Amyloid Formation by Human Carboxypeptidase D Transthyretin-like Domain under Physiological Conditions

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Javier Garcia-Pardo‡§, Ricardo Graña-Montes‡§, Marc Fernandez-Mendez‡§, Angels Ruyra†, Nerea Roher‡§, Francesc X. Aviles‡§, Julia Lorenzo‡§, and Salvador Ventura‡§

From the ‡Institut de Biotechnologia i Biomedicina, Departaments de §Bioquimica i Biologia Molecular and ¶Biologia Cel·lular, Immunologia i Fisiologia Animal, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

Background: Proteins can form amyloid aggregates from initially folded states.

Results: The transthyretin-like domain of human carboxypeptidase D forms amyloid aggregates without extensive unfolding.

Conclusion: The monomeric transthyretin fold has an inherent propensity to aggregate due to the presence of preformed amyloidogenic structural elements.

Significance: Generic aggregation from initially folded states would have a huge impact on cell proteostasis.

Protein aggregation is linked to a growing list of diseases, but it is also an intrinsic property of polypeptides, because the formation of functional globular proteins comes at the expense of an inherent aggregation propensity. Certain proteins can access aggregation-prone states from native-like conformations without the need to cross the energy barrier for unfolding. This is the case of transthyretin (TTR), a homotetrameric protein whose dissociation into its monomers initiates the aggregation cascade. Domains with structural homology to TTR exist in a number of proteins, including the M14B subfamily carboxypeptidases. We show here that the monomeric transthyretin-like domain of human carboxypeptidase D aggregates under close to physiological conditions into amyloid structures, with the population of folded but aggregation-prone states being controlled by the conformational stability of the domain. We thus confirm that the TTR fold keeps a generic residual aggregation propensity upon folding, resulting from the presence of preformed amyloidogenic β-strands in the native state. These structural elements should serve for functional/structural purposes, because they have not been purged out by evolution, but at the same time they put proteins like carboxypeptidase D at risk of aggregation in biological environments and thus can potentially lead to deposition diseases.

Carboxypeptidases (CPs) perform many diverse functions in the body by removing amino acids from the C termini of proteins and peptides. The following four subfamilies of CPs can be defined based on their sequential and structural homology: M14A, M14B, M14C, and M14D (1, 2). Among them, the M14B subfamily is composed of five catalytically active members that display a stringent specificity for cleaving C-terminal basic residues only. The other members of this subfamily are inactive, lacking essential catalytic residues (3, 4). All the members of this subfamily share a common structural architecture composed of a CP domain followed by a β-sandwich transthyretin-like (TTL) domain (5–9).

Carboxypeptidase D (CPD) is a member of the M14B subfamily that has a broad tissue distribution and functions in the processing of proteins and peptides in the secretory pathway (10). CPD was first discovered as a 180-kDa protein from duck, which binds hepatitis B viral particles (11, 12). Unlike all other members of the CP family, CPD contains three repeats followed by a transmembrane domain and a cytosolic tail. All three repeats contain a CP domain and a TTL domain. The function of these TTL domains is unknown, although it has been proposed that they could be involved in the regulation or oligomerization of the enzyme and/or in membrane binding (3).

TTL domains receive their names due to their structural, but not sequential, similarity to transthyretin (TTR), a transport protein that distributes the two thyroid hormones triiodothyronine and thyroxine, and retinol. TTR is a homotetrameric protein associated with senile systemic amyloidosis (13, 14) and familial amyloid polyneuropathy (15, 16). Dissociation of the TTR tetramer is a prerequisite for the development of these disorders (17–19). TTR tetrameric structure dissociates into dimers, which are unstable in the absence of additional quaternary interactions, explaining why TTR exists in primarily tetramer-monomer equilibrium (20, 21). The monomers constitute the building blocks for amyloid fibril formation (22–25).

TTR illustrates the generic overlap between interfaces and aggregation-prone regions in protein complexes, because many...
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of the interactions promoting the formation of functional complexes, including hydrophobic and electrostatic forces, can potentially favor abnormal intermolecular association as well (26, 27). Avoidance of nonfunctional interactions significantly influences the evolution of the physicochemical properties of proteins (28), and in general, soluble monomeric proteins tend to minimize the presence of hydrophobic, potentially dangerous patches at their surfaces (29). TTR and human TTL share the same fold but differ in their quaternary structure, providing a privileged model system to dissect the determinants of protein solubility from native states.

The conversion of folded proteins into amyloid assemblies generally requires nonphysiological conditions, such as extreme pH values (30, 31), organic co-solvents (32, 33), or high temperatures (34, 35). These destabilizing environments cause proteins to partially or fully unfold leading to the exposure of aggregation-prone regions, which are able to form intermolecular interactions, thus triggering aggregation (36). However, increasing evidence supports the existence of an alternative pathway in which the aggregation of globular proteins into amyloids can depart from conformational states directly accessible from the native state without the requirement of a large unfolding (37, 38). The population of these nearly native or native-like states (N*) accounts for an increased aggregation propensity of the protein leading to formation of amyloid assemblies. They usually correspond to metastable conformers accessible through fluctuations of the native state (37, 39). In these cases, amyloid aggregates are formed without transitions across the major energy barrier for unfolding. To date, only a reduced number of proteins have been shown to form amyloids under conditions that are close to the physiological environment (39–43). Here, we address the conformational and aggregate properties of a TTL domain from the first catalytic domain of human CPD (h-TTL) under close to native conditions and their functional implications, thus providing new insights on the interplay between the establishment of functional and deleterious protein interactions.

