Probing Solvent Accessibility of Transthyretin Amyloid by Solution NMR Spectroscopy*

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The human plasma protein transthyretin (TTR) may form fibrillar protein deposits that are associated with both inherited and idiopathic amyloidosis. The present study utilizes solution nuclear magnetic resonance spectroscopy, in combination with hydrogen/deuterium exchange, to determine residue-specific solvent protection factors within the fibrillar structure of the clinically relevant variant, TTR[Y114C]. This novel approach suggests a fibril core comprised of the six β-strands, A-B-E-F-G-H, which retains a native-like conformation. Strands C and D are dislocated from their native edge region and become solvent-exposed, leaving a new intermolecular association between strands F-G and H-H with a prolongation of these β-strands and, interestingly, with a possible shift in β-strand register of the subunit assembly. This finding may explain previous observations of a monomeric intermediate preceding fibril formation. A structural model based on our results is presented.

Amyloidosis represents a group of disorders in which endogenous proteins or peptides adopt anomalous folds and aggregate into elongated amyloid fibrils. At least 20 different human disease conditions are associated with protein deposits, of which Alzheimer’s disease and prion-associated disorders are among the most well known examples (1). Because the macroscopic properties of amyloid, in terms of aggregation and solubility, prevent the use of single crystal x-ray crystallography, as well as solution NMR spectroscopy, the atomic resolution of the molecular structure of amyloid fibrils is poorly understood. So far, structural information has mainly arisen from fiber diffraction studies (2), cryoelectron microscopy (3), mass spectrometry (4), and solid state NMR spectroscopy (5). From these data a general picture has emerged implying the occurrence of parallel or antiparallel β-sheets, in which the β-strands lie perpendicular to the direction of the long fibril axis. Familial amyloidotic polynuropathy is a hereditary form of amyloidosis caused by one of more than 70 different single point mutations in the 13.7-kDa human plasma protein transthyretin (TTR) (6). The clinical manifestation of familial amyloidotic polynuropathy is predominantly peripheral polynuropathy, affecting motor, sensory, and autonomic functions, with accumulation of amyloid deposits along the peripheral nerves and within the visceral organs. The TTR wild-type protein can also form amyloid in vivo and is associated with a senile form of disease affecting around 25% of the population above 80 years of age (7). The native fold of TTR has long since been established through x-ray diffraction studies (8), and these show that native TTR adopts a tetrameric arrangement, where each monomeric subunit contains eight β-strands, denoted A–H, arranged in a characteristic sandwich of two four-stranded β-sheets (DAGH and CBEF). Two monomeric subunits are associated via F-F and H-H interactions to form a dimer that in turn interacts in a face-to-face manner with another dimer to form the tetramer. Crystalline structures of several in vivo identified amyloidogenic mutants of TTR have been determined over the years. However, they show only subtle and minor variations in their three-dimensional structure compared with the native structure of wild-type TTR (9).

In this work we have focused on the characterization of the fibrillar structure of TTR. We have recently developed a general method using NMR spectroscopy in combination with hydrogen/deuterium (H/D) exchange to facilitate the identification of individual core residues within a fibril (10). The technique relies on the partial solvent protection of hydrogen-bonded amide protons throughout the length of the fibril. Backbone amide protons located on the exterior of the fibril are more solvent-accessible and thus experience a relatively higher solvent exchange rate compared with amide protons that are located inside the fibril core and shielded from the solvent. In practice, the method works by pulse labeling fully protonated fibrils in a D₂O solution over a given period of time. The resulting partially deuterated fibrils are subsequently converted into an NMR detectable monomeric state by resolubilization using a denaturing agent at low pH. This treatment traps the H/D exchange pattern originally present in the fibril and which can be detected via the soluble monomeric conformer. Apart from pinpointing the protected core residues of the fibril, the NMR method also allows for a specific quantification of their protection level. A detailed description of the method is presented elsewhere (10). A similar, but nonquantitative, approach was presented recently for fibrils derived from β₂-microglobulin (11).

