The Tetrameric Protein Transthyretin Dissociates to a Non-native Monomer in Solution

A NOVEL MODEL FOR AMYLOIDOGENESIS*

(Received for publication, February 16, 1999, and in revised form, August 25, 1999)

Alexandre Quintas‡§¶, Maria João M. Saraiva†, and Rui M. M. Brito‡‡¶‡‡¶

From the ‡Centro de Neurociências de Coimbra, Universidade de Coimbra, 3004-517 Coimbra, Portugal, the §Instituto Superior de Ciências da Saúde Sul, Quinta da Granja, 2825 Monte da Caparica, Portugal, the †Instituto de Ciências Biomédicas de Abel Salazar and the Amyloid Unit, Instituto for Molecular and Cellular Biology, Universidade do Porto, 4050 Porto, Portugal, and the **Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, 3049 Coimbra, Portugal

In amyloidosis, normally innocuous soluble proteins polymerize to form insoluble fibrils. Amyloid fibril formation and deposition have been associated with a wide range of diseases, including spongiform encephalopathies, Alzheimer’s disease, and familial amyloid polyneuropathies (FAP). In certain forms of FAP, the amyloid fibrils are mostly constituted by variants of transthyretin (TTR), a homotetrameric plasma protein implicated in the transport of thyroxine and retinol. The most common amyloidogenic TTR variant is V30M-TTR, and L55P-TTR is the variant associated with the most aggressive form of FAP. Recently, we reported that TTR dissociates to a monomeric species at pH 7.0 and nearly physiological ionic strengths (Quintas, A., Saraiva, M. J., and Brito, R. M. (1997) FEBS Lett. 418, 297–300). Here, we show that the tetramer dissociation is apparently irreversible; and based on intrinsic tryptophan fluorescence and fluorescence quenching experiments, we show that the monomeric species formed upon tetramer dissociation is non-native. We also show, based on 1-anilino-8-naphthalenesulfonate binding studies, that this monomeric species appears not to behave like a molten globule. These data allowed us to propose a model for TTR amyloidogenesis based on tetramer dissociation occurring naturally under commonly observed physiological solution conditions.

In amyloidosis, normally innocuous soluble proteins polymerize to form insoluble fibrils. Amyloid fibril formation and deposition have been associated with a wide range of diseases, including spongiform encephalopathies, Alzheimer’s disease, and familial amyloid polyneuropathies (FAP). In certain forms of FAP, the amyloid fibrils are mostly constituted by variants of transthyretin (TTR). The most common amyloidogenic TTR variant is V30M-TTR, and L55P-TTR is the variant associated with the most aggressive form of FAP. Human TTR is a protein that is found in the plasma and cerebrospinal fluid and that has been implicated in the transport of thyroxine and retinol in association with the retinol-binding protein. The concentration of TTR in serum ranges between 170 and 420 µg/ml, and in cerebrospinal fluid varies between 5 and 20 µg/ml (3). TTR is a homotetramer (Fig. 1A) with a total molecular mass of 55 kDa. The three-dimensional structure of the TTR subunit consists of two β-sheets with four β-strands each, forming a β-sandwich (Fig. 1B) (4, 5). The association of the four subunits forms a central channel with two thyroxine-binding sites (Fig. 1A) (4). Comparison between the crystal structures of WT- and V30M-TTR showed a very similar global fold for both proteins. Substitution of valine 30 by methionine forces the β-sheets of the monomer ~1 Å apart, resulting in the distortion of the thyroxine-binding cavity (5, 6). However, the small differences between the crystal structures of WT- and V30M-TTR have not clearly pointed out the causes for the amyloidogenicity of V30M-TTR. Very recently, based on the crystal structure of the highly amyloidogenic L55P-TTR, a model for TTR amyloid fibrils has been proposed consisting of a tubular structure with inner and outer diameters of ~30 and 100 Å, respectively, and four monomers per cross-section (7).

Westermark and co-workers (8) have reported the formation of amyloid by TTR in vitro at low pH. This observation has led to the proposal of a low pH environment as a prerequisite for amyloid formation in vivo (9). These authors proposed that the low pH medium (present for example in the lysosomes) would induce tetramer rearrangement and dissociation to a monomeric amyloidogenic intermediate with altered tertiary structure, which in turn would self-assemble to form amyloid fibrils (10). However, this proposal, with lysosomal involvement, implies the formation of amyloid intermediates intracellularly, which is not consistent with the observation that TTR amyloid deposits are extracellular (11). Furthermore, in FAP, amyloid deposition occurs predominantly in the peripheral nerves, and both TTR mRNA and protein are not detected in the cellular components of nerves. Thus, amyloid assembly and deposition must take place extracellularly. Additionally, at low pH, both amyloidogenic and non-amyloidogenic TTR variants form amyloid in vitro.

