Partial Denaturation of Transthyretin Is Sufficient for Amyloid Fibril Formation in Vitro[†,‡]

Wilfredo Colon and Jeffery W. Kelly*

Department of Chemistry, Texas A&M University, College Station, Texas 77843-3255

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ABSTRACT: Amyloid diseases are caused by the self-assembly of a given protein into an insoluble cross-β-sheet quaternary structural form which is pathogenic. An understanding of the biochemical mechanism of amyloid fibril formation should prove useful in understanding amyloid disease. Toward this end, a procedure for the conversion of the amyloidogenic protein transthyretin into amyloid fibrils under conditions which mimic the acidic environment of a lysosome has been developed. Association of a structured transthyretin denaturation intermediate is sufficient for amyloid fibril formation in vitro. The rate of fibril formation is pH dependent with significant rates being observed at pHs accessible within the lysosome (3.6–4.8). Far-UV CD spectroscopic studies suggest that transthyretin retains its secondary structural features at pHs where fibrils are formed. Near-UV CD studies demonstrate that transthyretin has retained the majority of its tertiary structure during fibril formation as well. Near-UV CD analysis in combination with glutaraldehyde cross-linking studies suggests that a pH-mediated tetramer to monomer transition is operative in the pH range where fibril formation occurs. The rate of fibril formation decreases markedly at pHs below pH 3.6, consistent with denaturation to a monomeric TTR intermediate which has lost its native tertiary structure and capability to form fibrils. It is difficult to specify with certainty which quaternary structural form of transthyretin is the amyloidogenic intermediate at this time. These difficulties arise because the maximal rate of fibril formation occurs at pH 3.6 where tetramer, traces of dimer, and significant amounts of monomer are observed. Aromatic UV analysis demonstrates the presence of a subtle tetramer rearrangement which is centered around pH 5.3, indicating that it is definitely not the native tetramer that associates into amyloid fibrils. Spectroscopic studies and cross-linking experiments are consistent with either a rearranged tetramer or a monomeric intermediate as the amyloid precursor. These studies demonstrate that TTR can self-assemble into transthyretin amyloid fibrils under acidic conditions similar to those found in a lysosome, supporting the feasibility of lysosomal and/or endosomal involvement in amyloid disease.

Amyloid fibril formation refers to the abnormal self-assembly of a given protein into an insoluble cross-β-sheet quaternary structural form (Cohen et al., 1983; Stone, 1990; Kisilevsky, 1983; Castano & Frangione, 1988; Lansbury, 1992). Experimental evidence supports the cause and effect relationship between amyloid fibrils and amyloid disease; however, the pathogenic mechanism remains unclear (Benson & Wallace, 1989). Understanding how a normally soluble protein is transformed into fibrils is a critical part of understanding the mechanism of related amyloid diseases. The X-ray diffraction data for amyloid fibrils composed of different amyloidogenic proteins are nearly identical, suggesting that fibrils derived from different proteins have the same major structural features (i.e., cross-β-structure) (Glenner et al., 1974). The similarities (fibril morphology and nerve pathology) between the fibrils composed of the A4 polypeptide (responsible for Alzheimer's disease) and transthyretin (TTR)[1] fibrils suggest that either would be acceptable choices to probe the biochemical mechanism of fibril formation in human amyloid disease (Glenner et al., 1974; Kowall et al., 1991; Benson & Wallace, 1989; Joachim et al., 1989; Selkoe, 1989, 1990). Transthyretin was chosen to study the biochemical mechanism of amyloid fibril formation because of its favorable physical properties and characteristics including the following: (i) its small size and well-characterized structure; (ii) its excellent solubility and conformational stability; (iii) its ready availability either from human plasma or from *Escherichia coli* expression systems; and (iv) because TTR is the causative agent in two related amyloid diseases (Benson, 1989; Blake et al., 1974, 1978; Furuya et al., 1991; McCutchen et al., 1992).

Familial amyloid polyneuropathy (FAP), which affects approximately 1 in 100 000 persons, and senile systemic amyloidosis (SSA), which affects, to some extent, 25% of the population aged more than 80 years, are transthyretin-based amyloid diseases (Benson, 1989). Normal transthyretin is found to compose the amyloid fibrils in patients with SSA. Conversely, 1 of 15 different single-site amino acid variants of TTR is the major component of the FAP fibrils (Benson, 1989; Benson & Wallace, 1989; Stone, 1990; Saraiva et al., 1983, 1984). The full-length transthyretin protein is the predominant polypeptide isolated from the fibrils in FAP. In SSA, both full-length TTR and fragments thereof are found in the fibrils [Westermark et al., 1990; see Wallace et al. (1986) and references cited therein]. Proteolytic processing does not appear to play a major role in FAP or SSA, contrary to Alzheimer's disease, where proteolytic processing seems to occur prior to amyloid fibril formation (Stone, 1990). Transthyretin is normally found in human serum where it plays a role in the transport of thyroxine and retinol, the latter

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* To whom correspondence should be addressed.

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[1] Abbreviations: TTR, transthyretin; CR, congo red; FAP, familial amyloid polyneuropathy; SSA, senile systemic amyloidosis; AD, Alzheimer's disease; CMC, critical micelle concentration; Z 3-14, *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate; OD, optical density; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; CD, circular dichroism.

