Characterization of Two Highly Amyloidogenic Mutants of Transthyretin[†]

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ABSTRACT: The plasma protein transthyretin (TTR) has the potential to form amyloid under certain conditions. More than 50 different point mutations have been associated with amyloid formation that occurs only in adults. It is not known what structural changes are introduced into the structure of this otherwise stable molecule that results in its aggregation into insoluble amyloid fibrils. On the basis of calculations of the frequency of known mutations over the polypeptide, we have constructed two mutants in the D-strand of the polypeptide. These molecules, containing either a deletion or a substitution at amino acid positions 53−55, were unstable and spontaneously formed aggregates upon storage in TBS (pH 7.6). The precipitates were shown to be amyloid by staining with thioflavin T and Congo Red. Their ultrastructure was very similar to that of amyloid fibrils deposited in the vitreous body of patients with familial amyloidotic polyneuropathy type 1 with an amino acid replacement in position 30 (TTRmet30). Like amyloid isolated from the vitreous body of the eye, the amyloid precipitates generated from the TTR mutants exposed a trypsin cleavage site between amino acid residues 48 and 49, while plasma TTRmet30 isolated from amyloidosis patients as well as wild-type TTR only showed minor trypsin sensitivity. Our data indicate that the mutants we have constructed are similar to amyloid precursors or may share structural properties with intermediates on a pathway leading to amyloid deposits of plasma TTR.

Transthyretin (TTR)[1] is a protein that in plasma forms a homotetramer with a molecular mass of 55 kDa. In plasma it serves as a transport protein for thyroxin from the thyroid gland to its target tissues. TTR has an internal channel with two binding sites for thyroxin interacting by negative cooperativity (Fergusson et al., 1975). Moreover, it forms a complex with retinol binding protein. The three-dimensional structure of TTR has been shown to contain subunit folds that form a globular structure of two β-sheets consisting of eight β-strands (Blake et al., 1978).

Two clinical forms of TTR-associated amyloidoses are known. Senile systemic amyloidosis is a nonhereditary disease and affects 25% of all males over the age of 80 years. Deposits are mainly found in the heart (Cornwell et al., 1988; Gorevic et al., 1989). This form has been taken as an evidence that TTR has an inherent ability to self-aggregate into amyloid deposits late in life. Point mutations enhance the amyloidogenic potential of TTR and leads to hereditary forms of TTR-associated amyloidosis occurring earlier in life. More than 50 different point mutations are known to be associated with amyloidosis (Saraiva, 1995). The most common is an exchange of valine 30 for methionine in the 127 amino acid long peptide (TTRmet30). This leads to familial amyloidotic polyneuropathy type I (FAP I), which is an autosomal dominant genetic disease with deposition of amyloid fibrils primarily along the peripheral nerves and to a lesser extent in kidneys, in liver, and in the eye; for a recent review, see Benson and Uemichi (1996).

The mechanisms leading to self-aggregation of native TTR into insoluble amyloid fibrils are not known. In the few cases where high-resolution structural studies of TTRmet30 have been performed, only minor changes have been observed, which have not given any clues as to the mechanisms of amyloid formation (Hamilton et al., 1992; Terry et al., 1993). It is generally believed that amyloid is formed from partly denatured proteins, possibly after partial proteolytic cleavage. Amyloid is a crystallike structure that can give a typical X-ray cross-β diffraction pattern (Burke et al., 1972; Glenner et al., 1980). The starting point of aggregation has been assumed to be a nucleation-dependent self-aggregation of partly misfolded proteins (Gajdusek, 1991; Kelly & Lansbury, 1994; Jarret & Lansbury, 1995). This model is supported by experimental studies, where it has been shown that partially denaturing TTR in acid conditions results in spontaneous appearance of amyloid deposits (Colon & Kelly, 1992). A correlation has been shown between the clinical aggressiveness of specific mutations and their ability to form amyloid *in vitro* (McCutchen et al., 1995).

Previously we have analyzed the distribution of point mutations along the polypeptide chain and noted that they were distributed in a broad peak over an "edge" region of the TTR subunit (Serpell et al., 1996). This constitutes the C- and D-strands of the TTR fold. From this analysis we suggested that this region of TTR contains a "hot spot" for amyloidogenic mutations, although mutations at other sites of the polypeptide can also result in genetic diseases with

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[1] Abbreviations: GuHCl, guanidine hydrochloride; SE-HPLC, size-exclusion high-pressure liquid chromatography; TBS, Tris-buffered saline; TTR, transthyretin; TTRmet30, valine for methionine exchange at residue 30 of transthyretin; FAP I, familial amyloidotic polyneuropathy, type I.

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amyloid deposits. Therefore we decided to introduce mutations in the edge region and focused on the short D-strand. Three amino acids in position 53−55 of TTR were either substituted or deleted and the resulting mutant forms of TTR were characterized in terms of their stability and their propensities to spontaneously form amyloid aggregates.

