X-ray Absorption Spectroscopy Reveals a Substantial Increase of Sulfur Oxidation in Transthyretin (TTR) upon Fibrillization*

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Transthyretin (TTR) amyloid fibrils are the main component of the amyloid deposits occurring in Familial Amyloidotic Polyneuropathy patients. This is 1 of 20 human proteins leading to protein aggregation disorders such as Alzheimer’s and Creutzfeldt-Jakob diseases. The structural details concerning the association of the protein molecules are essential for a better understanding of the disease and consequently the design of new strategies for diagnosis and therapeutics. Disulfide bonds are frequently considered essential for the stability of protein aggregates and since in the TTR monomers there is one cysteine residue, it is important to determine unambiguously the redox state of sulfur present in the fibrils. In this work we used x-ray spectroscopy to further characterize TTR amyloid fibrils. The sulfur K-edge absorption spectra for the wild type and some amyloidogenic TTR variants in the soluble and fibrillar forms were analyzed. Whereas in the soluble proteins the thiol group from cysteine (R-SH) and the thioether group from methionine (R-S-CH₃) are the most abundant forms, in the TTR fibrils there is a significant oxidation of sulfur to the sulfonate form in the cysteine residue and a partial oxidation of sulfur to sulfoxide in the methionine residues. Further interpretation of the data reveals that there are no disulfide bridges in the fibrillar samples and suggest conformational changes in the TTR molecule, namely in strand A and/or in its vicinity, upon fibril formation.

Amyloidoses are a group of protein misfolding diseases, which include spongiform encephalopathies, Alzheimer’s disease, and Familial Amyloidotic Polyneuropathy (FAP), all of them characterized by extracellular deposits of an insoluble fibrillar protein. Transthyretin (TTR) is the most abundant protein component of amyloid fibrils in the case of FAP patients. It is a plasma protein, with an extended β-sheet conformation, implicated in the transport of thyroxine and vitamin A. Over 80 TTR variants are described in the literature (1), most of them are pathogenic and related to amyloidosis. While V30M-TTR is the most frequent amyloidogenic variant, L55P-TTR is the variant associated with the most aggressive form of FAP described in the literature.

The molecular mechanisms involved in amyloid fibril formation are not yet well understood. Most of the proposed models refer to the dissociation of the tetrameric protein into an intermediate species that self-assembles leading to the insoluble fibril (2, 3). However, some controversy remains regarding this intermediate structure and in particular regarding its monomeric or dimeric conformational nature. For a better understanding of amyloid assembly, mutant TTR proteins containing disulfide bonds linking two monomers were designed, and their ability to form fibrils was tested. While some mutants did not form amyloid, which is consistent with the idea that the monomeric species are necessary for fibril formation (4), others do form fibrils, pointing to a dimeric nature of the intermediate species (5). Recently, scanning transmission electron microscopy (STEM) clarified this question because it revealed a mass-per-length of the protofilaments of −0.47 kDaÅ, which is consistent with the model where the monomer is the building block (3).

Insights into the mechanism of fibril formation were obtained by x-ray diffraction analysis of FAP amyloid fibrils. It was shown that fibrils exhibit a cross β-sheet structure (6), and two structural models were proposed: a continuous β-sheet helix (7) or an association of units with a structure close to the TTR monomer (8). The two models are obviously different, and neither of them refers to the packing interactions between the different subunits.

The possible role for the sulfur-containing amino acids in the fibril remains unknown. Based on the fact that Cys-10 is more exposed to the solvent in V30M-TTR, it was considered that amyloid fibrils could result from association of TTR molecules through disulfide bridges (9). This idea was supported by biochemical analysis of amyloid from homozygous and heterozygous individuals with the V30M-TTR mutation (10). However, it does not explain the formation of fibrils in the case of amyloidogenic C10R-TTR variant.