The present study applies the method described above to the
**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The pET-3a vector containing the gene encoding TTRY114C was transformed into Escherichia coli BL21 according to standard procedures. Uniformly ^15^N- or ^15^C-labeled protein was produced by growing cells in M9 minimal medium containing ^15^N/NaCl (Cambridge Isotope Laboratories) and ^15^C-[^12]glucose (Cambridge Isotope Laboratories) as required. Protein expression was induced using 0.4 mM isopropyl thiogalactopyranoside. To lyse the cells 10 mg of lysozyme/ml of pellet was added and incubated for 30 min. After lysis DNAs were added in the presence of Mg^2+ ions followed by centrifugation at 20,000 × g for 20 min. The supernatant was purified further using an anion exchange column (Mono Q HR 10/10 Amersham Biosciences) followed by gel filtration (Sephadex G-75, Superdex 16/10, Amersham Biosciences) using 20 mM phosphate buffer, pH 7.0. Pure fractions were pooled and concentrated using a spin column device with a 10-kDa filter (Amicon 10K, Millipore).

**Preparation of Amyloid Fibrils from TTRY114C**—To obtain amyloid fibrils a 3 mM solution was incubated in 20 mM phosphate buffer, pH 7.0, at 55 °C for 96 h. 1% of β-mercaptoethanol (BME) was included to prevent intermolecular disulfide bridge formation. The initially clear solution changed its appearance into a viscous gel, which is a characteristic feature of amyloid fibrils.

**Gel Electrophoresis**—Proteins were separated on a 16% polyacrylamide gel with a 4% stacking gel under constant voltage (native conditions) or in the presence of 0.1% SDS, according to standard procedures. 1% BME was included as a reducing agent. None of the samples was boiled. Under non-native conditions, samples were dissolved in 100 mM SDS loading buffer and separated further on a 16% SDS-PAGE according to standard procedures.

**Atomic Force Microscopy (AFM)**—The aggregated TTRY114C was diluted to 3.6 μM with distilled water and applied onto freshly cleaved ruby red mica (Goodfellow, Cambridge, UK). Samples were allowed to adsorb for 30 s, washed three times with distilled water, and air dried. The bound material was imaged with a Nanoscope IIIa multimode AFM (Digital Instruments Santa Barbara, USA) using Tapping Mode™ in air. A silicon probe was oscillated at 300–325 kHz, and images were collected at an optimized scan rate corresponding to 0.7–1 Hz. The image was flattened and presented in height mode using Nanoscope software (Digital Instruments).

**Circular Dichroism (CD) Spectroscopy**—CD spectra were recorded at 20 °C, using a CD-6 spectrometer (Jobin Yvon–spex France). Samples were prepared by incubation of 20 μM protein in 50 mM sodium phosphate buffer, pH 7.0. Acquired spectra were collected between 195 and 250 nm and base line corrected by subtraction of a buffer reference spectrum. The ellipticity is reported as mean residue molar ellipticity ([θ], in degree cm^2^/dmol•c) according to Equation 1,

\[
[\theta] = 100 \cdot [\theta]_n \cdot (C \cdot L \cdot N)
\]

(Eq. 1)

where [θ]_n is the ellipticity (degrees), C is the concentration of polypeptide (M), L is the optical path length (cm), and N is the number of amino acid residues in the polypeptide. Evaluation of the secondary structure content of the protein was carried out by using the R2D prediction program (15).

**Mass Spectrometry**—Mass spectrometric analysis in combination with H/D exchange was used to study the exchange rate of labile protons within the fibrillar state. The aqueous fibrillar solution with a monomeric concentration of 200 μM was diluted 10 times in D_2O and incubated for various lengths of time. To enable analysis the solution was exchanged rapidly through addition of equal volumes of an ice-cooled solubilizing buffer consisting of 99% acetonitrile and 1% formic acid. The sample was subsequently injected within 20 s into a quadrupole electrospray mass spectrometer (Waters). The number of protected sites was determined from the measured mass and the theoretically calculated mass of a fully deuterated monomeric species.

**NMR Spectroscopy and Sequential Backbone Assignment of TTR in SDS**—NMR experiments were carried out at 25 °C on a Bruker AVANCE spectrometer, operating at 600 MHz (proton frequency) and equipped with a 5-mm triple resonance, pulsed field, z-gradient cryo-probe. A typical NMR sample contained 0.3 mM TTR, 100 mM deuterated SDS (d-SDS, D_2O, 98%; Cambridge Isotope Laboratories) and 20 mM d-DTT in 95% H_2O, 5% D_2O at pH 3.05 or in D_2O at a pD of 3.45. The data were processed using NMRPipe (14) or XWINNMR (Bruker Biospin), although assignments were made in Ansigs for Windows (15). The sequence-specific backbone resonance assignment of TTR followed the standard triple resonance strategy for 15^N-labeled proteins, using two-dimensional 15^N HSQC and three-dimensional 15^N DIPSI-HSQC, 15^N NOESY-HSQC, CBCA(CO)NH, HNCACB, HCANH, HC(N)/CO, HNCO, HCN(A)/CO, and C(CO)NH experiments (16, 17).