MATERIALS AND METHODS

Enzymes, Oligonucleotides, and Isotopes. All restriction endonucleases, T4 DNA ligase, and DNA polymerase were purchased from New England Biolabs and used as recommended by the manufacturer. TaqI DNA polymerase was purchased from Perkin-Elmer Cetus. Oligonucleotides were synthesized by Symbicom AB (Umeå, Sweden) or by a department facility. All ^{35}S isotopes were from Amersham, U.K.

Construction of pETF1. Wild-type TTR cDNA inserted into the plasmid pPA7 (Wallace et al., 1985) was amplified by PCR. Flanking 5′ *Nde*I and 3′ *Bam*HI sites were introduced by ligation of the oligonucleotides 5′-GCG GCA TAT GGG ACC TAC GGG CAC CGGT-3′ and 5′-GCG GGA TCC TTA TTC CTT GGG ATT GGT GAC-3′, respectively. The amplified DNA was phenol-extracted and precipitated by ethanol. After *Nde*I and *Bam*HI digestion, the fragments generated were run on a 3% low-melting agarose gel (NuSieve GTG, FMC Bioproducts, Rockland, ME) and purified using Gene Clean as described by the manufacturer (Bio 101, La Jolla, CA). The purified DNA fragment was ligated with T4 DNA ligase to the *Nde*I/*Bam*HI-digested vector pET3a (Rosenberg et al., 1987). Competent DH5α cells were transformed according to Hanahan et al. (1983) after 2 h of incubation at room temperature. Screening of the clones were done by digestion of miniplasmid preparations with *Nde*I and *Bam*HI followed by agarose gel electrophoresis on 1% agarose gels (NuSieve GTG) and also by DNA sequencing. DNA from positive clones was used for further mutagenesis.

In Vitro Mutagenesis. The mutant TTR molecules were produced by site-directed mutagenesis as originally described by Kunkel et al. (1987). The *Escherichia coli* BW 313 (dut^-, ung^-) strain was used for production of uracil-containing single-stranded TTR DNA cloned into bacteriophage M13mp18. *E. coli* strain BMH71−18 (Kunkel et al., 1987) with an active uracil N-glycosylase was used as the final recipient for the mutagenized DNA.

Two mutants of the TTR D-strand were constructed with either a deletion of the three amino acids of the D-strand (deletion mutant, rTTRΔ53−55) or a substitution (substitution mutant, rTTRG53S,E54D,L55S) as described previously (Serpell et al., 1996). Briefly, two mismatched primers (for rTTRΔ53−55, 5′-CAG TTG TGA GCC CAT GAG ACT CAC TGG TTT TCC C-3′; for rTTRG53S,E54D,L55S, 5′-GTT GTG AGC CCA TGA GAA TCG CTA GAC TCA CTG GTT TTC-3′) were treated with kinase and annealed *in vitro* to the uracil-containing ssDNA. After second strand synthesis the DNA was transformed into BMH71−18. By using the primer 5′-CAC ATG CAT GGC CAC ATT-3′, the TTRmet30 variant was constructed in a similar way. All mutant TTR cDNAs were cloned into the expression vector pET3a and checked by DNA sequencing.

Isolation and Purification of Recombinant TTR Mutants and TTR from Plasma. The plasmids with the TTR constructs (the D-strand mutants, TTRmet30, and wild-type TTR cDNA) were transformed into competent *E. coli* BL21 cells (Hanahan, 1983). An overnight culture in LB medium supplemented with 100 μg/mL ampicillin was diluted to $OD_{600} = 0.15$ in 2× LB, 0,4% glucose, and 100 μg/mL ampicillin in a 20 L fermenter. Cells were grown at 37 °C until $OD_{600} = 0.8$ and then induced with 0.4 mM IPTG. Three hours after induction, the bacterial paste was collected by centrifugation.

Since the D-strand mutants form inclusion bodies, cells were lysed and inclusion bodies were isolated according to Sambrook et al. (1989) with slight modifications. Sonication was used instead of DNase treatment. The inclusion bodies were washed in buffer (50 mM Tris-HCl, pH 7.5, and 1 mM EDTA) and collected by centrifugation. The pellet was solubilized (0.01 M phosphate buffer, pH 7.2, 4 M urea, and 1% β-mercaptoethanol) and the remaining insoluble material was pelleted and discarded. After dialysis overnight at 4 °C against deionized water, the material was run on a DEAE-Sepharose FF column in 0.05 M Tris-HCl, pH 7.5, and eluted with a linear NaCl gradient (0−0.5 M). The main protein peak was collected, which contained a single protein band of 16 kDa in SDS−PAGE, confirmed by immunoblotting to be TTR (Serpell et al., 1996).

The bacterial strains expressing recombinant wild-type TTR and TTRmet30 do not form inclusion bodies. Cells were treated with lysozyme for 30 min at 37 °C and sonicated, and cell debris was removed by centrifugation. Ammonium sulfate was added to the clear lysate, which was stirred for 20 min. Following centrifugation at 20000*g* for 20 min, the supernatant was dialyzed overnight against 50 mM Tris-HCl, pH 7.5, and 0.05 M NaCl and run on a DEAE-Sepharose FF column as described above. The TTR-containing fractions were concentrated on a Diaflo PM10 filter (Amicon, Beverly, MA) and the final purification step involved preparative electrophoresis in 10% native polyacrylamide gel on a Model 491 Prep Cell as described by the manufacturer (Bio-Rad).

