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Amyloidogenic and anti-amyloidogenic properties of recombinant transthyretin variants

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ABBREVIATIONS: TTR = transthyretin; FAP = familial amyloid polyneuropathy; FAC = familial amyloid cardiopathy; AVO = amyloidotic vitreous opacities; SSA = senile systemic amyloidosis

Abstract

Most transthyretin (TTR) mutations lead to TTR amyloid depositions in patients with familial amyloidotic polyneuropathy and familial amyloidotic cardiomyopathy. However, though an amyloidogenic protein itself, TTR inhibits aggregation of Alzheimer's amyloid beta protein (Aβ) in vitro and in vivo. The pathogenic relationship between two amyloidogenic processes remains unclear. To understand how TTR mutations influence the ability of TTR to inhibit Aβ amyloidosis, forty-seven recombinant TTR variants were produced and analyzed. We showed that all recombinant proteins formed tetramers and were functional in thyroxine binding. Acid denaturation at pH 3.8 resulted in aggregation and fibril formation of all TTR variants. However, only TTR G42 and TTR P55 formed fibrils at pH 6.8. Most TTR variants bound to Aβ and inhibited Aβ aggregation in vitro. TTR variants S64, A71, Q89, V107, H114 and I122 revealed decreased binding to Aβ and decreased inhibition of Aβ aggregation. Only TTR G42 and TTR P55 completely failed to bind Aβ and to inhibit Aβ aggregation. We suggest that TTR variants characterized by decreased binding to Aβ or by decreased inhibition of Aβ aggregation in vitro may contribute to Aβ amyloid formation in vivo. These TTR variants might be important targets for epidemiological studies in Alzheimer's disease.

Introduction

Transthyretin (TTR), previously referred to as prealbumin, is known as a main transporter of thyroxine and retinol binding protein in mammals[1,2]. Circulating TTR is found in serum and cerebrospinal fluid as a symmetrical tetramer composed of four identical subunits of 127 amino acid each, that migrate as a complex at 55 kDa[1,2]. Presently, over eighty genetic TTR variants derived from single amino acid substitutions have been documented[3,4,5]. Most TTR variants were found in patients with two related clinical syndromes characterized by systemic TTR amyloid depositions in the peripheral nervous system: familial amyloidotic polyneuropathy (FAP) and familial amyloid cardiomyopathy (FAC). Interestingly, several TTR variants were not associated with amyloidosis: S6, R10, N90, R102, T109, and M119[3,4,5].

FAP and FAC were described as autosomal dominant inherited diseases caused by different TTR muta-

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tions[1,2,3,4,5]. However, the role of TTR mutations in TTR amyloidogenesis is not well understood. Although the region of amino acid residues 42–55 in the TTR monomer was considered as a ''hot spot region'', amyloidogenic mutations span the entire TTR molecule[3,6]. Moreover, TTR with an entirely normal amino acid sequence was found in patients with senile systemic amyloidosis[7]. In addition, synthetic peptides homologous to the sequence of normal TTR form amyloid-like fibrils *in vitro*[8]. Thus, the normal amino acid sequence of TTR has structural predispositions for amyloid formation.

Several reports suggested a role for TTR in catabolism of amyloid beta protein (Aβ), which is a main component of amyloid plaques in Alzheimer's disease[9,10,11,12]. These suggestions were largely based on the observations that TTR formed complexes with Aβ and inhibited Aβ aggregation and fibril formation *in vitro* and *in vivo*[9–15]. However, relationships between TTR and Aβ amyloidoses remain unclear, in part due to the difficulties in investigation of individual TTR variants. Indeed, most FAP and FAC patients were reported to be heterozygous carriers of TTR mutations. Therefore, we took an alternative approach for the isolation of individual homozygous TTR variants and generated recombinant TTR variants.

Here we describe an expression system which provides a high level production of recombinant TTR proteins, and a simple procedure for purification of these recombinant proteins. Forty-seven recombinant TTR variants were produced, purified, and characterized for their binding to thyroxine, pH-sensitivity to self-aggregation, and ability to inhibit Aβ aggregation.

