The Crystal Structure of Amyloidogenic Leu55 → Pro Transthyretin Variant Reveals a Possible Pathway for Transthyretin Polymerization into Amyloid Fibrils*

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Transthyretin (TTR), also known as thyroxin-binding prealbumin, is a plasma homotetrameric protein present in mammals, birds, and reptiles. It is synthesized in the liver, choroid plexus of the cerebral ventricles, and retina. The protein binds the complex retinol-retinol binding protein, preventing its glomerular filtration, and also polyhalogenated biphenyl compounds. The half-life of TTR is 2–3 days in humans, and the major site of degradation is the liver followed by the muscle and skin. Transthyretin is associated with amyloid deposition in several organs in particular peripheral nerves, cardiac tissue, and ocular vitreous. The majority of the transthyretin-associated amyloidoses are due to single amino acid substitutions, and in senile systemic amyloidosis, the non-mutated protein is present in the amyloid fibrils. Familial amyloidotic polyneuropathy is characterized by an autosomal dominant mode of inheritance, with the usual onset of clinical disease appearing about age 25–35. Peripheral and autonomic neuropathies are prominent and early features of the disease.

Several hypotheses have been suggested to explain the amyloidogenic properties of TTR that lead to neurotoxicity and organ dysfunction. It has been proposed that there is a conformational state, different from the one presented by the wild-type protein, prior to fibril formation. Therefore, x-ray crystallographic comparison studies of the structures of the wild-type and amyloidogenic TTR variants will contribute to explain the amyloidogenic potential of TTR. Defining these structural differences is important, not only in understanding the pathogenesis of the disease, but also in devising therapeutic agents to combat the disease.

Blake et al. reported the crystal structure of the wild-type protein. The protein crystallizes in space group P212121 with a dimer in the asymmetric unit. The TTR monomer, with 127 amino acid residues, contains eight strands, throughout H, of seven to eight residues in length. The exception is strand D, which is three residues in length. The eight strands form two sheets of four strands each, DAGH and CBEEF, arranged in a topology similar to the classic greek key barrel. Two monomers are related by a noncrystalllographic 2-fold axis, and they are joined along the FH border to form the dimer. The dimer is composed of two eight-stranded β-sheets, DAGH’G’A’D’ and CBEEF’E’B’C’. The tetramer consists of two dimers related by a crystallographic 2-fold axis. The connecting edges occur between the AB loop of one dimer with the H strand of the other dimer.

The x-ray crystallographic structures of the amyloidogenic variants TTR Met30 (13, 14), TTR Ile122 (15), and TTR Ser84 (16) revealed an overall structural homology with the wild-type protein. Only small differences were detected, including a higher spacing between DAGH and CBEEF sheets in the TTR Met30 monomer along with a movement of strand A exposing residue 10 to solvent (14). Damas et al. (15) reported an increase in the length of the hydrogen bonds between the Val122 → Ile TTR dimers. It was proposed that these changes could lead to the destabilization of the tetrameric structure, thus promoting the formation of an intermediate structure that polymerizes into amyloid fibrils.

Preliminary results, concerning the crystallization procedure of the highly clinically aggressive mutant Leu55 → Pro TTR, were reported previously (17). To learn more about the structure/pathogenesis relationship in TTR variants, the structure of amyloidogenic Leu55 → Pro TTR variant was refined, compared with wild-type TTR, and is described in the present work.
**Leu$^{55} \rightarrow$ Pro Transthyretin Crystal Structure**

### TABLE I

Refinement statistics for the Leu$^{55} \rightarrow$ Pro TTR crystal structure

The eight monomers in the asymmetric unit cell are referred as A, B, C, D, E, F, G, and H.

|                        | Leu$^{55} \rightarrow$ Pro TTR |
|------------------------|---------------------------------|
| Space group            | C2                              |
| Unit cell:             |                                 |
| $a$                    | 149.99 Å                        |
| $b$                    | 78.74 Å                         |
| $c$                    | 98.95 Å                         |
| $\beta$                | 100.5°                          |
| Resolution             | 15–2.7 Å                        |
| $R_{\text{cryst}}$ (all reflections)$^a$ | 19.9 |
| $R_{\text{cryst}}$ ($F_{\text{obs}} > 3\sigma$) | 18.1 |
| r.m.s. bond distances  | 0.019 Å                         |
| r.m.s. angle distances | 0.030 Å                         |
| r.m.s. planar 1–4 distances | 0.047 Å |
| Number of protein residues | 928 |
| Number of water molecules | 0 |
| Average $B$-factors (Å$^2$) | 23.7 (A), 32.3 (B), 25.0 (C), 24.1 (D), 31.7 (E), 30.8 (F), 32.4 (G), 27.1 (H) |

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$^a$ The crystallographic $R$-factor ($R_{\text{cryst}}$) is defined as: $R_{\text{cryst}} = \sum |F_{\text{obs}} - F_{\text{calc}}| / \sum |F_{\text{obs}}|$. 