Human plasma TTR was isolated from pooled human plasma using previously described procedures (Colon & Kelly, 1992). The final step was preparative electrophoresis as described above. TTR met30 mutant was isolated from heterozygous donor plasma according to the same procedure.

Gel Electrophoresis. Protein samples were analyzed either under denaturing conditions in the presence of β-mercaptoethanol and SDS according to the standard Laemmli procedure (1970) or in native conditions without SDS or reducing agent. Proteins were separated on a 4−22.5% polyacrylamide gradient gel with 4% stacking gel under constant voltage conditions.

Immunoblotting. Proteins were transfered to Immobilon-P membrane (Millipore) and the membrane was blocked with 5% skim milk. Immunodetection was performed with a polyclonal rabbit antibody to human TTR (Dako, Denmark; 1:1000 dilution) and HRP-labeled swine anti-rabbit IgG antibody (Dako; 1:1000 dilution). Detection was performed using enhanced chemiluminescence (ECL, Amersham, U.K.).

Glutaraldehyde Cross-Linking. Samples of 25 μL of TTR (1 mg/mL) in PBS were mixed with 10 μL of 2.5% glutaraldehyde solution and incubated at 37 °C for 1 min. The cross-linking reaction was terminated by the addition of 10 μL of 0.5 M sodium borohydride. Aliquots (10 μL) were analyzed by SDS−PAGE with or without boiling before loading as described in the results section.

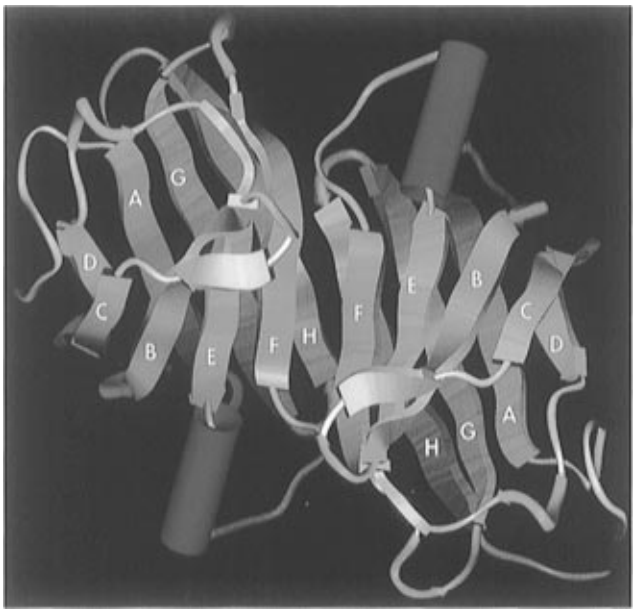

FIGURE 1: Ribbon diagram of the TTR dimer. The eight β strands are designated according to Blake et al. (1978). The small α helix is depicted in red, and the D-strand, the site for the mutations introduced, is shown in green. The coordinates were taken from Hamilton et al. (1993), and the figure was constructed using the Insight II program.

Size-Exclusion HPLC and Thioflavin T Binding Studies. SE-HPLC analysis was done on a ProteinPack I 125 7.5 × 300 mm column on a Waters chromatographic system with PBS as eluent. Flow rate was 0.3 mL/min and sample volume was 20 μL. Column calibration was done with bovine serum albumin, egg albumin, soybean trypsin inhibitor, and lysozyme obtained from Sigma.

Dye Binding Studies. Thioflavin T binding was measured by emission fluorescence at 482 nm, using 450 nm as excitation wavelength as described (LeVine, 1993). Congo Red binding was measured by the red shift from 490 nm according to Glenner et al. (1974).

TTR Aggregation Kinetics. TTR aggregation kinetics at 37 °C was recorded with an LKB Ultraspec spectrophotometer supplied with thermostatable cuvette holder with a magnetic stirrer at pH 4.0 according to Colon and Kelly (1992).

Electron Microscopy. The precipitates obtained after incubation of the recombinant TTR D-strand mutants in TBS for 12 h at 37 °C were washed with PBS and fixed in buffered 4% paraformaldehyde solution and postfixed with buffered OsO_4 solution (1.33% w/v). Agar-embedded samples were stained with 1% uranyl acetate overnight. Samples were then dehydrated with graded acetone series and embedded in a mixture of Epon-Araldite. Silver thin sections were prepared, stained with uranyl acetate and lead citrate, and examined under a Zeiss EM109 electron microscope.

Trypsin Digestion. A stock solution of trypsin was prepared by dissolving 10 mg of TPCK-treated trypsin (Sigma, St. Louis, MO) in 1 mL of 1 mM HCl. Before digestion, the stock solution was diluted 10 times in TBS and 2 μL of this solution was added to 30 μL of TTR solution (1 mg/mL) or amyloid suspended in TBS. Hydrolysis was performed at 37 °C during time periods indicated in figure legends.