In order to find out the role of sulfur in the aggregation mechanism leading to fibril formation it is important to determine unambiguously its oxidation state when the protein is in a soluble form and when it forms the amyloid fibrils. Sulfur K-edge x-ray absorption spectroscopy provides the analytical tool needed for that purpose. The chemical shift in the x-ray absorption edge and the energy of maximum absorption depend on the sulfur coordination and environment in the sample. The potential of this technique in the analysis of biological materials is enormous because no chemical pretreatment is needed,
and consequently there is no perturbation of the sulfur redox state. In fact, the oxidation state of sulfur in the human blood, plasma, and erythrocytes was examined for the first time in 1998 using this methodology (11). Later, preliminary studies concerning the acid/base equilibrium of the thiol group of papain, α-amylase, and human serum albumin have been reported (12).

In this work we use sulfur K-edge spectroscopy to examine the sulfur oxidation state of TTR in the soluble and polymerized forms of the protein in order to determine the possible role of the sulfur in intra and intermolecular interactions, namely through the presence or absence of disulfide bridges. Additionally, x-ray diffraction was used to confirm the fibrillar structures of the samples under study and to improve our knowledge about their molecular structure.

EXPERIMENTAL PROCEDURES

Preparation of the Protein Samples—All TTR mutants were produced in an Escherichia coli expression system, and the periplasmic space contents were obtained by osmotic shock. The supernatant was passed through DEAE-Sephadex, and the TTR-containing peaks were dialyzed overnight against water and then freeze-dried. Further purification was achieved by preparative gel electrophoresis. Finally, TTR solutions were dialyzed against water for 2 h. Protein concentration was determined using the Lowry method.

In vitro amyloid fibrils, except for the L55P-TTR, were formed by acidification. The proteins (2 mg/ml) were incubated with 0.05 M sodium acetate/0.1 M KCl, pH 5.6, for 48 h at room temperature. Then, the fibrils were sedimented by centrifugation at 15,000 × g for 20 min in a microcentrifuge, and the pellets were resuspended in MilliQ water and incubated at 37 °C. Fibril formation was achieved for all the TTR mutants except in the case of C10S-TTR, for which no significant behavior as revealed by the thioflavine-T assay and was designated by the Thioflavine-T binding assay, and immediately after the purification procedure thioflavin-T did not bind TTR. Then part of the protein sample was immediately stored at –20 °C. This sample, designated by L55P-TTR protein solution, was restored to room temperature immediately before the spectroscopy experiment. The other part of the protein sample was incubated at 37 °C for 2 weeks. Then the sample had amyloidogenic properties as revealed by the thioflavine-T assay and was designated by L55P-TTR fibril solution.

Thioflavine-T Binding Assay—Solutions of 10 µg of amyloid fibrils were prepared as described above. To each solution was added ThT, final concentration 30 µM, in 50 mM glycine/NaOH buffer, pH 9.0, in an assay volume of 1 ml. Excitation spectra were recorded by spectrophotometry (FP-770; JASCO) at 25 °C. Excitation and emission slits were set at 5 and 10 nm, respectively. The excitation spectra (400–500 nm) were taken with emission collected at 482.0 nm.

Sulfur K-edge XANES—Sulfur K-edge spectra were recorded at room temperature using synchrotron radiation, at ESRF, beamline ID21: a double Si (111) crystal monochromator allows to achieve an energy resolution of 10 eV. The sample fluorescence is collected either by a silicon photodiode or by a high purity germanium fluorescence detector.

The reference compounds cysteine, cystine, methionine, methionine sulfoxide, and anthraquinone-2-sulfonic acid were of reagent grade, purchased from Sigma and used as received. They were in the form of powder, and a small amount was dispersed in a very thin layer. Also, solutions of the reference compounds were prepared, and their spectra measured. No differences between the spectra of the compounds in the powder form and in solution were observed.

The protein samples (5–10 µl) were introduced in a special cell for liquid x-ray absorption spectroscopy (L-XANES). L-glutamine peptide and Sup35 peptide (14) were treated with a siliconizing reagent. A fibril solution of L55P-TTR (6 mg/ml) was drawn up into the siliconized glass capillary tubes that were then sealed at the top, placed into a 2-T magnet, and allowed to dry at room temperature. As soon as a thin line was formed, the capillary tubes were sealed with wax in order to retain some hydration of the samples. The x-ray diffraction patterns were only collected for the samples showing birefringence under cross-polarized light.