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via the TTR–retinol binding protein complex. Transthyretin is a tetrameric protein (MW 55K) composed of identical 127-residue subunits, each having a β-sheet structure known to 1.8-Å resolution by X-ray crystallography (Blake et al., 1974, 1978).

Smetana in 1927 proposed that lysosomes are intimately involved in amyloid fibril formation (Smetana, 1927). Lysosomes are organelles found inside cells and are responsible for protein turnover. Protein turnover is accomplished by targeting proteins to lysosomes wherein acidic denaturation and proteolysis take place. Cohen, Glenner, and others have shown that amyloid fibrils are present proximal to and inside lysosomes in reticuloendothelial cells in animals with amyloid disease (Shirahama & Cohen, 1975; Cohen et al., 1983; Shirahama et al., 1990; Westermark et al., 1990; Glenner et al., 1971). Recently it has been shown that lysosomal/endosomal processing of β-APP affords fragments which contain the amyloidogenic peptide A4, which composes the fibrils in Alzheimer's disease (Golde et al., 1992). It is unclear how a lysosome could facilitate amyloid fibril formation in transthyretin-based amyloid diseases since appreciable amounts of proteolysis are not observed. We hypothesize that the lysosome could achieve partial denaturation of transthyretin, affording a partially denatured transthyretin intermediate which could associate into amyloid fibrils faster than it could be degraded by proteolysis. In this paper, we examine the feasibility of such a mechanism using an in vitro approach to examine the physiochemical role that acid denaturation within the lysosome or endosome could play in amyloid fibril formation in vivo.

MATERIALS AND METHODS

Congo red and ammonium sulfate were purchased from Sigma, phenol was acquired from Fisher, and the detergent Z 3-14 was obtained from Calbiochem. Rabbit IgG antibody against human TTR was purchased from Accurate Antibodies. Other reagents used were of the highest purity available.

Transthyretin Isolation and Purification. Transthyretin was isolated and purified from pooled human plasma using a three-step process. A 5% phenol (750 mL) solution was added dropwise to 1 L of plasma containing NaCl (20% w/v). Most of the plasma proteins precipitated while the majority of transthyretin remained in solution. The protein was dialyzed against 0.05 M Tris, pH 7.5, for 24 h and concentrated 5-fold by ultrafiltration. TTR was precipitated by ammonium sulfate fractionation between 55 and 85% saturation. The ammonium sulfate pellet containing transthyretin was dissolved in 0.05 M Tris buffer, pH 7.5, dialyzed against the same buffer for 5 h, and loaded onto a DEAE-Sephacel column preequilibrated with the same Tris buffer. A 0–0.3 M NaCl gradient was applied, and transthyretin eluted as the major peak. A G-10 column eluted with H_2O was used to remove the salt, and the pooled fractions were then lyophilized to yield 80–105 mg of TTR. We verified that transthyretin was pure by SDS–PAGE (Laemmli, 1970). The integrity of the primary structure was verified by laser desorption mass spectrometry: observed MW = 13 761; expected MW = 13 762 (spectrum by Professor Brian Chait, The Rockefeller University).

Transthyretin Assay. This protein has no known enzymatic activity. The integrity of the tertiary and quaternary structure of TTR was verified by fluorescence quenching brought about by thyroxine binding (Nilsson et al., 1975). A Western blot analysis revealed that the antigenic sites of TTR were still intact after purification. SDS–PAGE (12%) provides a fast and reliable way to identify transthyretin in a mixture of plasma proteins. When boiled and unboiled samples in 2% SDS are loaded onto an SDS gel, transthyretin migrates through the gel as monomer or dimer, respectively.

Preparation of Stock Protein Solutions. Transthyretin was dissolved in 0.01 M phosphate buffer/0.1 M KCl, pH 7.5, affording a concentrated stock solution (10–15 mg/mL). Dilution of the stock solution with the desired buffer afforded a solution in which the exact TTR concentration was determined by measuring the absorbance at 280 nm using an extinction coefficient of 7.76×10^4 M^{-1} cm^{-1} [$A^{1\%} = 14.1$ (1-cm cell)] (Van Jaarsveld et al., 1973).

Monitoring the Structural Changes by Circular Dichroism Spectroscopy. Far-UV CD experiments were performed by incubating TTR solutions (0.2 mg/mL) in the appropriate buffer (0.05 M sodium acetate or sodium phosphate/0.1 M KCl) at 25 °C for ≥4 h in the absence or presence of Z 3-14 (0.05 mg/mL). Spectra were recorded in a 0.1-cm pathlength cell employing a Jasco 600A CD spectrometer. Near-UV CD experiments were carried out under identical conditions using a 0.6 mg/mL TTR solution in a 1.0-cm pathlength cell at 37 °C. Both near- and far-UV experiments were also performed as a function of time to monitor denaturation.

Monitoring Structural Changes by UV Spectroscopy as a Function of Time. TTR from the concentrated stock solution preequilibrated at 37 °C was rapidly added to an acetate buffer solution at the desired pH (0.05 M sodium acetate, 0.1 M KCl, 1.2 mM $CaCl_2$, and 0.5 mg/mL Z 3-14) at 37 °C in a UV quartz cell to obtain a 0.6 mg/mL TTR solution. The conformational changes occurring during the acid denaturation of TTR were monitored as a function of time at 285 nm in a Milton Roy Spectronic 3000 diode array UV spectrometer fitted with a thermoelectric cuvette holder having stirring capabilities.

