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Tryptophan Probes of TDP-43 C-Terminal Domain Amyloid Formation

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Abstract

Aggregated TAR DNA-binding protein 43 (TDP-43) forms the cytoplasmic hallmarks associated with patients suffering from amyotrophic lateral sclerosis and frontotemporal lobar degeneration with ubiquitin. Under normal conditions, TDP-43 is a 414-amino acid protein; however, aggregates are enriched with N-terminal truncations which contain residues 267–414, known as the C-terminal domain of TDP-43 (TDP-43_{CTD}). To gain residue-specific information on the aggregation process of TDP-43_{CTD}, we created three single-Trp containing mutants (W385F/W412F, W334F/W412F, and W334F/W385F) by substituting two of the three native Trp residues with Phe, yielding fluorescent probes at W334, W385, and W412, respectively. Aggregation kinetics, secondary structure, and fibril morphology were compared to the wild-type protein using thioflavin-T fluorescence, Raman spectroscopy, and transmission electron microscopy, respectively. While only W334 is determined to be in the proteinase-K resistant core, all three sites are sensitive reporters of aggregation, revealing site-specific differences. Interestingly, W334 exhibited unusual multistep Trp kinetics, pinpointing a distinctive role for W334 and its nearby region during aggregation. This behavior is retained even upon seeding, suggesting the observed spectral change is related to fibril growth. This work provides new insights into the aggregation mechanism of TDP-43_{CTD} and exemplifies the advantages of Trp as a site-specific environmentally sensitive fluorescent probe.

Graphical Abstract

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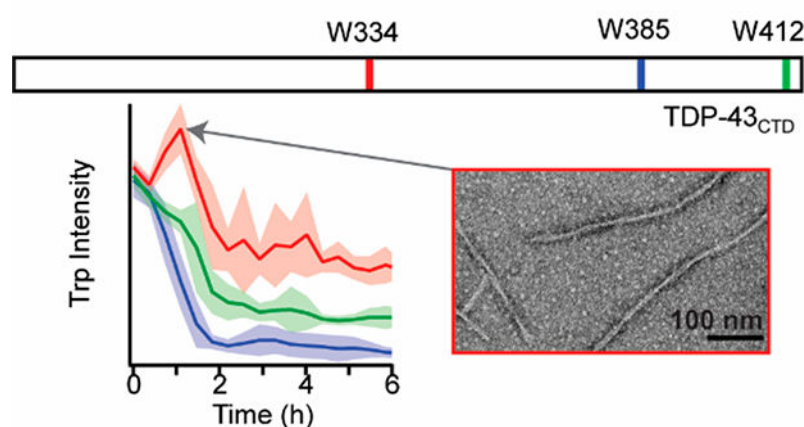
The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jpcb.1c00767>.

Details about protein expression and TEV protease purification, tables of MS analysis of PK digestion and Trp fit parameters, and Figures S1–S9, including protein characterization data as well as additional aggregation, TEM, and Raman data (PDF)



INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that causes motor neuron deterioration and eventual death via respiratory failure, usually within 3–5 years of diagnosis.¹ It is the most common fatal motor neuron disease, affecting about 16 000 Americans every year. Another neurodegenerative disease, frontotemporal lobar degeneration with ubiquitin (FTLD-U), a subgroup of frontotemporal dementia (FTD), is the most common form of early onset dementia and the leading cause of dementia after Alzheimer's disease.² In 2006, transactive response (TAR) DNA-binding protein of 43 kDa (TDP-43) was discovered to be the primary component of the inclusions in both ALS and FTLD-U (now known as FTLD-TDP).³ Since then, several other TDP-43 proteinopathies, such as limbic-predominant age-associated TDP-43 encephalopathy, have been defined.⁴ This information has motivated recent interest in the role TDP-43 aggregation plays in disease etiology.

TDP-43 is a 414-amino acid member of the heterogeneous ribonucleoprotein family⁵ shown to be necessary for embryonic development.⁶ TDP-43 is composed of a globular N-terminal domain (NTD) that allows for reversible dimerization, a nuclear localization signal (NLS), two RNA recognition motifs (RRM1 and RRM2), a nuclear export signal (NES), and an intrinsically disordered C-terminal domain (CTD), rich in glycine and serine residues with a prion-like, glutamine- and asparagine-rich segment (Figure 1a).^{7,8} While TDP-43 is primarily a nuclear protein, it is known to shuttle between the nucleus and cytoplasm during mRNA transport.⁹ Importantly, during cellular stress, TDP-43 protects mRNA by sequestering it in stress granules, which are liquid–liquid phase separated compartments in the cytosol.¹⁰ In ALS and FTLD-U, TDP-43 mislocalizes, becoming depleted in the nucleus and accumulating as insoluble aggregates in the cytosol.³ This phenotype has led to both loss-of-function and toxic gain-of-function hypotheses. Understanding the aggregation process of TDP-43 is important for both disease mechanisms.

TDP-43 found in ALS aggregates is heavily processed.⁷ Notably, it is often phosphorylated, ubiquitinated, and N-terminally truncated into 25- and 35-kDa C-terminal fragments. Although TDP-43 aggregates are generally characterized to be amorphous, a subset is