**EXPERIMENTAL PROCEDURES**

**Recombinant TTL Expression and Purification**—The transthyretin-like domain belonging to the first catalytic domain of human metallocarboxypeptidase D (residues 386–460), named here as h-TTL, was cloned into the pET-22B vector to encode a C-terminal hexahistidine fusion protein. For protein production, the plasmid was transformed into *Escherichia coli* BL21 (DE3) cells, which were then grown in 1 liter of lysogeny broth (LB) with 50 μg ml⁻¹ ampicillin at 37 °C and 250 rpm to an *A₆₀₀nm* of 0.5 to 0.6. When the cell reached this density, protein expression was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 16 h at 18 °C. Then the culture was centrifuged, and the cell pellet was frozen at −20 °C. After cell lysis by sonication in 1/50th the initial culture volume of 100 mM Tris-HCl, 0.5 mM NaCl buffer at pH 8.0, and the TTL protein was eluted in 3 column volumes of a 50 mM Tris-HCl, 0.15 mM NaCl at pH 8.0 buffer containing 500 mM imidazole. The recombinant protein was further purified on a Superdex 75 HR 10/30 column (GE Healthcare), and the protein buffer was exchanged on a Sephacryl G-25 column (GE Healthcare) to a 20 mM phosphate, 100 mM NaCl buffer at pH 8.0. The purified TTL protein was flash-frozen at −2.2 mg ml⁻¹ and stored at −80 °C.

**Intrinsic Fluorescence**—h-TTL intrinsic fluorescence was registered after equilibration at 25, 37, 42, 45, and 75 °C in a Jasco FP-8200 spectrofluorimeter by measuring Tyr emission spectra between 280 and 400 nm upon excitation at 268 nm. Slit widths were typically 5 nm for excitation and 5 nm for emission, and the spectra were acquired at 0.5-nm intervals, 1000 nm/min⁻¹ rate, and 0.1 s averaging time of 25 μM protein concentrations in 20 mM phosphate, pH 8.0, 100 mM NaCl buffer.

**Secondary Structure Analysis by Circular Dichroism (CD)**—h-TTL far-UV and near-UV CD spectra were recorded between 205 and 250 nm and 250 and 320 nm, respectively, at 25, 37, 42, 45, and 75 °C with a spectral resolution of 0.5 nm, using a Jasco 810 spectropolarimeter. Each spectrum was obtained by accumulating 20 scans of 25 μM protein sample in a 20 mM phosphate, 100 mM NaCl buffer at pH 8.0, in a 0.1 path-length quartz cell.

**Intrinsic Fluorescence Quenching Assays**—Quenching of h-TTL intrinsic fluorescence was analyzed by monitoring Tyr emission in the presence of acrylamide. Tyr fluorescent emission was recorded between 280 and 400 nm upon excitation at 268 nm and after equilibration at 25, 37, 42, 45, and 75 °C using 10 μM protein samples with final quencher concentrations ranging from 0 to 0.25 M in a Jasco FP-8200 spectrofluorimeter. Data were fitted to Equation 1,

\[
I_0 \Gamma = (1 + K_{sv}[Q])e^{-[Q]} \quad (\text{Eq. 1})
\]

where *I₀* and *I* are the fluorescence intensities in the absence and presence of a concentration of quencher [Q], *Kₚ₉* is the Stern-Volmer constant, and *V* is the static quenching constant.

**Thermal and Chemical Denaturation**—h-TTL thermal denaturation was monitored by following the changes in Tyr intrinsic fluorescence at 303 nm upon excitation at 268 nm, in bis-ANS binding at 485 nm upon excitation at 370 nm and in CD ellipticity at 235 nm. Signal change was recorded using a 1 °C/min⁻¹ gradient and protein concentrations ranging from 5 to 25 μM.

Chemical denaturation of h-TTL was followed by monitoring change in Tyr intrinsic fluorescence at 303 nm of 25 μM protein samples at different urea concentrations after equilibration at 25, 37, 42 or 45 °C. Samples fluorescence was recorded in the 280 to 400 nm range after excitation at 268 nm, using a Jasco FP-8200 spectrofluorimeter.

Experimental data of thermal and chemical denaturation were fitted to a two-state unfolding model where the signals of the folded and the unfolded state are linearly dependent on temperature or denaturant concentration, see Equations 2 and 3, respectively, using a nonlinear least squares algorithm provided with KaleidaGraph (Synergy Software).
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was evaluated by adding at \( T = 0 \) 10% (w/w) of amyloid fibrils preformed at 42 °C.

Binding to Amyloid Dyes—Th-T binding to h-TTL protein aggregates was measured by recording Th-T fluorescence using a Jasco FP-8200 spectrofluorimeter, with an excitation wavelength of 445 nm and an emission range between 460 and 600 nm. Spectra of dilutions were registered with a 25 \( \mu \)M final Th-T concentration in the absence or presence of 25 \( \mu \)M h-TTL aggregates at different temperatures in a 20 mM phosphate, 100 mM NaCl buffer at pH 8.0. For optical microscopy analysis, h-TTL protein aggregates were incubated for 1 h in the presence of 25 \( \mu \)M Th-T. After centrifugation at 14,000 \( \times \) g for 5 min, the precipitated fraction was placed on a microscope slide and sealed. Th-T fluorescence images were obtained under UV light with a fluorescence microscope (Leica Microsystems).

