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Modifications of transthyretin in amyloid fibrils: analysis of amyloid from homozygous and heterozygous individuals with the Met30 mutation

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The finding of individuals homozygous for FAP I (familial amyloidotic polyneuropathy, transthyretin TTRMet30) with amyloid deposits in the vitreous body, gave us access to a unique material lacking wild type transthyretin and contaminating proteins. Amyloid TTR is modified in several ways. Besides the full-length protein and its dimer form, two smaller bands were identified by SDS-PAGE and protein sequencing. One corresponded to a peptide starting at amino acid Thr49, the other was a mixture of two peptides starting at positions 1 and 3 in a 3:1 ratio. Upon reduction the amount of the TTR dimer decreased, the monomer amount increased, and the resulting monomers became available for carboxymethylation. Moreover, the mobility of the small band, which includes Cys10, increased upon reduction. This cysteine seemed to be involved in an interchain disulfide bridge both between intact TTR molecules and between small fragments. The same pattern was found in heterozygous fibril material although smaller amounts of the truncated peptides were found. Fibrils were formed both from normal and mutated TTR in heterozygotes. The significance of our results for amyloid formation is discussed.

Key words: amyloid fibrils/disulfide bonds/familial amyloidotic polyneuropathy/homozygosity/transthyretin

Introduction

Familial amyloidotic polyneuropathy (FAP) is one of a number of diseases caused by amyloid deposition (amyloidosis). Certain proteins or protein fragments precipitate as amyloid, a fibrillar aggregate with an anti-parallel β-pleated sheet structure. The affected protein in FAP type I is the plasma protein transthyretin, TTR, previously known as prealbumin (Costa *et al.*, 1978). In this disease amyloid is deposited in certain tissues, e.g. kidney, heart and along peripheral nerves (Benson and Wallace, 1989).

TTR is a protein of 127 amino acids in which four monomers are associated non-covalently to form a tetramer of 56 kDa (Blake *et al.*, 1978). Each monomer has a high degree of β-structure that contains eight β-strands in two parallel sheets of four strands each. It has been suggested that this β-structure probably predisposes the TTR molecule to form amyloid fibrils. Thus, most amyloid precursor protein molecules with two major domains of predicted β-configuration may form structures that generate a β-pleated amyloid sheet. However, it should be pointed out that unmutated TTR can also be amyloidogenic. In senile systemic amyloidosis no TTR mutation was found (Westermark *et al.*, 1990). This form of TTR amyloid affects ~25% of all individuals over 80 years in the form of heart deposits (Cornwell *et al.*, 1983).

More than 30 different point mutations of TTR have been described. Most of these mutations result in amyloid deposition in different organs and some along peripheral nerves. The reason for different organ distribution is not known. The mutations are found evenly all over the molecule and in themselves do not provide structural explanations for amyloid formation. Most of the mutations result in rather subtle changes of the TTR structure, but they may increase the probability of amyloid formation. Gajdusjek (1991) suggested that the conformational change needed for amyloid formation could be an induced nucleation and that a process related to crystal formation could induce fibril formation. Other amyloid diseases where mutations in the amyloid precursor protein have been implicated are FAP of Finnish type (gelsolin, Levy *et al.*, 1990), Alzheimer's disease (5% of the cases, Hardy and Allsop, 1991) and prion diseases, such as Creutzfeld Jacob's disease (5-10%) and Gerstmann Sträussler Scheinkers syndrome (Gajdusjek, 1991).

