Designing transthyretin mutants affecting tetrameric structure: implications in amyloidogenicity

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The molecular mechanisms that convert soluble transthyretin (TTR) tetramers into insoluble amyloid fibrils are still unknown; dissociation of the TTR tetramer is a pre-requisite for amyloid formation *in vitro* and involvement of monomers and/or dimers in fibril formation has been suggested by structural studies. We have designed four mutated proteins with the purpose of stabilizing [Ser¹¹⁷ → Cys (S117C) and Glu⁹² → Cys (E92C)] or destabilizing [Asp¹⁸ → Asn (D18N) and Leu¹¹⁰ → Ala (D110A)] the dimer/tetramer interactions in TTR, aiming at elucidating structural determinants in amyloidogenesis. The resistance of the mutated proteins to dissociation was analysed by HPLC studies of diluted TTR preparations. Both ‘stabilized’ mutants migrated as tetramers and, upon dilution, no other TTR species was observed, confirming the increased resistance to dissociation. For the ‘destabilized’ mutants, a mixture of tetrameric and monomeric forms co-existed at low dilution and the latter

increased upon 10-fold dilution. Both of the destabilizing mutants formed amyloid *in vitro* when acidified. This result indicated that both the AB loop of TTR, destabilized in D18N, and the hydrophobic interactions affecting the dimer–dimer interfaces in L110A are implicated in the stability of the tetrameric structure. The stabilized mutants, which were dimeric in nature through disulphide bonding, were unable to polymerize into amyloid, even at pH 3.2. When the amyloid formation assay was repeated in the presence of 2-mercaptoethanol, upon disruption of the S–S bridges of these stable dimers, amyloid fibril formation was observed. This experimental evidence suggests that monomers, rather than dimers, are the repeating structural subunit comprising the amyloid fibrils.

Key words: amyloid, amyloid neuropathy, thyroxine, transthyretin.

INTRODUCTION

Abnormal self-assembly of transthyretin (TTR) into amyloid fibrils has been implicated as the causative agent in familial amyloidotic polyneuropathy (FAP). The majority of TTR-associated amyloidoses is due to single amino-acid substitutions [1].

Plasma TTR is a homotetrameric protein, with a physiological role in the transport of the hormone thyroxine (T₄) and retinol. It was established by X-ray crystallography that two binding sites exist for T₄, which are located in a central channel formed by the interaction of the four monomers [2]. Although both binding sites are similar, they present different binding affinities for T₄ because of negative co-operativity of binding. Retinol-binding protein, which binds TTR for the transport of vitamin A, attaches to the exterior of the tetramer as revealed by the structure of the complex [3]. TTR has an extensive β -sheet structure making the molecule potentially amyloidogenic and mutations in its sequence increase the susceptibility to amyloid formation [4].

The molecular mechanism leading to the polymerization of TTR and its deposition as amyloid fibrils is unknown, although there is evidence to support the notion that fibril formation may develop from destabilization of the tetrameric form of the protein. This was revealed by crystallographic studies of amyloidogenic variants, namely Val¹²² → Ile (V122I etc.) and L55P [5,6], and in biophysical amyloid formation studies *in vitro* [7]. In order to elucidate the fibre structure in FAP more directly, X-ray

diffraction patterns from native vitreous TTR amyloid fibrils have been analysed. Although some authors have proposed a model based on a helical protofilament with dimensions suggesting a dimer/monomer size-unit for the fibril [8], others have postulated that the TTR monomer would constitute the unit structure of the protofilament [9]. Therefore information is needed on the importance of specific residues and/or domains that stabilize the tetrameric structure as well as on the unit structure of fibrils. Observations resulting from several studies lead us to design TTR mutants with weakened or strengthened dimer/tetramer interactions in order to address these issues.

It has been suggested that the substitution T119M might have a protective role in amyloidogenesis, indicated by the slower progress of the disease in heterozygote patients of TTR V30M-T119M [10]. Studies on the T119M variant [11] indicated that, contrary to most TTR variants, the substitution might induce stabilization of the tetrameric structure, resulting in lower amyloidogenic potential. In fact, comparative investigations on the resistance of TTR to dissociation into monomers, by isoelectric focusing, in serum samples from carriers of mutations V30M and T119M, and from heterozygotes with both mutations, revealed a striking difference in resistance to dissociation.