Binding of bis-ANS to h-TTL was evaluated by registering bis-ANS fluorescence between 400 and 650 nm after excitation at 370 nm in a Jasco FP-8200 spectrofluorimeter. Spectra were recorded at 25, 37, 42, 45, and 75 °C after diluting native h-TTL in a 20 mM phosphate, 100 mM NaCl buffer at pH 8.0 with bis-ANS. Final protein and dye concentrations were 25 and 2.5 \( \mu \)M, respectively.

FTIR Spectroscopy—Attenuated total reflectance Fourier-transformed infrared spectroscopy (ATR-FTIR) analysis of h-TTL aggregates after 14 days of incubation was performed using a Bruker Tensor 27 FTIR spectrometer (Bruker Optics) with a Golden Gate MKII ATR accessory. Each spectrum was measured at a spectral resolution of 2 cm\(^{-1}\). Infrared spectra between 1725 and 1575 cm\(^{-1}\) were fitted through overlapping Gaussian curves, and the amplitude, mass center, bandwidth at half of the maximum amplitude, and area for each Gaussian function were calculated employing the nonlinear peak-fitting program PeakFit (Systat software).

Transmission Electron Microscopy—Samples of h-TTL incubated at 37 and 42 °C for 14 days were diluted 10-fold and placed onto carbon-coated grids. After 5 min, grids were washed with distilled water and then negatively stained with 2% (w/v) uranyl acetate for 2 min. Micrographs were recorded in a Hitachi H-7000 transmission electron microscope (TEM) operated at 75-kV accelerating voltage.

TTL Aggregation-prone Peptide Preparation—A peptide with the sequence GTYNLT VibrLVTGYM, corresponding to the aggregation-prone region of h-TTL predicted using the WALTZ (47) algorithm, was purchased from EZBioLab, Inc., with a purity of 95.4%. Stock solutions were prepared at 5 mM in 1,1,1,3,3,3-hexafluoro-2-propanol (hexafluoroisopropanol), centrifuged at 15,000 \( \times \) g at 4 °C for 15 min and filtered through millex-GV 0.22-mm filters to remove pre-aggregated species. After removing hexafluoroisopropanol by evaporation, samples were stored at \(-80\) °C. For analysis, the peptide was resuspended in DMSO, further diluted to 100 \( \mu \)M in 20 mM phosphate, 100 mM NaCl buffer at pH 8.0 (with a maximum DMSO concentration of 5%), and finally bath-sonicated for 10 min. Peptide aggregation was carried out without agitation at 25 °C for 72 h, and the amyloid properties of peptide aggregates were analyzed as described above.

Liposome Preparation and Liposome Binding Assays—Liposomes were prepared by the thin film hydration method. Briefly,
1,2-didodecanoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dioleoyl-sn-glycero-3-phosphoric acid monosodium salt (DOPA), and cholesterol were dissolved in chloroform solutions and mixed in a round-bottom flask at the desired molar ratios for DOPA (0.0:0.5:0.5), DOPA/DLPC (0.25:0.25:0.5), and DLPC (0.5:0.0:0.5) liposomes. The organic solvent was removed by rotary evaporation to obtain a dry lipid film that was then hydrated with 20 mM phosphate, 100 mM NaCl buffer at pH 8.0 to give a lipid concentration of 10 mM. Multilamellar liposomes were formed by constant vortexing followed by extrusion in an Extruder (Lipex Biomembranes) through polycarbonate membranes (Avanti Polar Lipids) of variable pore size under nitrogen pressure. Liposomes were extruded in three steps as follows: first through a 0.8-μm pore diameter filter, then through a 0.4-μm filter, and finally through a 0.2-μm filter until the obtention of large unilamellar liposomes. The particle size distribution and potential of the final liposomal formulations were determined by dynamic light scattering at 25 °C using a Zetasizer NanoZS (Malvern Instruments).

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The intrinsic fluorescence of 25 μM h-TTL in 20 mM phosphate, 100 mM NaCl buffer at pH 8.0 was monitored after equilibration at 25 °C in the presence of DOPA, DOPA/DLPC, or DLPC liposomes at 0.25, 0.5, and 1.0 mM final concentration as described above. The aggregation of h-TTL in the presence of DOPA, DOPA/DLPC, and DLPC liposomes was evaluated at a 1 mM liposome concentration. Reactions were incubated at 42 °C for up to 3 days, and h-TTL aggregation was monitored as described above.

