost due to the overlapped emission spectra of the two tryptophans.¹⁶ To obtain site-specific information on changes in the Trp environment during structural rearrangements, we incorporated fluorotryptophan into the protein in place of Trp, and used ¹⁹F-NMR, which has high sensitivity, 100% natural abundance of the NMR-active nucleus and a large chemical shift dispersion range,¹⁷ to directly probe the local environment of the ¹⁹F nucleus in each fluorinated Trp. ¹⁹F-NMR has been applied to obtain site-specific structural information and conformational dynamics in a number of proteins.^{18–21} Here we report a ¹⁹F-NMR study of TTR, in conjunction with UV absorbance, Trp fluorescence spectroscopy and computational modeling. Two ¹⁹F probes, substituted at the 5- and 6-positions of the indole ring (Fig. 1C; 5FW and 6FW respectively) provide site-resolved structural information for Trp at residues 41 and 79. Acid-mediated aggregation assays and real-time ¹⁹F-NMR urea unfolding experiments show that WT-TTR containing 5FW (WT-5FW) is more stable than WT-TTR labeled by 6FW (WT-6FW). Backbone amide chemical shift perturbations indicate that remote structural changes not adjacent to either Trp site are more extensive in WT-6FW than WT-5FW, which could be related to the substituted fluorine at the 6-position in residue 79 (6F-W79). The 5-position substitution in this residue (5F-W79) appears to be better tolerated, consistent with the ¹⁹F-NMR observation and modeling results that 5F-W79 is solvent exposed whereas 6F-W79 is not. The site-specific information provided by the use of ¹⁹F-NMR with fluorotryptophan-substituted TTR, together with the changes in behavior induced by the position of the fluorine label at the 5- or 6-position, suggest that this approach will be particularly valuable in delineating the effects of disease-causing mutations on the delicately-balanced stability of the TTR tetramer.

Materials and Methods

Unless otherwise mentioned, 10 mM potassium phosphate and 100 mM KCl at pH 7.0 was used as buffer (NMR buffer) and experiments were performed at 298 K. Images of TTR structures were prepared using PyMOL.²²

Site-directed mutagenesis

Primers for W41Y (5'-ggctgctgatgacacctatgagccattgcctctgg-3' and 5'-ccagaggcaaatggctcataggtgtcatcagagcc-3') were ordered from IDT and the site-directed mutagenesis PCR was performed using the QuikChange Kit (Agilent), according to the manufacturer's instructions.

Protein expression and purification

The human TTR sequence that includes a N-terminal Met was encoded in a pET29a plasmid. A fresh transformation to the *E. coli* BL21 Star (DE3) strain was performed for every batch of protein expression. The M9 medium containing 1 g/L (¹⁴NH₄)₂SO₄ or (¹⁵NH₄)₂SO₄ (Cambridge Isotope Laboratories) and 3 g/L ¹²C-glucose was used in expression. Around 5 mL of the overnight M9 starter culture with 50 µg/mL kanamycin was used to inoculate a 50-mL M9 growth culture at 310 K. For ¹⁹F-labeling, 3 mg of 5FW or 6FW (Sigma) was added when the optical density at 600 nm (OD₆₀₀) reached 0.6 and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added 30 min later. Cell culture was then

expressed at 310 K for 4 hr. We found that the inclusion of glyphosate as an inhibitor of the shikimate aromatic amino acid biosynthetic pathway²³ during expression for 5FW- or 6FW-labeling was not necessary for TTR (Fig. S1). Incorporation of 5FW and 6FW WT-TTR and in a single-tryptophan mutant W41Y was nearly complete, as indicated by electrospray ionization mass spectrometry (Table S1). The cell pellet was re-suspended in a buffer of 50 mM Tris, 150 mM NaCl (pH 7.6) supplemented with ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor (Thermo Fisher), followed by ammonium sulfate precipitation twice (21% and 33%, w/v). The resultant TTR pellet was re-suspended and injected to a Superdex 75 and then a Capto Q ImpRes column (GE Healthcare Life Sciences) for purification. Protein fractions were pooled and exchanged to NMR buffer by a PD25 column (GE Healthcare Life Sciences).

Trp fluorescence

Trp fluorescence measurements were performed on a Fluorolog 3 spectrometer (Horiba/Jobin Yvon) in the L-format. Trp was excited at 280 nm and its emission was scanned from 320 nm to 420 nm with 1 nm as the wavelength interval. The slit width was 5 nm and the integration time was set as 0.2 s. The concentration of TTR was around 1 μ M in the measurements.

Circular dichroism (CD)

CD measurements were performed using an Aviv 62DS/202 CD spectrometer. Approximately 0.4 mg/mL protein was used in a 1-mm cuvette and the wavelength was scanned from 260 nm to 200 nm in 1-nm steps with an integration time of 2 s. The bandwidth was set at 2 nm. Raw ellipticity was averaged from three scans and converted to mean residue ellipticity (Fig. S2).