**H/D Exchange of TTRY114C Fibrils Analyzed by NMR**—The H/D exchange experiment on the amyloid fibrils was performed by a brief centrifugation of the aqueous amyloid solution, which enabled the removal of the supernatant. The remaining amyloid pellet (~100 μl) was subsequently diluted 10 times using D_2O containing 5 mM phosphate buffer, pH 7.4, and 10 mM d-DTT. To ensure the essentially complete removal of H_2O and BME the washing procedure was repeated twice. After a total of 10 min incubation in D_2O, which includes the duration of the buffer exchange procedure, fibrils were collected and dissolved through the addition of 0.5 ml of D_2O buffered to pH 3.45, containing 100 mM of d-SDS and 20 mM d-DTT. The protection of amide protons after dissolution of the fibrils was followed by a series of two-dimensional 15^N HSQC spectra, starting 10 min after the SDS solution was added. Each experiment was recorded in 15 min with six transients per increment and 1024 × 128 (t_1 × t_2) data points. To be able to monitor quantitatively the dissolution of the fibrils into a monomeric species—a one-dimensional proton spectrum was recorded prior to each HSQC spectrum.

To discriminate between exchanging protons within the fibril and the monomeric species a reference H/D experiment was carried out on the SDS monomer. A protein solution in H_2O (100 mM d-SDS and 20 mM d-DTT in 95% H_2O and 5% D_2O) was concentrated 10-fold followed by a rapid 10-fold dilution in a D_2O solution (100 mM d-SDS and 20 mM d-DTT in 100% D_2O) and the collection of an identical series of two-dimensional 15^N HSQC spectra as above.

**DATA Analysis and Fitting**—To monitor the post-trap H/D exchange after dissolution of the partly exchanged TTRY114C fibrils in D_2O, peak volumes from base-line corrected 15^N HSQC spectra were obtained by integration using Bruker XWINNMR 3.1 software routines and analyzed by the CurveExpert 1.37 program. The relative total monomer concentration of the solution was determined from integration of the completely exchangeable methyl region in a one-dimensional proton NMR spectrum, recorded directly prior to each individual 15^N HSQC spectrum. Consequently, the integrals of exchangeable amide proton cross-peaks in the 15^N HSQC spectra are corrected for the effective monomeric concentration at any specific time after dissolution of the fibrils. The post-trap decays of these corrected amide proton integral (I) of each protected residue in the monomeric state were fitted to a single exponential function,

\[
I(t) = I_0 \cdot e^{-k \cdot t} \cdot \exp\left(-\frac{t}{\tau}\right)
\]

(Eq. 2)

where k_{obs} is the post-trap H/D exchange rate and I_0 is the extrapolated proton integral at time zero. The latter describes the degree of protection of amide groups that survived the deuteration exchange period with D_2O in the fibril state, prior to resolubilization by SDS. The actual fraction of protection within the fibrillar state could thus be calculated by a comparison of corresponding peak integrals in a similarly treated, but fully protonated, reference spectrum. By definition the relative ratio between I_2 in the two spectra is 100% in absence of any H/D exchange process and 0% in conditions of complete H/D exchange.

**Structure Modeling**—A model of the fibril structure of TTRY114C was generated using the coordinates from wild-type TTR (PDB 1F41), the experimentally determined protection factors, and known intermolecular distances (3, 18, 19). The monomeric structure was modified.
Cys114 residue in TTRY114C, are complicating features both in wild-type TTR and TTRY114C, and the additional material had assembled as high molecular weight aggregates in lane 5. The presence of amyloid fibrils was verified by AFM. Fig. 1.

A. native and SDS-PAGE analysis of TTRY114C. Lanes 1–7, native PAGE analysis to follow aggregation of TTRY114C after incubation in 55 °C at various time intervals: lane 1, 0 h; lane 2, 0.5 h; lane 3, 2 h; lane 4, 6 h; lane 5, 12 h; lane 6, 48 h; lane 7, 96 h. Lane 8, SDS-PAGE showing TTRY114C fibrils dissolved in 100 mM SDS and separated on a 16% SDS-PAGE without previous boiling. In all samples 1% BME was included to avoid formation of disulfide bridges. This treatment effectively blocked intermolecular disulfide bridge formation, as shown by SDS-PAGE analysis where fibrils were dissolved in a 100 mM SDS solution without previous boiling of the protein sample. The well defined sharp band that can be seen in Fig. 1A, lane 8, corresponds to the expected size of a monomer.