RESULTS

STRUCTURAL SIMILITUDE BETWEEN HUMAN TTL AND THE TTR MONOMER—In the crystal structure of TTR, each monomer (A–D) is composed of two four-stranded β-sheets (with a DAGH and CBEF arrangement), which are connected by loops with a short α-helix located between β-strands E and F (Fig. 1) (49, 50). Duck CPD TTL shares topological similarity and connectivity with TTR over 78 Cα atoms (with a root mean square deviation of 2.4 Å) despite their low sequence similarity (18% identity) and TTL lacking strand eight present in TTR. The structure of the duck CPD TTL domain consists of a β-barrel made up by seven strands (β1 to β7) arranged in an antiparallel four-stranded (β3, β2, β5, and β6) and a mixed three-stranded β-sheet (β1, β4, and β7), both folded together enclosing a hydrophobic core (5–7). The same topology was found in the crystal structure of a short splicing variant of Droso phila melanogaster CPD (9). A similar transthyretin-like fold topology has been found in several diverse protein families, including dioxygenases, glutocetransferases, and glucoamylases (51–53). Because the three-dimensional structure of the TTL domain of human CPD is not available yet, we modeled it on top of solved TTL structures using I-TASSER. The resulting model and a structural alignment with human TTR are shown in Fig. 1.
Spectral Properties of Human TTL—We expressed and purified h-TTL. In gel filtration chromatography, the protein elutes as a single peak, with an apparent mass of 8.7 kDa, fairly close to the theoretical and mass spectrometry determined mass of 9.3 kDa, indicating that the domain remains as a monomer in solution (data not shown). We monitored its conformational properties in the native state by far- and near-UV circular dichroism (CD) and intrinsic fluorescence at pH 8.0 and 25 °C at a 25 μM protein concentration. The far-UV CD spectrum of h-TTL exhibits a single minimum at 217 nm consistent with an all-β-sheet structure (Fig. 2A). The spectrum exhibits a maximum at 236 nm (Fig. 2A), which is usually attributed to the contribution of Trp and Tyr side chains because the amide contributions in this region are generally negative. Because h-TTL lacks Trp residues, this signature can be univocally assigned to tyrosine (Tyr) side chains. Accordingly, the near-UV CD spectrum exhibits a single negative band in the 270–280-nm interval (Fig. 2B).

**FIGURE 2. Conformational analysis of h-TTL.** A, far-UV-CD; B, near-UV-CD; C, intrinsic fluorescence; D and E, bis-ANS binding; F, Stern-Volmer plots of the acrylamide quenching of tyrosine intrinsic fluorescence at 25 °C (black), 37 °C (green), 42 °C (orange), 45 °C (red), and 75 °C (purple). D and E, dashed lines represent free bis-ANS emission spectra.
Human TTL contains three Tyr residues at positions 38, 46, and 55. Tyr-38 is exposed to solvent, whereas Tyr-46 and Tyr-55 are buried in the h-TTL structure. We monitored the intrinsic fluorescence of Tyr residues by exciting the protein at 268 nm and recording fluorescence between 280 and 400 nm. The fluorescence spectrum of h-TTL exhibits a characteristic Tyr emission maximum at 303 nm (Fig. 2C).

**Thermal and Chemical Unfolding of Human TTL**—The thermal stability of h-TTL was analyzed by monitoring the changes with temperature of Tyr intrinsic fluorescence at 303 nm, in CD ellipticity at 235 nm, and in bis-ANS extrinsic fluorescence at 485 nm. The analyses were performed in the 15–75 °C range at pH 8.0 and 10 μM protein concentration (Fig. 3, A–C). A single cooperative transition was observed in all cases, and the data could be fitted to a two-state temperature-induced unfolding model (R > 0.99). Melting temperatures (T_m) of 55.0 ± 0.1, 55.2 ± 0.1, and 53.7 ± 0.1 °C were calculated from intrinsic fluorescence, CD, and bis-ANS fluorescence data, respectively, indicating that all the probes report on the same global unfolding process. No detectable protein aggregation occurred during melting in the 5–25 μM concentration range (data not show).

Thermal unfolding data indicate that the protein is essentially folded in the 25–45 °C temperature range. To further confirm this extent, we analyzed the far-UV, near-UV, and intrinsic fluorescence of h-TTL at 25, 37, 42, and 45 °C and compared them with that of the denatured protein at 75 °C. The fluorescence spectra of h-TTL in the 37–45 °C range are identical and slightly less intense than that at 25 °C (Fig. 2C); all them were different from the spectra exhibited by the unfolded protein, where a large increase of the fluorescence maximum was observed, indicating the exposure to solvent of previously protected Tyr residues (Fig. 2C). We monitored the presence of exposed hydrophobic clusters in the structure of h-TTL at these temperatures by measuring their binding to bis-ANS, a dye that increases its fluorescence emission upon interaction with these nonpolar regions. The spectra in the 25–42 °C range were essentially identical, with an increase in fluorescence emission of bis-ANS at 45 °C (Fig. 2D). Still, this change was much lower than the one observed for the unfolded protein at 75 °C (Fig. 2E). To further confirm the native-like conformation of h-TTL in the 25–45 °C range, we recorded the near- and far-UV CD spectra of the protein at 25, 37, 42, and 45 °C at pH 8.0. The spectra obtained at all these temperatures can be superposed in both the near- and far-UV regions and are clearly different from that of the unfolded state at 75 °C (Fig. 2, A and B). Finally, the relative accessibility of Tyr residues was analyzed by acrylamide quenching. Upward curving in Stern-Volmer plots is consistent with h-TTL Tyr residues being located in different environments, with both static and dynamic quenching contributions (Fig. 2F). The Stern-Volmer constant for h-TTL (KSV) at 25 °C is 10.8 ± 0.1 m⁻¹. Partial or total unfolding is usually associated with an increase in KSV values. This effect is observed at 45 °C (KSV = 12.6 ± 0.2 m⁻¹) and
especially at 75 °C ($K_{SV} = 17.5 \pm 0.2 \text{ m}^{-1}$). In contrast, $K_{SV}$ values of 9.9 ± 0.1 and 7.7 ± 0.2 m$^{-1}$ were calculated for the protein at 37 and 42 °C, respectively. Although the $K_{SV}$ constants obtained at different temperatures should be interpreted with caution, quenching experiments suggest that the protein remains in a compact conformation in the 25–42 °C temperature range.