![Electron density map](image)

*Fig. 1. $3F_{\text{obs}} \text{--} 2F_{\text{calc}}$ electron density map calculated around residue Pro$^{55}$, in Leu$^{55} \rightarrow$ Pro TTR variant.*
The crystallographic packing in the Leu55Pro transthyretin variant is illustrated in Fig. 3. This variant crystallizes nonisomorphously with wild-type TTR, unlike other amyloidogenic transthyretin variants, whose structures are described in the literature (13–16). The crystal asymmetric unit contains four dimers, which assemble into one tetramer in a general position and two dimers near the twofold axes. This non-crystallographic tetramer is formed through hydrogen bond interactions involving the AB loop and strand H, as observed for the wild-type TTR tetramer, although the length of the hydrogen bonds formed is longer (Table II), resulting in an overall less stable tetramer.

Impairment of dimer-dimer contacts, resulting in decreased tetramer stability, was reported previously from biochemical studies (23, 24). However, the present work is unique in providing a detailed information about the interatomic contacts between the pathologic Leu55 → Pro TTR dimers, because this is the only amyloidogenic variant, reported until now, with a root mean square (r.m.s.) deviation of 0.019 and 0.030 Å for angle-bonded distances. The overall temperature factor for the protein main chain atoms varies between 23.7 and 32.3 Å² for the eight monomers (Table I).

The Ramachandran plot calculated using PROCHECK (22) showed no residues with main-chain dihedral angles in disallowed regions: 89% of the non-glycine residues are in most favored regions, 10.3% in additional allowed regions, and 0.7% in generously allowed regions. The observed hydrogen-bonding pattern also indicates that the protein model obtained after refinement is correct. Fig. 1 shows the 3Fo − 2Fc electron density map in the region of the Leu55 → Pro mutation.

Comparison of the wild-type and Leu55 → Pro mutant TTR (Fig. 2) shows significant deviations between the Ca positions corresponding to the strand D (residues 54–56) and loop FG (residues 97–103). Minor deviations are also observed in α-helix and loop AB. In fact, in the mutant monomer, residues 54–56 belong to a long surface loop that connects strands C and E (according to the wild-type TTR nomenclature). This loop (residues 48–66) is involved in the crystallographic packing as illustrated in Fig. 3.

Thus, the Leu55 → Pro monomer structure is organized in seven strands and one α-helix, in a topology similar to the classic β-barrel.

**Intermolecular Interactions in Leu55 → Pro TTR Crystal Structure**—Leu55 → Pro TTR variant crystallizes nonisomorphously with wild-type TTR, unlike other amyloidogenic transthyretin variants, whose structures are described in the literature (13–16). The crystal asymmetric unit contains four dimers, which assemble into one tetramer in a general position and two dimers near the twofold axes. This non-crystallographic tetramer is formed through hydrogen bond interactions involving the AB loop and strand H, as observed for the wild-type TTR tetramer, although the length of the hydrogen bonds formed is longer (Table II), resulting in an overall less stable tetramer.

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Leu$^{55}$ → Pro Transthyretin Crystal Structure
**FIG. 3.** Leu\(^{55}\) \(\rightarrow\) Pro Transthyretin Crystal Structure

A, unit cell packing contacts. The a, b, c axes of the unit cell are indicated by solid lines (X, Y, Z, respectively). The asymmetric units, consisting of eight monomers, are shown in different colors. B, interaction loop CE \(\rightarrow\) loop CE from neighboring monomers. C, Leu\(^{55}\) \(\rightarrow\) Pro TTR monomer-monomer interactions: loop FG \(\leftrightarrow\) loop FG, and loop AB \(\leftrightarrow\) \(\alpha\)-helix. D, 3\(F_{\text{obs}}\) \(-\) 2\(F_{\text{calc}}\)
complete tetramer in the asymmetric unit.