considered to be amyloid,¹¹ which is a class of highly ordered protein aggregates found in over 30 human diseases.¹² Amyloids have a filamentous morphology with high β -sheet secondary structure content and a characteristic assembly of individual β -strands running perpendicular to the fibril axis, known as cross- β structure.¹³ Aggregation studies of TDP-43 have increasingly focused on the fragment constituting residues 267–414, known as the C-terminal domain of TDP-43 (TDP-43_{CTD}), for three main reasons.^{14–19} First, TDP-43_{CTD} appears to be a key aggregation modulator of the full-length protein.²⁰ Second, the overwhelming majority of disease-related mutations on the TDP-43 gene (*TARDBP*) are missense mutations in the CTD (Figure 1a).⁷ Finally, N-terminally truncated TDP-43 containing the full CTD is heavily enriched in ALS-related aggregates.^{3,21} To date, most research on the TDP-43_{CTD} aggregation *in vitro* has focused on small peptide fragments from the CTD.^{15,22–26} A few groups have studied the aggregation of the whole CTD, though its high aggregation propensity has generally led to the use of a His-tagged protein to enhance solubility.^{17–19,27–29} Furthermore, different experimental conditions have been used to facilitate these studies such as reduced temperature (≤ 25 °C),^{17,27–29} acidic pH,^{18,30} presence of urea as a chemical denaturant,^{29,31} and slow dilution by dialysis.^{19,28} While *in vitro* amyloid formation of TDP-43_{CTD} has been reported,^{27,29,31} this observation has been contested.¹⁹ Although very recently, cryo-electron microscopy (cryo-EM) results strongly support the ability of TDP-43_{CTD} to form amyloid fibrils.³⁰

Here, we have characterized TDP-43_{CTD} amyloid formation by using a tagless-TDP-43_{CTD} protein under physiologically relevant solution conditions at pH 7.4 and 37 °C. To gain residue-specific information during aggregation, we generated three single-Trp-containing mutants (W385F/W412F, W334F/W412F, and W334F/W385F) where two of the three native Trp residues were replaced with Phe, yielding fluorescent probes at W334, W385, and W412, respectively. Hereafter, we will simply refer to these mutants by their single-Trp positions. Because Trp fluorescence is sensitive to local environment,³² it serves as an intrinsic reporter of side chain burial upon protein–protein interactions during aggregation.^{33–35} As the hydrophobicity increases in its local surroundings, there is a shift to higher energy (blue-shift) in the Trp emission maximum (λ_{max}). Moreover, the quantum yield of Trp is responsive to fluorophore mobility, π – π interactions, and nearby charges.³⁶ To corroborate the Trp results, we employed an extrinsic fluorophore, thioflavin-T (ThT), widely regarded as the gold standard of amyloid probes.³⁷ Secondary structure, fibril morphology, and protease-resistance core were examined by Raman spectroscopy, transmission electron microscopy (TEM), and proteinase-K (PK) digestion, respectively.

Our data conclusively show that TDP-43_{CTD} forms amyloid fibrils *in vitro*. All three Trp sites are spectrally sensitive to fibril formation and, thus, are faithful aggregation reporters to varying degrees. Importantly, aggregation kinetics monitored by Trp emission indicated site-specific differences, pinpointing a distinctive role for W334 and its nearby region. The multistep kinetics exhibited by W334 are retained even upon seeding with preformed fibrils, which we interpret as conformational changes at or near W334 are necessary for fibril growth. This work demonstrates the utility of site-specific Trp probes in amyloid formation and reveals new insights into the aggregation mechanism of TDP-43_{CTD}.

MATERIALS AND METHODS

Materials.

Unless otherwise noted, all reagents used were purchased from Sigma-Aldrich.

Recombinant Protein Expression and Purification.

TDP-43_{CTD} plasmid was a gift from Nicolas Fawzi (Addgene plasmid # 98669).¹⁴ The construct consisted of an N-terminal Thio6 expression tag, histidine tag, and TEV cleavage site, followed by residues 267–414. Trp-to-Phe mutations (residues 334, 385, and 412) were done via a QuikChange site directed mutagenesis kit (Agilent) using the following primers: 5'-GCAAGAGCTCCGGTTTTGGCATGTAAC TCG-3' (W412F), 5'-GGT GCGGCAATCGGCTTTGGTAGCGCAAGCAATG-3' (W385F), and 5'-GCGCTGCAGTCTAGCTTTGGTATGATGGGCATG-3' (W334F). WT or single-Trp mutant TDP-43_{CTD} plasmid was transformed into *Escherichia coli* BL21 Star (DE3) (Fisher Scientific) and expressed as previously described.³⁸ Expression was performed in 1.5-L of LB media or in a 20-L BioFlo 4500 fermenter (NHLBI Protein Expression Facility). Detailed protocols can be found in the Supporting Information.

Cell pellets (~4 g) were resuspended in 50 mL of lysis buffer (20 mM Tris, 500 mM NaCl, 10 mM imidazole, 1 mM DTT, 1 mM PMSE, and pH 8.0). Cells were lysed via 3 cycles of 30-s sonication on ice with a 3 mm tapered microtip attached to a Branson Sonifier 450 (50% duty cycle, output control = 5) after which inclusion bodies were pelleted by centrifugation at $18\,000 \times g$ for 30 min at 4 °C. Inclusion bodies were resuspended in denaturing buffer (20 mM Tris, 500 mM NaCl, 10 mM imidazole, 1 mM DTT, 8 M urea, and pH 8.0) and centrifuged again at $18\,000 \times g$ for another 30 min at 4 °C to pellet any remaining insoluble cell debris. Protein was bound to a preequilibrated HisTrap FF 16/10 column (GE Healthcare), and eluted with a linear gradient of imidazole in denaturing buffer (20 mM Tris, 500 mM NaCl, 500 mM imidazole, 1 mM DTT, 8 M urea, and pH 8.0). TDP-43_{CTD}-containing fractions eluted at ~125 mM imidazole were pooled and desalted with a HiPrep 26/60 Desalting Column (GE Healthcare) into TEV-cleavage buffer (20 mM Tris, 750 mM guanidine hydrochloride, pH 8.0). We used a His-tagged TEV protease in a pRK793 plasmid (Gift from David Waugh, Addgene plasmid # 8827)³⁹ expressed in BL21-CodonPlus (DE3)-RIPL (Agilent Technologies, USA) to cleave the His-tag. TEV purification methods can be found in the Supporting Information. TEV protease was added to a final concentration of 30 μ g/mL in the presence of 1 mM DTT. The resulting solution was gently agitated overnight at 4 °C and then incubated with Qiagen Ni-NTA superflow agarose resin (1 mL for 25 mL protein solution) for 1 h with gentle agitation at 4 °C. Resin was removed by several rounds of gentle centrifugation ($1\,000 \times g$) and the supernatant decanted in order to remove the cleaved histidine-tag, histidine-tagged TEV protease, and any uncleaved TDP-43_{CTD}. After cleavage, the protein contains a three residue N-terminal overhang (GHM). The protein was found to be >95% pure as assessed by SDS-PAGE and LC-MS (Figure S1, NHLBI Biochemistry Core). Measured masses were: 14890.55 Da for WT and 14812.45 Da for W334, W385, and W412. Protein was concentrated to ~50–150 μ M, aliquoted, and snap frozen in liquid nitrogen. Protein was stored at –80 °C until use. All buffers were filtered (0.22 μ m).