We later analyzed the dependence of the conformational stability of h-TTL on the temperature by monitoring the resistance of the protein against chemical denaturation with urea at pH 8.0 and 25, 37, 42, or 45 °C at a 25 mM concentration by following intrinsic fluorescence changes at 303 nm at equilibrium (Fig. 4). A single detectable transition was observed in all cases, indicating that the protein unfolds cooperatively from an initially highly packed state. The main thermodynamic parameters of the unfolding reaction were calculated from the equilibrium curves assuming a two-state model ($R > 0.99$ in all cases). The thermodynamic stability of the domain exhibited a high dependence on the temperature (Table 1). At 25 °C, h-TTL exhibits a Gibbs free energy difference, extrapolated to absence of denaturant ($\Delta G^{H2O}$) of 6.12 ± 0.04 kcal mol$^{-1}$, with a half-transition denaturant concentration ([urea]$_{50%}$) of 5.83 M. At 37, 42, and 45 °C, the protein is destabilized by 1.43, 2.41, and 4.38 kcal mol$^{-1}$, respectively (Table 1). In a similar manner, the $m$-value decreases as temperature increases, indicating lower cooperativity of the unfolding reaction at higher temperatures (Table 1).

**Aggregate of Human TTL into Amyloid-like Structures**—Because dissociated TTR monomers have been shown to readily aggregate into amyloid-like assemblies (17), it was interesting to evaluate the aggregative properties of a conformationally similar, yet monomeric, domain. Human TTL was incubated at 100 μM for 14 days at pH 8.0 and 25, 37, or 42 °C to assess whether this domain aggregates into amyloid-like structures under close to physiological conditions and whether this process is dependent on the thermodynamic stability of the native state. No apparent aggregation was observed at 25 °C (data not shown). In contrast, we did observe the formation of protein aggregates that were stained with the amyloid-specific dye

### TABLE 1

**Thermodynamic characterization of h-TTL at different temperatures by intrinsic florescence**

| Temperature | $\Delta G^{H2O}$ kcal mol$^{-1}$ | $m$-value kcal mol$^{-1}$ m$^{-1}$ | [Urea]$_{50%}$ M |
|-------------|---------------------------------|---------------------------------|-----------------|
| 298/25      | 6.12 ± 0.04                     | 1.05 ± 0.01                     | 5.83            |
| 310/37      | 4.69 ± 0.04                     | 1.03 ± 0.01                     | 4.55            |
| 315/42      | 3.61 ± 0.08                     | 0.86 ± 0.02                     | 4.20            |
| 318/45      | 1.74 ± 0.13                     | 0.64 ± 0.03                     | 2.72            |

**FIGURE 5.** Morphological and conformational analysis of h-TTL aggregates. A, bright field (upper panels) and fluorescence (lower panels) imaging of h-TTL aggregates stained with Th-T after 14 days incubation at 37 and 42 °C (green and 42 °C (red); Free Th-T emission is represented as dashed lines. C, normalized IR spectra of native h-TTL (upper panel) and h-TTL aggregates incubated at 37 °C (middle panel) and 42 °C (lower panel). Colored lines represent different secondary structure elements arising from Gaussian deconvolution. D, representative TEM micrographs of h-TTL aggregates after 14 days of incubation.
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Th-T, yielding bright green-yellow fluorescence against a dark background when observed by fluorescence microscopy upon incubation at both 37 and 42 °C (Fig. 5A). Th-T fluorescence emission is enhanced in the presence of amyloid fibrils, and the same behavior is observed upon incubation of aggregated h-TTL with Th-T, especially for samples incubated at 42 °C, where the Th-T fluorescence at the 480-nm spectral maximum increases by 100-fold (Fig. 5B). No Th-T binding to soluble h-TTL was detected at any of the assayed temperatures prior to incubation (data not shown).

ATR-FTIR allowed us to address the structural features of both native and aggregated h-TTL. The absorbance spectra of native h-TTL in the amide I region is dominated by a band at 1637 cm⁻¹, in agreement with its all-β fold (Fig. 5C and Table 2). Deconvolution of the spectra demonstrates differences in the secondary structural content of the protein incubated at 37 and 42 °C (Fig. 5C and Table 2). In this way, although the IR spectrum at 37 °C is dominated by a signal at 1634 cm⁻¹, which is typically attributed to the β-sheet structure, the main signal at 42 °C is shifted to 1627 cm⁻¹, indicating shorter hydrogen bonding and therefore more densely packed β-sheet structures, compatible with intermolecular contacts in an amyloid fold (Table 2).

The morphological features of h-TTL samples were analyzed after incubation using TEM. As shown in Fig. 5D, we detected the presence of protein aggregates at both 37 and 42 °C. Nevertheless, the size and morphology of the aggregates formed at the two temperatures were significantly different. In good agreement with the Th-T binding and secondary structural data, small oligomer-like aggregates, which tended to clump together, were observed at 37 °C, whereas at 42 °C the protein assembled into dense amyloid-like fibrillar meshes.