Glutaraldehyde Cross-Linking Studies To Follow the Time Course of Denaturation. A 250-μL solution (0.3 mg/mL) of TTR in 0.05 M sodium acetate buffer/0.1 M KCl at the desired pH in the presence of 0.5 mg/mL Z 3-14 was incubated at 37 °C. After the incubation period, the pH was rapidly increased by adding 150 μL of 0.6 M phosphate buffer containing 0.5 mg/mL Z 3-14, pH 7.5 (final pH was 7.0). Immediately afterward, 8 μL of a 25% glutaraldehyde solution was added and allowed to react for 2.5 min. The cross-linking reaction was terminated by the addition of 10 μL of 2 M $NaBH_4$. A 40-μL sample was removed and mixed with 40 μL of 5% SDS sample buffer. The sample was boiled for 10 min and loaded onto a 12% SDS–PAGE gel, and the relative proportions of quaternary forms were determined by densitometry. Control experiments were performed by removing 40-μL samples right after neutralization but before cross-linking. These samples were mixed with 40 μL of 5% SDS sample buffer and loaded onto an SDS–PAGE gel (without heating the samples) to determine the amount of monomer present. Under these conditions, the monomer can be quantified as it cannot reassociate to tetramer with 0.5 mg/mL Z 3-14 present. In the presence of SDS, the tetrameric form of transthyretin is denatured to the dimer; hence, only the amount of monomer relative to dimer can be discerned.

Measuring the Rate of Amyloid Fibril Formation. The rate of transthyretin fibril formation was determined by diluting a stock TTR solution preincubated at 37 °C into 0.05 M sodium acetate buffer at the desired pH at 37 °C to obtain a final solution containing 0.6 mg/mL TTR (11 μM) (0.1 M KCl and 1.2 mM $CaCl_2$). The samples were incubated at 37 °C in a quartz cuvette while stirring slowly to avoid foaming.

The optical density (OD) at 330 nm was measured as a function of time, and the slope of this line was used to determine the rate of fibril formation. Renaturation-induced amyloid fibril formation rates were established by lowering the solution to pH 2.5 with HCl at 0 °C. The pH 2.5 solution was then incubated at 37 °C for 20 min before the pH was rapidly increased by adding a fixed amount of NaOH to bring the solution to the desired pH. The time dependence of the increase of the OD at 330 nm was used to obtain the rate of fibril formation. Optical density measures the total mass of the amyloid fibrils and cannot differentiate between the concentration and the molecular weight, both of which increase over the time course of the experiments. Hence, the rate of amyloidosis is reported in relative terms as ΔOD/min (Mulkerrin & Wetzel, 1989; Andreu & Timasheff, 1986).

Fibril Characterization. (A) Congo Red Binding. Transthyretin amyloid fibrils bind congo red as evidenced by a red shift of 5–10 nm in the UV absorbance spectrum of congo red (Glenner et al., 1974). Approximately 7 μmol of TTR fibrils was added to a 10 μM solution of congo red (0.05 M sodium phosphate buffer/0.1 M KCl, pH 7.5); a red-shifted congo red absorbance was observed in the UV spectra (500 nm). The congo red–TTR fibril suspension was centrifuged at 12 000 rpm for 10 min to afford a red pellet which was resuspended in water and recentrifuged. The resulting pellet remained red in color, and a suspension of this pellet yielded a characteristic red-shifted congo red–amyloid spectrum with a base-line shift due to light scattering. The fibril–congo red complex exhibits the expected green birefringence when viewed under a light microscope with a polarized light source.

(B) Electron Microscopy. A Zeiss C-10 electron microscope was used to examine the structure of the fibrils. Transthyretin fibrils formed at pH 4.0 were centrifuged and resuspended to remove soluble TTR. The fibrils were sonicated for 15–20 min in a Branson 1200 ultrasonic cleaning bath, placed as a diluted suspension on carbon-coated copper grids, and allowed to stand for 2 min before the excess solution was removed. The grid was treated with fresh 1% uranyl acetate (pH ≈4.5) for 2 min before the excess staining solution was removed by filter paper blotting, affording negatively-contrasted transthyretin amyloid fibrils (Shirahama & Cohen, 1967).

RESULTS

Dependence of the Rate of Amyloid Fibril Formation on pH. Acid-induced TTR amyloid fibril formation was monitored by the turbidity at 330 nm as a function of time at various pHs (Figure 1a). The rate (ΔOD/min) of amyloid formation was examined between pHs 3 and 5, where amyloid fibril formation was found to occur, at intervals of ≈0.3 pH unit. Amyloid fibril formation was observed both during denaturation and during reconstitution of transthyretin. Sustained amyloid formation was fastest at pH 3.6 during denaturation (Figure 1b). The greatest rate of fibril formation was observed during renaturation by jumping the pH to 4.5. Under these conditions, fibril formation was found to be ≥10 times faster than fibril formation facilitated by denaturation at pH 3.6 (data not shown). The time courses of amyloid fibril formation at four different pHs at 37 °C are shown in Figure 1a. These plots exhibit the presence of one or more kinetic phases during denaturation/fibril formation. Since denaturation is required to obtain fibrils from the tetramer, it is necessary to consider denaturation when discussing fibril formation. The data suggest that fibril formation is best described by a nucleated condensation polymerization mechanism in which several subunits must associate to form a