In the present study, four TTR mutants were designed with the aim of destabilizing (mutations D18N and L110A) or stabilizing (mutations S117C and E92C) the dimer/tetramer interactions in TTR, in the hope of elucidating the nature of the quaternary structure of the amyloidogenic intermediate in the fibrillogenesis cascade.

Abbreviations used: FAP, familial amyloidotic polyneuropathy; T₄, thyroxine; TTR, transthyretin; D18N, Asp¹⁸ → Asn; V30M, Val³⁰ → Met substitution etc.; ThT, thioflavin T.

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EXPERIMENTAL

Design of domain interface variants

The computer graphics software Turbo-Frodo [12] was used to predict the possible structural changes associated with specific mutations, D18N, E92C, L110A and S117C. By inserting the variant residues into the known molecular structures of the wild-type, L55P [6] and T119M [13] TTR molecules, we assessed the changes in hydrogen bonding and/or close contacts with other main-chain or side-chain atoms.

TTR mutagenesis and expression *in vitro*

The wild-type TTR cDNA, cloned into the pINTR vector, preceded by a sequence encoding the *OmpA* leader signal and flanked by *Bam*HI and *Pst*I sites [14], was used to produce mutant TTR molecules by site-directed mutagenesis (Quick Change site-directed mutagenesis kit; Stratagene). These mutants were constructed using two mismatched primers, introducing a 1–3 base substitution in the original sequence: D18N: 5' C-A-A-A-G-T-T-C-T-A-A-A-T-G-C-T-G-T-C-C-G 3', 5' C-G-G-A-C-A-G-C-A-T-T-T-A-G-A-A-C-T-T-T-G 3'; E92C: 5' G-A-G-C-A-T-G-C-A-T-G-C-G-T-G-G-T-A-T-T-C 3', 5' G-A-A-T-A-C-C-A-C-G-C-A-T-G-C-A-T-G-C-T-C 3'; L110A: 5' C-A-T-T-G-C-C-G-C-C-G-C-G-C-T-G-A-G-C-G-C-C 3', 5' G-G-G-G-C-T-C-A-G-C-G-C-G-G-C-G-G-C-A-A-T-G 3'; S117C: 5' C-T-A-C-T-C-C-T-A-T-T-G-T-A-C-C-A-C-G-G-C 3', 5' G-C-C-G-T-G-G-T-A-C-A-A-T-A-G-G-A-G-T-A-G 3'. All PCR-amplified products were treated with *Dpn*I to digest non-mutated parental DNA template, and one tenth of the reaction volume, containing the circular, mutated dsDNA, was used to transform *Epicurian coli* XL1-Blue supercompetent cells. Small DNA plasmid preparations were produced and tested by sequencing. Mutated TTR was expressed in an *Escherichia coli* expression system, as described in [14].

Isolation and purification of TTR variants

E. coli strain BL-21 was transformed with individual expression plasmids and the periplasmic-space contents were obtained by osmotic shock. The supernatant was fractionated on DEAE-Sephadex and the TTR-containing peaks were dialysed overnight against water and then freeze-dried. Further purification was achieved by preparative gel electrophoresis, followed by gel filtration on a 1.5 cm × 50 cm Bio-Rad column packed with Biogel P-100 (Bio-Rad) equilibrated with appropriate buffer (0.1 M KH_2PO_4 /0.1 M Na_2SO_4).

Gel electrophoresis

Proteins were analysed in either native or denaturing conditions. Native electrophoresis was carried out in 8% (w/v) acrylamide gels. Electrophoresis under denaturing conditions was performed in SDS/polyacrylamide (15% acrylamide, 0.1% SDS) gels, with or without heat treatment of samples and with or without the addition of 2-mercaptoethanol (0.1 M). Isoelectric focusing (7% acrylamide gels) was also performed under semi-denaturing conditions (4 M urea) [11].

Western blots

Proteins were transferred from gels on to nitrocellulose membranes (HybondTM-C pure; Amersham), using a Tris/glycine system, for 1 h at 1 mA/cm² of membrane. Proteins from native gels were transferred on to nitrocellulose membranes, using a Tris/boric acid system, for 90 min at 100 V. After blocking and

washing with PBS containing 0.05% (v/v) Tween-20, the membrane was incubated with rabbit anti-TTR polyclonal antibody (DAKO) (1:1000 dilution in PBS) for 1 h at room temperature. The membrane was then washed several times and incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (Binding Site) at 1:1000 dilution for 1 h at room temperature. TTR was visualized using an enhanced chemiluminescent method (Pierce).