Size-exclusion analysis

A Superdex 75 gel filtration column with a bed volume of 121 mL was used with NMR buffer as the running buffer at 0.7 mL/min. Approximately 100 μ L of 100 μ M protein was injected and the effective in-column concentration was around 1 μ M after dilution. The elution was monitored by absorbance at 280 nm. Consistent with the computational modeling, we found that both WT-5FW and WT-6FW are predominantly tetrameric at 1 NM, as is WT-TTR (Fig. S3).

Electrospray ionization time-of-flight (ESI-TOF) mass spectrometry

An ESI-TOF spectrometer (Agilent) was used to measure the molecular mass of Trp-labeled TTR. Around 5 μ L of 10 μ M TTR was injected for analysis. For mass deconvolution, a window of 10000 to 20000 was used with the molecular weight agreement as 0.05%.

Aggregation assay

The aggregation assay was performed as previously reported.⁹ Briefly, un-agitated TTR samples (0.2 mg/mL) were incubated at 310 K for 2 days in 50 mM sodium acetate and 100 mM KCl at pH 4.4. At specified time points, the optical density at 330 nm (OD₃₃₀) was measured after a thorough vortex mixing.

NMR experiments

¹⁹F-NMR measurements were performed on a Bruker Avance 601 spectrometer equipped with a QCI ¹H/¹⁹F-¹³C/¹⁵N quadruple resonance cryoprobe and a shielded z-gradient coil. The NMR buffer was extensively degassed with argon and NMR tubes were purged by argon before use. For ¹⁹F-peak assignments, 100 μM TTR was used in NMR buffer in 10% D₂O. For urea unfolding experiments, TTR in NMR buffer containing 10% D₂O was mixed with an equal volume of urea buffer (12 M urea, 50 mM potassium phosphate, 100 mM KCl, 1 mM EDTA, 10 mM dithiothreitol (DTT) at pH 7.0 with 10% D₂O) to initialize unfolding. ¹H-coupled ¹⁹F spectra were collected due to the negative nuclear Overhauser effects for 5FW/6FW.¹⁹ Spectra were acquired with 4k complex points using a pulse length of 10 μs, sweep width of 40 ppm, carrier offset at -120 ppm, and inter-scan relaxation delay of 1 s. A 10-Hz exponential line-broadening factor was applied to enhance signal/noise and the time domain data were zero-filled to 16k before Fourier transformation. The ¹⁹F chemical shifts were referenced using an internal standard (free 5FW for 6FW-labeled samples, free 6FW for 5FW-labeled samples). The ¹⁹F longitudinal relaxation time constant *T*₁ was measured using a standard inversion recovery pulse sequence with a relaxation delay of 5 s. Delays of 0.0001, 0.005, 0.01, 0.02, 0.05, 0.07, 0.1, 0.15, 0.2, 0.3, 0.5, 0.8, 1 and 2 s were used for unfolded TTR, delays of 0.02, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2 and 2.5 s were used for folded TTR. The peak intensity was fitted using a single exponential function using the nonlinear least square method.²⁴ Uncertainties were calculated as one standard deviation of 50 bootstrapped datasets.²⁵ Translational diffusion coefficients were measured on a Bruker Avance 900 MHz spectrometer using a transverse relaxation-optimized pulse field gradient experiment.²⁶ A total of 11 x-gradient strengths from 6.8 G/cm to 23.8 G/cm were used with a diffusion delay of 200 ms and recycle delay of 1 s. All NMR data were acquired by XWinNMR/TopSpin NMR and processed by NMRPipe²⁷ and results were analyzed and visualized by MATLAB and Python. CSP was calculated according to Ref²⁸ using $\sqrt{(\Delta^1\text{H})^2 + (\Delta^{15}\text{N}/5)^2}$.

¹⁹F-NMR unfolding kinetics analysis

The line shapes of the unfolded ¹⁹F peaks were fitted using the appropriate number of Lorentzian function(s)²⁹ and the integrated peak areas from the fitted Lorentzians were used to quantify the unfolding kinetics described by a single exponential function. The fitting uncertainty is reported as one standard deviation from 50 bootstrapped datasets. We noted that the numerical fitting of the time-dependent decrease in peak areas for peaks belonging to W41 and W79 in the folded species yields similar results (data not shown). The relative peak areas for unfolded W41 and W79 are expected to report the relative number of ¹⁹F nuclei at each Trp site due to the nearly identical *T*₁ time constants (Table S2).