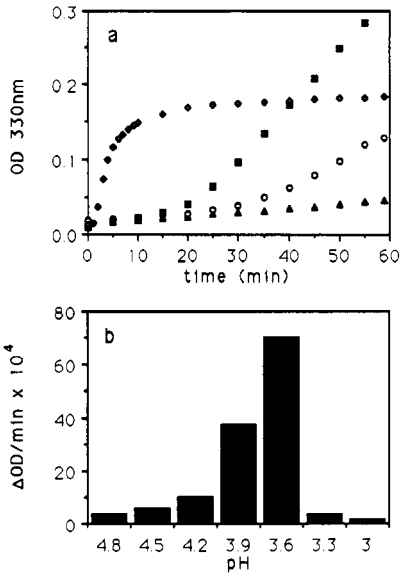

FIGURE 1: (a) Time course of TTR amyloid fibril formation at the following pHs; 4.5 (▲); 3.9 (○); 3.6 (■); and 3.3 (♦). The [TTR] was 0.6 mg/mL in 0.05 M acetate buffer, 0.1 M KCl, and 1.2 mM $CaCl_2$ at 37 °C. (b) Bar graph of the rate (ΔOD/min) of TTR amyloid formation as a function of pH at 37 °C for the longest sustained phase. The conditions listed in (a) were used here as well. The average rates of the three individual experiments with standard deviations are as follows (ΔOD/min): pH 4.8, $(3.7 \times 10^{-4}) \pm (1.4 \times 10^{-4})$; pH 4.5, $(5.6 \times 10^{-4}) \pm (3.7 \times 10^{-4})$; pH 4.2, $(1.0 \times 10^{-3}) \pm (3.8 \times 10^{-4})$; pH 3.9, $(3.7 \times 10^{-3}) \pm (1.2 \times 10^{-3})$; pH 3.6; $(7.0 \times 10^{-3}) \pm (1.9 \times 10^{-3})$; pH 3.3, $(3.6 \times 10^{-4}) \pm (3.3 \times 10^{-5})$; pH 3.0, $(1.4 \times 10^{-4}) \pm (2.6 \times 10^{-5})$.

nucleus or core before cooperative association takes place (Andreu & Timasheff, 1986). The time that it takes to assemble this core is dependent on the rate of denaturation to the amyloidogenic intermediate. Lag phases of differing durations are observed at pHs where fibril formation occurs. At low pH (3.6), denaturation affords the amyloidogenic intermediate quickly, facilitating rapid nucleation which is associated with a short lag phase. The lag phase is longer at pH 3.9 where denaturation to the amyloidogenic intermediate is slower. A log rate vs log concentration plot of fibril formation at pH 3.45 affords a line with a slope of 2.5, which is the reaction order with respect to the [TTR amyloidogenic intermediate] (data not shown). The rate of denaturation is very fast at pH 3.45; hence, complications arising from denaturation rates contribute less than at other pHs examined.

Dependence of the Rate of Denaturation on pH. The rate of TTR denaturation was probed using the absorbance at 285 nm, which served as a sensitive method to assess the environment of the tyrosine residues, a parameter sensitive to changes in tertiary and quaternary structure (Figure 2a). We have discovered that the zwitterionic detergent Z 3-14 (*N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate) inhibits amyloid fibril formation efficiently at 0.5 mg/mL during acid-induced denaturation and does not absorb at 280 nm. This micellar detergent is the only one of several nondenaturing detergents tested that facilitates reversible refolding after dilution. Dilution of the detergent concentration to ≤0.05 mg/mL during neutralization facilitates transthyretin reconstitution affording tetramer. Z 3-14 present at concentrations of 0.05–0.5 mg/mL has allowed us to probe the kinetics of denaturation without competitive amyloid fibril formation. Figure 2a shows the change in absorbance at 285 nm vs time as TTR (0.6 mg/mL) is denatured in acetate buffer at different pHs with added Z 3-14. The rate of denaturation increases as the pH is lowered. Light scattering is not observed in the

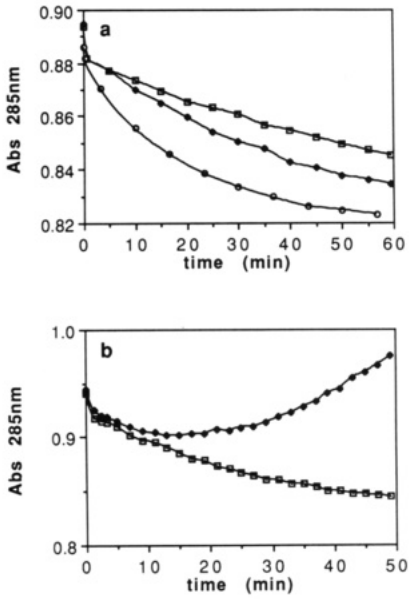

FIGURE 2: (a) Rate of TTR denaturation at pH 4.5 (□), 3.9 (♦), and 3.6 (○) is measured at 37 °C by the absorbance change at 285 nm as a function of time. [TTR] is 0.6 mg/mL in 0.05 M acetate buffer, 0.1 M KCl, and 1.2 mM $CaCl_2$ in the presence of 0.5 mg/mL Z 3-14 detergent. (b) Rate of TTR denaturation at pH 4.0 in the presence (□) and absence (♦) of Z 3-14. The experimental conditions are the same as those described in (a).

presence of Z 3-14, indicating that the detergent inhibits fibril formation. Fibril formation (via light scattering) is observed when Z 3-14 is not added (Figure 2b). Notice that light scattering starts to dominate this spectrum after approximately 10 min, which is consistent with the light-scattering experiments shown in Figure 1a. It is important to note that the time course of denaturation monitored by UV_{285nm} is not noticeably perturbed by the presence of Z 3-14 at pH 4.0 (Figure 2b).