T₄ binding assay

Soluble TTR samples (0.5–2 µg) were incubated with radio-labelled T₄ for 30 min and subjected to PAGE (8% acrylamide gels), as described in [15]. The gels were dried and autoradiographed.

HPLC

All TTR preparations destined for gel-filtration chromatography were diluted > 10 times in gel-filtration-chromatography buffer (20 mM sodium phosphate buffer/150 mM NaCl, pH 7.0) according to Quintas et al. [16]. Protein concentrations were determined spectrophotometrically at 280 nm, using an absorption coefficient of $7.76 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ based on a molecular mass of 55 kDa for TTR ($A_{280}^{1\%} = 14.1 \text{ mg}^{-1} \cdot \text{ml} \cdot \text{cm}^{-1}$). HPLC was performed on an Ultraspherogel SEC2000 column (Beckman) coupled to a high-precision pump (P-302) and a holochrome-280 UV detector (Gilson). Gel-filtration-chromatography buffer was used as eluent at a flow rate of 0.5 ml/min and each TTR preparation was injected in a volume of 200 µl. Before injection, the column was allowed to equilibrate with 2–5 column volumes of buffer and was washed with 0.5 M NaOH between injections. The protein standards used for calibration were: BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

Size-exclusion chromatography

Gel-filtration chromatography was performed on a Sephacryl S-200 1.0 cm × 100 cm high-resolution column (Bio-Rad) in 0.05 M sodium acetate buffer, pH 6.3 or pH 3.2. This system was previously calibrated using four protein standards: BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

Preparation of amyloid fibrils

A 0.2 mg/ml suspension of each mutant protein was made and 100 µg was incubated in 0.05 M sodium acetate/0.1 M KCl at various pH values ranging from 2.6–6.8 for 48 h at room temperature, to allow formation of amyloid fibrils [4,7]. To each solution was then added 500 µl of 50 mM glycine/NaOH, pH 9.0, and the amyloid fibrils were monitored for thioflavin T (ThT) fluorescence. Excitation spectra were recorded by spectrofluorimetry (FP-770; JASCO) at 25 °C with 30 µM ThT (Fluka) in 50 mM glycine/NaOH buffer, pH 9.0, in an assay volume of 1 ml. Excitation and emission slits were set at 5 nm and 10 nm respectively, and the excitation and emission wavelengths were 400 and 482 nm respectively. Electron microscopy was performed on samples of amyloid fibrils prepared from the wild-type TTR, as described in [4].

Alkylation of TTR

Soluble TTR samples (1 mg) were reduced and alkylated as described in [17].

RESULTS

Modelling

Four TTR mutant proteins were designed to weaken or strengthen tetramer/dimer interactions and their structural-functional properties were analysed. Two of these mutants,

Figure 1 Dimer–dimer interfaces in TTR

The ribbon representation of the wild-type human TTR was retrieved from the SwissProt Data Bank. **(A)** The close proximity of residues 18 and 20 (part of the AB loop), as well as 119 and 110 (facing the T₄ binding pocket) is demonstrated. **(B)** The proximity of Glu⁹² and Ser¹¹⁷ in the dimer is shown, which enables the formation of disulphide bridges when these residues are substituted with cysteine residues.

Figure 2 Functionality of mutant tetramers, concerning T₄ binding

Native PAGE of four recombinant TTR mutants previously incubated with radiolabelled T₄. Lanes 1–6: free T₄, human serum, E92C, S117C, L110A, D18N. Abbreviation: std, standard human serum.

designated ‘destabilized’ mutants, were unstable because of a fast dissociation into monomeric units (Figure 1A). In the wild-type protein, the side chain of Asp¹⁸ of one dimer is hydrogen bonded to the amino group of Asp²¹ of the other dimer within the tetrameric structure. In view of this, we reasoned that if Asp¹⁸ was replaced by a similar, but uncharged amino acid, this link should disappear, thus loosening the AB loop area. In variant T119M, there is a van der Waal’s interaction between amino acids Met¹¹⁹ and Leu¹¹⁰ belonging to different dimers, which strengthens the interaction between dimers in the T₄ binding pocket [13]. In the wild-type protein, Leu¹¹⁰ projects into the channel forming hydrophobic interactions with amino acids from another dimer. By introducing an alanine residue at position 110 these interactions should be weakened, promoting dissociation of the molecule.