¹⁹F-Trp model-free relaxation analysis

The contribution of dipole-dipole coupling (DD, the first term) and chemical shift anisotropy (CSA, the second term) to ¹⁹F *T*₁ and *T*₂ relaxation is described by the following equations:^{30, 31}

$$1/T_1 = \frac{K^2}{20} \rho \left(\frac{4}{3} \pi r_{\min}^{-3} \right) (J(\omega_H - \omega_F) + 3J(\omega_F) + 6J(\omega_H + \omega_F)) + \frac{6}{40} \omega_F^2 \delta_z^2 \left(1 + \frac{\eta^2}{3} \right) J(\omega_F)$$

(1)

$$1/T_2 = \frac{K^2}{40} \rho \left(\frac{4}{3} \pi r_{\min}^{-3} \right) (J(\omega_H - \omega_F) + 3J(\omega_F) + 6J(\omega_H + \omega_F) + 4J(0) + 6J(\omega_H)) + \frac{1}{40} \omega_F^2 \delta_z^2 \left(1 + \frac{\eta^2}{3} \right) (3J(\omega_F) + 4J(0))$$

(2)

where ρ is the density for proton-containing spheres in proteins as $5.73 \times 10^{28} \text{ m}^{-3}$ from Sarker et al.³² $r_{\min}$ is the minimal distance from the nearest proton (2.6 Å) used for the spherical integral taken from Luck et al.³¹ $J(\omega)$ is the spectral density functions described by Abragam³³ sampled at the angular frequencies of ^{19}F or ^1H nuclei (ω_H or ω_F at 14.1 T in our work) or linear combinations thereof, δ_z is the chemical anisotropy for ^{19}F -Trp, η is the corresponding asymmetry and $K = \mu_0 \gamma_H \gamma_F \hbar / 4\pi$, where μ_0 is the vacuum permeability constant, γ_x is the magnetogyric ratio for nucleus x and $\hbar$ is the reduced Planck constant. For 5FW, $\delta_z = -62.3$ ppm and $\eta = 0.24$.³¹ For 6FW, $\delta_z = -65.4$ ppm and $\eta = 0.37$.³⁴ The model-free formalism for spectral density from Lipari and Szabo^{35, 36} under the isotropic tumbling condition takes on the following form:

$$J(\omega) = \frac{2\tau_c}{1 + \omega^2 \tau_c^2} \quad (3)$$

where τ_c is the overall rotational correlation time for TTR, which can be approximated by:²⁹

$$\tau_c = 4\pi\eta r^3 / 3k_B T \quad (4)$$

where η is the viscosity of the solvent, r is the hydrodynamic radius of TTR, k_B is the Boltzmann constant and T is the temperature. Empirical formulas from Cho et al.³⁷ were used to correct for the viscosity in the presence of 10% (v/v) D_2O and values from Kawahara and Tanford³⁸ were used to account for 6 M urea. The hydrodynamic radius of TTR can be roughly estimated by:

$$r = \left(3\bar{V}M / 4\pi N_A \right)^{1/3} + r_w \quad (5)$$

where $\bar{V}$ is the specific volume ($0.73 \text{ cm}^3/\text{g}$), M is the molecular weight, N_A is the Avogadro's constant and r_w is the thickness of the hydration shell ($1.6\text{--}3.2 \text{ \AA}$).²⁹ Under these numerical conditions, the theoretical T_1 and T_2 values for ^{19}F in TTR can be predicted using equations (1)(2).

Molecular modeling

The starting structure was taken from the X-ray structures of WT-TTR PDB: 1BMZ³⁹ or PDB: 1F41⁴⁰ (results not shown). The corresponding X-ray symmetry (C_2 and D_2 , respectively) was used to create the tetramer structure from the crystallographic dimer structure. The choice of starting structures did not change the observation that the fluorine atoms can be tolerated by the TTR tetrameric structure in modeling. All 8 Trp residues in a TTR tetramer were replaced with 5FW or 6FW, the parameters of which were determined using AMBER Antechamber program using AM1-BCC model.^{41, 42} Each protein was placed in a cuboid TIP3 water⁴³ box larger than TTR by a minimal $10\text{-\AA}$ on all sides and Na^+ was used as the counter ion. Energy minimization was performed using AMBER 12 with ff12SB as the force field.⁴⁴ Solvent was minimized first with 500 cycles of steepest descent (SD) steps before conjugation gradient minimization. The maximal number of cycles of minimization was 10000. During the solvent minimization, all TTR residues were constrained by a force constant of $500 \text{ kcal/mol/\AA}^2$ around the X-ray structural positions. Constant volume periodic particle mesh Ewald⁴⁵ boundaries were used with the non-bonded cutoff set to $10 \text{ \AA}$. The system was minimized with 10 cycles of SD steps before the conjugation gradient minimization and the maximal step of minimization was 10000. The relative solvent accessible surface area (SASA) per residue was calculated using the GETAREA software⁴⁶ with the input structure as PDB: 1BMZ.³⁹ The radius of the water probe was set at $1.4 \text{ \AA}$.

Results

Modeling the TTR tetramer with fluorinated Trp

To evaluate whether the fluorine atoms at the 5- or 6-position of the indole ring could be tolerated by TTR without causing substantial overall structural changes, *in silico* modeling was first performed to incorporate either 5FW or 6FW at residue 41 and 79, followed by a global energy minimization. As expected, the slight increase in van der Waals radius upon replacement of hydrogen by fluorine ($\sim 0.3 \text{ \AA}$)⁴⁷ at these locations seems to be well tolerated by the tetrameric structure, leading to minimal structural changes in the energy-minimized models when compared to non-labeled WT-TTR (root-mean-square deviation, RMSD $\sim 0.3\text{--}0.4 \text{ \AA}$ for both WT-5FW and WT-6FW, Fig. 1A). However, modeling alone cannot exclude the possibility of subtle perturbations of the conformational dynamics of TTR (see below).