Aggregation Kinetics.

Stock solutions of TDP-43_{CTD} were thawed on ice and then passed through a 100-kDa Amicon filter (Millipore) to remove any preformed aggregates at 4 °C. Protein concentrations were determined on a Cary 300 Series UV–Vis Spectrometer (Agilent Technologies) using the appropriate molar absorptivity at 280 nm as determined by ProtParam (ExPasy): $\epsilon_{280\text{ nm}} = 17\,990\text{ M}^{-1}\text{ cm}^{-1}$ for WT and $\epsilon_{280\text{ nm}} = 6,990\text{ M}^{-1}\text{ cm}^{-1}$ for W334, W385, and W412. The protein was then diluted (at least 5 fold, final concentrations of guanidinium hydrochloride = 135–180 mM) into 10 mM NaPi, pH 7.4 buffer. The use of desalting columns was not desirable due to large sample loss ($\geq 70\%$). Reactions of 70 μL were then made into black 384-well polypropylene flat bottom plates (Greiner Bio-One) and sealed with a MicroAmp optical adhesive film (Thermo Fisher Scientific). Reactions were monitored using a SPARK Multimode Microplate reader (Tecan) maintained at 37 °C. The samples were allowed to come to temperature for 20 min prior to measurement. Trp and ThT fluorescence measurements were collected with excitation wavelengths of 280 and 415 nm and emission wavelengths of 350 and 480 nm, respectively. Only half of the samples contained ThT (5 μM) to ascertain that the presence of ThT had no effect on Trp kinetics. In quiescent conditions, the microplate was shaken linearly at 6 mm for 5 s in between each read. In shaking conditions, the microplate was continuously shaken linearly at 6 mm. Data shown are from independent experiments made up of at least four technical replicates for each sample condition. At least three independent experiments and at least two biological replicates for each condition were conducted.

Fluorescence Spectroscopy.

Trp and ThT emission spectra were measured using a Fluorolog FL-3 instrument (Horiba Scientific). Trp fluorescence was excited at 280 nm and monitored from 290 to 450 nm. Excitation and emission slits were 1 and 2 nm, respectively. Temperature was maintained at 25 °C using a temperature controlled sample holder and circulating water bath. Buffer backgrounds have been subtracted from all spectra. We observed large variations in Trp intensities of the fibrillar samples, which we attribute to differences in amounts recovered out of the microplate wells.

Trp spectra were fit with eq 1.^{40,41}

$$I(\lambda) = I_0 \exp \left[-\frac{\ln(2)}{\ln^2(\rho)} \ln^2 \left(1 + \frac{(\lambda - \lambda_{\max})(\rho^2 - 1)}{\rho \Gamma} \right) \right] \quad (1)$$

$\lambda_{\max}$ is the wavelength at which maximum intensity is observed, I_0 is the intensity at $\lambda_{\max}$, and Γ is the full width at half-maximum of the peak. The fit parameter ρ describes the asymmetry of the distribution. Fit parameters are reported in Table S2.

Raman Spectroscopy.

Raman spectra were collected using a home-built instrument as previously described.⁴² Samples were concentrated by centrifuging at $11\,000 \times g$ for 30 min, followed by deposition of a 20- μL droplet onto a #1 quartz coverslip (Electron Microscopy Sciences). Representative spectra are shown with each collection constituting 25 accumulations with 5-

s integration time. Data were processed by buffer subtraction using LabSpec 6 software (Horiba Scientific). For comparison, the amide-I peak was normalized using Igor 7.06 (Wavemetrics).

TEM.

TEM images were collected on a JEOL JEM 1200 EXII microscope equipped with an XR-60 digital camera (Advanced Microscopy Techniques) operating at 80 kV (NHLBI Electron Microscopy Core). Grids were prepared by depositing 5 μL of solution onto a 400-mesh copper grid with a Formvar/carbon film (Electron Microscopy Sciences) and allowing it to adhere for 1 min. Excess solution was wicked away using grade 1 Whatman filter paper (GE Healthcare). Grids were washed once with filtered deionized water. Grids were then stained for 10 s using 5 μL of 1% (w/v) uranyl acetate, followed by wicking with filter paper. Finally, grids were dried at RT before collecting images. Fibril widths were calculated manually using the measure tool of the image processing package FIJI (NIH).⁴³ Histograms were fit using the Quick Fit gauss function in Igor 7.06 (Wavemetrics).

RESULTS AND DISCUSSION

TDP-43_{CTD} Amyloid Formation.

When incubated under quiescent conditions at 37 °C, TDP-43_{CTD} aggregation occurs after just a few hours in pH 7.4 solution, as evidenced by ThT fluorescence-monitored kinetics (Figure 1b). A sigmoidal curve is observed for TDP-43_{CTD}, typical for amyloid formation. It is characterized by a lag phase, followed by a rapid growth phase and a plateau when equilibrium is reached. TDP-43_{CTD} amyloid fibril formation was verified via TEM, in which primarily twisted fibrils with widths between 8 and 15 nm were visualized (Figure 1c). In comparison, Trp emission decreases immediately without a distinctive lag phase; however, the time it takes to reach equilibrium is comparable to the ThT kinetics (Figure 1b). Trp spectrum measured postaggregation reveals a blue-shift of approximately 11 nm, suggesting that one or more of the three Trp residues are in a more hydrophobic environment (Figure 1d). Emission intensity is greatly reduced, consistent with the Trp kinetics measurement.