The packing interaction between the CE loops (residues 48–66) of neighboring monomers, belonging to different tetramers, forms the main crystal contact as illustrated in Fig. 3. Additionally, Arg21, from the AB loop of one monomer is H-bonded to the α-helix residue Gly83 of the neighboring monomer. Because of this interaction, the side chain of Arg21 is situated in good electron density, as shown in Fig. 3D. In contrast, Arg21 is disordered in wild-type TTR, because of inefficient hydrogen bonding (13). In wild-type TTR, Arg21, from monomer A, forms a salt bridge to Asp18 (2.94 Å), a van der Waals interaction with Tyr114 (2.99 Å), and a hydrogen bond to a water molecule with a high B-factor. In monomer B, Arg21 is modeled in a double conformation. The alternate position of the B21 side chain forms hydrogen bonds to Ser82 (2.64 Å) and to the hydroxyl group of TyrB78 (2.43 Å).

In addition to loop CE ↔ loop CE, loop AB ↔ α-helix interactions, there are other interactions, namely Ser100 (loop FG) ↔ Arg103 (loop FG), Ser100 (loop FG) ↔ Asn124 (C terminus). These interactions are not observed in the wild-type TTR. Therefore, they are referred as abnormal intersubunit contacts, to distinguish them from the intersubunit contacts that are also observed in wild-type TTR, which are designated as native.

The surface areas involved in the monomer-monomer and dimer-dimer interactions were calculated for the Leu55 → Pro and wild-type TTR (Table III). Whereas the intersubunit contacts between two monomers in a dimer do not differ between the variant and wild-type structures, the tetramer dimer-dimer interface diminishes in Leu55 → Pro TTR. It also evident that there is a tight monomer-monomer interaction and a much more tenuous dimer-dimer interface. This fact is experimentally confirmed by the observation that TTR dissociates to form 30-kDa dimers in SDS and 15-kDa monomers only after boiling in SDS with reducing agents (25). Furthermore, the interface between dimers of different tetramers (2524 Å2) is higher than the area of contact between the dimers in a tetramer (1569 and 1837 Å2, for the variant and wild-type protein, respectively).

Table IV summarizes the Leu55 → Pro TTR monomer-monomer hydrogen bonds.

**DISCUSSION**

The fibrillar structure resulting from the self-association of an abnormal conformation of TTR is thought to be the causative agent in familial amyloidotic polyneuropathy. However, the mechanism that converts normally soluble TTR tetramers into insoluble amyloid fibrils remains largely unknown.

Structural comparative studies of the native fold and abnormal conformations of TTR variants are expected to be very useful in developing therapeutic strategies for intervention in amyloid disease. In particular, the molecular characterization of the most aggressive TTR amyloidogenic variant, Leu55 → Pro TTR, may provide an important contribution to the characterization of the amyloidogenic process.

Studies concerning the kinetics of amyloid formation indicate that the Leu55 → Pro TTR variant exists in an amyloidogenic conformation at conditions, whereas the wild-type protein remains stable and non-amyloidogenic. It was observed that amyloid formation from the wild-type protein had an initial rate determining step, not diminished in the presence of electron density map calculated around residue Arg21, in Leu55 → Pro TTR. The electron density for the side chain of Arg21 is well defined in Leu55 → Pro TTR, because of inefficient hydrogen bonding.

**Table II**

| Hydrogen bonds | Leu55 → Pro TTR |
|---------------|---------------|
| Ala19 CO(A) ... HN Tyr114 sym | 2.94 | 3.22 |
| Gly22 CO(A) ... HN Val112 sym | 2.98 | 2.91 |
| Ala19 CO(B) ... HN Tyr114 sym | 2.92 | 3.18 |
| Gly22 CO(B) ... HN Val112 sym | 2.85 | 3.06 |
| Ala36 CO(C) ... HN Tyr114(F) | 3.49 |
| Gly32 CO(C) ... HN Val112 sym | 3.40 |
| Ala19 CO(D) ... HN Tyr114(E) | 3.18 |
| Gly22 CO(D) ... HN Val112 sym | 3.00 |
| Ala36 CO(E) ... HN Tyr114(D) | 3.06 |
| Gly22 CO(E) ... HN Val112 sym | 3.24 |
| Ala26 CO(F) ... HN Tyr114(C) | 3.30 |
| Gly22 CO(F) ... HN Val112 sym | 2.97 |
| Ala36 CO(G) ... HN Tyr114 sym | 3.33 |
| Gly22 CO(G) ... HN Val112 sym | 3.03 |
| Ala36 CO(H) ... HN Tyr114 sym | 3.05 |
| Gly32 CO(H) ... HN Val112 sym | 3.00 |

