Point mutations in the human plasma protein transthyretin are associated with the neurological disorder familial amyloidosis with polyneuropathy type 1. The disease is characterized by amyloid fibril deposits causing damage at the site of deposition. Substitution of two amino acids in the hydrophobic core of transthyretin lead to a mutant that was very prone to form amyloid. In addition, this mutant has also been shown to induce a toxic response on a neuroblastoma cell line. Renaturation of the transthyretin mutant at low temperature facilitated the isolation of an amyloid-forming intermediate state having the apparent size of a dimer. Increasing the temperature effectively enhanced the rate of interconversion from a partly denatured protein to mature amyloid. Using circular dichroism the β-sheet content of the formed mature fibrils was significantly lower than that of the native fold of transthyretin. Morphology studies using electron microscopy also indicated a temperature-dependent transformation from amorphous aggregates toward mature amyloid fibrils. In addition, 1-anilino-8-naphtalenosulfonate fluorescence studies suggested the loss of the thyroxin-binding channel within both the isolated intermediate and the mature fibrils.

Transthyretin (TTR) is a thyroid hormone transport protein and forms a complex with retinol binding protein in plasma (1, 2). Through undefined mechanisms, TTR can misfold into amyloid fibrils. This property is shared with a limited set of 20 other proteins. Although detailed molecular structures are currently unavailable, amyloid fibrils share many structural and morphological characteristics, which are not foreseen in the primary, secondary, or tertiary structure of the precursor proteins (3). The fact that all amyloid fibrils bind thioflavine-T and Congo red with specific spectroscopic characteristics indicates they have unifying physical properties. All amyloid fibrils consist of protofilaments wrapped around each other (3, 4). Moreover, they are all generally about 70–100 Å in diameter without branching points. Interestingly, fibrils are rich in β-structures that are usually arranged perpendicularly to the fibril axis (5). Familial amyloidosis with polyneuropathy type 1 (FAP1) is a genetic disorder with dominant inheritance but with low penetrance. More than 70 independent point mutations in TTR are known, and most of these are associated with clinical manifestation of amyloidosis (6). The age of onset in FAP1 patients varies between 20 to 60 years depending on the type of mutation (7, 8). However, mutations are not a necessary requirement for amyloid formation, because even the wild type TTR can form amyloid fibrils in elderly individuals, a condition known as senile systemic amyloidosis (9). Therefore, the ability to form amyloid is an inherent property of the polypeptide, with an increased risk of developing the disease being a result of amino acid substitutions.

The folding properties of TTR are complex, because a pronounced hysteresis exists between unfolding and refolding after denaturation in chaotropic agents (10), suggesting that kinetic barriers intervene between the two states. In addition, upon dilution to submicromolar concentrations, wild type TTR dissociates into monomers. Because this monomer has lost its native fold (11), individual monomeric units are thermodynamically unstable. Partial denaturation has also been postulated as a prerequisite for amyloid formation, and in the case of TTR experimental evidence has been presented to support this theory (12, 13). Moreover, the loss of the native fold of TTR upon amyloid formation (13–15) suggests that the folding pathways exhibit more than one stable minimum under physiological conditions.

X-ray diffraction methods, used to elucidate the native conformation of TTR, resulted in a well-defined structure showing a homotetramer were each subunit contains eight β-strands denoted A–H, each having a β-barrel sandwich fold. Two monomers associate via hydrogen bonds between the F–F and H–H interactions, whereas two dimers associate back to back (16). A ribbon structure of the dimer is depicted below in Fig. 1. Interestingly, crystal structures of naturally occurring amyloidogenic mutants only reveal subtle changes in the tertiary structure compared with the wild type protein (17). Increasing evidence supports the theory that a tetramer dissociation precedes amyloid formation. Low pH has been shown to induce TTR amyloid (18), and formation also correlates well with a dissociation of the tetramer into monomers (12). The tetramer dissociation rate of clinically severe mutations is also significantly increased compared with wild type TTR, which suggests a loss of stability in these mutant precursor proteins (13, 19). Moreover, a dilution of TTRL55P generates monomers that form protofibrils upon concentration (20).