Far- and near-UV circular dichroism spectroscopy was used to confirm our expectation that TTR was not fully denatured at pH 3.6, where amyloid fibril formation is fastest during denaturation. Transthyretin (0.2 mg/mL) was incubated at pH 3.6 for 4 h with added Z 3-14 detergent (0.05 mg/mL). The CD spectrum of this solution shows essentially no loss of secondary structure demonstrated by the nearly identical pH 7.5 and 3.6 CD spectra (data not shown). Glutaraldehyde cross-linking studies show 25–30% TTR monomer after 3 h, the remainder being tetramer and only traces of dimer (Figure 3). The near-UV CD spectra show no significant changes in the pH range of 7.5–3.75 over a time period of 24 h (data not shown). The near-UV CD spectra at pH 3.6 exhibit small but significant changes in the tertiary structure as a function of time (Figure 4). At pH 3.5, 50% of the tertiary structure was lost over 4 h, and at pH 3.3, the protein had lost >80% of its native tertiary structure over 4 h (Figure 5). These data strongly suggest that an intermediate with nativelike tertiary structure is the precursor to amyloid fibrils since amyloid fibril formation does not occur at pHs where the near-UV shows loss of native tertiary structure. It is critical to keep in mind that the CD spectra represent a mixture of states. The far-UV CD spectrum of TTR at pH 2.0 shows a small but significant increase in β-sheet secondary structure, consistent with denaturation and structural rearrangement to a compact molten globule-type conformation or "A-state" with an ill-defined tertiary structure which has been observed with other proteins (Figure 4, far-UV CD data not shown) (Goto et al., 1990).

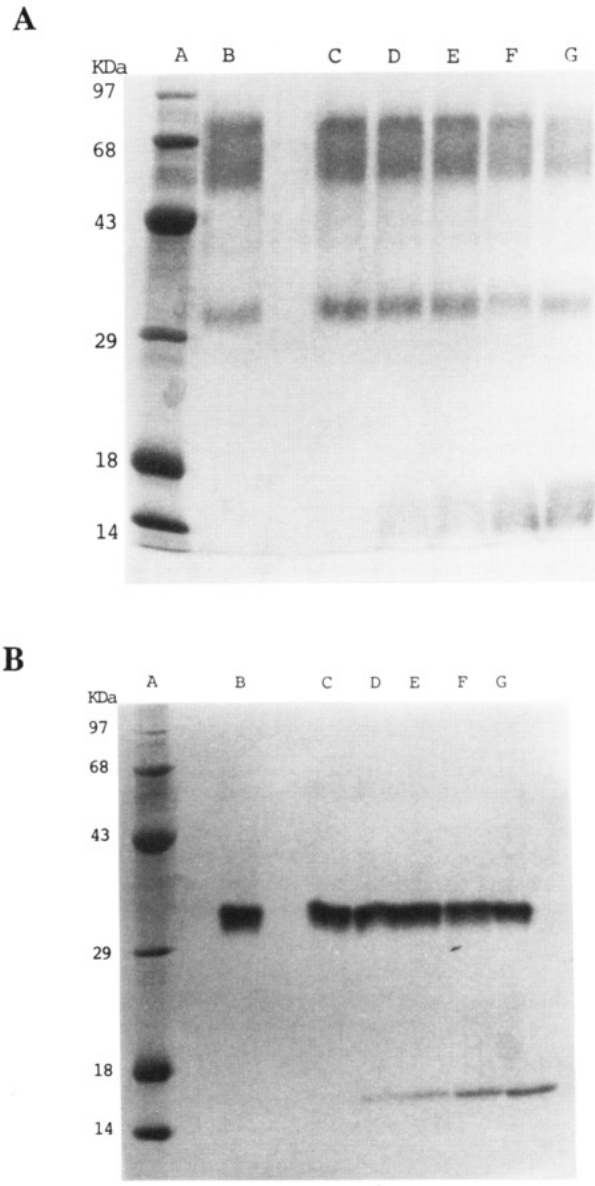

FIGURE 3: (A) SDS–PAGE gel stained with Coomassie Blue. Lanes A–G are MW standards (lane A), glutaraldehyde cross-linked TTR at pH 7.0 showing dimer and tetramer (dimer results from incomplete tetramer cross-linking; see results for details) (lane B), and glutaraldehyde cross-linked TTR after incubation at ph 3.6, 37 °C, for the indicated period (see Materials and Methods for details): for 1 min (lane C), 15 min (land D), 30 min (lane E), 90 min (lane F), and 180 min (lane G). (B) SDS–PAGE of an un-cross-linked TTR sample prepared under conditions described under Materials and Methods. Lanes A–G also represent the same as in (A).