Secondary structural characterization of the TDP-43_{CTD} fibrils was performed by Raman spectroscopy. A sharp and narrow amide-I peak is observed at 1666 cm^{-1} (Figure 1e), characteristic of β -sheet secondary structure as reported for other amyloids such as α -synuclein⁴² and A β ₁₋₄₀.⁴⁴ The formation of β -sheet structure is also indicated by the amide-III peak at 1230 cm^{-1} . To characterize the fibrils biochemically, we subjected TDP-43_{CTD} fibrils to limited proteolysis by PK, a broad spectrum protease commonly used to evaluate amyloid core structure (Figure S2).⁴⁵ While TDP-43_{CTD} aggregates have been shown to be resistant to PK degradation previously,²⁹ the exact residues of the core were not defined. Analysis of liquid chromatography–mass spectrometry data (Table S1) indicates that the fibrils are first attacked at the C-terminus, leaving a 104-residue protease resistant core spanning residues 267–371, which is underlined in Figure 1a. At higher concentrations of PK, a slightly smaller species appears, identified as residues 270–368, indicating that additional cutting can occur at the ends. Notably, of the three Trp residues, only W334 lies within this protected core. Interestingly, W334 has also been shown to be a critical residue

for phase-separation,¹⁶ forming part of transient helix during the process.¹⁴ Moreover, W334 was found to play a role in the aggregation of a TDP-43_{CTD} peptide fragment.²⁶

Conclusively, we show that TDP-43_{CTD} forms amyloid fibrils at neutral pH and physiological temperature. This observation was also verified under shaking conditions. While the aggregation time was greatly reduced by approximately a factor of 6, shaking did not significantly change the amount of fibrils formed or their morphologies (Figure S3). We postulate that when it has been reported that TDP-43_{CTD} forms only non-amyloid aggregates it may be a consequence of specific experimental conditions, such as high protein concentrations ($>100 \mu\text{M}$) that would favor amorphous aggregation over amyloid formation *in vitro*.¹⁹ Importantly, we find that intrinsic Trp fluorescence is a more sensitive probe of TDP-43_{CTD} aggregation compared to that of ThT. The early observation of Trp quenching suggests conformational changes and self-association is occurring during the lag phase where no apparent change is seen in ThT emission.

Amyloid Formation of Single-Trp TDP-43_{CTD} Variants.

In order to delineate the spectral contribution of each of the three tryptophan residues in TDP-43_{CTD} and to gain site-specific information, three single-Trp-containing TDP-43_{CTD} variants were generated by site-directed mutagenesis. In each mutant, all but one of the Trp side chains were substituted with Phe (W385F/W412F, W334F/W412F, W334F/W385F), yielding Trp at positions W334, W385, and W412. All mutants exhibited similar ThT aggregation kinetics to that of the WT (Figure 2a). Here, the aggregation experiments were performed under shaking conditions to facilitate faster acquisition timeframes. All fibrils showed an amide-I peak position at 1668 cm^{-1} (Figure 2b and Figure S4), and no obvious morphological differences were visualized by TEM (Figure 2c). In addition, consistent PK-degradation patterns were obtained for the mutants, suggesting similar core structures of the fibrils (Figure S2 and Table S1). However, we found that the final ThT fluorescence intensities were consistently higher for all three mutants as compared to WT. We determined that this was not due to differences in final fibril amounts, as all proteins aggregated to a similar extent based on SDS-PAGE analysis (Figure S5). Since we have no evidence of different fibril polymorphs as assessed by Raman spectroscopy, TEM, and limited-proteolysis, we speculate that the Phe mutations could be affecting ThT binding and/or rotational constraint as ThT has been shown to bind near aromatic residues.⁴⁶ Nevertheless, our conservative choice of Phe mutations resulted in minimal effects on kinetics and fibril structure. Thus, these variants can be used to reveal residue-specific kinetics and structural information on TDP-43_{CTD} amyloid formation.

Site-Specific Trp Probes of TDP-43_{CTD}.

In the soluble state, all mutants have identical emission spectra with λ_{max} at 347 nm (Table S2), as expected for an intrinsically disordered protein, where all Trp residues are similarly water-exposed (Figure 2d). However, upon amyloid formation, site-specific differences in the local environment of each Trp residue are revealed. While all spectra are blue-shifted, representing the increased hydrophobicity in an aggregated state, each mutant has a distinctive λ_{max} with W385 at $323 \pm 3.2 \text{ nm}$, W334 at $331 \pm 2.3 \text{ nm}$, and W412 at $334 \pm 2.6 \text{ nm}$ ($n \geq 5$). This suggests that W385 is in the most hydrophobic environment, hence the

most buried of the three residues, followed by W334 and W412. The spectral blue-shifts of 24 and 13 nm for W385 and W412, respectively, were unexpected as they were found outside the PK-resistant core (residues 267–371). However, they are in line with recent cryo-EM work from the Surewicz group, which shows that all three Trp residues are resolved in the fibril structure that comprises residues 276–414.³⁰ This is interesting in light of the PK digestion results, suggesting that the C-terminal domain of the amyloid structure is more vulnerable to protease attack with accessible cleavage sites.

W334 Fluorescence Exhibits Multiphasic Kinetics.