Materials and methods

Construction of TTR expressing plasmids

A full-length human TTR cDNA cloned in pUC18 was obtained from ATCC. To remove the ''leader sequence'' and introduce BamH1-Xho1 restriction enzyme sites flanking the TTR cDNA, we used polymerase chain reaction. A forward primer (5′cgggatccGGCCCTACGGGCACCGGTGAATCCA3′) contained a 5′-clamp (cg), a Bam H1 cloning site and a sequence encoding the amino-terminus of mature TTR. The reverse primer (5′ggcctcgagTCATTCCTTGGGATTGGTGACGACAG3′) contained a 5′- clamp (gg), an Xho1 cloning site, and a partial TTR cDNA sequence starting from stop codon. To provide periplasmic localization and secretion of the recombinant protein into the growth media, we cloned fragment of the TTR cDNA that lacked the ''leader sequence'' into the BamH1-Xho1 cloning sites of the pET-22b(+) plasmid which provided an amino-terminal bacterial *pelB* leader (Novagen). To avoid PCR-derived errors, the sequences from many individual TTR cDNA clones were verified by DNA sequencing. Only those clones whose DNA sequence exactly matched the authentic TTR cDNA sequence were employed. A DNA fragment of 33 bp between the end of the *pelB* leader and the first codon of mature TTR was deleted using method of site-directed mutagenesis described by Kunkel[16]. The same method of site-directed mutagenesis was used to generate point mutations. A detailed scheme for the construction of TTR expression plasmids is presented on Figure 1. As above, the sequence of all mutated DNAs was confirmed by DNA sequence analysis.

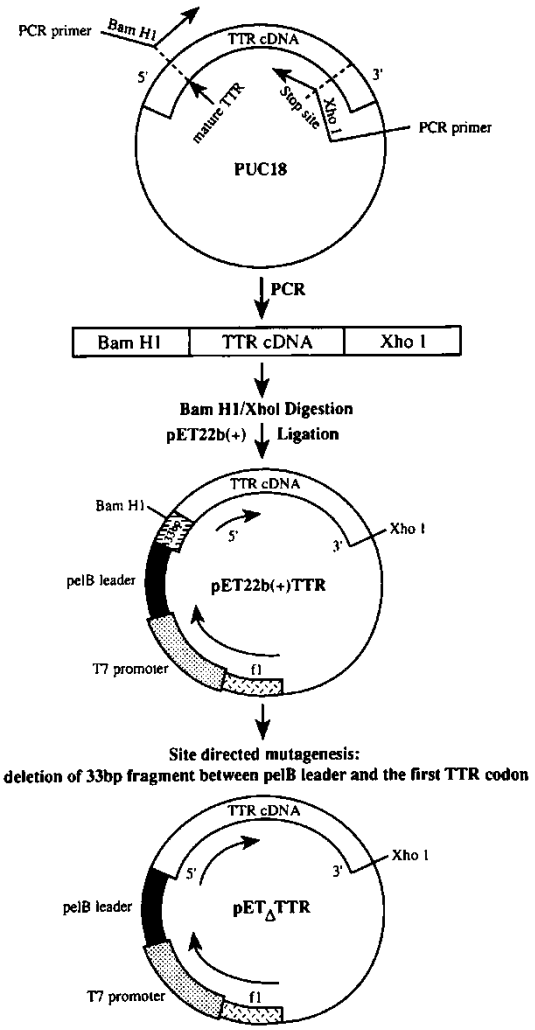

FIGURE 1: Scheme for the construction of the TTR expression plasmids

Isolation of recombinant TTR

Forty seven recombinant TTR variants were expressed in *E.coli* strain BL21 (DE3). The cells bearing TTR plasmids were grown at 37°C in 50 ml of LB broth up to 0.7–0.8 OD_{600}. TTR expression was induced by 1 mM IPTG with gentle shaking. After 3 h induction, the culture was centrifuged at 5000 x g and the supernatant was carefully removed from cell pellet. After a second centrifugation, LB media contained secreted TTR was subjected to ion-exchange chromatography on a 5 ml DEAE-Sephacel column equilibrated with 20 mM Tris-HCl, pH 7.2, 10 mM NaCl. Recombinant TTR was eluted at 0.3 M NaCl and concentrated using Centricon 30 concentrators (Amicon). After separation on SDS-polyacrylamide gels (SDS-PAGE), recombinant proteins were stained by Coomassie Brilliant Blue R-250 (Sigma) or transferred onto PVDF membranes (Bio-Rad) for Western blot analysis with ECL kit (Amersham). Using an anti-TTR antibody (Calbiochem), all recombinant TTR variants showed immunoreactive bands of the same molecular weight as purified plasma TTR purchased from Sigma (data not shown). Sequencing of TTR immobilized on PVDF membranes was performed by automated Edman degradation using Applied Biosystems 477A sequencer. Amino-terminal sequencing routinely showed the first ten amino acid residues of purified recombinant proteins completely matched those in the amino-terminal amino acid sequence of plasma TTR. Thus, the *pelB* leader sequence was properly processed and mature TTR was secreted.