The other two mutated proteins, referred to here as ‘stabilized’, were designed with the purpose of creating stronger tetramers through very stable dimers (Figure 1B). In tetrameric TTR, each monomer is composed of two four-stranded β -sheets (DAGH and CBEF), and the association of two monomers in a dimer results in two eight-stranded β -sheets (DAGHH’G’A’D’) and (CBEFF’E’B’C’). The introduction of cysteine residues at positions 92 and 117 of strands F and H, respectively, should create disulphide bridges between the two monomers from each dimer, stabilizing the tetramer through stronger dimeric interactions.

In the present work, different physical-chemical characteristics of ‘stabilized’/‘destabilized’ mutant proteins are reported.

Physico-chemical characterization of TTR mutants: tetramers, dimers and monomers

Functional tetramers

To assess whether the four mutants were still functional in T₄ binding, preparations of recombinant protein were incubated with radiolabelled T₄, and were compared with the wild-type protein in a human serum sample (Figure 2). The two mutants containing extra cysteine residues (S117C and E92C) had a tetrameric structure and retained their ability to bind T₄. This

Figure 3 Dissociation into monomers is favoured in the destabilized mutants

Top panels: HPLC elution patterns of wild-type TTR and L110A (Leu110Ala) at $3 \mu\text{M}$ concentration. The protein standards used to calibrate the column are indicated: 67 kDa, BSA; 43 kDa, ovalbumin; 25 kDa, chymotrypsinogen A; and 13.7 kDa, ribonuclease A. The bottom panel shows the eluted fractions of recombinant L110A separated by native PAGE. Lane 1, wild-type TTR; lanes 2–5, L110A tetramer elution (13–16 min); lanes 6–8, L110A monomer elution (22–28 min). The arrow indicates the direction of electrophoresis. T, tetramer; M, monomer.

experiment also showed that Cys¹⁰ did not form disulphide bonds with either Cys⁹² or Cys¹¹⁷, since both tetramers were functional. The D18N and L110A mutants, both designed to create instability in the TTR tetramer, exhibited contrasting behaviours. D18N was not affected in its ability to bind T_4 , whereas L110A did not bind the ligand. This result was not surprising, since, in the wild-type protein, Leu¹¹⁰ is highly involved in T_4 binding.

Resistance of mutated proteins to dissociation into monomers

The resistance to dissociation of both stabilized and destabilized mutant proteins was investigated (Figure 3). When the elution

pattern of $3 \mu\text{M}$ and $0.3 \mu\text{M}$ solutions of the mutants was compared with the wild-type TTR, a marked difference was observed. In the case of the wild-type protein at the highest concentration ($3 \mu\text{M}$), the only form isolated by chromatography corresponded to a tetrameric form of the protein, with a total absence of aggregates and/or monomers. When the preparation was diluted 10-fold, a second form was eluted, with a molecular mass of 14 kDa, which corresponded to the monomeric form of the protein, as reported by Quintas et al. [16]. The behaviour of the destabilized mutants was quite different, particularly L110A. When a $3 \mu\text{M}$ solution was subjected to gel filtration and the eluted peaks were analysed by native PAGE, it was apparent that a mix of the two forms already coexisted (Figure 3, middle and bottom panels). This observation contrasted with the elution pattern obtained for the wild-type TTR (Figure 3, top panel). Also, after a 10-fold dilution, the proportion of the monomeric relative to the tetrameric form increased from 0.25 to 0.4 (results not shown), increasing the equilibrium flow in the direction of the monomeric form in solution. This suggests that, in this mutant, the hydrophobic interactions are reduced, so affecting the dimer–dimer interfaces, which are unstable and dissociate into monomers much more easily than in the wild-type TTR. A similar elution pattern was obtained for D18N, although the increase in the monomeric form relative to the tetrameric, upon 10-fold dilution, was not as marked as for L110A (results not shown), implicating the AB loop in the stabilization of the tetramer.

The resistance of the stabilized mutants to dissociate into monomers is evident from the results shown in Figure 4 (first and second panels from the top), where not even a 10-fold dilution produced monomeric forms; this behaviour was expected, since under semi-denaturing isoelectric focusing (4 M urea), the two cysteine-linked mutants migrated as tetramers, whereas wild-type TTR presented both tetramers and monomers (results not shown). As a control, to assess whether the behaviour of these two mutants was being affected in any other way, apart from that due to the extra disulphide bridges, the same 10-fold dilution was performed after reduction and alkylation of the protein preparations. When formation of disulphide bridges was prevented, both stabilized mutants behaved as the wild-type TTR, exhibiting the tetrameric and monomeric forms (Figure 4, third panel).