Spontaneous formation of a mature amyloid under physiological conditions has not been reported for any of the TTR mutants that result in disease. However, several weeks of
incubation of the TTRL55P and TTRV30M mutants at 37 °C gave rise to protofilament structures, which are likely to constitute mature amyloid structures (21, 22). Therefore, the slow kinetics of amyloid formation by in vivo relevant mutants makes them unsuitable as an experimental model system to search for a biochemical understanding of the amyloid phenomenon and the mechanism behind its neurotoxicity. To overcome this limitation we have developed mutants with a potential to form amyloid far beyond the ability of naturally occurring TTR mutations (14). The morphology and the tinctorial properties of these fibrils are indistinguishable from ex vivo extracted amyloid. Nevertheless their physical and biochemical properties could differ from those found in nature. In the mutant design we focused on a previously identified mutational hot spot in a flexible area at the edge of the molecule (23–25). The crystal structure of the highly amyloidogenic variant TTR-D,2 clearly shows a displacement of the D-strand generating a novel β-strand interaction via hydrogen bonding in a new register (26). To extend this work, the two valines located at positions 14 and 16 were exchanged for asparagine and glutamate, respectively (TTR-A).3 These two valines are normally embedded in a highly hydrophobic environment within the native structure and form hydrogen bonds with the D-strand (16).

This TTR-A mutant has a very high tendency to aggregate into fibrillar structures. Furthermore, TTR-A amyloid also showed a cytotoxic effect on cultured neuronal cells4 and reacted with monoclonal antibodies with specificity for TTR-amyloid. These initial observations prompted us to characterize the folding pathway for this mutant. The results of this analysis are described herein. Renaturation studies identified a dimeric intermediate having a high content of random coil, which could be transformed into amyloid. The fibrils were soluble and appeared to have significantly less β-sheet structure than the native tetrameric fold. Moreover, the hydrophobic channel containing the thyroxin binding site was not preserved in either the dimer or the formed fibril (Fig. 1). In addition, the path from an intermediate molecule to mature amyloid were shown to be strictly controlled by temperature, and the morphology proceeded from amorphous aggregates to mature amyloid via the appearance of protofibrils in a condensation process.

**MATERIALS AND METHODS**

**Chemicals—**All chemicals used were of the highest analytical grade and generally purchased from Sigma-Aldrich (Stockholm, Sweden), unless otherwise indicated.

**In Vitro Mutagenesis—**The pET3a bacterial expression system (Novagen) was the parental derivative in all constructs. Enzymes were purchased from Bio labs In Vitro AB, Stockholm, Sweden. Oligonucleotides were purchased from, Life technology, Uppsala, Sweden. The TTR-A mutant was produced using polymerase chain reaction site-directed mutagenesis as described previously (14). The flanking primers used contained 5′-Ndel and 3′-BamHI sites, respectively, and the sequence was 5′-GCG GGA TCC TTA TTC CTT GGG ATT GGT GAC-3′ and 5′-GGG GGA TCC TTA TTC CTT GGG ATT GTT GAC GAC-3′ respectively. This generated a methionine in position –1. For the introduction of an asparagine and a glutamate in positions 14 and 16, respectively, the primer combination of 5′-CTG GCA TAT GGG ACC TAC GGG CAC CCG T-3′ and 5′-GGG GGA TCC TTA TTC GGT AGT ATT GAT GAC-3′ was used. The correct DNA sequence was verified using an ABI 377 sequencer.

**Purification of the Recombinants TTR Mutants—**Plasmids were transformed into competent Escherichia coli BL21 cells as previously described (14). The cells were lysed with lysozyme (1 mg/ml) for 30 min at room temperature and treated with DNase in the presence of 20 mM Mn2+ ions until the solution was no longer viscous. The TTR-A and TTR-D mutants formed inclusion bodies, which were washed three times with phosphate buffer (0.05 M, pH 7.5) and three times with 2 M GdnHCl through repeated centrifugation steps at 20,000 × g. The pellets were finally dissolved in 8 × GdnHCl, and debris was removed through centrifugation for 20 min at 20,000 × g followed by blocking of the free thiol group in position 10 as described below. The protein was then dialized against 20 mM phosphate buffer containing 50 mM NaCl. Final purity was ~95% as determined by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. Wild type TTR did not form inclusion bodies; this allowed the supernatant obtained after lysis of the cells to be directly purified using an anion column (MonoQ HR 10/10, Amersham Pharmacia Biotech, Uppsala, Sweden) in a NaCl gradient. 95% pure fractions were pooled and dialyzed against phosphate buffer (20 mM, pH 7.5) containing 50 mM NaCl.