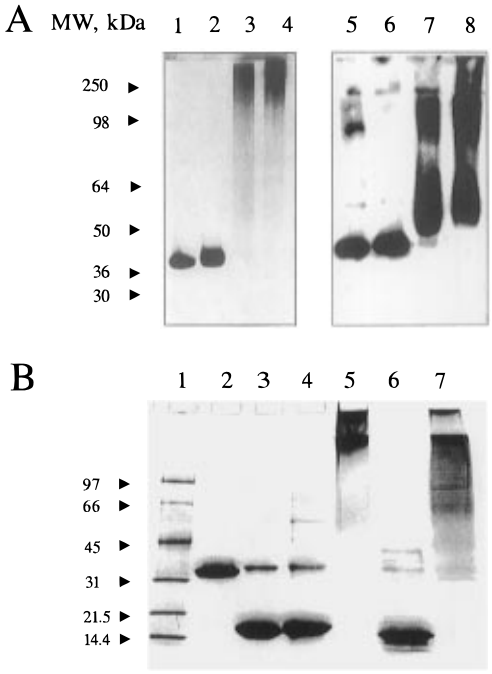

FIGURE 2: (A) Native PAGE analysis of plasma and recombinant TTR. Lanes 1−4, Coomassie staining, and lanes 5−8, immunoblotting of purified plasma wild-type TTR (lanes 1 and 5); recombinant wild-type TTR (lanes 2 and 6); recombinant TTR substitution mutant (lanes 3 and 7); and recombinant TTR deletion mutant (lanes 4 and 8). (B) SDS−PAGE analysis of cross-linked and non-cross-linked TTR followed by Coomassie staining. Lane 1, MW markers; lane 2, plasma wild-type TTR, nonboiled sample; lane 3, plasma wild-type TTR, boiled; lane 4, recombinant TTR substitution mutant, nonboiled; lane 5, recombinant TTR substitution mutant, cross-linked, nonboiled; lane 6, recombinant TTR deletion mutant, nonboiled; lane 7, recombinant TTR deletion mutant, cross-linked and nonboiled.

RESULTS

Two mutant TTR molecules were generated in this study in which the three amino acids in the short D-strand (residues 53−55) were either substituted or deleted as described (Serpell et al., 1996). In the substitution mutant, glycine, glutamic acid, and leucine were exchanged for serine, aspartic acid, and serine, respectively (cf. Figure 1). The mutants were expressed in *E. coli* under the control of the strong T7 promoter (Rosenberg et al., 1987) and extracted and purified by ion-exchange chromatography. Upon native PAGE analysis we found that the purified mutant proteins migrated as very broad bands while plasma-derived or recombinant wild-type TTR migrated as a single distinct band (Figure 2A, lanes 1−4). It should be emphasized that both D-strand mutants formed inclusion bodies in *E. coli* cells, in contrast to wild-type TTR and TTRmet30. Control experiments have shown that wild-type TTR dissolved in the same harsh condition (0.01 M phosphate buffer, pH 7.2, 4 M urea, and 1% β-mercaptoethanol) as that used for dissolution of inclusion bodies did not result in the formation of poorly resolved high molecular weight aggregates.

In order to identify the broad band, the two mutants and, as controls, purified wild-type TTR from the two sources were separated by native PAGE, blotted, and stained with a TTR-specific polyclonal antibody (Figure 2A, lanes 5−8). The broad bands could clearly be identified as TTR.

It has previously been demonstrated that TTR is a stable molecule that can resist dissociation into monomers in

moderately high concentrations of denaturing agents like SDS and GuHCl (Branch et al., 1971, 1972). When the two D-strand mutants of TTR were analyzed by SDS−PAGE, they dissociated into monomers of 16 kDa (Figure 2B, lanes 4 and 6). This dissociation occurred to the same degree in nonboiled as in boiled samples. Nonboiled wild-type TTR migrated as a 32 kDa peptide (Figure 2B, lane 2), which represents the migration rate of the normal TTR tetramer (Goldsteins et al., manuscript in preparation).

To exclude that the appearance of the high molecular weight aggregates in native PAGE was an artifact due to the electrophoretic separation conditions, the two mutants were cross-linked with glutaraldehyde and analyzed by SDS−PAGE. The distribution of aggregated forms was the same as that seen after separation by native PAGE (Figure 2A), while the non-cross-linked proteins dissociated into monomers (cf. Figure 2B, lanes 5 and 7 with lanes 4 and 6). This indicates that the high molecular weight aggregates were present in solution and did not form during electrophoresis. The mobility of the wild-type protein was not affected by glutaraldehyde cross-linking (data not shown).