**Table III**

| Surface areas (Å²) involved in the intersubunit contacts for Leu55 → Pro and wild-type TTR |
|-----------------------------------------|
| Hydrogen bonds | Leu55 → Pro TTR | TTR |
|---------------|----------------|-----|
| Main chain-main chain | Thr118 NH ... OC Tyr216 | Thr118 CO ... HN Tyr216 |
| | Tyr114 CO ... HN Ala110 | Val94 CO ... HN Glu89 |
| | Gly83 CO ... HN Ser80 | Glu89 NH ... OC Val94 |
| | Thr96 NH ... O ... Glu89 | Ser100 CO ... N ... Asp124 |
| | Thr116 CO ... HO ... Ser115 | Asp116 CO ... N ... Arg21 |
| | Lys90 CO ... N ... Arg11 | Gly83 CO ... N ... Arg21 |
| | Gly62 O ... N ... His112 | Gly62 O ... O ... Thr206 |
| | Gly62 O ... O ... Thr206 | His109 N ... O ... Glu92 |
| | Thr96 O ... O ... Glu92 | Thr96 O ... O ... Glu92 |
| | Asn124 N ... HO ... Ser120 | Tyr116 O(H) ... HO ... Tyr116 |
| | Tyr116 O(H) ... HO ... Ser116 | Ser85 O ... HO ... Ser85 |
| | Thr116 O ... N ... His112 | Thr119 O(H) ... HO ... Ser115 |
| | Ser112 O(H) ... HO ... Ser112 | Ser112 O(H) ... HO ... Ser112 |

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pre-formed fibrils, which could be associated with the conformational change of the protein into an amyloidogenic intermediate. However, the kinetics of amyloid formation from the Leu$_{55}$ → Pro TTR variant showed the absence of this lag time (26). This could suggest that Leu$_{55}$ → Pro TTR is already in an amyloidogenic conformation, which assembles immediately into amyloid fibrils, under the conditions tested. Furthermore, TTR with a triple deletion on strand D forms amyloid fibrils in vitro at neutral conditions (27).

The evidence for an amyloidogenic conformation for Leu$_{55}$ → Pro TTR is further corroborated by the experimental study of the acid denaturation pathway of some TTR variants, namely Thr$_{119}$ → Met, Val$_{122}$ → Met, and Leu$_{55}$ → Pro. It was observed that the amyloidogenicity of the variants was inversely correlated with the stability of the tetramer toward acid denaturation (26).

Thus, it seems likely that the Leu$_{55}$ → Pro TTR variant exists in an amyloidogenic conformation, with an increased tendency to self-association into amyloid fibrils, even at conditions where the wild-type protein remains stable. It is likely that the crystal structure here described for the Leu$_{55}$ → Pro TTR monomer resembles the amyloidogenic intermediate in the biochemical pathway that leads to the amyloid fibril deposition.

The data reported here indicate that the Leu$_{55}$ → Pro mutation changes the transthyretin secondary structure by the disruption of strand D: residues 54–56 belong to a long loop that connects β-strands C and E, according to the wild-type TTR nomenclature. This loop is involved in the crystallographic packing observed in the present crystal structure (Fig. 3, A and B).

Positional differences are also detected along the monomer-monomer and dimer-dimer interfaces. The hydrogen bonds between the AB loop of one dimer to the strand H of the other dimer (native dimer-dimer interface) are clearly longer than those described for the wild-type TTR tetramer, indicating a potentially less stable Leu$_{55}$ → Pro tetramer (Table II). This result was already pointed out for the amyloidogenic Val$_{122}$ → Ile TTR variant (15), and it is in agreement with biochemical studies concerning the tetramer stability of several TTR variants (23, 34). The surface areas associated with the native dimer-dimer interface, i.e. loop AB → strand H interaction, which are also an indication of the tetramer stability, are 14.2 and 16.5% of the total dimer surface area for Leu$_{55}$ → Pro TTR and wild-type TTR, respectively.