**Blocking of Free Thiol Groups—**Earlier unpublished observations for the TTR-A and TTR-D mutant have revealed a high propensity to form an undesirable intermolecular disulfide bridge involving the cysteine at position 10. To overcome this concern the proteins were solubilized in GdnHCl containing 20 mM β-mercaptoethanol. Addition of iodoacetamide to a final concentration of 50 mM selectively blocked the free thiol. Selectivity was routinely verified by mass spectroscopy.

**Size Exclusion Chromatography—**A 120-ml column containing Sephadex G-75 (Superdex G-75 16/60, Amersham Pharmacia Biotech) con-

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2 TTRG33S/E54D/L55S.
3 TTRV14N/V16E.
4 K. Andersson and E. Lundgren, manuscript in preparation.
nificant inhibitory effect on amyloid maturation. Therefore, investigation, this phenomenon has been shown to have a significant effect on the cysteine at position 10 was observed. In a parallel investigation the presence of an intermolecular disulfide bond involving the cysteine at position 10 was blocked by iodoacetamide in all TTR variants used in this study.

Identification of a Dimeric Intermediate—To follow the amyloid formation pathway of TTR-A, we wanted to use a non-aggregated form of the protein from which fibril formation could be induced. Previously it was reported (21) that the kinetics of TTR aggregation is markedly slowed by low temperature. Therefore, purified protein was dissolved in 8 M urea and loaded onto a gel filtration column previously equilibrated with a buffer of 20 mM phosphate buffer, pH 7.0, containing 50 mM NaCl and held at 4 °C. Gel filtration effectively removed the urea, and the protein eluted as a rather broad peak. The main fraction eluted from the column in various concentrations. All gel-filtration analyses were performed at 4 °C with a flow rate of 1 ml/min. The following proteins were used as molecular mass standards: thyroglobulin, 670 kDa; IgG, 158 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; trypsin inhibitor, 20 kDa; myoglobin, 17 kDa; and hen egg lysozyme, 14.4 kDa. Thyroglobulin and IgG migrated outside the linear range of the column and were therefore not included in the standard curve. The correlation coefficient (r²) between proteins of known molecular mass and their elution rates was calculated to be 0.9947.

Electron Microscopy—A solution of TTR-A protein was prepared in distilled water. A drop of each solution was placed for 30 s on freshly glow-discharged, 300-mesh copper grids coated with carbon. The solution was subsequently rinsed with water and negatively stained with 2% uranyl acetate. The grids were air-dried and examined using a Zeiss EM 900 electron microscope.

Trystophan Fluorescence—Intrinsic tryptophan fluorescence was employed to probe any structural changes arising both from the dissociation of the fibril or the refolding of the denatured molecule. Proteins were analyzed in 0.02 M phosphate-buffer M (pH 7.5) containing 50 mM NaCl. The final protein concentration was 0.02 mg/ml. Renaturation studies were initiated through a 100-fold dilution of a 2 mg/ml protein solution containing 8 M GdnHCl. A 295-nm excitation wavelength, known to be specific for tryptophan, was employed, and fluorescence emission spectra were collected between 307 and 430 nm. The wavelength shift was monitored using the integral midpoint of the curve. All measurements were performed at 20 °C in a 1-cm quartz cuvette. The slit widths for excitation and emission, respectively, were set to 3 and 5 nm.

1-Anilino-8-naphthalenesulfonate Fluorescence—Various concentrations of ANS were prepared in 20 mM phosphate buffer, pH 7.0, containing 50 mM NaCl and filtered through a 22-μm filter. The protein concentration was 1 μM, and the slit widths were set to 5 nm for the excitation and 10 nm for the emission. The temperature was maintained at 4 °C for all measurements.

Circular Dichroism (CD) Spectroscopy—The secondary structure was monitored using a Jobyn Yvon-Spex CD-6 spectrophotometer. The protein was dissolved in 20 mM phosphate buffer, pH 7.0, containing 50 mM NaCl and adjusted to a final concentration of 10 μM. All spectra were recorded from 198 to 260 nm, and the temperature was adjusted in intervals using a waterbath (NESLAB Instruments, Portsmouth) with a 15-min equilibration time between each new spectrum.