Photophysical characterization of fluoro-substituted Trp in TTR

We first characterized TTR labeled with fluorinated Trp by UV absorbance. Two $\pi \rightarrow \pi^*$ electronic transitions, $^1\text{L}_a$ and $^1\text{L}_b$, are responsible for the absorbance band of the Trp indole ring around 280 nm .^{16, 48} Consistent with a previous report,⁴⁹ the introduction of 5FW to WT-TTR leads to a slight red-shift (by 2 nm) of the 280 nm UV absorbance peak when compared to the unlabeled control (Fig. 2A). As previously noted,^{50, 51} it is likely that this

shift is caused by an energy level change in the 1L_b transition, which has a more structured absorbance band than 1L_a .^{16, 48} The intensity of the absorbance side-band at 300 nm relative to the main band at 280 nm appears to be correlated with the number of incorporated 5FW residues, which conveniently serves as a spectroscopic hallmark for qualitative estimation of labeling efficiency (Fig. S1). In the case of WT-6FW (Fig. 2B), the ~300 nm side-band is not as pronounced as that for WT-5FW, though it still appears to be correlated with the number of labeled tryptophans.

Intrinsic Trp fluorescence spectroscopy offers useful information regarding the local environments surrounding the indole ring.^{9, 16} In aqueous solvents, 1L_a (Fig. 2C, inset) is identified as the main emitting state for the un-substituted Trp as well as for 5FW and 6FW;⁵⁰ this electronic transition is more sensitive to solvent polarity changes than 1L_b .⁵² The 1L_a excited state in 5FW and 6FW has a larger dipole moment than that of un-substituted Trp.^{53, 54} Consequently, the excited state in fluoro-Trp derivatives is preferentially stabilized by solvation in polar environments compared to un-substituted Trp.⁵⁵ Thus, incorporation of 5FW or 6FW at a solvent-exposed site is expected to lead to more strongly red-shifted fluorescence emission than for a buried site. Indeed, the fluorescence emission peak for 5F-W79 and 6F-W79 in the W41Y mutant exhibits little red shift compared to the un-substituted W41Y (< 4 nm, Fig. 2C, D) since the indole ring at residue 79 is almost completely buried (1% relative side chain solvent accessible surface area, SASA, Fig. 3B, D). In sharp contrast, the emission of substituted Trp in either WT-5FW or WT-6FW, with an additional fluoro-Trp at residue 41, is red-shifted by ~15 nm compared to W41Y-5F-W79 and W41Y-6F-W79, which is due to the increased stabilization of the 1L_a excited state of the partially solvent-exposed indole ring at residue 41 (24% relative side chain, Fig. 3A, C). The red-shift in the fluorescence spectrum upon 5FW- or 6FW-substitution in TTR is similar to previous reports for other proteins.^{50, 56, 57} Thus, the photophysics of the two Trp residues matches well with their structural features, for both 5FW and 6FW probes.

Higher aggregation propensity for WT-6FW than WT-5FW

The similarity in the photophysics of TTR substituted with 5FW and 6FW, together with their high incorporation yields, and the similarity of their secondary structures monitored by circular dichroism (Fig. S2) led us to expect similar aggregation kinetics for TTR substituted with these two labeled tryptophans. To our surprise, we observed a substantial difference: WT-6FW aggregated much faster than WT, while WT-5FW aggregated more slowly than WT (Fig. 4).

^{19}F -NMR assignment of folded and unfolded TTR

The relative stability of the substituted TTRs was investigated by measuring the ^{19}F -NMR spectra of WT-5FW and WT-6FW. Two well-resolved ^{19}F resonances are observed for each sample and could be assigned using the W41Y mutant, for both folded and urea-unfolded TTR (Fig. 5). The perturbation caused by the W41Y mutation is minimal; chemical shift changes in the ^1H , ^{15}N -heteronuclear single quantum coherence (HSQC) spectra are small and are localized to residues in the nearby BC loop and its immediate environment (Fig. S4). No changes in the ^{19}F chemical shifts for W79 were observed between the spectra of the

W41Y and WT-5FW and WT-6FW samples (Fig. 5). Diffusion-ordered NMR experiments with ^{15}N -labeled WT-5FW and WT-6FW at 100 μM concentration showed that both are present as the tetramer (Table S3). The relatively broad ^{19}F resonances of the folded proteins are consistent with tetramer formation, and the observation of only two peaks in the ^{19}F NMR spectra indicates that the structure is symmetric in solution.