Incubation of both TTR D-strand mutants in TBS at 37 °C resulted in the formation of a gel-like precipitate. We have previously shown that such precipitates from both mutants give a cross-β X-ray diffraction signal and therefore represent amyloid (Serpell et al., 1996). This was confirmed by transmission electron microscopy of precipitate sections. Figure 3B shows distinct fibrils of the substitution mutant, which have a thickness ranging between 85 and 95 Å and lengths between 1000 and 2000 Å. Amyloid from the vitreous body (Figure 3A) was used as a control, showing somewhat thicker fibrils of approximately 100 Å. In both cases fibrils with sharp borders were visible both in cross-sections and in longitudinal sections in which they formed tubulelike structures. These structures were also seen in the mutants, although they were more rare in the deletion mutant (Figure 3C). Most of the fibrils formed from the deletion mutant were thicker and more dense than those from the vitreous body or the substitution mutant. Both mutants gave clear signals in both Congo Red and thioflavin T dye tests (Table 1). Thus, with Congo Red the absorbance peak shifted from 490 nm to 495−500 nm in the case of aggregated D-strand mutants and vitreous amyloid. Soluble TTR, independent of source, was not capable of giving this shift. Thioflavin T had a strong fluorescence emission peak at 482 nm which is characteristic for the unique stacked cross-β pleated sheet structure in amyloid (LeVine, 1995). This was seen with neither wild-type TTR nor soluble TTRmet30. Taken together, the substitution mutant clearly forms amyloid as judged from both morphological and dye binding criteria, while we cannot exclude that amyloid formed from the deletion mutant is admixed with less ordered aggregates.

When the substitution mutant of TTR was analyzed by SE-HPLC (Figure 4), it did not resolve as a single sharp peak similar to purified plasma TTR. A broad peak with an exclusion volume corresponding to aggregates of TTR oligomers appeared, followed by a small sharp peak with an exclusion volume characteristic for the TTR tetramer. SE-HPLC analysis of aggregation kinetic of this mutant showed a progressive increase in the degree of aggregation of the oligomeric fraction (Figure 4A) at 22 h compared to 3 h, while the tetrameric form remained unchanged. Similar results were obtained with the deletion mutant, although the aggregation occurred much earlier (data not shown).

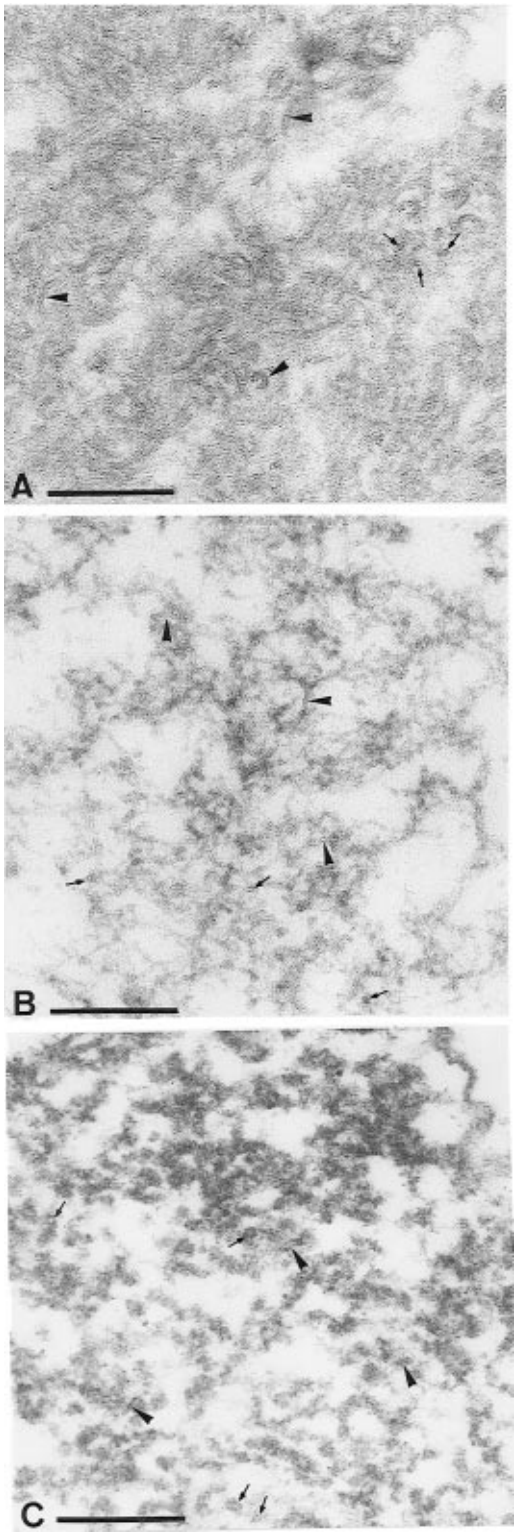

FIGURE 3: High-power electron micrographs of (A) amyloid isolated from the vitreous body, (B) precipitates generated from the TTR substitution mutant, and (C) precipitates generated from the deletion mutant. All materials consist of fine fibrils having tubulelike structures; i.e., they exhibit either a circular structure in cross sections (arrows) or double line structures in longitudinal sections (arrowheads). Scale bar = 0.2 μm.