RESULTS

Expression of TTR-A in E. coli resulted in the formation of inclusion bodies. In addition, during the course of this investigation the presence of an intermolecular disulfide bond involving the cysteine at position 10 was observed. In a parallel investigation, this phenomenon has been shown to have a significant inhibitory effect on amyloid maturation. (Therefore, to avoid putative misinterpretation of the results the thiol group of cysteine 10 was blocked by iodoacetamide in all TTR variants used in this study.)

Identification of a Dimeric Intermediate—To follow the amyloid formation pathway of TTR-A, we wanted to use a non-aggregated form of the protein from which fibril formation could be induced. Previously it was reported (21) that the kinetics of TTR aggregation is markedly slowed by low temperature. Therefore, purified protein was dissolved in 8 M urea and loaded onto a gel filtration column previously equilibrated with a buffer of 20 mM phosphate buffer, pH 7.0, containing 50 mM NaCl and held at 4 °C. Gel filtration effectively removed the urea, and the protein eluted as a rather broad peak. The main fraction of the elution volume corresponding to approximately half of the TTR tetramer size and was denoted TTR-A^DIMER (Fig. 2A). Protein molecular masses were determined using a standard curve based on a range of globular proteins defined under “Material and Methods.”

At 4 °C, the TTR-A^DIMER remained stable for 24 h with no significant aggregation as monitored by re-injection on the column (Fig. 2C). However, we subsequently noted at temperature-dependent aggregation kinetics. Following a brief incubation of the TTR-A^DIMER at 37 °C corresponding to 20 min, the protein rapidly aggregated to a molecular mass above 600 kDa (Fig. 2D). Aggregation was essentially irreversible, because reloaded aggregated material did not dissociate to low molecular masses. From this finding, we interpret that a high free energy barrier exists between the aggregated state and its free constituents under the experimental conditions used.

In addition, serial dilution of the TTR-A followed by elution on the size exclusion column revealed a concentration-depend-
ent effect on the aggregation state. The upper limit was set by aggregation, and we could only isolate the TTR-A_dimer at the highest concentration, ~20 μM. Interestingly, dissociation to a monomeric species could be observed at sub-micromolar concentrations (Fig. 2B).

To investigate whether or not the dimeric species was a general phenomenon concerning highly amyloidogenic TTR mutants, we also analyzed the previously described TTR-D^2 (14). Upon refolding of TTR-D on the size exclusion column, this molecule showed comparable behavior as observed for TTR-A, in particular, a dominant peak having the size of a TTR dimer around 30 kDa, which was denoted TTR-D_dimer (data not shown).

The dimeric peaks from refolding of both TTR-A and TTR-D showed a broadening, indicating a range of conformations with different exclusion volumes. From our analysis of serial dilutions at 4 °C we concluded that no rapid equilibrium between the dimeric and the monomeric species exists, because the molecular mass remained constant regardless of the protein concentration. The difference in molecular mass shown in Fig. 2B is simply just an effect of overlapping peaks.

Refolding of Wild Type TTR Also Renders a Dimeric Intermediate—Examination of wild type TTR refolding on a size-exclusion column gave rise to three defined peaks represented by molecular masses corresponding to a tetramer, a dimer, and a monomer. The tetramer had an apparent molecular mass of 55 kDa, which is in good agreement with its theoretical value. Under these conditions the dimer could only be observed and isolated in concentrations around 2 μM and showed a significant overlap with a monomeric species. This fact rendered it less suitable for further characterization. We noted that refolding at various protein concentrations significantly altered the proportions of each peak. In Fig. 2E a serial dilution of wild type TTR, where the intensity of the peak has been normalized against the monomeric species, is shown. Upon protein dilution, the tetrameric and dimeric species notably diminished in favor of a monomer. This is in accordance with thermodynamic laws and reflects the associative forces between the molecules. Because all peaks could be seen simultaneously and no major shifts in the molecular mass were observed we conclude that only slow exchange (hours) exists between the different species under these conditions. This correlates with the results of refolding experiments involving both TTR-A and TTR-D.