Literature reports^{58, 59} indicate that a downfield shift usually signifies a more buried ^{19}F probe. Figure 5B shows that both peaks in the WT-6FW spectrum are downfield shifted (6F-W41: -118.3 ppm, 6F-W79: -117.8 ppm) relative to free 6-fluorotryptophan (-121.6 ppm, shown as a reference in Fig. 5A). By contrast, the two peaks in the WT-5FW spectrum (Fig. 5A) move in opposite directions from free 5-fluorotryptophan (-124.8 ppm, shown as a reference in Fig. 5B): the 5F-W41 resonance moves downfield (-119.0 ppm) and the 5F-W79 peak shifts slightly upfield (-125.3 ppm). These chemical shifts can be rationalized based on the local environments of the Trp residues in the TTR tetramer: the downfield chemical shift changes for 5F-W41 and both peaks in WT-6FW are due to the burial of the ^{19}F -probes in these locations (Fig. 3A, C, D), whereas the upfield shift of the 5F-W79 resonance is due to the fact that although the indole of W79 is almost completely buried, the 5-position is solvent accessible (Fig. 3B). Our ^{19}F -NMR measurements clearly reveal atomic-level detail on the local environment of the Trp residues in TTR. As a control, ^{19}F -NMR spectra recorded with and without addition of the reference compound (5FW and 6FW) showed identical chemical shifts (Fig. S5).

When TTR is unfolded by urea, both peaks in WT-6FW and the 5F-W41 peak in WT-5FW move upfield by 4–5 ppm, indicating greater solvent accessibility. The 5F-W79 chemical shift difference between folded and unfolded states is only 0.5 ppm, confirming its high solvent accessibility in the folded state (Fig. 5). For both 5FW and 6FW probes, the ^{19}F chemical shifts of W79 and W41 differ only slightly (by 0.1 ppm) in the spectra of the unfolded protein.

^{19}F relaxation in the TTR tetramer

Measurement of the T_1 time constants of the ^{19}F resonances gives insights into the oligomeric state. Measured T_1 time constants and ^{19}F linewidths for the 5FW and 6FW probes in both folded and unfolded TTR are shown in Table S2. For folded tetrameric TTR, the predicted T_1 for 5FW or 6FW under isotropic tumbling conditions is 1.2–1.4 s, which is consistent with the experimental results (Table S2). The predicted line width (~ 70 Hz) in the absence of chemical exchange, however, is less than that observed (>100 Hz). Similar findings have been reported for the cyan fluorescent protein.¹⁹

The ^{19}F - T_1 values for unfolded TTR in the presence of 6 M urea decrease to ~ 0.7 s at 14.1 T and the linewidths are much narrower (~ 30 Hz, Table S2) than those of folded TTR for both 5FW and 6FW probes, consistent with a decrease in the rotational correlation time after unfolding. T_1 for the unfolded monomer is predicted to be 0.6–0.7 s at 14.1 T and 298 K with a line width around 30 Hz, in agreement with the experimental results (Table S2). The measured T_1 values, together with the diffusion coefficients shown in Table S3, are consistent with the presence of tetrameric TTR in the folded state and monomer in the unfolded state.

Urea-induced unfolding monitored by ^{19}F -Trp probes at two sites

NMR measurements are normally too slow to measure protein folding and unfolding transitions. However, tetrameric TTR is unfolded by urea on a slow ($\sim$ hours) time scale, which makes this system ideal for monitoring the unfolding reaction by real-time NMR. Tetrameric WT-5FW and WT-6FW TTR were unfolded by the addition of urea, and the unfolding kinetics were monitored by measuring the changes in ^{19}F peak areas belonging to the folded and unfolded protein as a function of time (Fig. 6A, B). The data were fitted to single exponential functions (Fig. 6C, D). Corrections were made for the $\sim$ 5–15 min dead time associated with pipetting, matching/tuning and shimming the probe. The incubation time with urea was limited to 4 days as previously reported for TTR.¹⁵ At a time resolution of 1 hr, the two Trp residues (41 and 79) report the same unfolding rates ($\sim$ 0.02 hr^{-1} for WT-5FW and $\sim$ 0.14 hr^{-1} for WT-6FW). The reported unfolding kinetics of WT-TTR by 6 M urea at 298K measured by Trp fluorescence is 0.031 hr^{-1} ,¹⁴ which is slightly greater than that for WT-5FW and smaller than that for WT-6FW. This ranking is fully consistent with the relative aggregation propensity for these three constructs (Fig. 4).