The most common form of hereditary TTR amyloidosis is TTRMet30, where valine at position 30 in the TTR protein is replaced by methionine. In most of the affected individuals carrying this TTR mutation, peripheral polyneuropathy is the prominent symptom. FAP I, TTRMet30 is a disease that exhibits autosomal dominant inheritance occurring in descendants from persons of Japanese (Tawara *et al.*, 1983), Portuguese (Saraiva *et al.*, 1984) and Swedish origin (Holmgren *et al.*, 1988a; Nakazato *et al.*, 1987). The Swedish form of this disease has a late onset (average 57 years) with low penetrance (Holmgren *et al.*, manuscript in preparation). In the Portuguese and Japanese form of the disease, the age of onset is earlier and the progress is more rapid. The reasons for these discrepancies are not known, but questions can be raised regarding which factor might protect the Swedish mutant gene carriers from the early onset of the disease by ~25 years compared with Portuguese and Japanese carriers. Death in this syndrome is most commonly due to gastrointestinal problems, myocardial or renal failure. The TTR gene has been localized to chromosome 18 and it is mainly expressed in the hepatocytes (Wallace *et al.*, 1985). Most of the patients are heterozygous but among the Swedish patients several homozygous individuals have been found (Holmgren *et al*, 1988a; Sandgren *et al.*, 1990, 1991). However, it must be noted that several of these suffer only from vitreous opacities. Furthermore, no gene dosage effect on the severity of symptoms has been found. Recently two TTRMet30 homozygotes have been reported from Turkey

(Skare *et al.*, 1990). In their family study, limited to 31 individuals in three generations, only homozygotes have FAP I symptoms with late onset (55 and 61 years, respectively).

To understand the mechanism for amyloid fibril formation it is important to know the amyloid protein composition with respect to the contribution by the mutant as well as normal TTR protein. In plasma from TTRMet30 heterozygotes ~55% of total TTR consists of normal TTR (Holmgren *et al.*, 1993). By peptide mapping Saraiva *et al.* (1984) showed that the fibrils isolated from the kidney from a FAP I TTRMet30 patient selectively accumulated the mutated protein. In ~10% of the Swedish FAP I patients amyloid deposits were found in the vitreous body of the eye (Sandgren *et al.*, 1991). We took advantage of the fact that the vitreous body is a relatively protein poor environment and could obtain almost pure amyloid fibrils with mild methods. Moreover, as some of the patients are homozygous for the TTRMet30 gene (Holmgren *et al*, 1988b; Sandgren *et al.*, 1990, 1991), we could obtain pure mutated protein. In this study, we have examined fibrils from the vitreous body from homozygous and heterozygous individuals. The TTR-Met30 mutation was identified by RFLP analysis as previously described (Holmgren *et al.*, 1988b). TTR proteins predominated and a significant proportion of the material was truncated with fragments starting at amino acid positions 1, 3 and 49. We present evidence for the formation of an interchain disulfide bridge and/or stabilization of tetramer interactions predisposing to fibrillar aggregates.

Results

Analysis of the proteins from the vitreous body

The proteins in the amyloid fibrils of the vitreous body were gently extracted and analysed using SDS–PAGE (Figure 1A) followed by immunostaining (Figure 1B) under reducing and non-reducing conditions. Two prominent protein bands were found under reducing conditions. The larger of these bands was identified as the TTR monomer. It had the same gel mobility as the purified TTR control plasma protein and it was easily detected by immunostaining using an anti-human TTR antibody. It migrated somewhat anomalously as a 17 kDa protein (calculated molecular weight is 13.8 kDa). A second prominent band migrated as a 14 kDa protein. It stained weakly with the anti-TTR human antibody used from material purified by HPLC (data not shown). This band was found to react strongly with an antibody against the senile form of TTR amyloid (data not shown). It has been shown that the main portion of amyloid from senile systemic amyloidosis consists of TTR fragments (Cornwell *et al.*, 1988). More of this 14 kDa protein and less of the TTR monomer were found in the samples from the homozygous individuals than from heterozygous individuals. Similar results were also obtained from our studies with two more homozygous and heterozygous individuals (data not shown).

Under non-reducing conditions we found the same bands as above, with decreased intensity of the 17 kDa band (Figure 1B, lanes 1 and 3). Furthermore, the intensity of a band corresponding to the size of the TTR dimer increased. Higher molecular weight bands that were recognized by the TTR antibody were found, probably representing TTR multimers. Some weakly TTR reactive bands, smaller than the TTR dimer, were also seen on the gel. A third, smaller protein band was also detected and it showed a dramatic mobility shift between non-reducing and reducing conditions and this band was more prominent from the homozygous than from the heterozygous material. However, it should be noted that the smallest fragment was not detected by the anti-human TTR antibody (Figure 1B) or the anti-human senile amyloid antibody (data not shown).