Glutaraldehyde cross-linking studies as a function of time show predominantly tetrameric transthyretin over the pH range of 4.8–3.8, whereas monomeric transthyretin begins to appear significantly at pH 3.6 where the maximal rate of fibril formation is observed (Z 3-14 amyloid inhibitor present) (Figure 3). At pH 3.6, the concentration of the monomeric intermediate increases with time, while the tetramer band decreases with time (Figure 3). The dimer seems to be a transient intermediate since it is not significantly populated at pHs between 7 and 3 (Figure 3). It is important to note that the majority of dimer that appears in the cross-linking gels in Figure 3 comes about due to incomplete cross–linking of the tetramer. When the cross-linking experiments are corrected for incomplete cross-linking of the tetramer, less than 0 ± 3% dimer is detected between pH 5 and 3. The tetramer band at pH 3.6 represents 70–75% of the total TTR

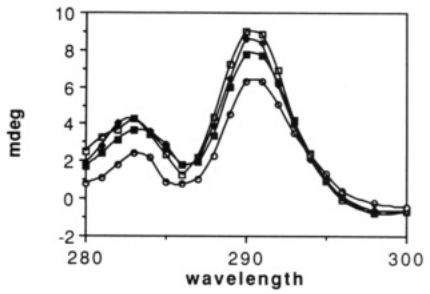

FIGURE 4: Near-UV CD spectra of a 0.6 mg/mL TTR solution in 0.05 M acetate buffer, 0.1 M KCl, and 0.05 mg/mL Z 3-14 at pH 3.6 incubated for the indicated time: 1 (□), 30 (♦), 90 (■), and 180 (○) min at 37 °C.

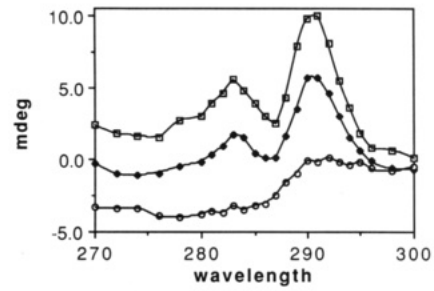

FIGURE 5: Near-UV CD spectra of a 0.6 mg/mL TTR solution in 0.05 M acetate buffer, 0.1 M KCl, and 0.05 mg/mL Z 3-14. Solutions were incubated for 4 h at 37 °C at the indicated pH: pH 3.8 (□), pH 3.5 (♦), and pH 3.3 (○).

after incubation for 3 h, the remainder being predominantly monomer with a trace of dimer, contrary to the cross-linking experiments at pH 7 where only the tetramer is present (Figure 3A). Two bands appear in the tetramer region presumably due to two different preferred cross-linked forms. This band doubling is not a result of heterogeneity in the protein. At concentrations of 0.5 mg/mL Z 3-14, the monomer cannot reassociate to the tetramer after the jump to pH 7. This allows us to quantitate the monomer concentration at low pH while doing the cross-linking at high pH, where cross-linking is efficient. We were concerned that cross-linking studies carried out under these conditions may not detect the dimer as it could possibly reassociate to afford tetramer. Therefore, we performed low-pH cross-linking experiments to ensure that this was not the case. Cross-linking studies carried out at pH 5.0–3.0 under optimized conditions revealed only traces of dimer and were quantitatively similar to those reported herein (obtained with the pH jump method), indicating that the dimer was indeed an unstable species.

Characterization of Amyloid Fibrils. Transthyretin amyloid fibrils were identified by their ability to bind congo red (data not shown). The TTR amyloid–congo red complex exhibits green birefringence when viewed in a light microscope having a polarized light source, consistent with what has been observed with other fibrils having an amyloid structure (Glenner et al., 1974; Klunk et al., 1989; Puchtler et al., 1962). We have compared our birefringence results to the high-resolution color photographs of the green birefringence observed in SSA patients. The photograph of the birefringence observed from myocardial amyloid samples is virtually identical to what we see in the case of TTR amyloid prepared by the in vitro method (data not shown) (Thomas, 1979).

Electron microscopy of negatively-contrasted transthyretin amyloid fibrils formed at pH 4.0 exhibits fibrils that are laterally associated (Figure 6B). After sonication, the fibrils dissociate from one another, affording individual fibrils (Figure 6A). The amyloid fibrils produced in vitro by acid-induced