Next, TDP-43_{CTD} aggregation kinetics were monitored by W334, W385, and W412 fluorescence (Figure 3a). All three sites exhibited quenching at 350 nm and reported on the process of amyloid formation. Further, the individual Trp signals together account for that observed for the WT protein (Figure 1b), suggesting that they are independent fluorophores. Like WT, W385, and W412 showed obvious sigmoidal curves with no distinctive lag phases. Contrastingly, the trajectory of W334 clearly deviated from the others with the appearance of a new kinetic phase after ~30 min, in which the fluorescence intensity builds to a maximum at 1 h. The subsequent fluorescence decrease is similar to those of the other mutants, reaching saturation after 2 h. This new phase occurs during the elongation phase as measured by ThT fluorescence (Figure 2a).

At the time point when maximum W334 intensity is reached, the microplate reader was stopped to collect the full Trp emission spectrum (Figure 3b, t_{int}). This revealed a blue-shifted spectrum from soluble protein (Figure 3b, t_0) with an average λ_{max} of 338 ± 3.2 nm ($n = 5$), indicating that it is only partially water-exposed, becoming more protected in a nonpolar environment.⁴⁷ The increased intensity could represent a conformation with restricted side chain mobility.⁴⁸ It is also possible that W334 is closer to a positive charge in this intermediate state which could increase its quantum yield.³⁶

The increase of fluorescence intensity at 350 nm persisted under quiescent conditions at varying protein concentrations (5–20 μM , Figure 3c) and is not due to the presence of guanidine hydrochloride (Figure S6). Similar protein concentration dependence experiments were performed for the WT protein (Figure S7). For both W334 and WT, there were no apparent protein concentration effects on aggregation kinetics with no reduction in lag time. This is unusual for amyloid formation; however, this behavior has been reported for other systems such as apolipoprotein C–III and the Pmel17 repeat domain.^{33,34} As noted before, in the absence of agitation, the rate of aggregation significantly decreases, the lag time increases from ~30 min to 3 h as determined by ThT fluorescence (Figure 3d).

W334 Kinetics under Seeding Conditions.

Since the typical amyloid formation process is thought to occur via a nucleation-dependent elongation mechanism,⁴⁹ the observed early transition could reflect the formation of an intermediate (*e.g.*, oligomers) or initial fibril growth processes. To shed light on the nature of this kinetic phase, we examined whether the W334 enhancement would be retained if we accelerated the aggregation by seeding with preformed fibrils. Seeding is a common technique in the amyloid field in which a small amount of preformed fibrils are introduced

to the soluble protein. This provides a template for the soluble protein and the nucleation or lag-phase of aggregation is shortened or abrogated. An aggregation intermediate thus would be bypassed as the protein can directly add to the ends of seeds (i.e., elongation) or surface-catalyzed polymerization can occur (i.e., secondary nucleation⁵⁰).

Upon the addition of preformed fibrils (10% v/v), accelerated aggregation is evident for W334 (Figure 4a and 4b, black curves). Seeding results in the reduction of both lag and growth phases. However, it is noted that the seeds are not particularly potent as the lag phase is not completely abrogated. This seeding behavior was also confirmed for the WT protein, and it did not appear to change fibril morphology (Figure S8). Consistent with ThT, Trp-monitored kinetics are also enhanced (Figure 4a and 4b, red curves). The intermediate phase of W334 still persists in the presence of seeding, though its duration is shortened. Strikingly, in both unseeded and seeded reactions, this Trp intensity increase occurs concomitantly with the ThT growth period (Figure 4a and 4b, yellow area). This suggests that the fluorescence increase is not indicative of an aggregation intermediate, but rather W334 is sensitive to conformational changes occurring during fibril elongation and maturation. The fluorescence spectrum taken at this intermediate time-point (Figure 4c) exhibited the expected blue shift, with a λ_{max} of 342 nm and intensity enhancement. This change could represent residue packing and rearrangement of the amyloid core during fibril growth.

TEM Reveals Fibrillar Structures during the Intermediate Time-point.

The persistence of the W334 spectral intermediate upon seeding indicates that it is reporting on conformational changes during fibril growth and maturation. To provide direct evidence of fibrils at this intermediate time-point (t_{int}), we extracted and examined the seeded W334 sample at 4.5 h (Figure 4b, arrow) by TEM. Indeed, only fibrillar structures were seen (Figure 4d), supportive of our conclusion that W334 is reporting on changes related to fibril growth. We evaluated the WT protein at a comparable time, which also confirmed the sole presence of fibrillar material (Figure S9). To rule out that we are not simply imaging the seeds, we examined the intermediate time-point in the unseeded samples. We turned to shaking conditions to facilitate measurement as the intermediate phase occurs in an hour. Notably, the fibrils taken at t_{int} were significantly thinner than fibrils taken at the end of the aggregation for W334 (Figure 5) and WT (Figure S10). Thus, we refer them to immature vs. mature fibrils. Both rod-like and twisted immature fibrils were present for W334 and WT (Figure S10). For W334, histograms show a center of 4.9 ± 1.0 nm for the immature fibrils taken at 1 h and a center at 9.5 ± 1.3 nm for fibrils taken at the end of the reaction (Figure 5c). For WT, the difference between the two time-points is less pronounced (7.0 ± 1.4 nm vs. 9.1 ± 1.4 nm, Figure S10c). This may point to a difference in side-chain packing between W334 and WT. However, since thinner fibrils are present at the intermediate time-point for both WT and W334, we suggest that the fibril maturation process is occurring in the WT protein as well.

This unique sensitivity of W334 pinpoints that conformational changes in its nearby region play a vital role in amyloid formation. We propose that in early stages of aggregation, W334 is solvent-exposed as the protein is soluble and disordered. As self-association begins to occur, W334 begins to quench, perhaps due to contact with the peptide backbone.³² Then, as

filaments start to form, W334 side-chain mobility is restricted as it becomes partially buried, causing an increase in quantum yield and a blue-shift, respectively. Upon fibril maturation, W334 is fully buried in a parallel in-register conformation³⁰ and its fluorescence is reduced due to π - π stacking. This maturation may be the expansion of the amyloid core to include more residues or simply reflect conformational rearrangements as the proteins seek to adopt the most stable fold as more subunits add during fibril growth.