Identification of fibril proteins by amino acid sequencing

To determine further the nature of the main protein bands from homozygous and heterozygous fibril material was separated using 20% SDS–PAGE, blotted onto an Immobilon filter, stained and sequenced (Figure 2). The first 12 amino acids of the 17 kDa band were found to be identical

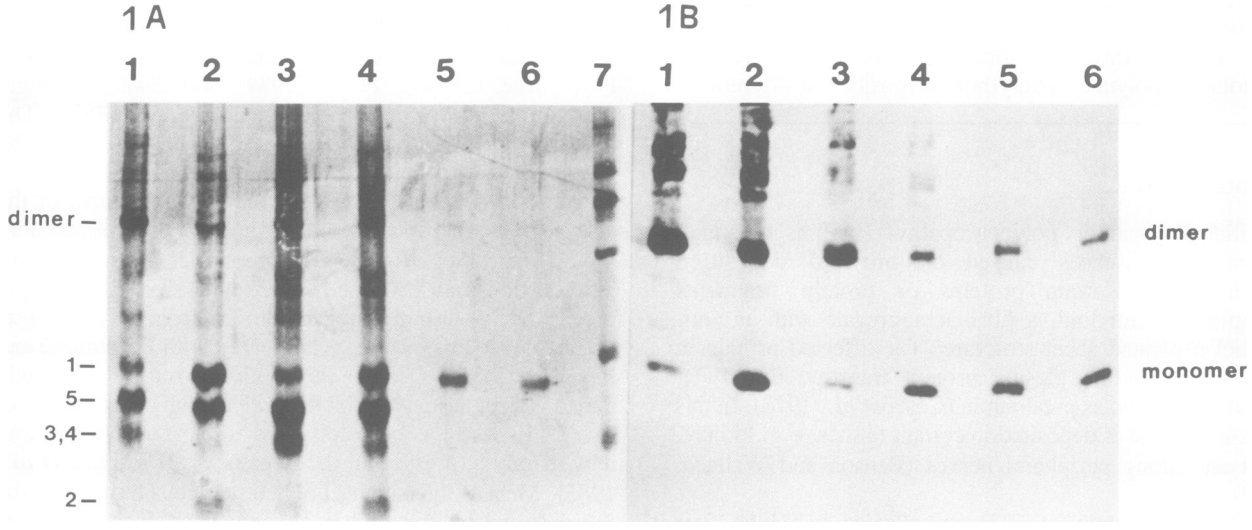

Fig. 1. Analysis of proteins from the vitreous body. **A.** 20% SDS–PAGE. **B.** Immunoblotting. Lanes 1–4, vitreous samples from individuals carrying the TTRMet30 mutation: lane 1, heterozygous non-reducing conditions; lane 2, heterozygous reducing conditions; lane 3, homozygous non-reducing conditions; lane 4, homozygous reducing conditions. Lanes 5–6, TTR from plasma: lane 5, non-reducing conditions; lane 6, reducing conditions; lane 7, molecular weight standards (from the bottom, proteins of 14.4, 21.5, 31, 45 and 97.4 kDa (omitted in the gel for the immunoblotting). The positions of the TTR dimer and monomer are marked on the figure. The numbering of the bands to the left are as in Table I.

to the TTR protein sequence (Table I, sequence 1a and b). The prominent band, migrating as a 14 kDa protein, was found to be a fragment of TTR, since its 10 N-terminal amino acids corresponded to the published sequence (Kanda *et al.*, 1974), starting at the Thr49 position (49–127 fragment). This sequence was determined independently using two different homozygous individuals (sequence 5a and b).