Stability Studies in GdnHCl Monitored by Tryptophan Fluorescence Spectroscopy—To elucidate structural information and to investigate the overall stability of the highly amyloidogenic mutants, intrinsic tryptophan fluorescence was employed during unfolding and refolding. TTR contains two tryptophans at positions 41 and 79. Denaturation curves of wild type TTR showed a red shift from 342 to 358 nm when the GdnHCl concentration was increased from 0 to 8 M. The main transition was observed between 6 and 7 M (Fig. 3A), that reached a maximum between 55 and 60 °C (Fig. 4). This was also associated with a change in fold, the secondary structure of the formed aggregates.

Far UV CD Analysis—We observed high molecular aggregates that did not dissociate through dilution under physiological conditions. To investigate whether or not this phenomenon was also associated with a change in fold, the secondary structure was monitored using CD spectroscopy. TTR-A_dimer was subjected to a temperature scan ranging from 5 to 95 °C (Fig. 4B). The CD spectra showed a significant shift from a partially structured molecule toward a molecule with a higher β-sheet content (Fig. 4A) that reached a maximum between 55 and 60 °C (Fig. 4B). However, at temperatures above 60 °C, a loss of β-sheet structure was seen (Fig. 4B), and at 95 °C a high content of random coil was obtained (data not shown). Nevertheless, the highest observed β-sheet content was significantly lower than that of the native fold of wild type TTR (Fig. 4A). We used a reversed scan between 55 and 5 °C to verify that the acquisition of β-structure was essentially irreversible (Fig. 4B). Significantly, TTR-D displayed the same behavior whereas native wild type TTR was essentially unaffected by temperature shifts between 5 and 60 °C (data not shown). This was ex-

![Fig. 3. GdnHCl denaturation and renaturation monitored by tryptophan fluorescence.](http://www.jbc.org)
expected, because the native tetramer does not melt until temperatures are near the boiling point (27).

ANS Binding—The fluorescent dye ANS was used to identify solvent-exposed hydrophobic areas. Because these are more prevalent within partly denatured structures, the amount of bound ANS indicates the degree of unfolding. The local environment around the ANS molecule contributes to its fluorescent properties, and a hydrophobic localization gives a higher intensity and displays a blue shift compared with a solvent-exposed surface-bound molecule. In addition, it is known that two ANS molecules associate tightly to the thyroxin T4 binding site, located in the hydrophobic core of the native TTR tetramer, resulting in a strong fluorescence intensity (28). Upon mixing a constant amount of protein with various concentrations of ANS, wild type TTR gave a strong fluorescence intensity even at a low ANS/monomer ratio in clear agreement with its described occupancy of the T4 binding channel (Fig. 5A). At saturation only seven ANS molecules/monomer were found, indicative of a well folded molecule (Fig. 5A). In contrast, the TTR-ADIMER exhibited a low fluorescence intensity, and an approximate linear relationship between ANS concentration and intensity was observed (Fig. 5A). Equilibrium was obtained at a ratio of ~50 ANS per monomer (Fig. 5A). In addition, the spectrum showed a red shift of about 10 nm compared with wild type TTR (Fig. 5B). Moreover, the aggregated state of TTR-ADIMER showed a strong fluorescence intensity and a blue shift (Fig. 5B). ANS binding to wild type TTR was not affected by a preceding incubation at 55 °C (data not shown). Taken together these data support the notion that the TTR-ADIMER is highly unstructured, and the fold becomes more defined once the protein aggregates. Moreover, it seems that the hydrophobic channel is not preserved or inaccessible to ANS within the fibril.

Tinctorial Properties and Morphology—Spectroscopic analysis of the aggregates obtained from the TTR-A mutant gave a positive signal using both Congo red and thioflavine-T (data not shown). Using electron microscopy, a marked difference was observed between aggregates obtained after incubation at different temperatures. After 30 min in 25 °C only amorphous aggregates could be observed (Fig. 6A). Furthermore, prolonged incubation for 24 h rendered a condensation of the amorphous aggregates and some fibril structures, mostly resembling protofilaments (3, 21) with a diameter around 50 Å, could be discerned (Fig. 6B). However, after 72 h at 25 °C a high percentage of fibrils was observed with a more condensed structure and more intense borders defining the edges of the fibrils (Fig. 6C). Their diameter ranged between 85 and 95 Å, consistent with mature fibrils. Nevertheless, they differed in structure from ex vivo isolated material, being shorter in length and also notably curved. This phenomenon has previously been described for the Alzheimer Aβ-peptide and denoted protofilaments (29), and the morphology has recently also been described in the case of TTR (20). Upon prolonged incubation at room temperature no significant change of the morphology was detected. From CD analysis we showed that an increase in temperature of the TTR-ADIMER generated more β-structures. Because these are known to be more prevalent within amyloid structures, we analyzed the morphology of TTR-ADIMER after incubation at 55 °C. This treatment rapidly shortened the maturation time, and a transition from amorphous aggregates to mature fibrils, having a condensed and straighter structure, was observed even after 10 min (Fig. 6). Clearly, these aggregates were mature amyloid. Furthermore, given their diameter measurement, 90 Å, they were likely to consist of laterally arranged protofilaments (3). All morphology studies have been confirmed using atomic force microscopy (data not shown).
DISCUSSION