Our results and conclusion are supported by the literature, where previous work also suggested that residue W334 is involved in the aggregation of a fragment of TDP-43_{CTD}.²⁶ Specifically, it was shown that W334 formed a key interaction with Q343, stabilizing self-association and fibrillation. Of note, the recent fibril structure from the Surewicz group finds W334 at the interface between the expanded N-terminal region of TDP-43_{CTD} and the planar C-terminal region, which could explain as to why this interfacial residue is particularly sensitive to fibril growth.³⁰ However, TDP-43_{CTD} fibril polymorphism may exist, and thus, we cannot be certain that the same structure is formed here under our solution conditions. Nevertheless, these observations support an important role for W334 and its nearby region for aggregation. Furthermore, this residue is also implicated in TDP-43 phase-separation.^{14,16,51} There are also four ALS-related mutations flanking W334 (Figure 1a), highlighting a pathological connection. Moving forward, Trp fluorescence could be a valuable tool to examine the effects of disease mutations in the regions surrounding W334 and W385. The presence of many Phe residues would also allow for the introduction of new Trp locations into TDP-43_{CTD}, such as the N-terminal region, which lies within the PK-resistant core, to explore the other regions of the protein.

CONCLUSION

This work establishes that TDP-43_{CTD} rapidly forms amyloid aggregates at low concentrations (5 to 20 μ M) at physiologically relevant pH and temperature. We show that the introduction of Phe mutations to generate single-Trp containing TDP-43_{CTD} variants did not significantly perturb amyloid formation. All three Trp residues reliably reported on aggregation, including W385 and W412, which lie outside the PK-core. Moreover, Trp fluorescence proved to be a more sensitive reporter of amyloid formation compared that to the commonly used ThT probe and offered site-specific spectral responses. Notably, W334 exhibited unique kinetics with an apparent intermediate phase of fluorescence intensity increase, which we attribute to conformational changes experienced during fibril elongation and maturation based on the fact that it could not be bypassed by seeding and the presence of thin filaments at that time. Collectively, our results reveal a distinctive role for W334 and its nearby region for fibril growth, offering new molecular insights into TDP-43_{CTD} amyloid formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

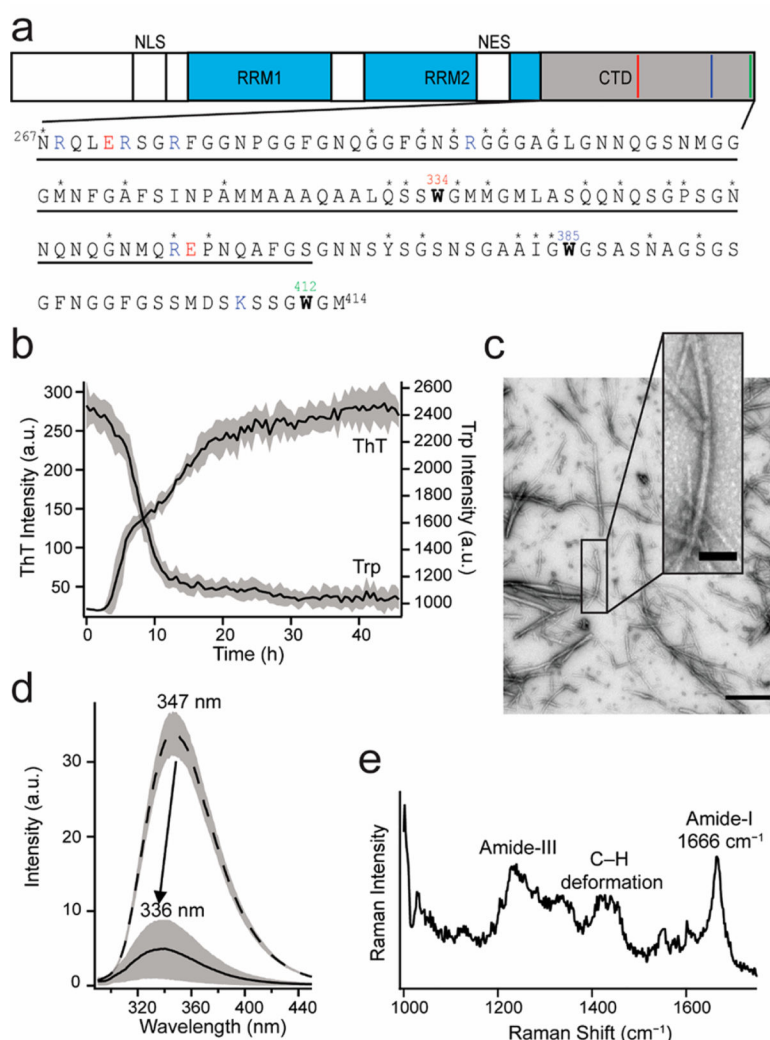
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**Figure 1.**

TDP-43_{CTD} forms amyloid fibrils at physiological pH and temperature. (a) Schematic representation of the full length TDP-43, showing the locations of different domains: nuclear localization sequence (NLS), RNA recognition motifs (RRM1 and RRM2), nuclear export sequence (NES) and C-terminal domain (CTD). The primary sequence of TDP-43_{CTD} is shown with acidic and basic residues colored red and blue, respectively. Trp sites (W334, W385, and W412) used in this study are indicated. ALS disease-related mutation locations are marked by asterisks. Underlined region corresponds to the PK-resistant core determined by LC-MS (Table S1). (b) Aggregation kinetics of WT TDP-43_{CTD} monitored by ThT and Trp fluorescence ([TDP-43_{CTD}] = 10 μ M, [ThT] = 5 μ M in 10 mM NaPi, 145 mM GuHCl, pH 7.4, and quiescent conditions at 37 °C). Lines and shading represent the mean and standard deviation, respectively (n = 5). (c) TEM image of WT TDP-43_{CTD} fibrils. Scale bar indicates 500 nm. An expanded view is also shown (scale bar is 100 nm). (d) Trp fluorescence before (dashed line) and after (solid line) WT TDP-43_{CTD} aggregation. Lines and shading represent the mean and standard deviation, respectively (n = 5). (e) Raman

spectrum of WT TDP-43_{CTD} fibrils. Peaks attributed to the amide-I band (1666 cm^{-1}), amide-III, and C–H deformation are indicated.