Dimeric TTR species

The dimeric nature of S117C and E92C TTR variants was determined by gel-filtration chromatography, comparing the elution pattern of protein solutions incubated at pH 3.2 with those incubated at pH 6.3. At pH 6.3 all the proteins were eluted in the form of tetramers. Figure 4 (bottom panel) shows that, at pH 3.2, the only species of the stabilized mutants isolated was eluted at 28 min and corresponded to the dimer, with a mass of 28 kDa, and with a total absence of aggregates and/or monomers. Under acidic pH, both stabilized mutants are dimers, whereas wild-type TTR dissociates into monomers and precipitates as amyloid, as reported by Bonifácio et al. [4] and Lai et al. [7]. Precipitates were also formed with the destabilized mutants (see below).

Susceptibility to amyloid formation

All four mutants were tested for their susceptibility to amyloid formation upon acidification (leading to dissociation and aggregation into fibrils) by measuring the fluorescence of protein solutions, previously incubated at various pH (2.3–6.3), after the addition of ThT. It was observed that both of the destabilized

Figure 4 Dimeric nature of stabilized mutants at pH 3.2

Gel-filtration chromatograms of 3 μ M S117C (Ser117Cys) run at pH 6.3 (top panel), 0.3 μ M S117C at pH 6.3 (second panel), 0.3 μ M reduced and alkylated (R + A) S117C run at pH 6.3 (third panel) and 3 μ M S117C run at pH 3.2 (bottom panel). The tetrameric form of the mutant was eluted at 20 min (top panel), the dimeric form at 28 min (bottom panel) and the monomeric form at 31 min, indicated by an arrow (third panel). The protein standards used to calibrate the column were: 67 kDa, BSA; 43 kDa, ovalbumin; 25 kDa, chymotrypsinogen A; 13.7 kDa, ribonuclease A. Similar data were obtained for E92C (results not shown).

mutants, D18N and L110A, had a similar susceptibility to amyloid formation as the wild-type protein, requiring an acidic

Figure 5 Monomers are required for amyloid formation

Graphic representation of the data obtained in amyloid formation with protein preparations of S117C upon incubation at pH 2.3–6.2, with (solid line) or without ($\blacktriangle$) 2-mercaptoethanol and tested for ThT fluorescence. Each point corresponds to the maximum peak obtained at 440 nm upon ThT binding. Similar data were obtained for E92C (results not shown). IRF, intensity of relative fluorescence.

pH, below pH 4.5, to form fibrils (results not shown). However, when E92C and S117C were tested for their susceptibility to amyloid formation, no fibrils were observed, even at pH 3.2. When the amyloid-formation assay was repeated in the presence of 2-mercaptoethanol to disrupt the dimers, amyloid-fibril formation occurred (Figure 5), indicating that monomers rather than dimers are necessary for fibrillogenesis.

DISCUSSION

To date, no unifying model for TTR amyloid formation under physiological conditions has been developed, but all current hypotheses assume that the mutations in TTR affect the structural equilibrium of the molecule, destabilizing the tetramer in favour of intermediates which exhibit amyloidogenic potential. Colon and Kelly [18] first presented biochemical evidence of the existence of amyloidogenic intermediates after partial acid denaturation, creating a model for fibril formation. They proposed that TTR could self-assemble into amyloid-like fibrils in an acidic environment (simulating the lysosome), with the more amyloidogenic variants requiring less extreme conditions. X-ray diffraction studies on amyloid fibrils extracted from vitreous humour lead to different models. In one case, it is explicitly stated that monomers are the units which compose the fibrils [9], whereas, in a previous work [8], a continuous β -sheet helix composed of monomers or dimers was proposed.

The aim of the present work was to clarify the structure of the amyloid-forming units. Based on X-ray data of several TTR structures, we built highly stable tetramers (stabilized mutants) with covalent disulphide bridges between monomers of the same dimer.