Structural perturbations probed by backbone amide chemical shifts

The different behavior of WT-5FW and WT-6FW proteins was further analyzed by measuring the backbone amide chemical shift perturbation (CSP) between the fluoro-Trp substituted proteins and WT-TTR using ^1H - ^{15}N HSQC (Fig. S6). A plot of the CSP according to the amino acid sequence is shown in Figure 7A and B. The residues that display significant CSP (greater than one or two standard deviations) are mapped on the modeled structures of WT-5FW and WT-6FW in Figure 8A and B. The chemical shift perturbations caused by 5F-W41 and 5F-W79 substitution are localized to the immediate environment of the tryptophans, especially W41. In contrast, the chemical shift perturbations caused by 6FW substitution of both tryptophans are more widely spread through the protein structure, with large changes in chemical shift for residues near W79. Considering that the mutation of W79, not W41, destabilizes the tetrameric TTR,⁹ we hypothesized that the 6F-W79 substitution is responsible for the more extensive structural perturbations in WT-6FW. The W41Y mutant was used to test this hypothesis. Comparison of the ^1H - ^{15}N HSQC spectra of W41Y and W41Y-6F-W79 shows that the 6-fluoro atom on W79 causes large chemical shift perturbations for residues in the AB loop, EF loop, and H strand (Fig. 7C, 8C,D, Fig. S4). Labeling W41Y with 6FW rendered it more aggregation-prone (Fig. S7), suggesting a correlation between structural changes in these regions and TTR aggregation propensity.

Discussion

Much of our information on the stability and folding of TTR comes from intrinsic Trp fluorescence measurements in the absence and presence of denaturants.^{14, 60} In this work, we complement the Trp fluorescence measurements with ^{19}F -NMR by using two commercially available fluoro-Trp probes (5FW and 6FW). The urea denaturation of TTR tetramer over hours or days to yield unfolded TTR monomer can be readily followed by monitoring the ^{19}F -NMR signals in real time. Since the unfolding of the TTR monomer is 5–6 orders of magnitude faster than the TTR tetramer,^{14, 15, 61} no unfolding intermediate

could be identified in the ^{19}F -NMR unfolding assays under our experimental conditions. By comparing ^{19}F -NMR urea unfolding experiments and turbidity aggregation assays, we were able to show that the ^{19}F -labels can reliably report on the relative kinetic stabilities of TTR variants. In addition, ^{19}F -NMR offers an advantage by providing site-specific information on structure and stability. Two well-resolved ^{19}F resonances were observed for W41 and W79 using either the 5FW or 6FW probe with distinct site-dependent solvent accessibilities. By contrast, such residue-specific information is absent in Trp fluorescence measurements. The intrinsic Trp emission is very broad at room temperature in aqueous solutions, which, without mutagenesis, prevents deconvolution of each contributing Trp spectrum.¹⁶ For ^{19}F -NMR with fluorinated Trp, not only are the resonances of the individual Trp residues in folded TTR well separated, but they are also dispersed in the unfolded state, allowing direct quantification of the relative populations of the unfolded and folded states, without the need for small-molecule probes such as resveratrol¹⁴ or stilbene vinyl sulfonamide.⁶² The ^{19}F -probes at residues 41 and 79 report nearly identical unfolding kinetics, indicating cooperative global unfolding of the TTR tetramer at the time resolution of 1hr reported in this work.

^{19}F -NMR can also probe position-dependent structural properties for each Trp site in TTR. For W41, the ~3–6ppm downfield shift of the ^{19}F resonances relative to the corresponding free compounds suggests that both the 5- and 6- positions of the indole are buried within the structure of the TTR tetramer. On the other hand, the 5- and 6-positions on the indole at residue 79 have very different local environments within the TTR tetramer that result in distinct chemical shift changes relative to free 5FW and 6FW (Fig. 5): although the bulk of the W79 side chain is buried in both proteins, the fluorine atom at the 6-position is buried whereas that in the 5-position is solvent-exposed (Fig. 3B, D). By comparison, the fluorescence emission spectra are nearly indistinguishable for W41Y-5FW/W41Y-6FW and WT-5FW/WT-6FW (compare Fig. 2C with Fig. 2D). This is because the dipole moment is an electronic property of the whole indole π -electron system; therefore the position-dependent solvent accessibility at 5- or 6-position on the substituted indole does not play a critical role in differentiating the corresponding emission spectra in TTR, but such solvent accessibility or chemical environmental difference among ^{19}F nuclei is clearly distinguishable by ^{19}F -NMR.

Interestingly, we noted an unexpected stability difference between WT-5FW and WT-6FW TTR, in which both Trp residues were substituted. As shown by turbidity and ^{19}F -NMR unfolding assays, the WT-5FW tetramer is kinetically more stable than WT-6FW. The increased aggregation propensity appears to be correlated with fluorotryptophan incorporation at W79. Previous studies have shown that in contrast to the W41F mutation, which has little effect on tetramer stability, the W79F mutation greatly destabilizes the TTR tetramer.⁹ This is consistent with the aggregation propensities measured here; W41Y TTR exhibits similar aggregation properties to WT TTR, whereas substitution of 6F-Trp at position 79 of the W41Y mutant greatly increases its aggregation propensity (Fig. S7). Notably, the effects of fluorotryptophan substitution on TTR aggregation depend on the site of fluorine incorporation in the W79 indole ring, with 6F-W79 promoting aggregation while 5F-W79 does not. We suggest that structural rearrangements required to accommodate 6F-

W79 may be linked to the reduced kinetic stability in WT-6FW, whereas the 5F-W79 substitution can be more readily accommodated since it is solvent-exposed (Fig. 3B).