Congo Red based aggregation assay

100 μM Aβ_{1-42} (US peptide) in deionized H_2O was sonicated for 15 sec, filtered through Millex-GV 0.22mm filters (Millipore) and incubated for 24–36 h at 37°C in PBS (150 mM NaCl/10 mM Na phosphate buffer, pH 7.4) alone or with recombinant TTR (20 μM) in a total volume of 50 μl per well in 96-well polyvinyl microplates (Costar). Aggregation of TTR and TTR variants was performed at 300–600 μM, a significantly higher concentration than that used for Aβ_{1-42} ending experiments. Fibril formation was also monitored by electron microscopy. After the desired time of incubation, 150 μl of PBS and 2 μl of Congo Red (5 μM) were added to each sample followed by incubation at room temperature in the dark for 10 min. The microplate was spun at 3000 rpm for 40 min, the supernatant discarded and absorbance of red pellets was measured in ''Microplate reader'' at 490 nm. The percent of absorbance was determined by comparing the average of duplicate samples to the Congo Red blank. A similar procedure was employed for analysis of recombinant TTR aggregation after acid denaturation in phosphate buffers. Aggregation assays employing Thioflavin-T[9], gave similar results to this Congo Red binding assay.

Thyroxine binding assay

For immunoprecipitation of TTR-^{125}I- thyroxine complexes, 200 ng of recombinant TTR were incubated with ^{125}I- thyroxine (1 μCi) and anti-rabbit TTR antibody (dilution 1:200) (Calbiochem) in PBS at room temperature for 8 h. After incubation, PEG 6000 was added up to a final concentration of 10% and incubation was continued overnight. Bound thyroxine was collected by centrifugation at 7000 rpm for 20 min followed by three additional cycles of washing with PBS and collection by centrifugation.

Aβ binding assay

Synthetic Aβ_{1-42} was labeled by using ^{125}I Bolton-Hunter reagent (Amersham) as recommended by the manufacturer. For binding assay, 0.5 μg of TTR were incubated in 20 μl PBS with 10^5 dpm ^{125}I-Aβ (specific activity 3–6 x 10^6 dpm/mg) at 37°C. After incubation, samples were immediately electrophoresed in non-denaturing gradient PAGE (4–20%) (Bio-Rad). The distribution of radioactivity in dried gels was analyzed using GS-250 Molecular Imager (Bio-Rad) and Chemi-Doc system (Bio-Rad).

Electron microscopy

Samples containing preincubated recombinant TTR or Aβ were applied to carbon-coated copper grids, negatively stained with uranyl acetate, and visualized with JEOL 200cx electron microscope operating at 80 kV. Samples were examined and photographed at magnifications of 60,000x.

Results

To produce recombinant TTR, we used the pET22b (+)-based expression system (Novagen) that provides secretion of recombinant proteins into the media by utilizing the bacterial *pelB* leader sequence (Figure 1). Total yield of recombinant TTR in the growth media reached up to 50 mg/l. SDS-PAGE analysis and densitometry demonstrated that recombinant TTR represents approximately 70–80% of total protein in the conditioned media (Figure 2). We did not detect TTR bands in the growth media of non-transformed BL21 (DE3) *E. coli* cells. One step DEAE chromatography yielded TTR that was greater than 95% pure by gel electrophoresis. Amino-terminal sequencing showed that the first 10 amino acids of purified recombinant protein completely matched the N-terminal sequence of plasma TTR. Thus, the *pelB*

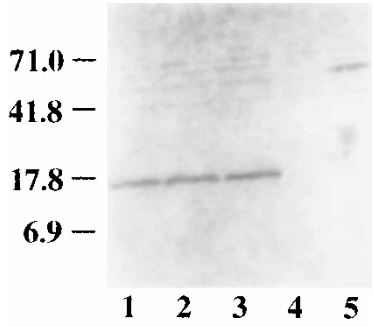

FIGURE 2: Electrophoretic analysis of secreted recombinant TTR. Recombinant TTR variants were expressed in *E.coli* strain BL21 (DE3) and growth media was analyzed in 12% SDS-PAGE. Coomassie Blue-stained gels: lanes 1, 2, 3 - different clones expressing recombinant TTR; lane 4 - control BL21 (DE3) cells that did not contain TTR plasmids. Samples loaded on lanes 1, 2, 3 and 4 were boiled for 5 min in 2% SDS before electrophoresis. Lane 5 – conditioned growth media of TTR expressing clone that was loaded without SDS treatment and boiling. The relative molecular masses (kDa) of protein markers are shown on the left.