Thioflavin T binding was measured in samples taken from the material eluted from the SE-HPLC column. Figure 4B shows that only the high molecular weight oligomeric form gave a positive signal, while no peak was seen corresponding

Table 1: Dye Binding Test of Precipitates from Different TTR Sources

source	Congo Red bound[a] (M)	thioflavin T fluorescence intensity[b]
vitreous body	$(1.49 \pm 0.2) \times 10^{-6}$	1480 ± 180
substitution mutant	$(1.22 \pm 0.3) \times 10^{-6}$	1060 ± 140
deletion mutant	$(1.43 \pm 0.3) \times 10^{-6}$	1110 ± 150

[a] Measured according to Lai et al. (1996). [b] Measured at 482 nm, arbitrary units normalized for protein content.

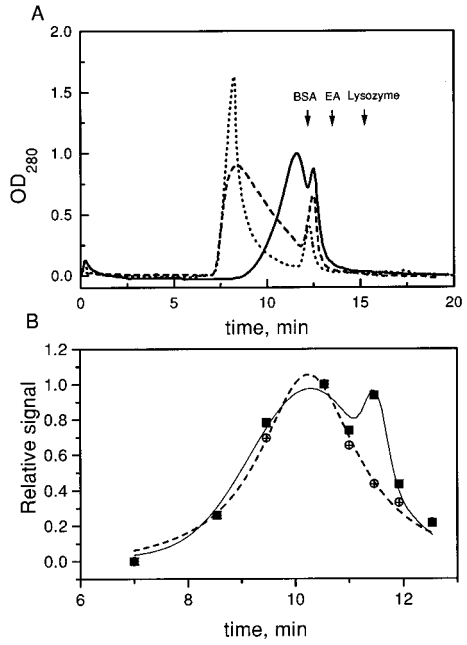

FIGURE 4: (A) SE-HPLC analysis of TTR aggregation. Initial exclusion profile (solid line), as well as exclusion profile after incubation at 37 °C in TBS for 3 h (dashed line) and 22 h (dotted line) are shown. (B) Recombinant TTR substitution mutant was subjected to SE-HPLC separation. Fractions of 1 mL were withdrawn and protein concentration (OD_{280}, solid line) and thioflavin T binding (dashed line, fluorescence at 482 nm in relative units) were measured.

to the sharp protein peak representing the putative tetrameric TTR.

TTR has previously been shown to form amyloid at low pH (Colon & Kelly, 1992). McCutchen et al. (1993, 1995) have shown that the rate of aggregation correlated with the severity of disease associated with different mutants. In order to compare the relative stability of the D-strand mutants with wild-type TTR and TTRmet30, we followed the protocol of these investigators. We could demonstrate (Figure 5) that the D-strand substitution mutant formed aggregates much more rapidly than either wild-type TTR or TTRmet30. The curve showing the rate of aggregation of the substitution mutant collapsed after a few minutes due to massive precipitation. In the case of the deletion mutant, aggregates formed immediately at pH 4.0 and therefore no recording could be done.

The high rate of aggregation and the amyloidogenicity of the mutant TTR molecules described here has impeded more elaborate structural studies. On the basis of a previous observation that TTR extracted from amyloid in the vitreous body was cleaved at a putative serine protease cleavage site (Thylén et al., 1993), we decided to test for exposed trypsin cleavage sites on different TTR molecules. In the experiment shown in Figure 6, we asked whether this site is differentially

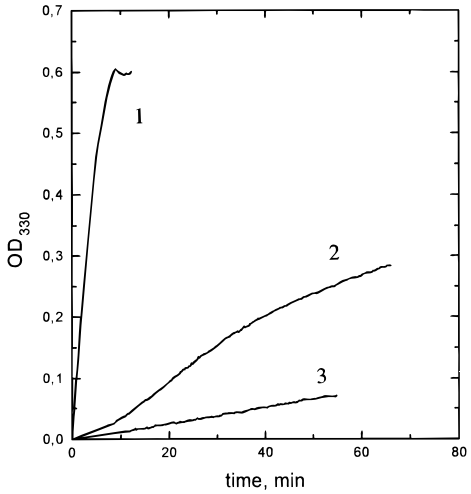

FIGURE 5: Kinetics of TTR aggregation in 0.05 M acetate buffer with 0.1 M KCl, pH 4.0. (1) Recombinant TTR substitution mutant; (2), plasma TTR met30; (3), plasma wild-type TTR.

exposed in different TTR molecules. As described (Thylén et al., 1993), TTR isolated from vitreous amyloid contains TTR monomers, multimers, and TTR fragments of discrete lengths: one large fragment (Thr49−Glu127) and one smaller fragment (Gly1−Lys48). Very little TTR monomer remained after incubation of vitreous amyloid with trypsin for 30 min as analyzed by SDS−PAGE (Figure 6B, lanes 1 and 2) . Instead, the amount of the band corresponding to fragment Thr49−Glu127 increased. Normal plasma TTR is nearly resistant to trypsin cleavage under the same conditions. The amount of the smaller Gly1−Lys48 fragment is too small to be detected under the conditions used.