Two sequences were obtained from the smaller band: one sequence started at position 1 of the TTR protein sequence and the other at position 3. In the homozygous material the proportion was 3:1 between these two fragments and in the heterozygous material the proportion was 1:1 (sequence 4a and b). Analysis was performed both with reduced (sequence 2a and b) and non-reduced samples (sequence 3a and b) from the small fragment of the homozygous material. In both cases the same sequence and fragmentation pattern was observed. Two N-terminal sequences were also found for the TTR monomer, but the proportion between the sequence starting at position 1 and 3 was 1:2 in this case.

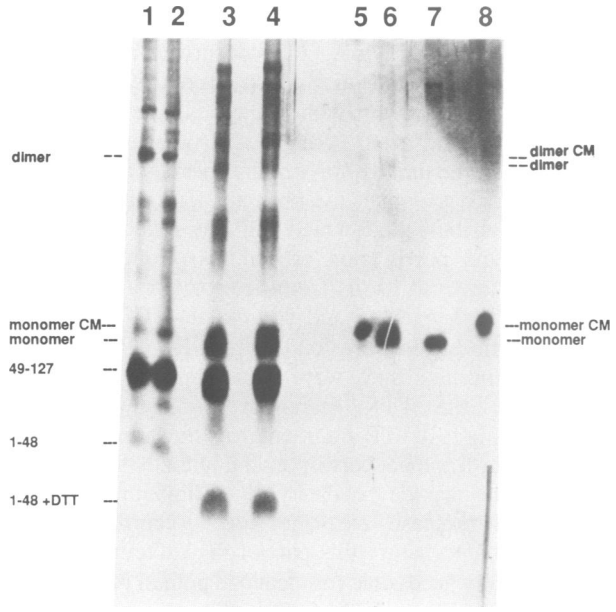

Fig. 2. SDS–PAGE separation of carboxymethylated and non-carboxymethylated fibril samples. Lanes 1–4, homozygous fibril material: lane 1, carboxymethylated; lane 2, untreated; lane 3, reduced; lane 4, carboxymethylated and reduced; lanes 5–8, plasma TTR: lane 5, carboxymethylated; lane 6, untreated; lane 7, reduced; lane 8, carboxymethylated and reduced. The positions for the carboxymethylated dimer (CM), the dimer, the carboxymethylated monomer (CM), the monomer, the 49–127 fragment, the 1–48 fragment and the 1–48 fragment in the presence of DTT are marked on the figure.

Carboxymethylation

Iodoacetic acid treatment was used to determine whether the cysteine residues in the fibril were available for carboxymethylation or blocked by disulfide bonding. Carboxymethylation with iodoacetic acid introduces a new acidic group at each Cys residue not involved in a disulfide bond. Thus, electrophoretic separation of carboxymethylated and non-carboxymethylated Cys-containing species can be feasible (Creighton, 1974). Fibril samples from a homozygous individual and plasma TTR were separated using 20% SDS–PAGE after carboxymethylation (Figure 2, lanes 1 and 5), untreated (lanes 2 and 6), reduced (lanes 3 and 7) and carboxymethylated and reduced (lanes 4 and 8). Iodoacetic acid treatment of the samples resulted in a mobility shift for the TTR monomer, which made it possible to distinguish between the non-carboxymethylated and carboxymethylated TTR. With plasma TTR the same pattern was also seen after carboxymethylation both for non-reduced and reduced samples (lanes 5 and 8 respectively), indicating that the cysteines are free and not involved in disulfide bridges. When fibrillar material was used no change in mobility was seen for the dimer after carboxymethylation but the monomer (a weak band) shifted mobility (lane 1). If the sample was then reduced after carboxymethylation (lane 4) two monomer bands were found. The amount of the new band was approximately twice the amount of the carboxymethylated monomer. When the samples were