The x-ray diffraction data were collected at ESRF, beamline ID13, using a 0.975 Å wavelength beam and a beam size of 5 µm. Patterns were recorded on a MAR research imaging plate during exposure times of 10–60 s. The background was recorded and subtracted to the diffraction pattern for elimination of the air scattering effects, using the software package FIT2D (13). The sample to detector distance was calibrated with silver behenate.

RESULTS

X-ray Fiber Diffraction—Glass capillary tubes of 0.5-mm diameter were treated with a siliconizing reagent. A fibril solution of L55P-TTR (6 mg/ml) was drawn up into the siliconized glass capillary tubes that were then sealed at the top, placed into a 2-T magnet, and allowed to dry at room temperature. As soon as a thin disk was formed, the capillary tubes were sealed with wax in order to retain some hydration of the sample. The x-ray diffraction patterns were only collected for the samples showing birefringence under cross-polarized light.

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FIG. 1. X-ray diffraction patterns from L55P-TTR amyloid fibrils. Specimen-to-film distance was 245 mm and the exposure time 30 s.

was always between 4 and 10 s per point.

The background was removed from the spectra by subtracting the medium value of the pre-edge horizontal region. Normalization was achieved by dividing the spectra by the medium value of the far post-edge flat region.

The proportion of the different forms of sulfur in the samples was estimated by simulation of the protein spectra using a linear combination of the spectra of the reference samples. The sum of the squares of the residuals between the original and the simulations was calculated in the region from 2.470 keV to 2.485 keV and divided by the number of experimental points measured in that region. The obtained value was designated by relative error. The percentages of the reference compounds found to give the minimum relative error were judged to be the best fit of the data.

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RESULTS

X-ray Fiber Diffraction—All the samples were examined by x-ray diffraction, and the data always revealed a cross-β pattern, characteristic of the amyloid material. Data from L55P-TTR is shown in Fig. 1, with a sharp 4.85 Å reflection on the meridian and a diffuse equatorial 10.3 Å reflection. The spacing and relative intensities of the observed meridional and equatorial reflections are listed in Table I.

The meridional reflections, 4.85 and 4.15 Å, may be indexed as the 6th and 7th order of a pseudo-period of 29.1 Å. In previous FAP fiber x-ray diffraction studies was calculated a 29-Å reflection. The spacing and relative intensities of the observed meridional and equatorial reflections are listed in Table I.

The meridional reflections, 4.85 and 4.15 Å, may be indexed as the 6th and 7th order of a pseudo-period of 29.1 Å. In previous FAP fiber x-ray diffraction studies was calculated a 29-Å minimum-repeating distance and based on that it was proposed that the monomer was the building block of amyloid fibrils (8). Later, it was confirmed by STEM that transthyretin fibrillogenesis entails the assembly of monomers (3). However, the 4.85 and 4.65 Å reflections may also represent sampling of a 4.75 Å reflection, corresponding to the backbone separation of the neighboring main chains. The 4.15 Å reflection could be the second order of an 8.3 Å reflection, also observed in the poly-L-glutamine peptide and Sup35 peptide (14). Recently EPR
measurements of spin-labeled TTR mutants suggest a distance of ~8 Å between subunits interfaces in the fibrils (15) that could originate that reflection.

The three observed meridional reflections indexed as Bragg reflections, fit a repeating unit of 116.3 Å ± 0.1, close to the 115.5 Å already proposed (7). This 4 × higher period (4 × 29 = 116 Å), can be due to a twist in the β-strands along the fibril axis, leading to the 116 Å repeat.