leader sequence was properly processed and secreted recombinant TTR represented mature protein. As seen in Figure 2, secreted recombinant TTR is a tetrameric protein that migrates as a 55 kDa tetramer and migrates as a 14 kDa monomer after boiling in SDS-containing loading buffer. An intact, tetrameric structure of TTR is required for thyroxine binding and represents an important criterion for evaluation of functional activity of recombinant TTR[2,17].

We did not find significant differences in thyroxine binding between wild type recombinant TTR and commercial TTR from human plasma (Table 1). Thus, both PAGE analysis and thyroxine binding show that secreted recombinant TTR is a functional tetrameric protein. Table 1 demonstrates that most recombinant TTR variants had the same binding to thyroxine as wild type recombinant TTR. Significantly decreased binding to thyroxine was detected for M30, P55, G54, V107, I50, S84, H58, R58 and increased binding for S6, M119 and T109 TTR variants.

In order to compare amyloidogenicity of recombinant TTR variants, we analyzed TTR aggregation at pH 3.8, 4.8, 6.2, and 6.8 using the Congo Red spectrophotometric assay. These experiments were based on previous observations that partial acid denaturation of TTR *in vitro* results in TTR aggregation and fibril formation[17,18,19]. Complete aggregation of all recombinant TTR variants was induced by pH 3.8 at 37^{0}C during 60 min (data not shown). Therefore, results obtained from the Congo Red assay at these experimental conditions were considered as 100% TTR aggregation. Kelly and Lansbury[6] reported that wild-type TTR formed fibrils at pH 3.8 and suggested that TTR variants also form fibrils at this pH. Strong aggregation of recombinant TTR was also observed at pH 4.8. Most recombinant TTR variants including wild type TTR did not aggregate at pH 6.2. Aggregation exceeding 30% was only observed for M30, L30, G42, I50, M50, I50, P55, R58, Q89, C114, H114, and Y116. At pH 6.8, significant aggregation was detected for M 30 (58.0%), G42 (49.9%), I50 (38.4%), P55 (88.0%). Electron microscopic analysis showed that aggregates of wild type TTR at pH 3.8 (Figure 3a) and aggregates of TTR G42 (Figure 3b) and TTR P55 (Figure 3c) at pH 6.8 represent typical fibrillar structures. In contrast, aggregates of TTR variants L30, M30 and I50 at pH 6.8 (Figure 3d, 3e and 3f) represented mostly amorphous masses without visible fibrillar structures.

Next, we examined the effect of recombinant TTR on Aβ aggregation. *In vitro*, wild type TTR inhibits Aβ amyloid formation[9] but the effects of mutant TTR variants on Aβ aggregation are unknown. Our studies showed that most recombinant TTR variants formed complexes with Aβ (Figure 4) and inhibited Aβ aggregation (Table 1). Several TTR variants (S64, A71, Q89, V107, H114 and I122) revealed decreased binding to Aβ and decreased inhibition of Aβ aggregation (Figure 4, Table 1). Only two TTR variants, G42 and P55, completely failed to bind Aβ (Figure 4) and failed to inhibit Aβ aggregation (Table 1). Moreover, both proteins increased the amount of Congo Red positive aggregates as compared with control samples, which did not contain these TTR variants.

Discussion

Most TTR mutations lead to deposition of TTR as an amyloid in patients with FAP (Familial Amyloidotic Polyneuropathy) and FAC (Familial Amyloidotic Cardiomyopathy). Surprisingly, TTR inhibits Aβ amyloid formation *in vitro*[9,10] and *in vivo*[11,12,13,14] even though TTR itself can form amyloid fibrils. The pathogenic relationship between two types of amyloidogenic processes is not well understood, mainly due to the lack of homozygous TTR variants in population studies. An alternative approach for isolation of individual homozygous TTR variants is based on the generation of recombinant TTR. Several studies demonstrated that recombinant TTR produced in *E. coli*, has normal structural characteristics and normal thyroxine binding activity[20,21,22]. Different systems for bacterial expression of recombinant TTR have been developed[19,20,21,22]. Despite high levels of recombinant protein expression, the described systems have serious limitations with