Table I. Amino acid sequencing of TTR fibrillar material

																														Sequence no
Aminoacid no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16/	47	48	49	50	51	52	53	54	55	56	57	58	59	
Aminoacid	G	P	T	G	T	G	E	S	K	C	P	L	M	V	K	V/	G	K	T	S	E	S	G	E	L	H	G	L	T	
Obtained	G	P	T	G	T	G	E	S	K	C	P	L																		1a
sequence			T	G	T	G	E	S	K	C	P	L	M	V																1b
"	G	P	T	G	T	G	E	S	K	C	P	L																		2a
"			T	G	T	G	E	S	K	C	P	L	M	V																2b
"	G	P	T	G																										3a
"			T	G																										3b
"	G	P	T	G	T	G	E	S	K	C	P																			4a
"			T	G	T	G	E	S	K	C	P	L	M																	4b
"																			T	S	E	S	G	E	L	H	G	L		5a
"																			T	S	E	S	G	E	L	X	G			5b

Table I shows the sequences that were obtained from SDS–PAGE, after blotting onto a Immobilon PVDF filter and sequencing. The relevant part of the TTR sequence (Kanda *et al.*, 1974) is shown on the top of the table. Two sequences (a and b) were obtained from the bands 1–4. The proportion between the sequences starting at amino acids 1 and 3 are given within brackets. Sequence 1 was from the band in the size of the TTR non-reduced monomer (1:2, homozygous); sequence 2, reduced small fragment (3:1, homozygous); sequence 3, non-reduced fragment (3:1 homozygous); sequence 4, non-reduced fragment (1:1 heterozygous). Sequence 5a and b were from the large fragment (homozygous) from two different homozygotes. In one of the determinations (5b) amino acid 56 could not be determined and is therefore marked with an x.

reduced, but not carboxymethylated, only one monomer band was observed (lane 3). This indicated the presence of a disulfide bond between monomers in the fibril material.

Fibril composition in heterozygous individuals

To determine if the fibril was formed from mutated TTR, and if both mutated TTR and normal TTR contributed to the fibril formation in heterozygotes, cyanogen bromide (CNBr) cleavages were performed. CNBr cleaves proteins at methionine residues. There are two methionine residues in the mutated TTR (Met13 and Met30) and one methionine in normal TTR (Met13, Kanda *et al.*, 1974). Thus, such cleavage can result in a large band of 114 amino acids from wild type TTR and of 97 amino acids from TTRMet30 in addition to smaller bands. To get a complete cleavage at position 13 in the fibril TTR, incubation with CNBr in 70% formic acid had to be continued for up to 36 h. This resulted in some degradation of the proteins, especially of plasma TTR (Figure 3, lane 5), where the 114 amino acids band is barely visible. From heterozygous fibril material we could identify two new large bands after cleavage, both of which were larger than the 49–127 TTR fragment; one was similar to the new band obtained after CNBr cleavage of plasma TTR and the other was smaller (lanes 3 and 4). The amount of the larger band (114 amino acids) was estimated to be 20–30% of the smaller band (97 amino acids). However, only the smaller CNBr band was obtained from the homozygous individual (lanes 1 and 2). As a comparison uncleaved homozygous fibril proteins are shown in lane 7 and plasma TTR in lane 8. This clearly indicates that both normal and mutated TTR are contained within the fibril. Smaller quantities of the CNBr fragment were found than of the 49–127 fragment from the homozygote. In the heterozygote more of the 49–127 fragment was found than the 114 amino acid fragment, which means that the wild type protein is also truncated. Cleavage products of the smaller bands corresponding to amino acids 1–48 and 3–48 could not be detected due to the small amount and to probable washing off during fixing and silver staining of the gel.