Sulfur K-edge XANES—The K-edge spectra of cysteine, cysteine, methionine, methionine sulfoxide, and anthraquinone-2-sulfonic acid are shown in Fig. 2. The energies of maximum absorption for these compounds are 2.4730, 2.4723, 2.4731, 2.4759, and 2.4809 keV, respectively. The scans were run with a step size of 0.00017 keV near the edge region, and the measured values are in agreement with those found in the literature (11, 16). The shapes of the spectra are clearly different except for cysteine and methionine that look very similar. Moreover, as described in previous studies (16), it can be observed that the higher the energy of maximum absorption the greater is the peak area.

The sulfur K-edge spectra of the WT-, V30M-, L55P-, and C10S-TTR samples are shown in Fig. 3. Some conclusions arise immediately from the observation of the spectra. There are no S–S bonds present in the samples as neither spectra shows the cysteine characteristic peak. The three energies of maximum absorption observed are 2.473 keV, 2.476 keV, and 2.481 keV indicating the presence of cysteine and/or methionine, methionine sulfoxide, and sulfonated cysteine. Although the shapes of the spectrum of TTR variants are roughly similar, there are pronounced differences between the non-polymerized and the polymerized forms: in the fibrillar samples there is a large amount of oxidized sulfur (stronger peak at 2.4759 keV from the S-sulfoxide and stronger peak at 2.4809 keV from the S-sulfonated).

Since more detailed interpretations demand a quantitative analysis, the experimental protein spectra were simulated by linear combination of the spectra from the reference samples. The simulated spectra are shown in Fig. 4, and the results of the fits and corresponding relative errors are presented in Table II.

TTR is a homotetramer with 127 amino acids per monomer. Cys-10 and Met-13 are the only sulfur-containing amino acids in the wild-type protein and L55P-TTR. V30M-TTR contains one cysteine and two methionines and variant C10S-TTR contains only one methionine.

In the non-polymerized protein samples there is a higher fraction of cysteines oxidized to the sulfonated form (24–28%) than methionines oxidized to the sulfoxide form (10% or less), which is consistent with Cys-10 being more exposed to the solvent than Met-13 as revealed by the x-ray crystallographic structures of these variants (9, 17, 18). According to the crystal structure of V30M-TTR variant, the extra methionine, Met-30, is buried in the molecule and not exposed to the solvent. Therefore in this case, the methionine sulfoxide probably results only from the partial oxidation of Met-13 as it will be the case for the wild type and the other variant proteins. The presence of the

| Meridional | Equatorial | Relative intensity |
|------------|------------|--------------------|
| d(obs) Å   | d(obs) Å   |                    |
| 12.5       | 4.85       | s                  |
| 10.3       | 4.65       | m                  |
| 6.56       | 4.15       | s                  |

**Table I**

**Spacing of equatorial and meridional reflections**

**FIG. 2.** Sulfur K-edge XANES spectra for the reference compounds.

**FIG. 3.** Sulfur K-edge XANES spectra of protein solutions (a) and fibril solutions (V30M-, WT-, and L55P-TTR) (b). V30M-TTR spectra are present in blue, WT-TTR in black, L55P-TTR in red and C10S-TTR in green.

S-sulfonated in C10S-TTR variant is residual (2%) and most improbable since no cysteines are present in the protein. The corresponding peak, which is very small, should arise from the multiple scattering effects at the high energy end of the spectra (11).
The data concerning the cysteine residue show that there is a slight increase in the S-sulfonated content from the wild-type protein (24%) to V30M-TTR (27%) to L55P-TTR (28%). In fact, it was already reported that sulfur K-edge spectroscopy detects changes of 5% in the thiol-to-disulfide ratio (11). In our experiments, we were able to obtain a higher accuracy in the thiol-to-sulfonate ratio, 2%, because the energy peaks were not so close to each other. However, the observed differences are near the sensitivity of the experimental method.

To further understand the accuracy of the simulated spectra and their relative errors the experimental spectrum of the soluble L55P-TTR was compared with the spectra simulated with the parameters that best fit the results of the four TTR mutants in the soluble form. These spectra and corresponding errors are presented in Table II. The percentages of the different forms of sulfur used in the simulations are presented in Table II.