Table 1. Characterization of recombinant TTR variants

Mutation	Clinical syndrome*	Rel. T4 binding % *	Aβ aggregation (%)	TTR aggregation (%) pH 4.8	pH 6.2	pH 6.8
S6		145.2 ± 18.21	25.6 ± 3.03	80.6 ± 7.30	ND	ND
R10		85.3 ± 3.38	37.4 ± 4.49	55.8 ± 4.15	ND	ND
A30	FAP	82.9 ± 6.23	41.6 ± 1.93	100	ND	ND
M30	FAP	28.1 ± 2.95	48.5 ± 7.42	100	58.3 ± 6.41	58.0 ± 6.80
L30	FAP	92.4 ± 5.81	35.5 ± 3.76	100	38.2 ± 7.05	18.4 ± 2.55
L33	FAP	99.7 ± 15.15	37.0 ± 6.86	100	ND	ND
I33	FAP	78.2 ± 13.06	43.9 ± 4.13	100	ND	ND
P36	FAP	72.7 ± 2.68	47.6 ± 2.06	100	10.1 ± 4.24	ND
G42	FAP	87.5 ± 7.37	130.3 ± 9.18	100	56.7 ± 5.81	49.9 ± 7.14
T45	FAC	74.1 ± 16.02	35.2 ± 4.57	100	5.0 ± 2.07	ND
D45	FAC	69.5 ± 4.49	37.5 ± 6.32	100	ND	ND
V47	FAP-CTS	64.6 ± 5.04	44.5 ± 6.78	100	ND	ND
R47	FAP	64.8 ± 4.55	33.6 ± 12.98	100	ND	ND
A47	FAP	72.9 ± 4.94	28.9 ± 2.09	100	ND	ND
A49	FAP	83.9 ± 18.82	37.8 ± 2.16	100	7.1 ± 1.47	ND
I50	FAC	43.9 ± 2.74	55.6 ± 3.82	100	88.2 ± 6.02	38.4 ± 12.43
R50	FAP	NA	41.1 ± 7.83	100	20.1 ± 4.68	ND
M50	FAP	65.8 ± 7.65	31.4 ± 3.60	100	32.3 ± 3.41	ND
P52	FAP	66.9 ± 6.70	47.6 ± 2.06	100	ND	ND
G54	FAP	19.2 ± 1.21	42.8 ± 1.47	65.4 ± 4.67	15.6 ± 5.95	ND
P55	FAP	7.6 ± 1.04	153.2 ± 12.51	100	100	88.0 ± 8.87
R58	FAP-CTS	55.2 ± 6.36	27.9 ± 2.29	88.6 ± 6.16	72.5 ± 8.90	ND
H58	FAP-CTS	51.3 ± 3.73	33.6 ± 7.91	100	ND	ND
K59	FAC	88.4 ± 8.48	NA	100	ND	ND
A60	FAC	86.2 ± 5.45	26.7 ± 3.38	100	ND	ND
K61	FAP	91.4 ± 9.49	42.3 ± 4.51	81.6 ± 5.82	ND	ND
L64	FAP	82.5 ± 7.45	35.3 ± 5.95	100	ND	ND
S64	FAP	76.7 ± 1.83	55.7 ± 10.93	100	88.1 ± 9.15	ND
L68	FAC	79.4 ± 10.06	32.1 ± 5.97	100	ND	ND
H69	AVO	81.1 ± 7.63	31.8 ± 5.92	100	ND	ND
N70	FAP-CTS	NA	NA	100	76.8 ± 4.83	ND
A71	FAP	77.9 ± 6.58	58.9 ± 9.56	100	ND	ND
Y77	FAP	NA	NA	100	ND	ND
S84	FAP-CTS	46.0 ± 6.67	45.1 ± 4.00	100	ND	ND
N84	AVO	65.7 ± 7.42	48.5 ± 6.19	100	ND	ND
Q89	FAC	NA	63.4 ± 8.31	100	58.3 ± 2.20	ND
N90		96.5 ± 6.32	36.7 ± 1.37	100	ND	ND
G97	FAP	80.4 ± 4.66	40.8 ± 5.20	91.6 ± 4.85	ND	ND

(*continued overleaf*)