The therapeutic challenge in all forms of amyloidosis is to prevent fibril formation and/or dissolve previously formed fibrils. To achieve this goal an increased knowledge about the properties of amyloid and the pathways leading to its formation is necessary. In particular, key areas of study include identifying putative precursor folds, intermediates, and interacting areas within the fibril.

In this study we have shown that two single-amino acid substitutions in the hydrophobic A-strand of TTR is highly unfavorable for the native tetrameric conformation. In addition, these mutations generate an unstable molecule that is prone to form amyloid even at physiological pH and ionic strength. Prior to amyloid formation, however, a stable dimeric species could be trapped at low temperature. This discovery is in good agreement with a recent finding (30) suggesting that the native dimeric interactions are preserved within the amyloid fibril. At sufficiently low concentration also a monomeric species was obtained that likely reflects the interacting forces within the dimer. The dimeric fraction could then be transformed into different stages of aggregation that increase the temperature. Using electron microscopy, a transition could be observed from amorphous aggregates to mature fibrils via protofibrils. Thus, the pathway from a populated precursor toward mature amyloid involves several less ordered states that are likely to be kinetically locked in local free energy minima on the reaction co-ordinate. The mature fibrils apparently lost the hydrophobic channel present in the native tetramer and displayed a significantly reduced secondary structure as compared with the native tetramer fold. Attempts to perform a ligand stabilization using thyroxin and various analogues, which have previously been successfully performed on tetrameric TTR variants (31), failed when using the isolated intermediate of TTR-A. The same approach was also tested using retinol binding protein without success. In both cases the results indicate that the ligand binding areas are affected or that formation of a stable tetramer from TTR-A is impossible under these conditions.

The propensity of proteins to aggregate into amyloid is expected to be unfavorable from an evolutionary point. To eliminate the risk of an entrapment into an aggregated state, the exposure time of sticky epitopes during folding should be minimized. The initial events during protein folding are generally believed to proceed through a less specific rapid hydrophobic collapse followed by a rearrangement into the native structure (32).

From our data, the hydrophobic collapse also appears to be present within amyloid formation. We interpret these findings to indicate that a destabilized native structure favors novel intra- and intermolecular interactions and leads to amyloid formation. Depending on the shape of the free energy landscape between the unfolded and the native state of a protein, intermediates may or may not emerge. In a smooth energy landscape only the precursor and the final state may be significantly populated. A general scheme concerning the formation of amyloid is that it proceeds via an energetically unfavorable nucleation step followed by a more favorable growth phase. Thus a lag precedes a log phase, during which the amyloid fibrils grow at the ends (33). In addition, depending on the amino acid sequence and different physical parameters, the conditions favoring intermediate structures with enhanced ability to form an elongating $\beta$-sheet vary between different proteins, as do the folding forces counteracting them.

It has been speculated that structures of amyloid could be induced from almost any peptide sequence, given the correct physical parameters. In support of this hypothesis, two human...
sequences have been identified, which have the ability to form amyloid in vitro but are not thought to be associated with a clinical disease (34, 35). It follows that a physiological role for amyloid structures in insects has been demonstrated (36). Partial unfolding of the protein is a necessary requirement for the aggregation of TTR into amyloid fibrils (12). The effect of point mutations on the overall stability has been widely investigated, and a correlation between their clinical aggressiveness and tetrameric instability is established (13, 37).