Here, we show that the TTR tetramer dissociates to non-native monomeric species at pH 7 and nearly physiological ionic strengths, and the monomeric species may self-assemble into high molecular mass aggregates. Our data allowed us to propose a novel model for amyloidogenesis by TTR under com-
TRANSTHYRETIN DISSOCIATION AND AMYLOIDOGENESIS

EXPERIMENTAL PROCEDURES

Materials—ANS, potassium iodide (KI), guanidinium hydrochloride, and all other chemicals were of the highest purity commercially available and were purchased from Sigma.

Protein Sample Preparation and Monomer Identification—Recombinant WT- and V30M-TTR were produced in an Escherichia coli expression system (12) and purified as described previously (13). Protein concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient of 7.76 × 10^4 M⁻¹ cm⁻¹ (14). Protein dilutions were prepared in 20 mM sodium phosphate buffer and 150 mM sodium chloride, pH 7.0. When needed, TTR samples were re-concentrated by ultrafiltration on a Centricon-3 (Amicon, Inc.) with a molecular mass cutoff of 3 kDa.

Tetrameric and monomeric species of all TTR variants were isolated by gel filtration chromatography performed on an Amersham Pharamacia Biotech FPLC Superdex-75 HR column coupled to an Amersham Pharmacia Biotech high precision F-500 pump and a UV detector equipped with a deuterium lamp and an integrator from Konik Instruments. The column was equilibrated with 20 mM sodium phosphate buffer and 150 mM sodium chloride, pH 7.0, and was frequently cleaned with 0.5 M NaOH. All runs were performed at a flow rate of 0.4 ml/min. Apparent molecular masses were calculated by interpolation on an elution volume versus log(molecular mass) calibration curve for four protein standards: bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa). TTR monomeric species were further identified by ultrafiltration experiments performed on CentriPor centrifuge concentrators (Spectrum Science, Inc.) with a molecular mass cutoff of 15 kDa and by SDS-polyacrylamide gel electrophoresis performed on an Amersham Pharmacia Biotech PhastSystem.

Fluorescence—Fluorescence experiments were performed on a SPEX Industries Fluorolog spectrophluorometer. For intrinsic tryptophan fluorescence studies and fluorescence quenching studies by KI, an excitation wavelength of 280 nm was used, and the excitation and emission slits were set to 1.5 and 2 nm, respectively. Quenching studies were performed by adding small aliquots of a freshly prepared stock solution of KI to 0.5–1 μM protein solutions. For ANS binding studies, an excitation wavelength of 370 nm, excitation slits of 1.5 nm, and emission slits of 2 nm were used. Final sample dilution in the KI and ANS titrations never exceeded 1%. All fluorescence studies were performed at a controlled temperature of 25.0 °C and on a 1.0-cm path length cuvette.

Solvent Accessibility—Solvent-accessible surface area calculations were performed using the coordinates from the refined crystal structures of WT- and V30M-TTR (5) and the program GRASP (15) running on an SGI Octane workstation. The solvent-accessible surface area of the TTR tryptophan residues was compared with the solvent-accessible surface area of tryptophan in the tripeptide Gly-Trp-Gly to evaluate the relative solvent exposure of each one of the tryptophan residues in the tetramer and native monomeric subunits of WT- and V30M-TTR.