![Experimental and simulated sulfur K-edge XANES spectra for the TTR samples](image)

**Fig. 4.** Experimental and simulated sulfur K-edge XANES spectra for the TTR samples (thick lines, experimental; thin lines, simulation). Soluble WT-TTR (a), V30M-TTR (b), L55P-TTR (c), and C10S-TTR (d); and fibrillar WT-TTR (e), V30M-TTR (f), and L55P-TTR (g). The percentages of the different forms of sulfur used in the simulations are presented in Table II.

| Sample       | Relative errora | Reduced S | Sulfoxide | Sulfonate | Cys/Met | Cys/CysS | Met/MetS |
|--------------|-----------------|-----------|-----------|-----------|---------|----------|----------|
| Soluble proteins |                 |           |           |           |         |          |          |
| V30M-TTR     | 0.0020          | 86        | 5         | 9         | 1/2     | 73/27    | 92/8     |
| L55P-TTR     | 0.0024          | 81        | 5         | 14        | 1/1     | 72/28    | 96/10    |
| WT-TTR       | 0.0033          | 84        | 4         | 12        | 1/1     | 76/24    | 90/10    |
| C10S-TTR     | 0.0053          | 97        | 1         | 2         | 0/1     | 99/1     |          |
| Fibrils      |                 |           |           |           |         |          |          |
| V30M-TTR     | 0.0077          | 51        | 19        | 31        | 1/2     | 79/3     | 72/28    |
| L55P-TTR     | 0.0065          | 46        | 16        | 38        | 1/1     | 24/76    | 68/32    |
| WT-TTR       | 0.0048          | 58        | 16        | 26        | 1/1     | 52/48    | 68/32    |

a Sum of the squares of the residuals between the original and the simulations in the region from 2.470 to 2.485 keV divided by the number of experimental points measured.

The data concerning the cysteine residue show that there is a slight increase in the S-sulfonated content from the wild-type protein (24%) to V30M-TTR (27%) to L55P-TTR (28%). In fact, it was already reported that sulfur K-edge spectroscopy detects changes of ~5% in the thiol-to-disulfide ratio (11). In our experiments, we were able to obtain a higher accuracy in the thiol-to-sulfonate ratio, ~2%, because the energy peaks were not so close to each other. However, the observed differences are near the sensitivity of the experimental method.

To further understand the accuracy of the simulated spectra and their relative errors the experimental spectrum of the soluble L55P-TTR was compared with the spectra simulated with the parameters that best fit the results of the four TTR mutants in the soluble form. These spectra and corresponding errors are presented in Fig. 5. When the L55P-TTR spectrum was simulated with the parameters obtained for the best fit of WT-TTR (84% reduced sulfur, 4% sulfoxide, and 12% sulfonate), V30M-TTR (86% reduced sulfur, 5% sulfoxide, and 9% sulfonate) and C10S-TTR (97% reduced sulfur, 1% sulfoxide, and 2% sulfonate), the relative errors (0.0083, 0.019, and 0.059, respectively) were over three times the error associated with the spectrum that represents the best fit for L55P-TTR, which is 0.0024.

The results suggest that L55P-TTR, the most aggressive variant described in the literature until now, is the variant with the higher content of sulfonated sulfur (28%). Analysis of the fibrillar material reveals that the percentage of sulfur from the methionine residues that are in the sulfoxide form (V30M-TTR: 28%, L55P-TTR and WT-TTR: 32%) are approximately 3–4× higher than the corresponding value in the soluble proteins (V30M-TTR: 8%, L55P-TTR and WT-TTR: 10%). The cysteines are even more oxidized: in V30M-TTR variant 93% are in the sulfonated form, in L55P-TTR 76% and in WT-TTR protein 48% of the cysteines are oxidized. It is worth mentioning that L55P-TTR variant was not polymerized by acidification. This indicates that cysteine and methionine oxidation is not an artifact resulting from partial denaturation due to acidification but results from structural alterations during or upon polymerization.

**Thioflavine-T Binding Assay**—In order to improve our understanding about the role of sulfur oxidation in the self-assembly of TTR protein, the susceptibility to amyloid formation of reduced WT-TTR was monitored using the thioflavine-T binding assay.