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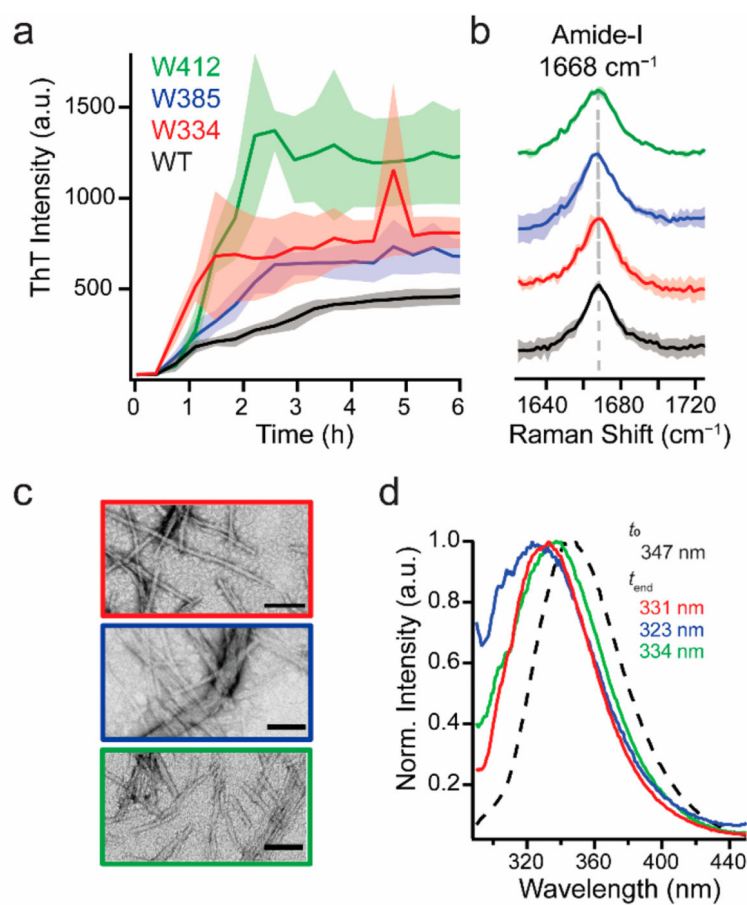


Figure 2.

Characterization of single-Trp mutants of TDP-43_{CTD}. (a) Comparison of aggregation kinetics of W334 (red), W385 (blue), W412 (green), and WT (black) TDP-43_{CTD} monitored by ThT fluorescence ([protein] = 10 μ M, [ThT] = 5 μ M in 10 mM NaPi, 160 mM GuHCl, and pH 7.4 under shaking conditions at 37 °C). Lines and shading represent the mean and standard deviation, respectively ($n \geq 4$). (b) Comparison of amide-I peaks of W334 (red), W385 (blue), W412 (green), and WT (black) fibrils. Spectra are normalized and offset for clarity. Dashed line indicates 1668 cm⁻¹. Lines and shading represent the mean and standard deviation, respectively ($n = 3$). Full spectra are shown in Figure S4. (c) Representative TEM images of W334 (red), W385 (blue), and W412 (green) fibrils. Scale bars are 100 nm. (d) Average Trp fluorescence spectra measured after aggregation (t_{end}) of W334 (red), W385 (blue), and W412 (green) ($n \geq 5$). Spectra are normalized to their λ_{max} . For comparison, only one representative spectrum taken before aggregation (dashed black line, t_0) is shown for clarity because all three mutant spectra overlay. λ_{max} values are also indicated.

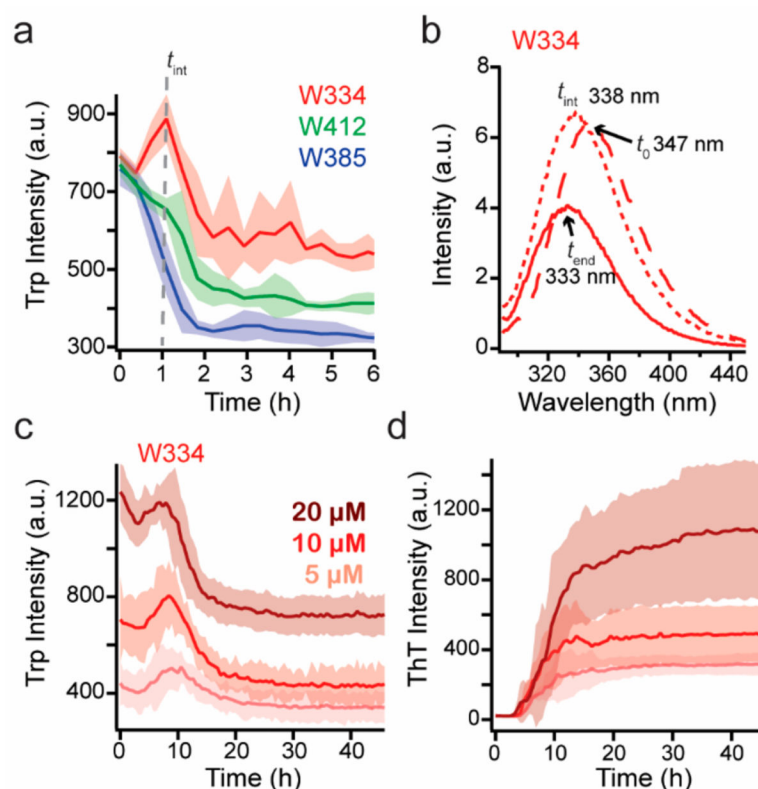


Figure 3.