A band with similar electrophoretic mobility as the Thr49−Glu127 fragment was also seen when precipitates from both substitution and deletion D-strand mutant TTR were digested with trypsin. Only data from the substitution mutant are shown in Figure 6B.

In contrast, the soluble form of D-strand mutant TTR was completely hydrolyzed by trypsin (Figure 6A,C), while recombinant wild-type TTR and TTRmet30 were found to be almost completely resistant to trypsin cleavage under these conditions. Thus TTR in amyloid carries an accessible trypsin cleavage site in the loop connecting the C- and D-strands of the molecule that is protected in the native wild-type protein. By tryptophan fluorescence after denaturation in GuHCl (0−8 M), we were not able to show any significant differences between wild-type TTR, TTRmet30, and the D-strand mutants (data not shown).

DISCUSSION

It is not known how amyloidogenic proteins initiate self-aggregation and form amyloid fibrils. Two pathogenic factors are recognized: first, many of these proteins have point mutations and several of them are trimmed down to smaller fragments by proteolysis. Second, it is believed that fibril development is preceded by destabilization and partial misfolding.

Few studies have been reported on structural changes in mutant amyloidogenic TTR variants, and those have not given any clues as to the possible mechanisms of how fibril formation might initiate (Hamilton et al., 1992; Terry et al., 1993). The rate of spontaneous *in vitro* amyloid formation at low pH differs between different mutant TTR molecules.

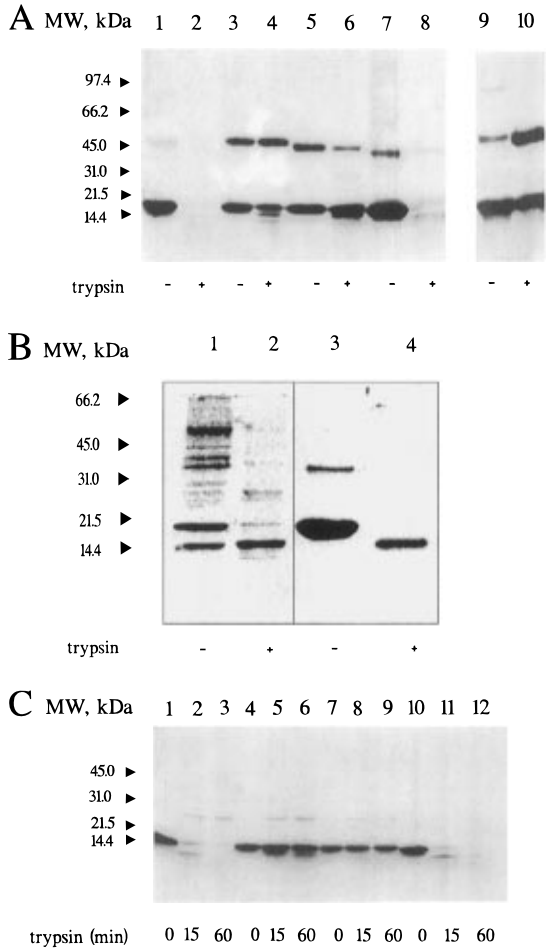

FIGURE 6: Sensitivity of different TTR molecules to trypsin cleavage analyzed by SDS−PAGE and Coomassie staining. (A) The TTR samples were analyzed after trypsin cleavage during 30 min at 37 °C. Lanes 1 and 2, recombinant TTR substitution mutant; lanes 3 and 4, recombinant wild-type TTR; lanes 5 and 6, recombinant TTRmet30; lanes 7 and 8, recombinant deletion mutant of TTR; lanes 9 and 10, wild-type plasma TTR. (B) SDS−PAGE analysis of *in vivo* and *in vitro* amyloid after treatment with trypsin as in panel A. Lanes 1 and 2, vitreous amyloid; lanes 3 and 4, amyloid formed from the recombinant substitution mutant. (C) Sensitivity to trypsin cleavage with time. The different TTR species were incubated without or with trypsin for 15 and 60 min at 37 °C. Lanes 1−3, substitution mutant; lanes 4−6, recombinant wild-type TTR, lanes 7−9, recombinant TTRmet30; lanes 10−12, recombinant deletion mutant.

The interpretation is that amino acid replacements at different sites of the polypeptide lead to differential stability (McCutchen et al., 1995). A model has been proposed suggesting that destabilization and partial misfolding of TTR is the basis for the generation of amyloid (Kelly & Lansbury, 1994). These authors proposed that the whole edge of the molecule with the C- and D-strands loops out before TTR starts to self-aggregate.

In this study we have generated two mutants, in which the three amino acids in the D-strand of the TTR subunit (Blake et al., 1978) were either deleted or substituted (cf. Figure 1). These mutants have a strong tendency to self-aggregate under physiological conditions and precipitate into insoluble gel-like aggregates. The D-strand mutants migrated in native PAGE as poorly defined broad bands larger than the TTR tetramer.