CD Spectra Suggest Less Secondary Structure within the TTR Fibrils Compared with the Native State—Far UV CD spectroscopy was used to investigate further the structural properties of TTRY114C. The nearly identical CD spectra observed for the native form of TTRY114C and TTR wild-type (see Fig. 2) were analyzed by the K2D prediction program (13) and resulted in an estimated β-sheet content of 42 and 40%, respectively. This is in agreement with a recently presented crystal structure of TTRY114C showing only subtle differences from the native TTR wild-type (12). TTRY114C fibrils formed at a protein concentration around 20 μM at pH 7 (verified by AFM) did not cause significant turbidity, a fact that allows for secondary structure analysis by means of CD spectroscopy together with the K2D program (13). The estimated β-sheet content of the fibrillar fold is ~26%, which, compared with the native fold, corresponds to a loss of nearly 40% of β-sheet in favor of a more random coil structure. The result is in accordance with a previous study on TTR wild-type amyloid (23) and is further evidence that the internal structural organization of the amyloid state differs significantly from that of the native state.

RESULTS

TTRY114C Forms Amyloid upon Incubation at an Elevated Temperature—The mutant protein TTRY114C was expressed in E. coli and purified as a native tetramer (12). As a result of the mutation Tyr114 → Cys114 the tetrameric fold of the protein is destabilized and consequently acquires an enhanced propensity to form amyloid, and thereby achieves a higher stability against denaturants. To monitor the H/D exchange pattern of amide protons in the fibril, using our methodological tools, it is important to achieve rapid dissociation of the fibrils into a monomeric species at a rate faster than the intrinsic rate of H/D exchange. Several different agents were tested for optimal dissolution. The requirements were best fulfilled in an aqueous solution containing SDS, as illustrated by SDS-PAGE analysis where the fibrils are rapidly dissociated into a monomeric species (the well defined band in Fig. 1A, lane 8).

To trap the H/D exchange pattern of the fibril, dissolution was carried out in SDS/D2O solution buffered to a pH of 3.45, where the intrinsic solvent exchange rate is at a minimum. SDS is also known to induce α-helical conformation, which further prolongs the post-trap exchange times within these structured regions of the monomer. A CD spectrum of fibrils dissolved in 100 mM SDS at pH 3.05 (corresponding to pD 3.45) is shown in Fig. 2. The appearance of a strong absorption minimum at 210 nm, and to some extent at 222 nm, is charac...
Illustrated as the number of protected amide protons within the fibril as a function of incubation time in D_2O. Spectra B and C were recorded 10 and 40 min after dissolution, respectively. Assignments are indicated where available.

The amyloid fibrils obtained from TTRY114C at protein concentrations of around 1 mM exhibited a gel-like appearance and were easily pelleted from the gel solution through centrifugation. The exchange of solvent-accessible protons was fitted to a single exponential function, making it possible, characteristic for a protein with α-helical secondary structure and estimated to 42% by the K2D program (13), clearly visualizing the increased content of helices under these conditions.