Dependence of the Aggregation Kinetics of Human TTL on the Temperature—We analyzed the kinetics of h-TTL aggregation and its dependence on the temperature by monitoring its Th-T binding over time. As shown in Fig. 6A, subtle changes in temperature result in dramatic effects on the aggregation regime. The kinetics of amyloid fibril formation usually follows a sigmoidal behavior characterized by three kinetic stages as follows: 1) lag phase, 2) exponential growth phase, and 3) plateau phase. These phases, characteristic of most amyloid processes, reflect a nucleation-polymerization mechanism. The h-TTL aggregation kinetics at 42 °C can be rationalized according to this mechanism and fitted to an autocatalytic equation, with nucleation and elongation constants of 0.11 and 33.8 (10⁶ min⁻¹), respectively, and a lag time of 1410 min. Lowering the temperature to 37 °C makes the aggregation reaction exceedingly slow, reaching only 10% of the maximal Th-T binding recorded at 42 °C after 14 days. In contrast, increasing the temperature just by 3 °C, to 45 °C, exacerbates aggregation, abrogating the lag phase of the reaction.

We compared Th-T signal during aggregation at 42 °C with both intrinsic and bis-ANS fluorescence at each of the reaction stages (Fig. 7). The low Th-T signal during the lag phase correlates with small changes in protein intrinsic fluorescence, suggesting that h-TTL does not suffer extensive unfolding at this stage. In contrast, bis-ANS fluorescence increases significantly during nucleation, indicating the formation of new hydrophobic clusters that likely reflect the assembly of native-like h-TTL into small oligomers (54). Like the Th-T signal, intrinsic fluorescence progressively increases at the growth and plateau stages, indicating that the h-TTL Tyr residues are in a different protein environment in the native and fibrillar states. The increase in intrinsic protein fluorescence is common to many amyloidogenic processes and seems to respond to the more hydrophobic environment that the amyloid fibril provides to aromatic residues as well as to their stacking (55), which is in

| Band | Area | Band | Area | Band | Area | Structure           |
|------|------|------|------|------|------|--------------------|
| cm⁻¹ | %    | cm⁻¹ | %    | cm⁻¹ | %    |                    |
| 1610 | 8    | 1600 | 4    | 1604 | 12   | Tyrosine ring      |
| 1637 | 47   | 1634 | 68   | 1627 | 44   | β-sheet            |
| 1668 | 45   | 1666 | 24   | 1659 | 44   | Loop/β-turn/bend/α-helix |
|      | 1686 | 4    |      |      |      | β-Sheet            |

A. FIGURE 6. h-TTL aggregation kinetics. A, fraction of aggregated h-TTL, measured as Th-T binding, as function of time at 37 °C (black), 42 °C (green), and 45 °C (orange). The inset shows Th-T emission spectrum of h-TTL aggregates after 14 days of incubation at 37 °C (black) and 42 °C (green). B, aggregation reaction performed at 25 °C in the absence (black) or in the presence (green) of 10% of fibrils (w/w) performed at 42 °C.
agreement with the observed concomitant increase in bis-ANS binding.

We further analyzed whether the amyloid fibrils formed at 42 °C were able to seed the aggregation of soluble h-TTL at 25 °C, where no apparent aggregation occurs spontaneously. Interestingly enough, the presence of 10% preformed fibrils promotes the formation of Th-T-positive aggregates following a classical sigmoidal curve (Fig. 6B). This suggests that the fibrillar state is able to recognize one or more regions in the structure of the folded protein and subsequently to promote the incorporation of the complete soluble protein into the fibril.

**Human TTL Displays a Highly Amyloidogenic Short Sequence Stretch**—It is now accepted that specific and continuous protein segments nucleate amyloid-like reactions and participate in the formation of the β-core of the mature fibrils (56). Different computational methods have been developed to predict those sequential stretches (57). Here, we used WALTZ, which exploits a position-specific scoring matrix deduced from the biophysical and structural analysis of the amyloid properties of a large set of hexapeptides, to identify amyloid-aggregating sequences (47). We identified a single amyloidogenic sequence stretch in h-TTL, encompassing residues Gly-44 to Met-56 (Fig. 8A), which overlaps with β-sheet 5 and includes part of the loops at its N- and C-terminal sides (Fig. 8B). To assess whether this region has the ability to self-assemble and act as a possible nucleation element in the aggregation process of h-TTL, we synthesized and characterized the amyloidogenic properties of the corresponding peptide (GTYNLTVVLTGYM). The peptide was incubated at 100 μM, pH 8.0, and at 25 °C. In these conditions, the solution becomes slightly cloudy after 1 min. The formation of fibrillar structures with size and morphology compatible with an amyloid nature could be observed by TEM upon 3 days of incubation (Fig. 8C). We analyzed the secondary structural content of the fibrils by ATR-FTIR in the amide I region of the spectrum (Fig. 8D). The absorbance spectrum in this region was dominated by a peak at 1626 cm−1 that indicates the presence of the intermolecular β-sheet. Finally, Th-T binding (Fig. 8E) confirms the amyloidogenic properties of the fibrillar structures formed by the most aggregation-prone sequence of h-TTR.