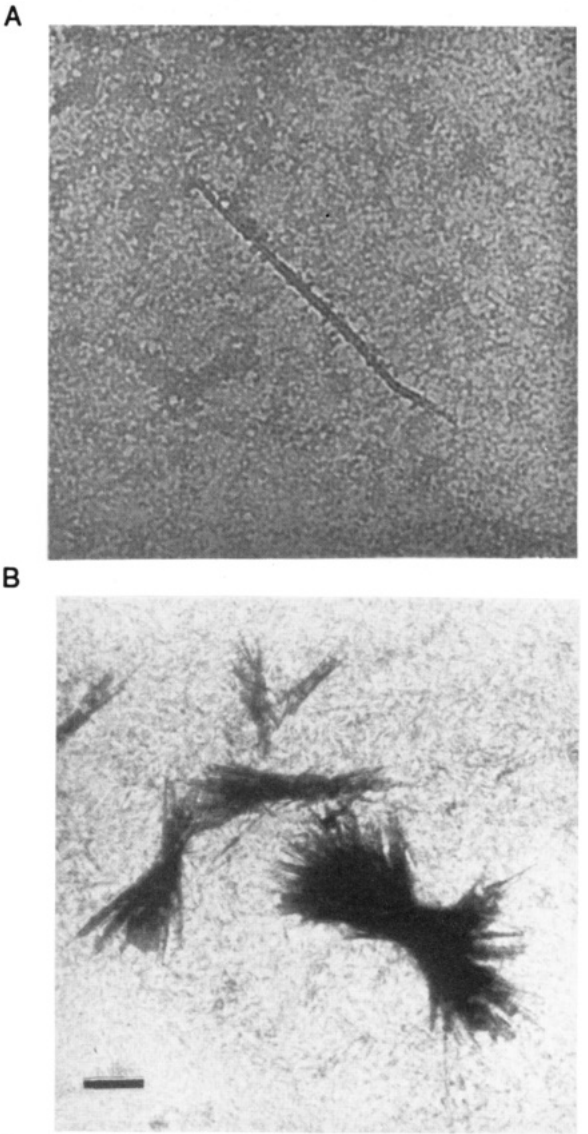

FIGURE 6: (A) Electron micrograph of a negatively-contrasted (1% uranyl acetate) fibril produced by incubating a 0.6 mg/mL solution of TTR at pH 4.0 for 1 h before sonication; fibril dimensions = 60 Å × 2400 Å; magnification 128000×. (B) Laterally associated TTR amyloid fibrils produced in vitro (see Materials and Methods for more detail): magnification 20000×; bar, 4000 Å.

denaturation accurately reproduce the macrostructure of the fibrils isolated from patients suffering from FAP or SSA (Glenner et al., 1974).

DISCUSSION

The results of kinetic light-scattering experiments in vitro demonstrate that transthyretin fibril formation is pH dependent and is fastest at pHs accessible within the lysosome in vivo (4.8–3.6) (Figure 1b) (Holtzman, 1989). Identifying with certainty the nature of the transthyretin denaturation intermediate capable of forming amyloid fibrils at low pH is complicated by the fact that fibril formation occurs over a pH range which is close to the pH where a TTR tetramer to monomer transition is operating. Our experimental data are consistent with either a structured tetrameric or a structured monomeric denaturation intermediate having nativelike tertiary and secondary structure as the likely amyloidogenic precursor. The dimer is the least likely amyloidogenic intermediate since we cannot detect a significant concentration of this intermediate over the amyloid-forming pH range (4.8–3.6) (Figure 3).

Examination of the transthyretin denaturation pathway is made possible owing to the discovery of the micellar detergent Z 3-14 which inhibits competing amyloid fibril formation without significantly affecting the denaturation pathway. Preliminary attempts to study the denaturation of transthyretin were hindered by aggregation. In spite of these difficulties, Branch demonstrated that transthyretin was not denatured by urea and found that >6 M Gu-HCl was required to unfold this protein (Branch et al., 1971). Our evidence that Z 3-14 does not significantly change the acid denaturation pathway is based on two observations: (i) after the acid-mediated denaturation of TTR in the presence of 0.05 mg/mL Z 3-14, the protein can be reconstituted by rapidly increasing the pH to 7; (ii) the denaturation time courses monitored by UV spectroscopy are virtually identical in the absence or presence of Z 3-14 until light scattering dominates the spectra in the former case (Figure 2b). Detergents have been successfully employed to prevent aggregation during the reconstitution of multidomain proteins such as rhodanese and have been used extensively by Horowitz and co-workers [see Tandon and Horowitz (1989) and references cited therein]. The Z 3-14 detergent that proved effective in our studies was first used to solubilize membrane proteins (Gonenne & Ernst, 1978). The micellar detergent presumably inhibits fibril formation by binding to the relatively hydrophobic amyloidogenic intermediate.

The tetramer is the only transthyretin quaternary form observed by glutaraldehyde cross-linking studies above pH 5.0 (data not shown). The tetrameric form of TTR undergoes a subtle rearrangement at pH 5.5 as the pH is lowered which is detected through the Tyr absorbance at 285 nm (data not shown). This rules out the native tetramer as the amyloidogenic intermediate. Since the dimer concentration never increases in the fibril-forming pH range, it is unlikely that it is the amyloid fibril precursor (Figure 3).

The presence of tetrameric and monomeric transthyretin were detected by glutaraldehyde cross-linking studies in the pH range where fibril formation was fastest (Figure 3). The rate of amyloid fibril formation increases as the pH is lowered to 3.6 during denaturation as does the monomer concentration at the expense of the tetrameric TTR as determined by time-dependent glutaraldehyde cross-linking experiments (Figures 1 and 3). The far-UV CD spectrum of transthyretin remains the same from pH 7.5 to 3.75 after incubation for 4 h, suggesting that the amyloidogenic intermediate has native amounts of β-sheet secondary structure. The near-UV CD spectrum of TTR is mostly native-like even at pH 3.6 (Figure 4). The time course of TTR denaturation at pH 3.6 by near-UV CD seen in Figure 4 shows moderate changes in the CD spectrum after 30 min (25% change at 3 h) consistent with an intermediate that has most of its tertiary structure intact. Neither the spectroscopic experiments nor the cross-linking experiments allow one to conclusively identify the amyloidogenic intermediate from the two most likely candidates, i.e., the structured rearranged tetramer and the structured monomer. Strong arguments can be made for each.