In addition to the native dimer-dimer intermolecular interactions, which are common to Leu$_{55}$ → Pro and wild-type TTR, there are other interactions between neighboring Leu$_{55}$ → Pro TTR dimers. These include the loop CE ↔ loop CE, loop AB ↔ α-helix, loop FG ↔ loop FG. The corresponding area is 23% of the Leu$_{55}$ → Pro TTR dimer total surface area (Table III). This indicates that the abnormal dimer-dimer interface is considerably more extensive than the native dimer-dimer interface, and therefore it may provide the driving force for TTR polymerization into amyloid fibrils.

Fig. 3A shows the arrangement of the Leu$_{55}$ → Pro TTR molecules in the unit cell, with the asymmetric units, composed of eight monomers, colored differently. The overall force in the assembly of the units is the intermolecular interaction between the CE loops (residues 48–66) from the different units (Fig. 3, A and B) and the hydrogen bonding between the AB loop and the α-helix of the nearest neighbor (Fig. 3, C and D). The FG loop (residues 97–103), which differs in all amyloidogenic variants and which is the same in wild-type and non-amyloidogenic TTRs, is also involved in the Leu$_{55}$ → Pro TTR monomer interactions. Ser100, from one monomer, forms hydrogen bonds with Arg$_{93}$ or Asn$_{124}$ from a neighboring monomer (Table IV).

These intermolecular interactions between neighboring Leu$_{55}$ → Pro TTR monomers may be important for the self-association of TTR.

The crystal packing presented in Fig. 3A clearly shows several channels running parallel to each other, thus suggesting a tubular structure that might be similar to the TTR amyloid fibril structure. The protein structure, around each channel, which can be considered as an intermediate structure for TTR amyloid fibril, is composed of four monomers per cross-section. The inner and outer diameters of this structure are 30 and 100 Å, respectively, and its wall is constituted by eight monomers. Four monomers are at one level, and the other four are at a level half way below or above a pseudo-unit cell, with dimension a = 84 Å, along the fibril direction (Fig. 4). The hydrogen bonding direction of the β-pleated sheets is about 45° to the proposed fibril direction. In the usual cross-β structure, reported for amyloid fibrils, the hydrogen bonding direction is parallel to the fibril axis. However, it is possible that when fibrils are assembled, and assuming that the tetrameric structure was disrupted, the movement between units is not so much restricted, and they may rotate relatively to the fibril axis approaching the situation of the cross-β structure.

This model agrees with the results from electron micro-

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**Figure 4. A stereographic view of the tubular Leu$_{55}$ → Pro TTR structure.** The diagram was built using graphics software package O (Jones et al., 1991). The a, b, c axes of the unit cell are indicated by solid lines (X, Y, Z, respectively).
graphs image reconstruction, which showed that TTR amyloid fibrils have a cross-section with four protofilaments arranged around a central hollow core (28). We have now presented information about the contacts between the units in the model, which is important because it provides insight into the development of strategies to inhibit the pathogenic process. Additionally, the reported diameter dimension of the amyloid fibril is similar to the diameter of the tubular structure proposed in this work.

Saraiva et al. (10) reported that the region corresponding to the strand D in Leu\(^{55}\) \rightarrow Pro TTR is exposed, according to monoclonal antibodies binding studies. It is interesting to note that in the Leu\(^{55}\) \rightarrow Pro TTR crystal structure, this region forms the outer part of the asymmetric units that assemble into the tubular structure in the unit cell (Fig. 3, A and B).

We believe that the model presented here, which derives from the analysis of the crystal structure of Leu\(^{55}\) \rightarrow Pro TTR variant, is a good approach to the molecular interactions present in TTR amyloid fibrils. The crystallographic packing analysis indicates putative interactions between units in the TTR amyloid (Table IV, Figs. 3 and 4). In particular, residues Arg\(^{21}\), Gly\(^{83}\), His\(^{56}\), Glu\(^{62}\), Ser\(^{100}\), Arg\(^{103}\), and Asn\(^{124}\) play an important role in Leu\(^{55}\) \rightarrow Pro TTR intersubunit interface and may be involved in amyloid fibril quaternary interactions.

Site-directed mutagenesis studies, together with amyloid fibril formation in vitro of the constructed mutants, are under way to reinforce and pave the way to the development of therapeutic agents that avoid the adoption of an amyloidogenic conformation.

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