Sequential Backbone Assignment of TTRY114C in SDS Solution—A prerequisite for the indirect analysis of the exchange pattern of the TTRY114C fibril is the assignment of protein resonances from the monomeric species found in SDS solution. The 15N HSQC of a 0.3 mM protein sample, dissolved in 100 mM d-SDS, 1 mM d-DTT, and recorded at 25 °C at pH 3.05 showed a spectrum with fairly broad resonances and a limited dispersion of chemical shifts (Fig. 3A). Such an appearance is expected for an α-helical protein in complex with a SDS micelle, with a total estimated molecular mass of at least 30 kDa. Sequence-specific resonance assignments were obtained using standard assignment techniques, together with a set of three-dimensional triple resonance experiments. Taking the absence of an amide group on the N-terminal methionine and the 8 prolines into account, a total number of 119 amide backbone resonances should be visible in a 15N HSQC. Of these 119 resonances 83 were unambiguously assigned, but the assignment of residues Glu72 and Asp74 were ambiguous. Slow conformational exchange was observed in regions 98–100 °C at pD 7.4 for 10 min to facilitate exchange of solvent-accessible protons. The observed exchange rates are in accord with similar investigations on TTRwt (24). Our mass spectrometry data suggest that around 45 amide protons remain protected within the fibrillar state of TTRY114C after 10 min of H/D exchange. To perform analysis by NMR, the fibrils were likewise subjected to a 10-min incubation period in D_2O solvent, followed by rapid dissociation into NMR-detectable monomers by the addition of a 10-fold volume excess of buffered D_2O solution to the pelleted fibrils, directly followed by vigorous shaking and re-collection of the pellet by centrifugation. The whole procedure was repeated twice, which effectively removed residual H_2O and BME. Mass spectrometry measurements showed that the bulk of the fast exchanging, solvent-exposed, amide protons were substituted by deuterons within 10 min from the start of incubation (fibrils incubated in D_2O). After that time a plateau of significantly slower exchange was reached (Fig. 4A). The observed exchange rates are in accordance with similar investigations on TTRwt (24). Our mass spectrometry data suggest that around 45 amide protons remained protected within the fibrillar state of TTRY114C after 10 min of H/D exchange. To perform analysis by NMR, the fibrils were subjected to a 10-min incubation period in D_2O solvent, followed by rapid dissociation into NMR-detectable monomers by the addition of a D_2O solution containing 100 mM d-SDS and 10 mM d-DTT, buffered to pD 3.45 (see “Experimental Procedures”).

Fig. 3B shows a selected region of the first 15N HSQC spectrum recorded 10 min after resolubilization of the fibrils into monomers. Approximately 50 amide hydrogens were found to be protected from solvent exchange. To obtain a quantitative value for the protection level of each preserved residue within the fibril, the post-trap exchange in the monomeric state was followed through a series of 15N HSQC experiments recorded at regular time intervals. Fig. 3C shows the 15N HSQC spectrum obtained 40 min after dissolution. The nondeuterated reference spectrum of TTRY114C is shown in Fig. 3A. The post-trap decay of the amide proton integral of each protected residue was fitted to a single exponential function, making it possible,
TABLE I