Table 1. (*continued*)

Mutation	Clinical syndrome*	Rel. T4 binding % *	Aβ aggregation (%)	TTR aggregation (%) pH 4.8	pH 6.2	pH 6.8
M111	FAC	71.5 $\pm$ 2.08	27.9 $\pm$ 1.47	68.8 $\pm$ 4.96	ND	ND
R102		103.6 $\pm$ 4.97	18.7 $\pm$ 5.33	NA	NA	NA
V107	FAP	33.4 $\pm$ 2.66	69.8 $\pm$ 7.27	NA	NA	NA
T109		286.3 $\pm$ 17.51	42.4 $\pm$ 4.45	ND	ND	ND
C114	FAP	75.7 $\pm$ 2.81	42.0 $\pm$ 2.19	100	64.4 $\pm$ 10.32	4.0 $\pm$ 0.50
H114	FAP-CTS	69.2 $\pm$ 7.31	53.1 $\pm$ 4.45	68.6 $\pm$ 3.17	63.6 $\pm$ 5.71	12.3 $\pm$ 2.55
Y116	FAP	NA	NA	88.5 $\pm$ 2.65	58.7 $\pm$ 12.06	20.8 $\pm$ 4.41
M119		166.6 $\pm$ 9.14	33.3 $\pm$ 3.70	100	ND	ND
I122	FAC, SSA	78.3 $\pm$ 9.15	68.4 $\pm$ 4.46	100	ND	ND
Wild type	TTR	100.0	28.8 $\pm$ 4.02	64.8 $\pm$ 5.17	11.0 $\pm$ 1.80	ND
Plasma	TTR	114.4 $\pm$ 3.86	23.7 $\pm$ 4.68	66.2 $\pm$ 7.03	ND	ND

FAP, familial amyloid polyneuropathy; FAC, familial amyloid cardiomyopathy; FAP-CTS, familial amyloid polyneuropathy with carpal tunnel syndrome; AVO, amyloidotic vitreous opacitieis; SSA, senile systemic amyloidosis; T4, thyroxine; NA, non-analyzed; ND, non-detected. T4 binding to TTR variants was compared with wild type recombinant TTR by placing a value of 100% for T4 binding to wild type recombinant TTR. The percent of Aβ aggregation was determined by placing a value of 100% for Aβ aggregation without TTR. The percent of TTR aggregation was determined by comparison with the aggregation of wild type recombinant TTR (0.5 mg/ml, 9 μM) at pH 3.8, which was considered as 100% of total TTR aggregation. All results represent means of three separate experiments $\pm$ SD.

regard to purification of recombinant protein from cell lysates.

The main advantage of our system is that recombinant TTR is secreted into growth media and can be easily purified using one step DEAE chromatography. We showed that secreted recombinant TTR formed tetramers and was completely functional in thyroxine binding. Significantly decreased binding to thyroxine was detected for M30, I50, G54, P55, H58, R58, S84, and V107. Only TTR variants S6, M119, and T109 had higher affinity to thyroxine than wild type TTR. Similar results for TTR variants M30, H58, S84, S6, M119, and T109 were previously demonstrated by other groups[20,23,24]. Conflicting results were reported where the TTR variant S6 isolated from human plasma displayed a fourfold increased affinity to thyroxine[25]. However, recombinant S6 TTR with an amino-terminal substitution of G for M had the same affinity to thyroxine as wild type recombinant TTR[20]. We detected increased affinity to thyroxine for recombinant S6 TTR, which did not have any additional amino acid substitutions. Although the amino-terminus of TTR is not directly involved in the formation of the thyroxine binding pocket, it is quite possible that conformational changes caused by both M1 and S6 substitutions modified the tetrameric structure of TTR and its affinity for thyroxine.

Current hypotheses of TTR amyloidosis suggest that intralysosomal proteolysis or lysosomal acid-mediated denaturation might cause the abnormal assembly of TTR monomers into TTR fibrils[6,26,27,28,29]. Kelly and Colon reported that TTR self-assembles into TTR-amyloid fibrils under acidic conditions similar to those found in lysosomes[18]. This group proposed that TTR mutations significantly alter tetramer stability and increase amyloidogenicity of TTR variants[30]. Quintas and colleagues confirmed these observations and showed that the amyloidogenic potential of transthyretin variants M30, P55 and M119 correlates with their tendency to aggregate in solution[31]. Our results demonstrated that mutations spanning two ''hot spot'' regions in the TTR monomer spanning positions 30–55 (11 variants in Table 1) and 70–116 (5 variants in Table 1) resulted in aggregation of recombinant TTR at pH 6.2–6.8. EM analysis of TTR aggregates at pH 6.8 demonstrated characteristic TTR fibrils for G42 and P55. Using the same experimental conditions, TTR variants L30, M30 and I50 formed only Congo-Red positive amorphous masses without visible fibrillar structures. Thus, the ability of TTR P55 and TTR