RESULTS

TRANSTHYRETIN DISSOCIATION—Tetrameric and monomeric species of WT- and V30M-TTR were isolated by gel filtration chromatography based on the dissociation behavior of the tetramers, previously reported (16). Fig. 2A shows gel filtration elution profiles of WT- and V30M-TTR with the characteristic equilibrium between major species in solution at pH 7, room temperature, and a total protein concentration of 0.3 μM (calculated as the tetramer). The chromatograms show the presence of two peaks with elution volumes of 8.8 and 15.2 ml, corresponding to apparent molecular masses of 60 kDa (the tetramer) and 5.9 kDa (a monomeric species, referred to hereafter as M5.9) (2). The monomeric nature of this M5.9 species has also been confirmed by SDS-polyacrylamide gel electrophoresis and ultrafiltration experiments performed on centrifuge concentrators with a molecular mass cutoff of 15 kDa. In the ultrafiltration experiments, a solution with approximately equimolar quantities of tetramer and M5.9 WT-TTR was placed in the upper chamber of the concentrator and centrifuged at 1500 × g for 1.5 h. Gel filtration chromatography of the contents of the upper and lower chambers showed the presence of mostly tetramer in the upper chamber and mostly M5.9 species in the lower chamber. Taking into consideration the molecular mass cutoff of the membrane used (15 kDa) and the molecular masses of the TTR dimer and tetramer (27.5 and 55 kDa respectively), we concluded that the M5.9 species is in fact monomeric. Additionally, SDS-polyacrylamide gel electrophoresis experiments (Fig. 3) clearly showed that whereas non-boiled L55P-TTR tetramer ran as a dimer on the gel, as previously reported for WT-TTR by others (17, 18), non-boiled M5.9 L55P-TTR ran as a monomer with an apparent molecular mass of ~15 kDa. These experiments clearly show the monomeric nature of the M5.9 species.

TTR tetrameric and monomeric (M5.9) species were isolated by gel filtration chromatography to characterize them by intrinsic tryptophan fluorescence, iodide fluorescence quenching, and ANS binding. To isolate tetrameric species, TTR samples were applied to the chromatography column at high protein concentrations (>1 mg/ml). To isolate the major monomeric species (M5.9), TTR samples were applied to the chromatography column at a concentration below 0.05 mg/ml. Fig. 2B shows gel filtration chromatograms of WT- and V30M-TTR tetramers after being studied by fluorescence, indicating that in both cases the tetramers were ~99% pure. Fig. 2C shows gel filtration chromatograms of the M5.9 samples for WT- and V30M-TTR after collection from the preparative run of gel filtration chromatography, re-concentration by ultrafiltration, fluorescence assays, and re-injection into the chromatography column. The ultrafiltration was performed on centrifuge concentrators with a molecular mass cutoff of 3 kDa. For WT-TTR, the re-concentration process of the M5.9 species led to the appearance of another monomeric species (M2.8), with an elution volume of 17.2 ml. However, in V30M-TTR, only the M5.9 species was observed after the re-concentration process. The conversion between M5.9 and other species was highly dependent on total protein concentration and the TTR variant being studied. Interestingly, re-concentration of the M5.9 species up to concentrations of 2 μM (corresponding to 0.5 μM tetramer) (Fig. 2C) did not...
not produce tetrameric species, indicating that the dissociation equilibrium of the tetramer seems to be irreversible at nearly physiological pH, which may suggest a monomeric species conformationally altered from the native TTR subunit.

To evaluate the interconversion between molecular species of TTR in solution, we incubated, at 37 °C for several days, tetrameric WT-TTR at a highly amyloidogenic variant (L55P-TTR). Fig. 4 shows a time course for the interconversion of the L55P-TTR tetramer to several other species at pH 7 and 37 °C, followed by gel filtration chromatography. It is clear that initially the tetramer dissociated to small amounts of M5.9 monomer (Fig. 4A), which, after 24 h of incubation at 37 °C, led to the formation of several other monomeric species (Fig. 4B). Incubation of the L55P-TTR tetramer at 37 °C and pH 7 for periods of up to 2 weeks led to a significant decrease in the tetramer concentration, an increase in the number and amount of monomeric species, and the formation in solution of high molecular mass aggregates with elution volumes of 6.0 and 6.8 ml (Fig. 4C). The formation of high molecular mass aggregates following the appearance of monomeric species in solution (Fig. 4) and the direct dissociation of these aggregates to monomeric species (16) strongly suggest that the TTR aggregates are assembled from one or more types of monomeric species. These data agree with and extend to pH 7 the recently reported results of analytical ultracentrifugation studies that showed aggregate formation after a few hours of TTR incubation at low pH (19). Additionally, when late eluting species (Fig. 4C) were collected and re-injected into the gel filtration column, they eluted as a mixture of species corresponding to the initial and M5.9 elution positions, showing interconversion between these monomeric species.