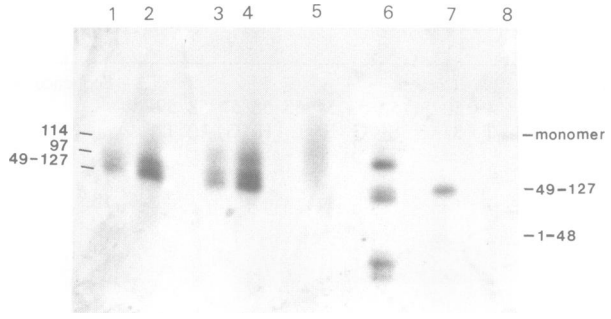

Fig. 3. SDS–PAGE separation of CNBr-cleaved fibril samples. Lanes 1–2, homozygous cleaved samples (1.5 and 5 μg); lanes 3–4, heterozygous cleaved samples (1.5 and 5 μg); lane 5, TTR cleaved; lane 6, size marker; lane 7, homozygous uncleaved; lane 8, TTR uncleaved. To the left of the figure are shown the positions for the two CNBr fragments (114 and 97 amino acids respectively) and the 49–127 TTR fragment. To the right is shown the position of the TTR monomer, the 49–127 and 1–48 fragments respectively. The molecular weight standards (lane 6) correspond (from the top) to 16.9, 14.4, 6.2, 2.5 and 1.7 kDa respectively.

Discussion

The aim of this investigation was to study modifications of TTR in amyloid fibrils that could give clues to the mechanism of fibril formation. TTR is an amyloidogenic protein that often precipitates into amyloid fibrils in elderly individuals. Several point mutations are described that appear to increase the risk of fibril formation, as symptoms can appear from early adulthood to late middle age, but never from childhood. Several authors have extracted amyloid from different tissues using rather harsh methods. We were able to take advantage of the presence of amyloid fibrils occurring in the protein poor environment of the vitreous body from a few individuals who were homozygous with respect to the amino acid substitution at position 30 in TTR (type 1 FAP). We therefore had the unique opportunity to study pure mutated amyloid fibrils to which the normal, non-mutated protein did not contribute and which did not require extensive extraction and purification procedures. Comparison was made with amyloid fibrils from heterozygous individuals, expressing both mutated and non-mutated TTR.

Three main bands were found after SDS–PAGE of the fibril protein and they were identified by amino acid sequencing: the TTR monomer, a prominent band corresponding to a TTR fragment starting at amino acid 49 and a smaller fragment corresponding to the N-terminal part of TTR. The peptide bonds Lys48–Thr49 in TTR could fit with a protease cleavage site. According to the 3-dimensional structure this part of the protein is exposed and potentially accessible for cleavage both in native (Blake *et al.*, 1978) and TTRMet30 (Terry *et al.*, 1993). Truncations resulting in fragments starting at the same or nearby positions (Ser46 and Ser52) have also been reported by Cornwell *et al.* (1988) and Westermark *et al.* (1990) in senile systemic amyloidosis and at position 49 in FAP I (Pras *et al.*, 1983).

The large fragment starting at position 49 was weakly recognized by the rabbit anti-human TTR antibody used only after HPLC purification (data not shown). It was only detectable using an antibody prepared against TTR from senile systemic amyloid. This antibody mainly recognizes TTR fragments starting at amino acids 46, 49 and 52 (Pitkäinen *et al.*, 1984). Whether the truncation opens up cryptic epitopes or whether fibrillar components prevented the immunoreaction requires further studies.

We found that the small band consisted of two peptides, one starting at the N-terminal glycine and the other at amino acid Thr3. Three times the amount of the peptides was found from the longer of these peptides in the homozygous material, whereas approximately the same amount of these peptides were found in the heterozygous material. It seems that normal TTR is more prone to a ragged N-terminus than the TTRMet30 monomer. Ragged N-termini involving up to two amino acids, have been seen in normal plasma TTR [5–10% of the monomers starting at amino acids 2 and 3, Pettersson *et al.* (1987)] and up to five amino acids in different amyloid fibrils (Tawara *et al.*, 1983; Dwulet and Benson, 1986; Wallace *et al.*, 1986). As ragged N-termini are also seen in unrelated proteins (Jörnwall *et al.*, 1986), it could be a secondary phenomenon. It could also have been introduced upon amyloid purification with little relevance for amyloid formation.

Two of the peptides seen by SDS−PAGE analysis seemed to contain disulfide bonds. The intensity of the band corresponding to the TTR dimer decreased under reducing conditions compared with non-reducing conditions, while the amount of the intact monomer appearing as a 17 kDa band increased. Moreover, the cysteines in the dimer were not available for carboxymethylation as after reduction the mobility of the resulting monomers was the same as that of the non-carboxymethylated monomer. Judging from staining intensities, at least two-thirds of the TTR monomers were engaged in an intersubunit disulfide bridge. In the case of the small fragment a dramatic decrease in mobility was seen upon reduction. The same sequences in the 12 N-terminal amino acids were seen, excluding sequence differences as an explanation for the mobility shift.