**Binding to Membranes Modulates Human TTL Aggregation**—It has been proposed that h-TTL might be involved in binding of CPD to the cell membrane, and in fact other TTR-like proteins have been shown to serve this function (59). The presence of biological membranes may strongly affect the aggregation of amyloidogenic proteins (60). We addressed whether this is the case for h-TTL using liposomes as mimics of natural cell membranes. The effect of negatively charged liposomes (DOPA), neutral liposomes (DLPC), and liposomes containing an equimolar mixture of charged and neutral lipids (DOPA/DLPC) was assessed. As shown in Fig. 9A, the effect of liposomes on the kinetics of h-TTL aggregation at 42 °C is dramatically dependent on their charge. The presence of DLPC liposomes exerts a strong inhibitory effect on h-TTL aggregation. DLPC liposomes with a ζ potential of −1.6 quench the fluorescence of Tyr residues in h-TTL in a concentration-dependent manner (Fig. 9B), suggesting that this inhibitory effect might be mediated by liposome interaction with aromatic/hydrophobic residues in the native h-TTL state. The presence of DOPA-containing liposomes with a ζ potential of −46 strongly accelerates the reaction completely abrogating the lag phase. h-TTL is an acidic protein with a pI of 5.6, and therefore a generic adsorption to liposomes facilitated by the presence of a negative surface potential in the membrane is not expected. However, inspection of the charge distribution of the h-TTL surface indicates an asymmetric localization of the negative and...
positive residues, which are placed in opposed faces of the protein (Fig. 9D). The amyloidogenic β-strand 5 is located in the positively charged side. Therefore, it is tempting to hypothesize that the strongly pro-aggregational effect exerted by negatively charged liposomes might be related to a preferential orientation of h-TTL relative to the lipidic surface, affecting either directly or indirectly the microenvironment of the most amyloidogenic region in h-TTL. In this orientation, the negatively charged side where the exposed Tyr-38 resides will remain apart from the membrane, explaining the lower fluorescence quenching exerted by DOPA-containing liposomes (Fig. 9D).

**DISCUSSION**

All the members of the CP M14B subfamily share a β-sandwich TTL domain at the C terminus, the function of which remains uncertain. Here, we show that the globular h-TTL domain displays an intrinsic propensity to aggregate into amyloid-like conformations, a property shared with the structurally homologous TTR monomer. The spectroscopic probes indicate that, at 42 °C, h-TTL has structural properties that are very similar to those of the native state at 25 °C. However, at 25 °C, the protein remains soluble, and at 42 °C the protein aggregates into amyloid-like structures following characteristic sigmoidal kinetics. Before aggregation, the protein appears to retain a secondary structure identical to that of the native state, and Tyr side chains report on the existence of a compact conformation. Moreover, the binding to bis-ANS remains unaltered relative to the native structure, ruling out the emergence of additional hydrophobic patches on the surface at 42 °C.

The protein is destabilized at 42 °C, with a ΔG° of 3.61 kcal mol⁻¹, significantly smaller than the ΔG° of 6.12 kcal mol⁻¹ measured at 25 °C. Although h-TTL still exhibits an unfolding cooperativity characteristic of folded states at 42 °C and spectroscopic properties indistinguishable of that of the protein at 25 °C, the m-value suggests an overall lower cooperativity of the protein at 42 °C, which might result from transient fluctuations around the native conformation at this temperature. In a similar manner to what has been proposed for lysozyme, a hyperthermophilic acylphosphatase, superoxide dismutase 1, TTR, and β₂-microglobulin (37), they will likely be these conformers,
in dynamic equilibrium with the native state, that would trigger the aggregation reaction. Accordingly, the aggregation rate is strongly dependent on the temperature, being very slow at 37 °C, where the protein is 1.08 kcal mol\(^{-1}\) more stable than at 42 °C, and extremely fast at 45 °C, where it is 1.87 kcal mol\(^{-1}\) less stable. In fact, despite that the secondary structural content of h-TTL at 45 °C is equivalent to that of the protein in the 25–42 °C range, both Tyr intrinsic fluorescence and ANS are higher, indicating partial exposure of previously hidden hydrophobic residues, a conformational feature that abrogates the lag phase of the aggregation, thus promoting the immediate incorporation of the protein into Th-T-positive aggregates. Despite that the increase in aggregation rates of initially unfolded amyloidogenic proteins with the temperature is a well known phenomenon (61), the dependence of the reaction on the temperature observed in these cases is much lower than the one reported here for h-TTL. This responds to the fact that, for this domain, the temperature sharply tunes the conformational stability and therefore controls the degree of structural fluctuations leading to the transient population of aggregation-susceptible conformers. Therefore, as recently proposed for the Src homology 3 domain, it is likely that the structures sampled by the monomer under native conditions encode not only the structures in the fibril state but also the rate of fibril formation (39). Alternatively, it can be that a fraction of the low populated unfolded species in equilibrium with the native ensemble might constitute a reservoir of conformations responsible for fibrillation, whose concentration would depend on the thermodynamic stability of the protein. In any case, our results indicate no global unfolding of the protein population occurs in the lag phase of the reaction, where the formation of oligomeric species is already taking place.