A soluble WT-TTR sample (100 μg) was reduced with dithiothreitol (10 mM), and the procedure to amyloid fibril formation by acidification was followed. The reducing agent was removed from the fiber solution just before the thioflavine-T binding assay in order to avoid possible quenching of the spectra. The results show that the reduced protein solution had strong susceptibility to fibril formation (Fig. 6).
Although many aspects of amyloid diseases are known, a molecular model describing the interactions between amyloidogenic units at high resolution, e.g., revealing the contacts between different atoms that are responsible for a highly stable structure, as it is the case of the amyloid fibrils, does not exist. This is a priority for defining how to design a drug that either (i) directly binds to a contact region and destroys the interaction or (ii) rather occupies the binding site of one amyloidogenic unit, avoiding its contact with a nearby unit inhibiting amyloid formation.

In this work we conclude that in TTR amyloid fibrils, Cys-10, the only cysteine residue present in the TTR protein, does not form disulfide bridges with other molecules and that sulfur-containing amino acids, namely Cys-10 and Met-13, are significantly oxidized upon polymerization. The oxidation of these amino acids may occur before fiber formation or in the mature fiber. In fact, there are two possibilities: oxidation of both amino acids leads to a more polar region capable of forming hydrogen bonds with other molecules and this leads to amyloid fibers; or aggregation leads to conformational changes in the protein that expose Cys-10 and Met-13 to the solvent allowing their subsequent oxidation. However, oxidation of both amino acids is not required for fibril formation as revealed by the strong susceptibility of reduced protein samples to form amyloid. Sulfur oxidation might result as a consequence of structural alterations during protein aggregation exposing the cysteines and methionines to the solvent.

Although cysteine-free TTR mutants are capable of amyloid fibril formation (19), showing that disulfide linkages are not required for fibrillogenesis, there are a number of studies relating the amyloidogenic behavior of TTR with the oxidation state of sulfur from their cysteine residues. This relation is not clear yet, since some results point to an increased amyloidogenicity of sulfonated transthyretin (20), while others indicate that the stability of the tetramer is increased by binding of sulfite to Cys-10 (21). Nevertheless, S-sulfonated transthyretin has been detected in serum and it was reported that it is significantly more abundant in diseased individuals (22, 23). The data show that a significant fraction of the thiol group of cysteines present in the soluble TTR samples is in the sulfonate form. A quantitative study of the sulfonic and sulfonic acid content was carried out in different proteins leading to the conclusion that a significant number of cysteines is irreversibly oxidized. In fact, one in four cysteines was estimated as being reactive to oxidants (24).

Examination of the protein models available in the Protein Data Bank (25) shows that only 2.5% of the structures contain
oxidized cysteines. However, in a significant amount of the protein structures the cysteines are buried and not exposed to the solvent. Furthermore, in some models the cysteic acid was represented by three water molecules around the sulfur, and therefore its oxidation state is not immediately obvious. In the x-ray crystallographic structures of TTR proteins, the oxidation of Cys-10 has not been detected. This might be explained by the fact that in those structures the N-terminal is disordered and Cys-10 is in most cases the first residue to be observed. In fact large temperature factors were assigned to the sulfur atom of that residue. In human serum, sulfated TTR as well as cysteinylated TTR were detected using mass spectroscopy (22).