These mutants appear to be unable to form a correct, detergent-stable tetrameric quaternary structure, most likely due to a failure in the native fold of the polypeptide chain. A minor fraction of the protein still associated as tetramers that did not aggregate further, while the major fraction formed soluble high molecular weight aggregates that polymerized into amyloid fibrils.

The precipitates qualified as amyloid by several criteria. Both D-strand mutants have been reported to give the typical cross-β structure upon X-ray diffraction (Serpell et al., 1996). They bound thioflavin T, which upon spectrofluorography resulted in an emission maximum diagnostic for amyloid according to LeVine (1993). Congo Red staining showed the classical red shift. Ultrastructurally the precipitates showed a similar fibrillar organization as that seen in control amyloid isolated from the vitreous body, while in the case of the substitution mutant the fibrils were thinner than those from the *in vivo* material. This may be due to the fact that *in vivo* amyloid has been shown to be decorated with molecules like the serum amyloid P component and glucose aminoglycans (Hawkins et al., 1990).

The deletion mutant differed from the substitution mutant in the sense that it was more unstable and aggregated at a much higher rate. Consequently it could not be subjected to all experiments conducted with the substitution mutant. Although both thioflavin T and Congo Red binding give a diagnostic signal for amyloid formation, the transmission electron microscopy results were less clear with this mutant. Both structures typical of amyloid occurring in the positive control as well as more dense, less ordered fibrillike structures were seen. We concluded that the substitution mutant was more advantageous to work with, since amyloid could be generated in a more controlled way. This was not unexpected, as a deletion of three amino acids might induce more dramatic structural changes than three amino acid replacements.

On the basis of our analysis, we speculate that the amino acid changes introduced in TTR in the experiments described here have created unstable protein molecules, which might have adopted a conformation leading to spontaneous amyloid formation. We think that these structurally modified mutants share some characteristics of intermediates in a pathway from native TTR molecules to TTR in amyloid and represent amyloid precursor molecules.

We have previously demonstrated a fragment of TTR in vitreous amyloid comprising amino acids Thr49−Glu127 (Thylén et al., 1993). Fragments of the same size were obtained after trypsin cleavage of amyloid obtained both from the two D-strand mutants and from vitreous amyloid, indicating that the same serine protease cleavage site was used. This suggests a structural modification of TTR in amyloid, since this cleavage site is exposed neither in wild-type TTR nor in TTRmet30 purified from plasma. The appearance of a distinct polypeptide fragment upon trypsin cleavage seems to be a characteristic marker for TTR in amyloid fibrils.

The D-strand mutants were completely cleaved in solution but not when packed in amyloid fibrils. It is not unexpected that the mutant proteins are completely cleaved after an initial proteolytic attack, while in amyloid fibrils other trypsin cleavage sites are hidden, which rescues them from further degradation. Wild-type TTR, as well as TTRmet30 at physiological pH does not have an exposed cleavage site on the C and D loop. The minor cleavage observed in the *E.*

coli-derived TTR molecules is not significant for the argument (cf. Figure 6, panels A and C).

We can here show that TTR mutated in the D-strand is structurally altered by two criteria. It is more easily dissociated by detergent and has an accessible trypsin cleavage site. We could not show any changes by tryptophan fluorescence after GuHCl denaturing, which could be interpreted as an equilibrium between a native and an altered fold, where the native fold is dominating.

The results presented here support a model for generation of amyloid from TTR which implies that one necessary condition is a destabilizing mutation. Lai et al. (1996) have presented data showing that mutations of TTR lead to formation of monomeric amyloidogenic intermediates. The experimental model of these authors suggests a low-pH-dependent pathway for amyloid formation, implying that it correlates with formation of a monomer of TTR. Our data show that the D-strand mutants formed monomers in SDS. However, this was not the case without detergent in a physiological pH range; rather, they readily formed aggregates larger than the TTR tetramer before amyloid precipitates appeared. Our study supports the proposal that in TTR amyloid there is a loss of the structural integrity of the C- and D-strands (Kelly & Lansbury, 1994; Lai et al., 1996). We propose that this structural alteration could also destabilize the quartenary structure, leading to formation of modified amyloidogenic dimers that are associated by the antiparallel organization of the F- and H-strands of the native molecule. We will in separate experiments show that TTR dimers easily form amyloid in a physiological pH range (to be reported elsewhere).

We do not know which factors govern formation of amyloidogenic intermediates from mutated TTR proteins. Pathological chaperones have been proposed for other amyloidogenic peptides (Wisniewski & Frangione, 1992), and proteolytic cleavage has been proposed to be a necessary prerequisite for amyloid formation of TTR (Benson & Uemichi, 1996). Our interpretation of the trypsin cleavage data is that the proposed disordered structure of the C- and D-strands has as a consequence that it loops out from the amyloid fibril, leading to the appearance of a trypsin cleavage site between amino acids Lys-48 and Thr-49. The model does not imply that proteolytic cleavage is necessary for amyloid formation but rather is a consequence of it, which is supported by a previous finding from our laboratory that only a fraction of TTR in vitreous amyloid is fragmented (Thylén et al., 1993).

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