The time courses of amyloid fibril formation (Figure 1a) as a function of pH are consistent with denaturation-modulated assembly of a TTR denaturation intermediate. At pH 4.5, 3.9, and 3.6, denaturation to the amyloidogenic intermediate appears to be the rate-determining step in fibril formation. The assembly process at pH 4.5 is very slow, presumably due to the low steady-state concentration of the amyloidogenic denaturation intermediate. At pH 3.9, denaturation is faster, and two phases are observed: (1) a lag phase (see Results for an explanation) and (2) a cooperative assembly phase. At pH 3.6, denaturation to the amyloidogenic intermediate is even faster as supported by the shorter duration of the lag phase and the increased rate of fibril formation, suggesting that the concentration of the amyloidogenic intermediate is higher. At pH 3.3, a very short lag phase is observed followed by rapid fibril formation, an indication that denaturation to the amyloidogenic intermediate is very fast. However, in the time frame observed, it can be seen that fibril formation reaches a stationary phase after about 10 min, consistent with denaturation beyond the structured amyloidogenic intermediate capable of fibril formation (Figure 1a). Denaturation beyond the transthyretin amyloidogenic intermediate is supported by the near-UV CD studies at pH 3.3 which exhibit loss of native tertiary structure after denaturation (Figure 5).

The change in electrostatic interactions caused by protonating ionizable groups in TTR is expected to change the structure during denaturation (Perry et al., 1989). The acid-induced denaturation of monomeric proteins has received considerable attention by Fink and others (Goto et al., 1990). Briefly, they have found that the acid-induced unfolding of proteins is often incomplete when compared to the Gu-HCl-induced denaturation. Around pH 2, proteins are maximally protonated and unfolded under conditions of low ionic strength. The addition of acid or salt to the maximally unfolded protein at pH 2 results in a cooperative transition to a compact state having a large amount of secondary structure and disordered tertiary structure called the "A-state". This state is very similar to the molten globule state reported for several proteins (Goto et al., 1990). Anion binding to the positively charged groups has been shown to be responsible for the formation of the A-state. Preliminary studies suggest that TTR does not readily unfold; rather, it appears to adopt an "A-state" structure as evidenced by the loss of tertiary structure and a small increase in the amount of β-sheet secondary structure at pHs below 3 (Figure 5, far-UV results not shown). Detailed TTR denaturation studies are underway to completely characterize the denaturation pathway.

Our data support the concept that the intralysosomal/endosomal pH is sufficient to form transthyretin amyloid fibrils (Holtzman, 1989). It is likely that amyloid fibril formation occurs in lysosomes/endosomes that are improperly functioning. One could imagine that fibril formation occurs in a lysosome that may be overloaded, protease deficient, at an abnormally low pH, or otherwise defective. Invoking a denaturation intermediate as the precursor to amyloid fibril formation is a new idea; contrarily, the concept that a conformational intermediate on the folding pathway of a given protein can associate has been amply demonstrated in the literature. In most cases, folding intermediates associate to form amorphous aggregates (Zettlmeissl et al., 1979); however, in some cases, highly ordered associations of conformational intermediates can occur (London et al., 1974; Brems, 1988; Turkewitz et al., 1988; King, 1986). That protein reconstitution and aggregation (or denaturation and aggregation) are competitive processes has been verified by the elegant work of Jaenicke, Goldberg, Brems, Horowitz, and King, which has inspired this investigation (Jaenicke, 1987; Zettlmeissl et al., 1979; Brems, 1988; King, 1986; London et al., 1974).

CONCLUDING REMARKS

These in vitro studies demonstrate that transthyretin can self-assemble into transthyretin amyloid fibrils in the pH range accessible to lysosomes in vivo, supporting the feasibility of lysosomal involvement in human amyloid disease (Holtzman,

1989). Partial denaturation alone is sufficient to effect amyloid fibril formation in vitro, explaining the role that the lysosome could play in amyloid diseases where proteolysis of the precursor is not observed. Identifying with certainty the nature of the transthyretin denaturation intermediate capable of forming amyloid fibrils is complicated by the fact that fibril formation occurs over a pH range which is close to the pH where the tetramer to monomer transition is operating. Our experimental data are consistent with either a rearranged tetrameric or a monomeric TTR denaturation intermediate having nativelike tertiary and secondary structure as the likely amyloidogenic precursor. Further experiments are underway to definitively identify this intermediate and to further characterize the denaturation pathway utilizing the amyloid inhibitor Z 3-14.

ADDED IN PROOF

During the course of this study, Westermark and colleagues reported that concentrated transthyretin solutions (10 mg/mL) and two peptidic fragments of transthyretin formed amyloid-like fibrils in vitro when dissolved in a 10% acetic acid solution (pH = 2.3) (Gustavsson et al., 1991). We have reproduced their experiments and have found predominantly aggregated TTR that does not have an amyloid structure as reported. If the copper grids used in electron microscopy are searched thoroughly, amyloid fibrils can be found, but they represent only a small percentage of the total amount of transthyretin present. Under our conditions (pH 4.0), we see predominantly amyloid fibrils in the EM experiment. Since Westermark's experiments were carried out at pH 2.3, where TTR has lost its tertiary structure, we reason that the small amount of fibrils produced in Westermark's experiments originate from the amyloidogenic intermediate which is transiently populated during denaturation at pH 2.3. The high concentrations of TTR used in these experiments (10 mg/mL) make this explanation plausible.

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