The structural perturbations caused by the fluorotryptophan substitutions were probed using ^1H - ^{15}N HSQC spectra. The 5F and 6F substitutions on W41 appear to cause only localized structural perturbations in the immediate vicinity of the tryptophan ring. The changes in the ^1H - ^{15}N HSQC spectra caused by the W41Y mutation and the 5F-W41 substitution are similar and are localized to residues located in the BC loop or in contact with it (Fig. 7A, Fig. S4). Turbidity assays show that the localized structural perturbations detected by NMR do not lead to tetramer destabilization or enhanced aggregation (Fig. 4, Fig. S7).

To probe the structural perturbations caused by 6F-W79 substitution, chemical shift differences between the ^1H - ^{15}N HSQC spectra of the W41Y mutant and W41Y-6F-W79 were evaluated (Fig. 7C, Fig. 8C,D). Large chemical shift perturbations are observed for residues in the AB loop, H88, and S117, with smaller changes in the EF region near W79. Similar shift perturbations are observed in the HSQC spectrum of WT-6FW (where both W41 and W79 are substituted by 6FW) relative to that of the WT protein, with additional shift changes for residues located close to W41. The chemical shift changes in the AB loop, which forms part of the weak dimer interface, most likely reflect subtle structural perturbations that arise from steric clash between the 6-fluoro atom on W79 and the side chains of D18 and R21 (Fig. 9A). The perturbations appear to propagate from the AB loop across the DAGH β -sheet to S117 in the H strand, which forms part of the strong dimer interface (Fig. 9B).^{63, 64} Alternatively, the 6F atom could potentially hydrogen bond to V20NH based on their proximity in the modeled structure (Fig. 9A). However, this mechanism is less likely given that fluorine is a very weak hydrogen bond acceptor.⁶⁵ These structural perturbations associated with 6-fluoro substitution at W79 are more extensive than those arising from 5FW labeling, and led to destabilization of the TTR tetramer and enhanced aggregation.

The structural perturbations in the AB loop caused by substitution of W79 by 6F-W79 are likely to be quite subtle, given that the radius of the fluorine atom is only 0.3 Å larger than the hydrogen it replaces. Nevertheless, the changes are sufficient to perturb the subunit packing across the weak dimer interface, destabilize the tetramer, and increase the propensity for aggregation. It is noteworthy that many of the chemical shift perturbations observed for WT-6FW involve residues that are linked to TTR pathogenic mutations. The AB loop, which forms part of the weak dimer interface, contains pathogenic variants of D18G, A19D, V20I, S23N, P24S A25T and V28M.^{2, 66} Among these mutants, D18G and A25T are highly unstable and aggregation-prone.^{60, 61} The observed chemical shift changes also suggest that the AB loop perturbations may propagate across the DAGH β -sheet to A109, L110 and V71, and thence to S117 in the strong dimer interface (Fig. 8B, C, D). Alternatively, perturbations of the AB loop could propagate to H88. The 6F-W79 substitution also appears to perturb a water-bridged hydrogen bond network involving H88^{63, 64} (Fig. 9B), which has been suggested to play a role in stabilizing tetrameric TTR and may serve as a structural basis for pH-dependent conformational changes.^{63, 67} Notably, four of these perturbed distal sites (V71, H88, A109 and S117) are associated with

pathogenic TTR variants.^{68–70} In addition, mutations of residues L111, S112, P113, and Y114 in the GH loop, which packs against the W79 indole ring and contacts the AB loop to form part of the weak dimer interface, are also pathogenic.^{2, 71}

In summary, we have introduced two convenient ¹⁹F probes of 5FW and 6FW at two distinct sites (W41 and W79) in the TTR tetramer. ¹⁹F-NMR affords the direct observation of residue-level and position-resolved magnetization signals in both folded and unfolded states of TTR, and reveals urea-mediated unfolding kinetics that is well correlated with the acid-mediated aggregation. We anticipate that the ¹⁹F-NMR approaches reported here will be generally applicable to assorted TTR pathogenic mutants to provide valuable and quantitative insights into their relative stabilities and to probe the fibrillization process, where the site-dependent changes are expected to be critical.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

TTR structures. (A) Overlaid X-ray structure of WT-TTR (gray, PDB: 1BMZ)³⁹ and modeled structures of WT-5FW (red) and WT-6FW (cyan). The heavy atoms of residues 41 and 79 are shown by green and orange spheres, respectively. (B) Structure of a TTR monomeric unit (PDB: 1BMZ) showing the topology of the two four-stranded β -sheets (CBEF and DAGH). (C) The chemical structures of 5FW and 6FW with the indole ring numbering indicated on 5FW.

Figure 2.