Further insight to the structure of amyloid fibrils can be obtained from the crystallographic structure of the TTR variants and the results here reported. Met-13 is not buried in the molecule but still partially protected by Lys-15, Glu-54, His-56, Arg-104, and Thr-106. Cys-10 is at the external surface of the molecule and also somehow protected, although not as much as Met-13. It is flanked by the main chain of Leu-12 and Met-13, the side chain of Arg-104, and by the main chain between His-56 and Thr-60 as shown in Fig. 7. The crystal structure of GSS/E54D/L55S-TTR, a constructed variant that polymerizes spontaneously at physiological conditions, revealed a structural change of the edge strand D exposing Met-13 (26). Also in the highly amyloidogenic L55P-TTR variant, a movement of strand D leading to a long loop between strands C and E was observed (18). This loop contains the His-56–Thr-60 region that mediates the access of the solvent to Cys-10 and to Met-13. The effect of the His-56–Thr-60 region over the accessibility of Met-13 to the solvent was determined for the x-ray crystallographic structures of WT- (PDB ID: 1F41) and L55P-TTR (PDB ID: 5TTR), using Turbo-Frodo (27). The accessible surface of Met-13 in the WT-TTR is 26 Å², half of the accessible surface of Met-13 in the L55P-TTR, which is 52 Å². The reported values are the averaged results for the accessible surfaces of all Met-13 in the crystallographic asymmetric unit. The accessible surface of Cys-10 was not calculated since the N-terminal in these TTR crystal structures is disordered.

Since in the fibrils we observe a substantial increase in the number of Cys-10 and Met-13 amino acids in the oxidized form, we propose that this occurs due to a movement of the protein main chain in the edge region His-56–Thr-60, as it happens in the case of L55P-TTR. In β2-microglobulin, another β-sandwich protein, it was reported that a restructuring of a β bulge that separates two short β strands leads to a protein with a longer strand located at the edge of the molecule and increased amyloidogenic properties (28, 29). Besides this rearrangement of the edge strand, the authors reported a rotation of His-51 increasing the hydrogen bonding potential of that strand and therefore allowing the formation of intermolecular interactions. In the case of TTR, also His-56, located at the edge β-strand that we believe is modified for fibril assembly, has a different conformation in WT- and L55P-TTR (Fig. 7). Furthermore, a contact between this amino acid and a glutamic acid of a nearby molecule was reported as an abnormal interaction that might be important for amyloid formation (18). Recently hydrogen/deuterium exchange combined with NMR analysis was used to identify the stable core of β2-microglobulin amyloid fibrils and suggests a loss of structure at the edge of the native β-sheet during self-assembly of the protein (30). In a different study, it was revealed that six of the seven strands present in this β-sandwich protein are protected from H/D exchange in the fiber structure (31).

According to the CATH-Protein Structure Classification (32), TTR and β2-microglobulin belong to the main β-class of proteins, both have a sandwich architecture and an immunoglobulin-like topology. Therefore, a high level of structure similarity exists between the two proteins, and it is most probable that, as in the case of TTR, the two β-sheet cores are maintained in the fibrils. An assembly mechanism, where two edge strands are moved away and the penultimate strands A and B are exposed was recently proposed for TTR (15). This would certainly explain oxidation of cysteine (near strand A) and methionine (on strand D) as well.

The x-ray diffraction patterns of TTR amyloid fibrils revealed the classic β-structure with the hydrogen bonds within the β-sheets parallel to the fiber axis. The presence of a broad reflection at 10 Å indicates that the fiber is composed of two β-sheets (14) as previously proposed. Along the fiber axis, the 29 Å repeating unit may correspond to a monomeric species, although only the 6th and higher order reflections are detected. The lower order reflections were not seen and are not usually detected (7, 8), which may indicate that the fundamental spacing of 29 Å includes an ordered region with the β-chains perpendicular to the fibril axis, the core of the native protein, and a disordered domain (8) that may be due to the structural alterations in the edge strands.

We conclude that in TTR amyloid fibrils the repeating units are composed of two β-sheets, resembling the TTR monomer, and that a structural alteration of the protein D-strand and the DE-loop lead to a disordered region that exposes Cys-10 and Met-13 to the solvent. We believe that the newly exposed surface is responsible for promoting protein aggregation. Furthermore, Cys-10 does not form disulfide bridges, and therefore such bridges are not responsible for the highly packed structure present in the fibrils. The thioflavine-T binding assays also show that the reduced protein is susceptible to amyloid formation by acidification. Therefore oxidation, at the native protein state, does not seem to promote amyloid fibril formation. Future studies involve the use of compounds that specifically bind the sulfur-containing amino acids in order to test their role in protein aggregation.

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