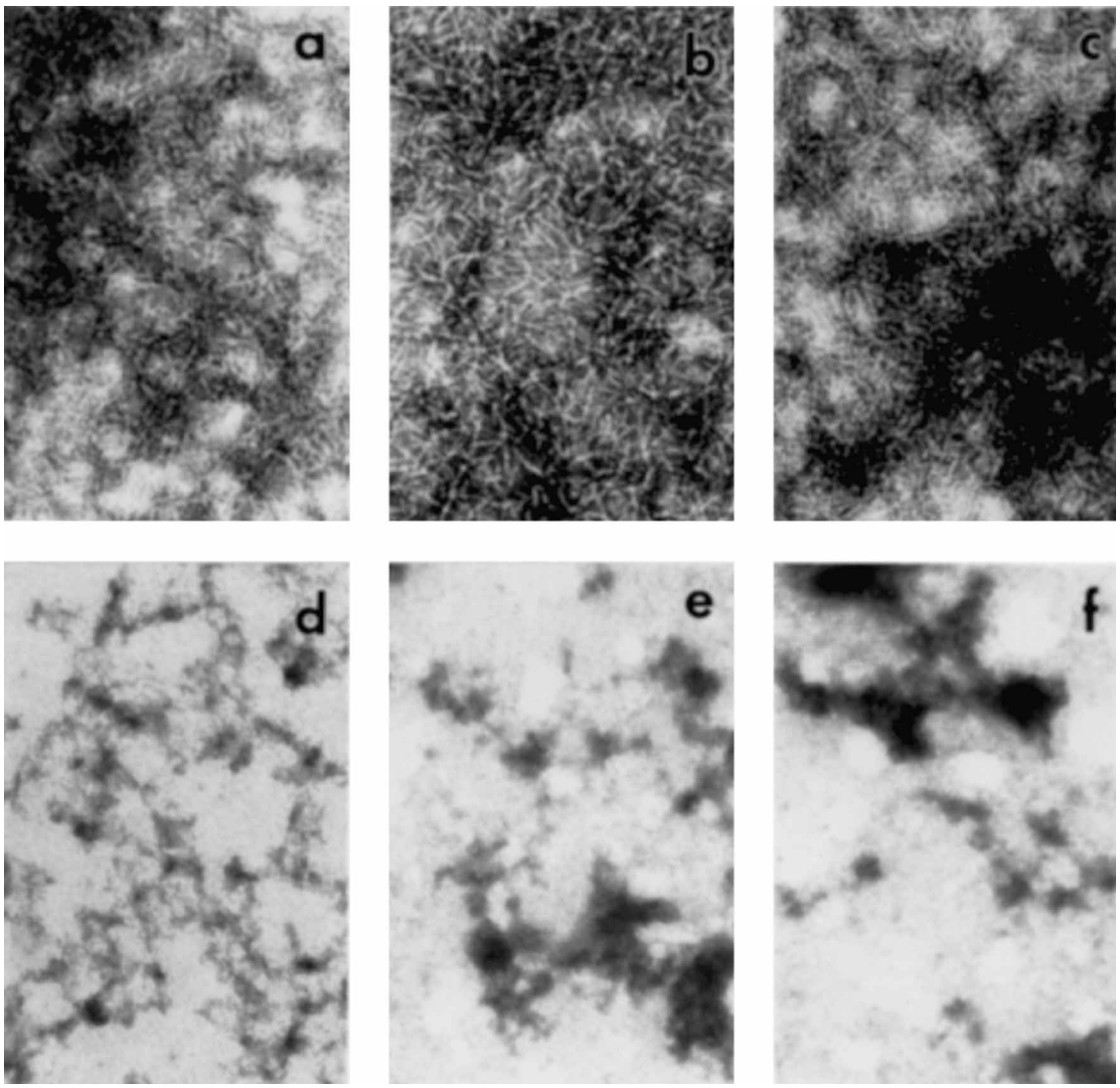

FIGURE 3: Electron micrographs of recombinant TTR aggregates. Aggregates of TTR and TTR variants at 2 mg/ml (36 μM) formed at different pH's and viewed at 60,000 x. a) Wild-type TTR fibrils formed at pH 3.8; b) P55 TTR fibrils formed at pH 6.8; c) G42 TTR fibrils formed at pH 6.8; d) L30 TTR aggregates formed at pH 6.8; e) M30 TTR aggregates formed at pH 6.8; f) I50 TTR aggregates formed at pH 6.8.