As there is only one cysteine (at position 10) per monomer and the cysteines are separated by 26 Å (Blake *et al.*, 1978), intramolecular disulfide bridge formation is not possible. Therefore the observed disulfide bridges must be intermolecular disulfide bonds. A model for amyloid formation based on X-ray crystallography data is presented by Terry *et al.* (1993). According to their model two cysteines from one TTR molecule are linked to two cysteines in another TTR molecule, by disulfide bridges, thus making a long chain of tetramers. The finding of a covalent bond via a cysteinyl residue between subunits was described by Felding *et al.* (1985) for senile amyloidosis and FAP. The TTR fragments that we have observed could have been formed from proteolytic cleavage at positions 48−49 from already formed amyloid, resulting in disulfide bridges also between the small 1−48 fragments. Other bands were also detected by SDS−PAGE. It is likely that some of them could be the TTR monomer linked to different forms of the fragments as the difference in the band pattern is seen between reduced and non-reduced samples.

In Creutzfeldt−Jacob disease, another form of amyloidosis, it was found that there was an increased number of disease cases among individuals homozygous at a specific point mutation in the prion protein (Palmer *et al.*, 1991). Their data suggested that the prion protein formed homodimers which may be important for the efficiency of the conversion into amyloid. The authors speculated that heterozygous individuals may be partially protected against the disease because of the increased number of heterodimers less prone to amyloid formation. In the case of TTR one should expect for stoichiometric reasons a certain frequency of homotetramers TTRMet30 mutation in heterozygous patients.

In order to understand the mechanism for amyloid fibril formation it is important to know the amyloid protein composition with regard to contributions by mutant as well as normal TTR protein. Several studies have addressed this question. In plasma from our heterozygous TTRMet30 patients, ~45% of total TTR is TTRMet30 (Holmgren *et al.*, 1993). The total level of TTR in these patients is ~75% of the level in normal individuals. In the two homozygotes tested the total TTR level is about the same as the total level of TTRMet30 in heterozygous individuals. Saraiva *et al.* (1984) suggested that the variant TTRMet30 selectively deposits in tissue as amyloid of which at least 90% of the TTR was mutated. In other types of FAP amyloid, both mutated and normal TTR were found. Wallace *et al.* (1986) found that the mutant protein in TTRThr60 fibrils made up two-thirds of the constituent protein. In plasma 40−45% of the TTR was mutated. Dwulet and Benson (1986) also found that about two-thirds of the fibrillar protein was mutated in the case of Indiana/Swiss type of FAP (position 84).

Our CNBr cleavage data shows that the amyloid fibrils consist of both normal and mutated TTR in the heterozygous individual. In our case normal TTR seems to make up 20−30% of the fibril, which is in accordance with what has been observed for TTRThr60 and TTRSer84 by others (Dwulet and Benson, 1986; Wallace *et al.*, 1986). Thus, several forms of TTR-associated amyloidosis have fibrils with a mixture of mutated and normal TTR subunits. The nucleation model of Gajdusjek (1991) predicted that once the amyloid formation has been initiated, normal TTR could also be deposited. Our finding that homozygous individuals are not more severely affected and may even be totally healthy up to old age (Holmgren *et al.*, 1988a,b), as well as the fact that children are seldom affected, supports the idea of a random event. Once the process has been initiated, it could also recruit native subunits of TTR tetramers containing different proportions of mutated subunits.

Thus, our data show an interchain disulfide bridge in TTR from fibrils, but not in TTR from plasma. Moreover, we demonstrate cleavage resulting in a peptide starting at position 49 and a smaller N-terminal fragment. As the two fragments also occur in heterozygous individuals we assume that it is a secondary phenomenon rather than important for the development of the fibrils. In the accompanying paper Terry *et al.* (1993) show that the mutation at position 30 results in conformational changes resulting in greater exposure of Cys10. We present evidence that disulfide bridges exist between subunits in TTR, which could enhance the probability for amyloid deposition. The alternatives that the interchain disulfide bridge could stabilize the formation of an inter-tetrameric association leading to amyloid deposition, or even could be etiologically involved, could be experimentally tested.