W334 fluorescence reveals distinctive aggregation kinetics. (a) Comparison of aggregation kinetics monitored by W334 (red), W385 (blue), and W412 (green) fluorescence ([protein] = 10 μ M in 10 mM NaPi, 160 mM GuHCl, and pH 7.4 under shaking conditions at 37 °C).

Lines and shading represent the mean and standard deviation, respectively ($n = 10$). Dashed line indicates when samples were removed for fluorescence measurement at the intermediate time point. (b) Corresponding W334 spectrum taken at intermediate time point (dotted line, t_{int}) compared to before (dashed line, t_0) and after (solid line, t_{end}) aggregation. λ_{max} values are also indicated. Comparison of (c) Trp and (d) ThT aggregation kinetics of W334 TDP-43_{CTD} at 5, 10, and 20 μ M (10 mM NaPi, 180 mM GuHCl, and pH 7.4 at 37 °C, under quiescent conditions). Lines and shading represent the mean and standard deviation ($n \geq 5$).

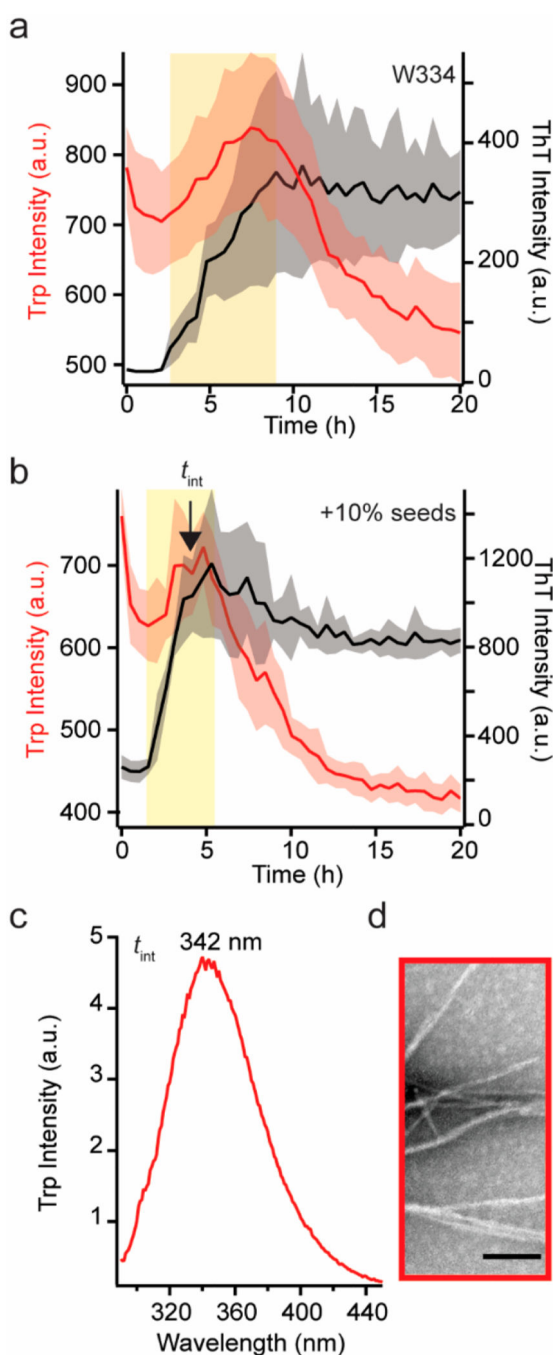


Figure 4.

Spectroscopic intermediate present after seeding. Comparison of ThT (black) and Trp (red) aggregation kinetics of W334 TDP-43_{CTD} in the (a) absence and (b) presence of 10% v/v preformed W334 seeds ([protein] = 10 μ M, in 10 mM NaPi, 180 mM GuHCl, and pH 7.4 at 37 $^{\circ}$ C, under quiescent conditions). Lines and shading represent the mean and standard deviation ($n \geq 5$). An arrow indicates the time point when samples were removed for fluorescence and TEM measurements (t_{int}). Yellow areas indicate ThT growth periods.

Corresponding (c) W334 spectrum and (d) TEM taken at t_{int} . λ_{max} value is indicated. Scale bar is 100 nm.

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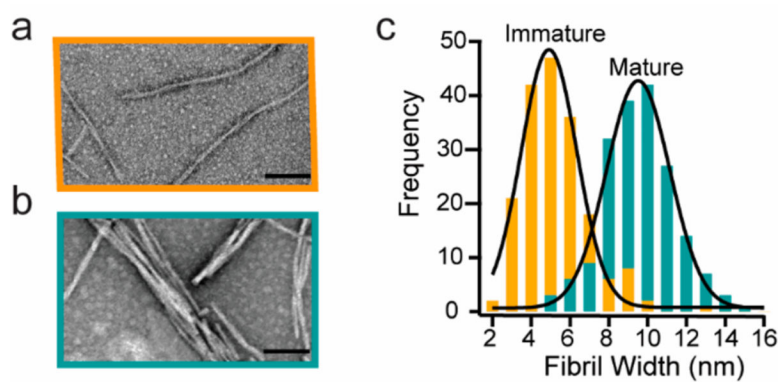


Figure 5. Thin fibrillar structures are found at intermediate time-point. TEM images of W334 TDP-43_{CTD} at (a) ~1 h of incubation (orange, immature) and (b) ≥24 h of incubation (teal, mature) ([protein] = 10 μ M, in 10 mM NaPi, and pH 7.4 at 37 °C, under shaking conditions). Scale bars are 100 nm. (c) Histograms of immature (orange) and mature (teal) fibril width (n = 185). Gaussian fits are shown as black lines.