G42 to form fibrils in model experiments correlate with high amyloidogenic potentials of these transthyretin variants *in vivo*[36,37].

Recent findings showed that TTR levels in cerebrospinal fluid were significantly decreased in late onset Alzheimer's disease patients and negatively correlated with senile plaque abundance[32,33]. Another notable example of the role of TTR in Aβ amyloidosis is added by observations that mutant TTR may contribute to the development of sporadic inclusion body myositis (s-IBM). S-IBM has many Alzheimer's disease features including Aβ amyloid deposits and clusters of tau-containing paired helical filaments[34]. Recently, Askanas and colleagues described a 70-year-old African-American patient homozygous for the TTR variant I122 who had both cardiac amyloidosis and sporadic inclusion body myositis (s-IBM)[35]. These authors suggested that TTR I122 does not enhance Aβ catabolism, thereby promoting Aβ amyloidogenesis. Interestingly, data presented here indicated that recombinant TTR variant I122 inhibits Aβ aggregation less efficiently than wild type recombinant TTR (Table 1). Together, these data raise the possibility that normal TTR participates in the clearance and catabolism of Aβ and may be one of the factors preventing Aβ amyloidosis *in vivo*.

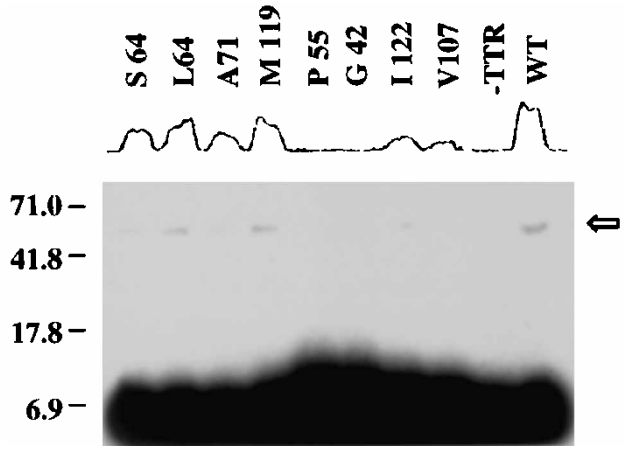

FIGURE 4: Detection of recombinant TTR- Aβ complexes. Autoradiogram of non-denaturing gradient PAGE (4–20%) (Bio-Rad) demonstrates distribution of ^{125}I-Aβ_{1-42} after incubation with recombinant TTR variants (200 ng). Scanning of X-ray films was performed using ChemiDoc system (Bio-Rad). Arrow shows direction of scanning. The relative molecular masses (kDa) of protein markers are shown on the left.

Among forty-seven investigated recombinant TTR variants, only G42 and P55 comletely failed to bind Aβ and to inhibit Aβ aggregation. TTR variant P55 does not bind thyroxine and is known as a highly amyloidogenic protein with an unstable tetrameric structure[6,36]. Although G42 is also a highly amyloidogenic protein[37], it binds thyroxine and does not reveal unstable tetrameric structure. We previously showed that E42 is a key amino acid in the Aβ binding domain of TTR[10]. Therefore, it is reasonable to propose that inability of TTR G42 to interact with Aβ may result from structural changes in the Aβ binding domain. We also can not exclude the possibility that G42 and P55 rapidly aggregate *in vitro* and that the aggregated proteins fail to interact with Aβ.

In summary, our results present a basis for studying the pathogenic relationship between two amyloidogenic processes. Our results showed that amyloidogenic mutations in TTR might affect the interaction of TTR with Aβ and lead to the inability of aggregated TTR proteins to prevent Aβ polymerization. In view of the fact that TTR sequesters circulating Aβ[38,39] and inhibits Aβ amyloidosis *in vivo*[11,12,13,14], our results might be particularly relevant for epidemiological studies in Alzheimer's disease.

Acknowledgments

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