### Intrinsic Fluorescence—Analysis of the intrinsic tryptophan fluorescence spectra (Fig. 5) of the tetrameric and monomeric species of WT- and V30M-TTR, isolated by gel filtration chromatography (Fig. 2, B and C), revealed that the tetrameric species of the two TTR variants have very similar emission maxima at ~340 nm (Fig. 5). However, the fluorescence spectra of the M5.9 WT- and V30M-TTR samples show different blue-shifted emission maxima at 330 and 335 nm, respectively (Fig. 5). The tryptophan emission maxima at ~340 nm observed for both TTR tetramers are indicative of partially buried tryptophans. TTR has two tryptophan residues per protein subunit at positions 41 and 79. Using site-directed mutagenesis, it has been shown that Trp79 exhibits very low fluorescence at pH 7 and that most of the intrinsic fluorescence is due to Trp41 (10). The solvent-accessible surface areas of Trp41 and Trp79 in tetrameric WT-TTR are ~83.0 and 2.5 Å², respectively (Table I), showing that the silent tryptophan is almost totally buried in the protein interior, whereas the observable tryptophan is partially exposed to the solvent. The solvent-accessible surface area of a totally exposed tryptophan residue in the model tripeptide Gly-Trp-Gly is 243.2 Å². Thus, in the tetrameric form of WT-TTR, Trp41 has a solvent exposure of 34.1%, and Trp79 has a solvent exposure of just 1.0%. The values of solvent exposure for tetrameric V30M-TTR are very similar to those obtained for WT-TTR (Table I). This agrees well with the observation of a partially buried tryptophan responsible for the emission maximum at 340 nm in tetrameric WT- and V30M-TTR (Fig. 5).

The fluorescence emission maxima at 330 and 335 nm for M5.9 WT- and V30M-TTR, respectively, are in apparent contradiction with what was expected for a native TTR monomer. In fact, the tryptophan solvent-accessible surface areas calculated from the native TTR subunit in the crystal structures of WT- and V30M-TTR are very similar to those calculated for the tetramers (Table I). According to this, the emission maximum of a native monomer should be very close to the emission maximum of the tetramer, i.e. 340 nm. The significant blue shifts of 10 and 5 nm observed for M5.9 WT- and V30M-TTR suggest that they are non-native monomeric species. The observed blue shifts can be due to a lower solvent exposure of the tryptophans and consequently a more hydrophobic environment around the tryptophan residues or to fluorescence quenching of the tryptophan on the red side of the emission peak.
envelope of the native tetramer (Trp 41). Both of these hypotheses are consistent with structural changes around the tryptophans’ environment and with an altered structure of the M5.9 species when compared with the native TTR subunit. The larger line width at half-height of the emission spectrum of M5.9 WT-TTR sample is most probably due to the presence of a significant amount of M2.8 WT-TTR (Figs. 2C and 5). This species has a red-shifted emission maximum relative to the M5.9 species and the tetramer (data not shown); and thus, it is not responsible for the large blue shift observed for M5.9 WT-TTR. The larger blue shift of the M5.9 WT-TTR emission spectrum compared with the M5.9 V30M-TTR emission spectrum is mostly probably due to different solvent exposures of the tryptophans in M5.9 WT- and V30M-TTR. This difference in the emission maximum of M5.9 WT- and V30M-TTR may indicate important structural or dynamic changes between these two monomeric species, which are not observed in the tetrameric forms.

We have already shown that the tetrameric form of a highly amyloidogenic TTR variant (L55P-TTR) dissociated to the M5.9 species, which in turn led to the formation of several other monomeric species (Fig. 4). Fig. 6 shows a comparison between the fluorescence emission spectra of tetrameric and some of these monomeric forms of L55P-TTR. The emission maximum of the tetramer is 340 nm, like what was observed for the tetrameric forms of WT- and V30M-TTR. However, the emission maxima of the late eluting monomeric species (Fig. 4C), between 352 and 359 nm, are all red-shifted relative to the emission maxima of the tetramer, indicating a more polar environment around the tryptophan residues probably due to a higher solvent exposure of the tryptophans in these monomeric species. These data suggest that the late eluting monomeric species (Fig. 4C), derived from the M5.9 species, are probably partially unfolded.