UV absorbance and fluorescence spectroscopic characterizations of unlabeled and labeled TTR variants. (A, B) Comparison of normalized absorbance spectra of 5FW-labeled (A) and 6FW-labeled TTR (B). The absorbance side band with fluorinated Trp is labeled with arrows. (C, D) Comparison of normalized tryptophan fluorescence emission spectra in various TTR variants with 5FW-labeled TTR in (C) and 6FW-labeled TTR in (D). Labeling of the solvent-exposed W41 with either 5FW or 6FW shows a red-shifted emission (~ 15 nm) indicated with arrows, which is due to larger solvation stabilization of 1L_a transition in substituted Trp. Inset in C: the two electronic transitions (1L_a and 1L_b) are indicated with arrows on the structure of indole, adapted from Lakowicz.¹⁶

Figure 3. Surface close-up of the two 5FW (A, B) and 6FW (C, D) residues in WT-5FW and WT-6FW, derived from the modeled structures shown in Figure 1. The indole side chain at W41 (green spheres) is partially solvent accessible with 5- (red, A) fully buried and 6-position (red, C) partially buried. In the case of W79 (orange spheres), most of the indole, including the 6-position (red, D) is structurally buried except for the substituted fluorine atom on the 5-position, which is solvent exposed (red, B).

Figure 4. Aggregation kinetics of TTR variants monitored by optical density at 330 nm (OD₃₃₀) at 310 K. WT-6FW aggregates faster than WT-5FW. The error bars represent one standard deviation from three independent measurements.

Figure 5.

^{19}F -NMR spectra of WT-5FW and W41Y-5FW in NMR buffer and 6 M urea (A), and WT-6FW and W41Y-6FW in NMR buffer and 6 M urea (B). A trace amount of free 6FW and 5FW was used as an internal standard for referencing in (A) and (B), respectively. The intensity is normalized to the highest peak. Base lines are offset for clarity. As a control, identical ^{19}F chemical shifts were observed with and without addition of the reference compound (5FW and 6FW, Fig. S5).

Figure 6.

Time-dependent changes in ^{19}F peak intensity during unfolding of WT-5FW (A) and WT-6FW (B) in 6 M urea. Each spectrum was recorded for one hour and the spectra are displayed in chronological order colored from blue to red. (C, D). Unfolding kinetics quantified by peak areas for unfolded W41 and W79 resonances. C. WT-5FW. Open red circles: 5F-W41 (unfolding rate $0.02 \pm 0.001 \text{ hr}^{-1}$); filled red circles: 5F-W79 (unfolding rate $0.02 \pm 0.001 \text{ hr}^{-1}$); black crosses: 6FW internal standard. D. WT-6FW. Open blue circles: 6F-W41 (unfolding rate $0.14 \pm 0.004 \text{ hr}^{-1}$); filled blue circles: 6F-W79 (unfolding rate $0.14 \pm 0.004 \text{ hr}^{-1}$); black crosses: 5FW internal standard. Single exponential fits are shown in black. In both labeled samples, the unfolding rates reported by W41 and W79 are indistinguishable. Uncertainties calculated as one standard deviation from 50 bootstrapped datasets. For reference, the peak areas for the internal standards remain constant during the measurements.

Figure 7.

Backbone amide chemical shift comparisons of WT-5FW and WT-6FW. (A, B) Weighted average backbone amide CSP comparison for WT-5FW (A) and WT-6FW (B) with WT. (C) The CSP comparison for W41Y-6FW with W41Y. The dashed lines denote one or two standard deviations. Letter a denotes ambiguous CSP, x for missing peaks and p for proline residues. Two Trp residues at 41 and 79 and secondary structures are labeled on top of figure.

Figure 8.

CSP perturbations mapped on TTR structure. Residues with weighted average ^1H , ^{15}N CSP larger than one or two standard deviations (σ , from Figure 7) close to W79 (magenta $> 2\sigma$; pink $> 1\sigma$, A, B, C) and or to W41 (deep teal $> 2\sigma$; pale cyan $> 1\sigma$, A, B) and are mapped onto the modeled 1BMZ-based structures of WT-5FW (A) and WT-6FW (B, C) in Ca sphere presentation, respectively. TTR tetramer is colored in light gray and residues 41 and 79 are shown as green and orange spheres, respectively. The fluorine atom is colored in red at 5- in (A) and 6-position in (B, C), respectively. (D) Close-up of the central hydrophobic cavity in (C) with perturbed residues by the 6F-W79 substituent.

Figure 9.

Close-up of structural perturbations caused by fluoro-Trp labeling. (A) Possible perturbations of V20NH and side chains of D18 and R21 by 6F-W79 in the modeled WT-6FW structure. (B) Water-mediated hydrogen bond network around W79 and H88. The ND1 atom in the side chain of H88 is connected to the ϵ amide in the indole ring of W79 via two waters. The NE2 in H88 also forms a water-mediated hydrogen bond with the OG1 in T118 from a neighboring protomer across the strong dimer interface (PDB ID: 3U2J from Yokoyama et al.⁶³). S117 next to T118 is perturbed by 6F-W79.