FIGURE 9. Effect of liposome composition on h-TTL aggregation. A, aggregation kinetics at 42 °C, followed by Th-T binding, h-TTL alone (blue), and in the presence of 1 mM DOPA (red), DOPA/DLPC (1:1) (black), or DLPC liposomes (orange). Intrinsic fluorescence spectra are of h-TTL alone (blue) and in the presence of 0.1 mM (red), 0.5 mM (black), or 1.0 mM (orange), the presence of neutral DLPC (B), and of highly negatively charged DOPA liposomes (C). D, electrostatic surface potential distribution and ribbon representation of h-TTL (in the same orientation) shown in red at the location of the APR detected by Waltz. Two views are shown related by a 180° rotation around the z axis. Blue indicates positive and red indicates negative charge potential. a.u., arbitrary units.
Most of the information we have on the aggregation of globular proteins has been obtained under strong denaturing conditions, in which polypeptide chains are significantly unfolded or populate molten globules. Although the assays performed under these conditions have provided important mechanistic insights into amyloid fibril formation (62), it is also true that the protein repertoire in living organisms would hardly face these environments. Our results are, in line with recent data, obtained from structurally and sequentially unrelated protein models, which suggest that protein aggregation and subsequent amyloid formation can occur under conditions where a protein populates conformations close to its native state but slightly destabilized (37, 63). Cellular stress can suffice to promote such destabilization, increasing conformational fluctuations and/or the transient population of partially unfolded conformers, which could trigger aggregation if they exceeded their critical concentration for nucleation. At 25 °C, the h-TTL is stable enough to skip aggregation, but the protein already aggregates at 37 °C, indicating that the side reaction can occur under mild physiological conditions. This is consistent with h-TTL having a highly amyloidogenic preformed β-strand. Local fluctuations around this structural element would likely allow anomalous intermolecular interactions between h-TTL monomers, leading to the formation of an aggregated β-sheet structure without extensive unfolding. This is the mechanism driving the formation of amyloids by TTR, in which dissociation of the tetrameric structure results in the direct exposure to the solvent of preformed β-strands previously involved in inter-subunit contacts at the interface of the complex. It has been suggested that, upon tetramer dissociation, the TTR monomer experiences only a local unfolding transition, mainly involving the external C and D strands, with β-strands AGH and BEF retaining largely a native-like folded conformation (23). Moreover, the core structure of the fibrils has been shown to involve the AGH and BEF sheets of the monomer in a native-like conformation, in such a way that interactions between B and A strands as well as between F and H strands from adjacent molecules account for the cross-β-structure of TTR fibrils (64). The fact that in h-TTL aggregation occurs without requiring any extensive unfolding suggests that the docking of preformed β-sheets might also account for the formation of amyloid-like structures in this structurally homologous domain. Despite its intrinsic aggregation propensity, the h-TTL domain remains soluble at 25 °C, where the protein is energetically more stable. This suggests that for pathological proteins sharing the same mechanism for amyloid formation, small compounds able to stabilize the native state and therefore reduce the population of aggregation-competent transient conformations might find therapeutic application (65). However, it is also true that even when they are in energetically stable conformations, these proteins are aggregation-susceptible, because, as shown here for h-TTL, the presence of small concentrations of preformed aggregates suffices to trigger their self-assembly reactions.

As shown in Fig. 10, we predict that all TTL domains of the CPs in the M14B subfamily exhibit at least one preformed, highly amyloidogenic β-strand, as predicted with Waltz. According to our data, this puts the respective domains at risk of aggregation. Because the formation of intracellular deposits reduces cell fitness, during the course of the evolution proteins have adopted sequential and structural strategies to escape from protein aggregation (28, 66, 67). However, in certain cases, especially for all-β-sheet proteins, the presence of preformed...

**FIGURE 10.** APRs detected in the transthyretin-like domains of the M14B subfamily members. APRs (in red) were detected employing Waltz in the TTL domains as follows: A, CPD (CP domains I, II and III); B, CPM; C, CPN; D, carboxypeptidase E (CPE); E, carboxypeptidase Z (CPZ); F, adipocyte enhancer-binding protein 1 (AEBP1); G, CPX-1; and H, CPX-2. CPM and CPN structures correspond to Protein Data Bank atomic coordinates deposited under accession codes 1UWY and 2NSM, respectively. The remaining ribbon representations correspond to models generated using the I-TASSER server.
Amyloidogenic structural elements cannot be completely avoided because they are needed for functional purposes, like the formation of native intermolecular interfaces, as in TTR. This argues that the aggregation-prone face of h-TTL might serve to contact other molecules. It has been suggested that TTL domains might play a role in the binding the M14B subfamily CPs to membranes. Although it is true that h-TTL might bind to neutral membranes and that the interactions abrogate its aggregation, it is also true that most biological membranes display a negative charge which, due to the asymmetric distribution of the charges at the h-TTL surface, would likely force the preferential orientation of the aggregation-prone face of the protein toward the membrane, resulting in a high aggregation propensity, as shown here for negatively charged liposomes. This questions the putative membrane-binding role of TTL domains in CPs, which should therefore serve as alternative functions that have yet to be discovered.

The aggregation mechanism described here for the first time for a native monomeric TTR-like protein is found also in a number of initially soluble globular proteins associated with protein deposition diseases and might be in fact quite generic for folds displaying preformed amyloidogenic elements in their structures, essentially β-sheets. This emphasizes the crucial role played by the protein quality machinery to preclude the aggregation of globular proteins under stress conditions that might decrease their conformational stability (58).

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