**Iodide Quenching of Fluorescence**—To further characterize the differences in solvent-accessible surface area between the tetrameric and M5.9 forms of WT- and V30M-TTR, we performed iodide fluorescence quenching experiments. Iodide is a large anion thought to have access only to surface-exposed Trp residues (20). Iodide fluorescence quenching experiments on tetrameric and M5.9 WT- and V30M-TTR were analyzed assuming the absence of static quenching and using the Stern-Volmer formalism, $F_0/F = 1 + K_{SV}[Q]$, where $F_0$ and $F$ are the fluorescence intensities in the absence and presence of quencher Q, and $K_{SV}$ is the Stern-Volmer constant (Fig. 7) (21). The Stern-Volmer plots for tetrameric WT- and V30M-TTR show positive slopes with Stern-Volmer constants of 5.3 and 6.4 M$^{-1}$, respectively. For comparison, we determined the Stern-Volmer constant for denatured WT-TTR in the presence of 6 M guanidinium chloride and found a value of 8.1 M$^{-1}$ (Fig. 7). These results agree with those from intrinsic tryptophan fluorescence experiments, indicating that in WT- and V30M-TTR tetramers, the Trp residues responsible for the observed fluorescence are partially exposed to the solvent. On
Solvent-accessible surface areas of the tryptophan residues in transthyretin, calculated using the program GRASP (15) and the x-ray coordinates for WT-TTR and V30M-TTR (5).

<table>
<thead>
<tr>
<th></th>
<th>WT-TTR</th>
<th>V30M-TTR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tetramer</td>
<td>Subunit</td>
</tr>
<tr>
<td>Trp$^{11}$</td>
<td>83.0 Å$^2$</td>
<td>84.2 Å$^2$</td>
</tr>
<tr>
<td></td>
<td>(34.1%)$^a$</td>
<td>(34.6%)$^a$</td>
</tr>
<tr>
<td>Trp$^{79}$</td>
<td>2.53 Å$^2$</td>
<td>3.13 Å$^2$</td>
</tr>
<tr>
<td></td>
<td>(1.0%)$^a$</td>
<td>(1.3%)$^a$</td>
</tr>
</tbody>
</table>

$^a$ In parentheses are indicated the relative solvent-accessible surface areas of the tryptophan residues calculated as the ratio between the solvent-accessible surface areas of the tryptophans in TTR and in the model tripeptide Gly-Trp-Gly, expressed as a percentage. 0 and 100% are indicative of no exposure and total exposure, respectively, of the Trp residue to the solvent.

The L55P-TTR tetramer concentration was 1.6 μM, and the L55P-TTR monomeric species concentrations were close to 1 μM. The excitation wavelength was 280 nm. A.U., arbitrary units.

**Fig. 6.** Intrinsic tryptophan emission spectra at pH 7 of the L55P-TTR tetramer and M16.2, M7.8, M5.1, and M3.2 monomeric species. The L55P-TTR tetramer concentration was 1.6 μM, and the L55P-TTR monomeric species concentrations were close to 1 μM. The excitation wavelength was 280 nm. A.U., arbitrary units.

**Fig. 7.** Stern-Volmer plot of fluorescence quenching by iodide of tetrameric WT (○) and V30M-TTR (■), M5.9 WT (●), and V30M-TTR (□), and WT-TTR in the presence of 6 M guanidinium chloride (×). The protein concentrations were 1.0 μM for tetrameric WT- and V30M-TTR, 2.0 μM for M5.9 WT- and V30M-TTR, and 0.9 μM for WT-TTR in the presence of 6 M guanidinium chloride. The excitation wavelength was 280 nm for all TTR species. The fluorescence emission maxima were 340 nm for tetrameric WT- and V30M-TTR, 330 nm for M5.9 WT-TTR, 335 nm for M5.9 V30M-TTR, and 355 nm for WT-TTR in the presence of 6 M guanidinium chloride. $F_0$ and $F$ are the fluorescence intensities in the absence and presence of KI, respectively.

Very accessible to the solvent.