The association of the two TTR dimers in the tetrameric structure forms a 16-stranded β -barrel 55 Å long [19]. TTR has only one cysteine (Cys¹⁰) residue in each identical subunit and its location is near the entrance of the central channel of the molecule. Both isolated recombinant TTR stabilized mutants bound T₄ in qualitative assays (Figure 2), which clearly shows that there are no extra disulphide bridges between the cysteine residues introduced into these mutants and the native Cys¹⁰ at

the entrance of the T₄ binding pocket. The dimeric structure was conserved even at very low pH and no amyloid fibrils were formed *in vitro* from these dimeric species. Thus our studies demonstrated that monomers, rather than dimers, are the repeating structural subunit composing the amyloid fibrils, corroborating the model described by Inouye et al. [9], based on X-ray analyses of vitreous fibrils from FAP patients.

Exploring the new surfaces exposed in the packing contacts revealed by the crystal structure of L55P [6], we designed a mutant with an asparagine residue substituted for an aspartate at position 18. Asp¹⁸ has close contacts with amino acids in positions 20 and 21, holding the tetrameric structure closely packed (Figure 1A). When an uncharged amino acid is introduced, the AB loop must become looser and disconnected from the body of the tetramer, which might explain the occurrence, upon dilution in the sub-micromolar range (3 μ M), of a mixture of tetrameric and monomeric forms. This tetramer is unstable, although, because it still binds T₄, no large changes have occurred in the binding channel. The AB loop seems to represent one of the key domains in the stabilization of the TTR tetramer, since the slightest alteration that might displace this area of the molecule, thus affecting the native fold of the structure, promotes dissociation into monomers. Two amyloidogenic mutants affecting Asp¹⁸, which are associated with the clinical phenotypes D18E and D18G, have been described. Mutations in other parts of the molecule might cause a modification in this area, as does L55P.

So far, there are more than 70 mutations described for TTR. The variant T119M is of particular interest with regard to T₄ binding. In this variant, a methionine residue replaces threonine at position 119 in the TTR monomer. The amino acid at position 119 is located deep inside the T₄ binding channel, and is postulated to interact with both the 4'-hydroxy group and the 5'-iodine of T₄ [20]. Substitution of a polar amino acid for a non-polar one may allow the T₄ molecule to interact more closely with the other amino acids involved in the binding cleft and thus lead to the stronger binding observed. In addition, the three-dimensional structure of variant T119M shows that the substitution of a methionine for a threonine residue at position 119 induces a movement of residue Leu¹¹⁰, pushing it into a position closer to the ligand. This structural alteration may strengthen the binding interactions of TTR with the T₄ phenolic ring, leading to the observed increase in the binding affinity of the protein [13].

Our results, with regard to the susceptibility of L110A to dissociate into monomers, showed that, at 3 μ M dilution, and by comparison with the wild-type protein, two clear forms coexisted in solution in the relative proportions of 0.75 tetrameric form to 0.25 monomeric form (Figure 3). When the preparations were diluted 10-fold, these relative values were altered to 0.6 and 0.4, corresponding to the tetrameric and monomeric forms in solution respectively. These results reflect the importance of Leu¹¹⁰, not only in maintaining the T₄ binding properties of native TTR, but also in strengthening the contacts between dimers. The location of this residue, deep in the hydrophobic core of the tetramer, in the dimer-dimer interface, favoured a fast dissociation into monomers upon the substitution of a leucine for an alanine residue, suggesting that a high number of bonds were broken or severely affected.

The experimental evidence of this work show that monomers, rather than dimers, are the repeating structural subunits which comprise the amyloid fibrils; it highlights also the importance of specific areas and residues for stabilizing the tetrameric structure of TTR. In particular, contacts between dimers and

destabilization of the AB loop seem pivotal. This information will help in designing strategies for therapeutic intervention in FAP.