Materials and methods

Gel analysis and immunoblotting

The amyloid fibrils from vitrectomies were collected in 0.9% NaCl and stored frozen in 1 ml portions at −20°C. Upon thawing the fibrils were centrifuged (15 000 r.p.m. for 25 min at 4°C), dissolved in 2×sample buffer (3 M urea, 2.5% SDS, 0.05 M Tris pH 6.8 and 0.05% bromophenol blue), treated at 95°C for 10 min, loaded and run on 20% SDS−polyacrylamide gels (SDS−PAGE; Dreyfuss *et al.*, 1984) under reducing conditions [0.1 M dithiothreitol (DTT; Sigma) at room temperature for 30 min] and non-reducing conditions. 1 M DTT was stored frozen in small aliquots. Freeze-dried plasma TTR (Sigma, St Louis, USA) was redissolved in H_2O and frozen in small aliquots for single use and used as a control. The samples were run in duplicate; one gel was used for the silver staining of the proteins (See and Jackowski, 1989) and one gel for immunoblotting, essentially as described by Harlow and Lane (1988). The primary antibody was rabbit anti-human TTR (DAKO, Denmark) and the secondary antibody was goat anti-rabbit IgG horseradish peroxidase (Bio-Rad). For development of the blot 4-chloro-1-naphthol (Bio-Rad) was used.

Amino acid sequencing

About 30 μg of fibrillar protein (centrifuged as above) and plasma TTR (size marker) were prepared in duplicate and dissolved in 2×sample buffer. To half of the samples 0.1 M DTT was added and reduction was performed at room temperature for 30 min. The samples were treated at 95°C for 10 min, loaded and run on a 20% SDS−polyacrylamide gel that had been

prerun overnight in the dark. All the solutions used had been filtrated through a 0.45 μm filter. The proteins were electroblotted onto a Immobilon PVDF membrane (Millipore) for 75 min, essentially as described by Matsudaira (1987). The proteins on the membrane were visualized by Coomassie blue R-250 staining. The filter was then stored at −20°C until used. Sequence analysis were made on an Applied Biosystems 477A Pulsed Liquid Phase sequencer with an online PTH 120A Analyser. Sequencing was performed with regular cycle programs and chemicals from the manufacturer. Initial yield calculated from a sequenced standard protein, β-lactoglobulin 47 and 25% respectively. Repetitive yield 97−97.5%.

Iodoacetic treatment

Iodoacetic treatment (10 mM) was performed in 2× sample buffer with pH adjusted to pH 8.5 at 37°C for 30 min. The samples were then divided and DTT was added to half to give a concentration of 0.1 M and incubation was continued for 30 min at 37°C. Untreated samples and samples to which DTT only was added, were also incubated for 1 h at 37°C in the dark. The pH was adjusted to 6.8 and the samples were treated at 95°C for 10 min. The samples were then applied to a 20% SDS−polyacrylamide gel and run until the bromophenol blue dye marker had left the gel. The gel was then silver stained.

CNBr cleavage

The fibril samples (~15 μg) were centrifuged as above, solubilized in 2×sample buffer and vacuum dried. The fibrils were then dissolved in 50 μl 70% formic acid containing 15 mg CNBr and left at room temperature for 18−36 h in the dark. After 18 h 25 μl of the samples were removed, 1 ml distilled water was added and the samples were vacuum dried. To the samples were added 2×sample buffer. The fibril samples were divided (1/5 and 4/5) into two tubes. Water and sample buffer were added to adjust the volume. The samples were then treated at 95°C for 10 min, loaded and run on a 20% SDS−PAGE gel. The gel was then silver stained (See and Jackowski, 1989). A second gel was run on material cleaved for 36 h.

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