**ANS Binding**—To evaluate the structural integrity of the M5.9 monomeric species and to compare the binding properties of the tetrameric and monomeric species of WT- and V30M-TTR, we performed ANS binding experiments (Fig. 8). ANS fluorescence is highly sensitive to the hydrophobicity of the surrounding chemical environment and has been widely used to probe apolar binding sites in proteins and, more recently, as a qualitative identification tool for protein molten globules (22, 23). Free ANS fluoresces weakly in aqueous solution, but an apolar environment tends to increase the quantum yield and to blue-shift the emission maximum of ANS. Fig. 8 (A and B) shows the effect on the fluorescence emission spectra of increasing concentrations of ANS in the presence of WT- and V30M-TTR tetramers. The emission maxima of ANS in the presence of WT- and V30M-TTR tetramers are 467 and 472 nm, respectively, showing a markedly blue shift from the emission maximum of free ANS at ~520 nm. The fluorescence intensity also increased >100-fold in the presence of the TTR tetramers. A nonlinear fit to the binding data (Fig. 8, A and B, insets) yielded $K_D$ values of 1.2 μM for the WT-TTR tetramer and 2.6 μM for the V30M-TTR tetramer, in agreement with previous studies on WT-TTR (24). Competition studies have shown that ANS binds to the same sites as thyroxine in WT-TTR (24). The thyroxine-binding site is highly apolar, which justifies the increase in fluorescence quantum yield and the blue shift of the emission maximum of ANS upon binding to tetrameric TTR.

Fig. 8 (C and D) shows the effect on the fluorescence emission spectra of stepwise addition of ANS to M5.9 WT- and V30M-TTR. In both cases, the emission maximum is 520 nm, and the emission intensity varied linearly with ANS concentration (Fig. 8, C and D, insets), showing that ANS does not bind or binds very weakly to the M5.9 species. The dissociation of the TTR tetramer destroyed the thyroxine-binding site, which is located in the central cavity formed by the four TTR subunits (Fig. 1A). Thus, high affinity binding of ANS to the thyroxine-binding region in the monomeric forms would not be expected. Additionally, these results also suggest that M5.9 WT- and V30M-TTR do not behave like a molten globule, which usually shows binding affinity for ANS.

**DISCUSSION**

The molecular mechanisms for amyloid fibril formation have recently been the target of increasing attention due to their central role in several human and animal pathologies. Here, we present a novel model for amyloidogenesis by TTR based on the dissociation behavior of the tetrameric protein under nearly physiological conditions as studied by gel filtration chromatography, ultrafiltration, tryptophan intrinsic fluorescence, fluorescence quenching by iodide, and ANS binding.

Gel filtration chromatography experiments with WT-TTR and the amyloidogenic variant V30M-TTR showed that the native tetrameric form of these proteins dissociates to a monomeric species (M5.9) at nearly physiological pH (Fig. 2) (16).
Intrinsic tryptophan fluorescence (Fig. 5) and fluorescence quenching by iodide (Fig. 7) of the tetrameric and M5.9 species revealed altered spectroscopic properties of the tryptophan residues and thus a structural change in the M5.9 species in comparison with the native subunit. Additionally, re-concentration of the M5.9 species up to concentrations of 0.03 mg/ml (0.5 μM, calculated as the tetramer), where >50% of the tetramer would be expected, did not show any detectable amount of tetrameric species (Fig. 2C). These results may indicate that the tetramer dissociates irreversibly to a non-native monomeric species at nearly physiological pH. The formation of this non-native monomer upon tetramer dissociation may be due to the exposure to the solvent of the large hydrophobic surface in the intersubunit interface. Thus, the entropic pressure to hide this hydrophobic surface from the solvent may be responsible for the structural changes occurring in the TTR subunits upon tetramer dissociation. These structural changes may differ between amyloidogenic and non-amyloidogenic variants of TTR.

**Fig. 8.** ANS titration, followed by fluorescence, of tetrameric WT-TTR (A) and V30M-TTR (B) and M5.9 WT-TTR (C) and V30M-TTR (D). The protein concentrations were ~2.2 μM for tetrameric WT-TTR (A) and V30M-TTR (B) and ~2.0 μM for M5.9 WT-TTR (C) and V30M-TTR (D). The excitation wavelength was 370 nm. The insets in A and B show the degree of saturation (calculated as $F/F_{\text{max}}$) as a function of ANS concentration. In C and D, the insets show the fluorescence intensity, at the emission maximum, as a function of ANS concentration. CPS, counts per second.