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REFERENCES

- Saraiva, M. J. M. (1996) Molecular genetics of Familial Amyloidotic Polyneuropathy. *J. Peripher. Nerv. Syst.* **1**, 179–188
- Wojtczak, A., Cody, V., Luft, J. R. and Paugborn, W. (1996) Structures of human transthyretin complexed with thyroxine at 2.0 Å resolution and 3', 5'-dinitro-*N*-acetyl-L-thyroxine at 2.2 Å resolution. *Acta Cryst.* **D52**, 758–765
- Monaco, H. L., Rizzi, M. and Coda, A. (1995) Structure of a complex of two plasma proteins: transthyretin and retinol-binding protein. *Science* **268**, 1039–1041
- Bonifácio, M. J., Sakaki, Y. and Saraiva, M. J. (1996) *In vitro* amyloid formation from transthyretin: the influence of ions and the amyloidogenicity of TTR variants. *Biochim. Biophys. Acta* **1316**, 35–42
- Damas, A. M., Ribeiro, S., Lamzin, V. S., Palha, J. A. and Saraiva, M. J. (1996) Structure of Val122Ile variant transthyretin – a cardiomyopathic mutant. *Acta Cryst.* **D52**, 966–972
- Sebastião, M. P., Saraiva, M. J. M. and Damas, A. M. (1998) The crystal structure of amyloidogenic Leu55Pro transthyretin variant reveals a possible pathway for transthyretin polymerisation into amyloid fibrils. *J. Biol. Chem.* **273**, 24715–24722
- Lai, Z., Colón, W. and Kelly, J. W. (1996) The acid-mediated denaturation pathway of transthyretin yields a conformational intermediate that can self-assemble into amyloid. *Biochemistry* **35**, 6470–6482
- Blake, C. and Serpell, L. (1996) Synchrotron X-ray studies suggest that the core of the transthyretin amyloid fibril is a continuous β -sheet helix. *Structure* **4**, 989–998
- Inouye, H., Domingues, F. S., Damas, A. M., Saraiva, M. J., Lundgren, E., Sandgren, O. and Kirschner, D. A. (1998) Analysis of X-ray diffraction patterns from amyloid of biopsied vitreous humor and kidney of transthyretin (TTR) Met30 Familial Amyloidotic Polyneuropathy (FAP) patients: axially arrayed TTR monomers constitute the protofilament. *Amyloid* **5**, 163–174
- Coelho, T., Chorão, R., Sousa, A., Alves, I. L., Torres, M. F. and Saraiva, M. J. M. (1996) Compound heterozygote of transthyretin Met30 and transthyretin Met119 are protected from the devastating effects of Familial Amyloid Polyneuropathy. *Neuromuscular Dis.* **6**, S20
- Alves, I. L., Hays, M. T. and Saraiva, M. J. M. (1996) Comparative stability and clearance of Met30 transthyretin and Met119 transthyretin. *Eur. J. Biochem.* **249**, 662–668
- Roussel, A. and Cambillau, C. (1989). In *Turbo-Frodo. Silicon Graphics Geometry Partners Directory*. pp. 77–78. Silicon Graphics, Mountain View, CA, U.S.A.
- Almeida, M. R., Damas, A. M., Lans, M. C., Brouwer, A. and Saraiva, M. J. (1997) Thyroxine binding to transthyretin Met119. *Endocrine* **6**, 309–215
- Furuya, H., Saraiva, M. J. M., Gawinowicz, M. A., Alves, I. L., Costa, P. P., Sasaki, H., Goto, I. and Sakaki, Y. (1991) Production of recombinant human transthyretin with biological activities toward the understanding of the molecular basis of Familial Amyloidotic Polyneuropathy (FAP). *Biochemistry* **30**, 2415–2421
- Saraiva, M. J. M., Costa, P. P. and Goodman, D. S. (1988) Transthyretin (prealbumin) in familial amyloidotic polyneuropathy: genetic and functional aspects. *Adv. Neurol.* **48**, 201–210
- Quintas, A., Saraiva, M. J. M. and Brito, R. M. M. (1997) The amyloidogenic potential of transthyretin variants correlates with their tendency to aggregate in solution. *FEBS Lett.* **418**, 297–300
- Saraiva, M. J. M., Birken, S., Costa, P. P. and Goodman, D. S. (1984) Amyloid fibril protein in familial amyloidotic polyneuropathy, Portuguese type. Definition of a molecular abnormality in transthyretin (prealbumin). *J. Clin. Invest.* **74**, 104–119
- Colón, W. and Kelly, J. W. (1992) Partial denaturation of transthyretin is sufficient for amyloid fibril formation *in vitro*. *Biochemistry* **31**, 8654–8660
- Blake, C. C. F., Geisow, M. J., Oatley, S. J., Rérat, B. and Rérat, C. (1978) Structure of prealbumin: secondary, tertiary and quaternary interactions determined by Fourier refinement at 1.8 Å. *J. Mol. Biol.* **121**, 339–356
- Blake, C. C. F., Burridge, J. M. and Oatley, S. J. (1978) X-ray analysis of thyroid hormone binding to prealbumin. *Biochem. Soc. Trans.* **6**, 1114–1118