As the FAP1 mutants of TTR cause rather subtle changes in overall stability and are not able to form mature amyloid under physiological pH and ion strength, we created mutants with an amyloidogenic potential beyond those found in vivo. Our approach was to significantly destabilize the native fold by introducing more than one point mutation. The fact that this mutant (TTR-A) spontaneously aggregated into fibrils and induced a toxic effect on cultured neuronal cells, showed unequivocally that TTR-A is a suitable model system for studying the molecular mechanisms underlying amyloid formation. The rate of amyloid formation from wild type TTR was reported to be significantly reduced by lowering the temperature (21). Therefore, unfolded TTR-A could be renatured at low temperature in a buffer representing physiological conditions with respect to pH and salt concentration. This tendency of mutants to aggregate into amyloid was directly proportional to the precursor protein concentration and temperature. The aggregates formed mature amyloid after the appearance of protofibrils, and their rate of formation was dramatically enhanced by an increase in temperature (Fig. 6D). This finding was also observed with the highly amyloidogenic TTR-D mutant generated in an earlier study (14). In addition, TTR-D also formed a dimeric precursor molecule.

The relative high proportion of the dimeric intermediate most likely arose from the destabilization of the native tetrameric structure upon introduction of the amino acid substitutions. This was further supported by the observation that the wild type TTR also formed a dimeric intermediate after refolding at low temperature. However, a relatively large proportion of dimer compared with the tetramer was only formed at low protein concentrations. This implied that the conditions shifting the equilibrium from a tetramer enhances the folding pathway. Moreover, a previous report (10), suggesting a reformation of the native structure of wild type TTR in chaotropic agent at pH 7, correlates well with a re-association of the tetramer. These data suggest that the refolding is a co-operative process that depends on intermolecular contacts between four monomers. Once the native tetramer is formed, it appears to be locked by high kinetic barriers, which likely explains the observed hysteresis.

Size exclusion chromatography of wild type TTR clearly shows that the tetrameric state is not involved in any rapid interconversion between other states. In addition, a rapid equilibrium could not be seen between the dimeric and the monomeric species. Data thereby implicate that all the observed folding intermediates are separated by kinetic barriers under the conditions used in this study. Unfortunately, the dimeric species from wild type TTR could only be isolated at very low concentration, where its chromatography peak significantly overlapped with that of the monomer and could therefore not be subjected to further analysis.

Structural studies suggested that the dimeric species differs from the native structure. Spectroscopic analysis revealed also that it contains significantly less structure than the native fold. Moreover, in a temperature scan monitored by CD, induction of a predominantly β-sheet structure was observed proportional to the temperature increase. Furthermore, support for an increase in structure upon aggregation was gained using ANS, which is a fluorescent dye used to identify exposed hydrophobic areas. This analysis showed a high ANS/monomer ratio in the mutant upon saturation compared with the native wild type TTR fold, which indicates a large proportion of random coil structures. However, upon aggregation, this ratio is lowered suggesting a structural change (Fig. 5A). Both the TTR-A(D1118) and the mature fibrils appear to have significantly more exposed hydrophobic areas than the native wild type TTR. Interestingly, the characteristic spectral changes caused by ANS binding in the hormone binding channel of wild type TTR (28) are not observed for the TTR-A(D1118) nor within the fibril state, implicating that the channel is neither preserved nor accessible.

We believe that the path from TTR-A(D1118) to an amyloid fold is initiated by a rather promiscuous association of β-strands (a schematic view is given in Fig. 7). This assumption could also explain the initial formation of amorphous aggregates as a kinetic trap, when the aggregates become too big. Thermodynamically, this block could be explained by entropic forces as a further optimization of β-strands with non-β-strand register, implying that they first must dissociate to form a novel interaction. Because the hydrophobic effect is lowered upon an increase in temperature (38), the observed block is likely to be caused mainly by entropic forces, and the associating stretches of the polypeptide are therefore likely to be hydrophobic. This scenario has previously been described in terms of an amyloid folding funnel, where initial aggregates are formed as a broad ensemble of multiple inter- and, in this case, intramolecular β-strand registers (39). The width of the funnel corresponds to the entropy of the molecule (number of different registers), whereas the depth reflects the contact energy. The maturation of an amyloid fibril would then correspond to how “deep” into the funnel the species has reached. Consequently, mature amyloid corresponds to a very limited amount of allowed β-strand register assemblies, perhaps only one. Ongoing investigations will reveal the sequence of the remaining secondary structure in the fibril with the potential to target these with amyloid inhibitors.

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