**Fig. 9.** Proposed model for amyloidogenesis by transthyretin. At pH 7.0, the TTR tetramer dissociates irreversibly to a non-native monomer (M5.9), which is in equilibrium with other partially unfolded species and soluble aggregates, which in turn may form amyloid fibrils.
Transthyretin Dissociation and Amyloidogenesis

Incubation at 37 °C of a highly amyloidogenic TTR variant, L55P-TTR, followed by gel filtration chromatography and fluorescence, showed the interconversion of the M5.9 species to several other partially unfolded monomeric species (Figs. 4 and 6). Additionally, the formation of non-native monomeric species seems to be the initial step in the pathway of self-assembly, leading to the formation of high molecular mass aggregates (Fig. 4C). We have also previously shown that high molecular mass aggregates formed by amyloidogenic TTR variants dissociate directly to the M5.9 species upon dilution (16).

Based on the observations above, we propose a novel model for amyloid fibril formation by TTR under nearly physiological conditions (Fig. 9). The first and very important step is the irreversible dissociation of tetrameric TTR to a non-native monomer (M5.9). This irreversible step may allow accumulation of the M5.9 species, which is then interconverted to other partially unfolded monomeric species. The presence of non-native monomeric species in solution leads to the formation of high molecular mass soluble aggregates. These soluble aggregates may dissociate directly to non-native monomeric species upon dilution. Thus, it seems that a complex equilibrium between non-native monomeric species, partially unfolded monomeric species, and soluble aggregates may exist at nearly physiological pH. These soluble aggregates may constitute the seeding units for amyloid fibril formation. Lundgren and co-workers (18) showed that formation of soluble aggregates in highly amyloidogenic variants of TTR correlates with the formation of amyloid precipitates. The recently reported results of analytical ultracentrifugation studies show soluble aggregate formation after a few hours of TTR incubation at low pH, preceding the formation of amyloid protofibrils (19). Moreover, it has been shown by x-ray diffraction of TTR amyloid fibers that axially arrayed TTR monomers constitute the fiber protofilament (25).

The TTR protein concentration in the serum ranges between 0.17 and 0.42 mg/ml (3.1–7.6 μM) (3), which is consistent with a high percentage of tetramer, as has been commonly indicated in the literature. However, the apparent irreversibility of the tetramer-to-monomer dissociation process may lead to the accumulation in some tissues of non-native monomeric species, some of them potentially amyloidogenic, allowing aggregate formation and slow amyloid fiber growth. Thus, the amyloidogenic potential of some TTR variants could be related to different tendencies of their non-native monomeric species to be converted into monomeric amyloidogenic intermediates, which in turn could self-aggregate and eventually form amyloid fibrils. This could also justify the different FAP onset ages produced by different TTR variants.

Previously, other authors proposed that a low pH environment (present, for example, in the lysosomes) would be a prerequisite for amyloid formation in vivo (9). These authors proposed that the low pH medium would induce tetramer rearrangement and dissociation to a monomeric amyloidogenic intermediate with altered tertiary structure, which in turn would self-assemble to form amyloid fibrils (10). However, this proposal implies the formation of the amyloid intermediates intracellularly, which is not consistent with the observation that TTR amyloid deposits are extracellular (11). Furthermore, in FAP, amyloid deposition occurs predominantly in the peripheral nerves, and both TTR mRNA and protein are not detected in the cellular components of nerves. Thus, amyloid assembly and deposition must take place extracellularly. Here, we showed that the formation of a non-native TTR monomer may occur at pH 7 and therefore does not require lysosomal participation.

Our model for TTR amyloidogenesis is based on the natural tendency shown by TTR to irreversibly dissociate to a non-native monomeric species under nearly physiological conditions. Thus, tetramer stability to dissociation could play some role in the amyloidogenic potential of the TTR variants (17, 26, 27), but more important, the structure, stability, and/or dynamics of the non-native monomeric species may play a crucial role in aggregate formation and potentially amyloidogenesis. Some of the TTR mutations could perturb protein stability and favor structural fluctuations of the monomeric species to an extent that could lead initially to the formation of soluble aggregates of these monomeric species and, at a later stage, to formation of insoluble amyloid fibrils.

Acknowledgment—We thank Paul Moreira for technical assistance in preparing recombinant transthyretin.

REFERENCES

The Tetrameric Protein Transthyretin Dissociates to a Non-native Monomer in Solution: A NOVEL MODEL FOR AMYLOIDOGENESIS
Alexandre Quintas, Maria João M. Saraiva and Rui M. M. Brito

doi: 10.1074/jbc.274.46.32943

Access the most updated version of this article at http://www.jbc.org/content/274/46/32943

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 27 references, 4 of which can be accessed free at http://www.jbc.org/content/274/46/32943.full.html#ref-list-1