| Residue | Relative solvent protection (%) | Secondary structure |
|---------|---------------------------------|---------------------|
| Gly\(^3\) | 0 | N-terminal |
| Thr\(^4\) | 0 | N-terminal |
| Gly\(^5\) | 0 | N-terminal |
| Thr\(^6\) | 0 | N-terminal |
| Gly\(^7\) | 0 | N-terminal |
| Glu\(^8\) | 0 | N-terminal |
| Asp\(^9\) | 0 | N-terminal |
| Ala\(^{10A}\) | (16) | \(\beta\)-Sheet A |
| Ala\(^{10B}\) | 24 | \(\beta\)-Strand B |
| Ile\(^{10C}\) | 100 | \(\beta\)-Strand B |
| Val\(^{11}\) | 35 | \(\beta\)-Strand B |
| Ala\(^{12}\) | 46 | \(\beta\)-Strand B |
| Val\(^{13}\) | 84 | \(\beta\)-Strand B |
| His\(^{14}\) | 69 | \(\beta\)-Strand B |
| Val\(^{15}\) | 71 | \(\beta\)-Strand B |
| Phe\(^{16}\) | 72 | \(\beta\)-Strand B |
| Arg\(^{17}\) | 33 | \(\beta\)-Strand B |
| Lys\(^{18}\) | 0 | \(\beta\)-Strand B |
| Ala\(^{19A}\) | (78) | \(\beta\)-Strand B |
| Ala\(^{19B}\) | (5) | Loop BC |
| Asp\(^{20}\) | 48 | Loop BC |
| Trp\(^{21}\) | 0 | \(\beta\)-Strand C |
| Ala\(^{22}\) | (16) | \(\beta\)-Strand C |
| Ser\(^{23}\) | 0 | \(\beta\)-Strand C |
| Gly\(^{24}\) | 0 | \(\beta\)-Strand C |
| Lys\(^{25}\) | (46) | \(\beta\)-Strand C |
| Thr\(^{26}\) | 0 | \(\beta\)-Strand C |
| Ser\(^{27}\) | (12) | Loop CD |
| Glu\(^{28}\) | 0 | Loop CD |
| Ser\(^{29}\) | 0 | Loop CD |
| Gly\(^{30}\) | 0 | \(\beta\)-Strand D |
| Val\(^{31}\) | (5) | \(\beta\)-Strand D |
| Lys\(^{32}\) | 0 | \(\beta\)-Strand D |
| Thr\(^{33}\) | 0 | \(\beta\)-Strand D |
| Val\(^{34}\) | 52 | Loop DE |
| Leu\(^{35}\) | (78) | Loop DE |
| Glu\(^{36}\) | 46/62 | \(\beta\)-Strand E |
| Asp\(^{37}\) | 46/62 | \(\beta\)-Strand E |
| Ala\(^{38}\) | 27 | \(\alpha\)-Helix |
| Lys\(^{39}\) | 0 | \(\alpha\)-Helix |
| Phe\(^{40}\) | 0 | Loop EF |
| Lys\(^{41}\) | 0 | Loop EF |
| His\(^{42}\) | 0 | Loop EF |
| Glu\(^{43}\) | 0 | Loop EF |
| Ala\(^{44}\) | 17 | Loop EF |
| Ala\(^{45A}\) | 94 | \(\beta\)-Strand F |
| Ala\(^{45B}\) | (12) | \(\beta\)-Strand F |
| Val\(^{46}\) | 69 | \(\beta\)-Strand F |
| Val\(^{47}\) | 96 | \(\beta\)-Strand F |
| Phe\(^{48}\) | 46 | \(\beta\)-Strand F |
| Thr\(^{49}\) | 51 | \(\beta\)-Strand F |
| Ala\(^{50}\) | 100 | \(\beta\)-Strand F |
| Asp\(^{51}\) | 68 | Loop PG |
| Asp\(^{52}\) | 0 | Loop PG |
| Ser\(^{53}\) | 0 | Loop PG |
| Glu\(^{54}\) | 0 | Loop PG |
| Glu\(^{55}\) | 68 | \(\beta\)-Strand G |
| Ala\(^{56}\) | (78) | \(\beta\)-Strand G |
| Ala\(^{57}\) | 89 | \(\beta\)-Strand G |
| Lys\(^{58}\) | 81 | \(\beta\)-Strand G |
| Thr\(^{59}\) | 73 | \(\beta\)-Strand H |
| Thr\(^{60}\) | 45 | \(\beta\)-Strand H |
| Ala\(^{61}\) | 100 | \(\beta\)-Strand H |
| Val\(^{62}\) | 52 | \(\beta\)-Strand H |
| Val\(^{63}\) | 40 | \(\beta\)-Strand H |
| Thr\(^{64}\) | 22 | \(\beta\)-Strand H |
| Asp\(^{65}\) | Yes | C-terminal |
| Glu\(^{66}\) | (46) | C-terminal |

Experimentally determined relative solvent protection factors of TTRY114C amyloid fibrils

The values of protection are expressed as fractional values, with 0 meaning complete exchange of the amide proton within 10 min of incubation in D_2O and 100 meaning full protection against deuterium exchange during the preincubation time. Only those residues that exhibit a detectable H/D exchange rate within SDS monomers are listed. For each residue the corresponding secondary structure within the native form is indicated. Overlapping residues are indicated with identical superscript characters, and their protection levels are written within parentheses. * indicates ambiguously assigned residues; # indicates the inability to fit an exponential function to the decay rate. § means that the actual fitted value was 121, 103, and 102% for Ile\(^{10}\), Ala\(^{30}\), and Ala\(^{10B}\), respectively.

![Fig. 5. Mapping of the observed protection factors of the fibril onto the native structure of TTR.](image-url)

A ribbon model of the TTR monomer derived from the crystal structure of the wild-type protein (PDB 1F41) and with its eight \(\beta\)-strands labeled from A to H is shown. Also, the N and C termini are indicated with large capital letters. The observed relative solvent protection factors found for residues within TTRY114C fibrils are mapped onto the native structure with the following color code: navy blue for complete and light blue for weak protection; red, unprotected; yellow, protected residue with ambiguous assignment; gray, undetermined. The image was prepared using MOL-MOL (20).

by extrapolation, to obtain the amide proton integral at time zero of fibril solubilization. This initial value represents the degree of protection each residue experienced in the fibrillar state. A comparison with a fully protonated reference sample then gives the percentage of protection within the fibrillar state: by definition a value of 100% in absence of any H/D exchange process, and a minimum of 0% under conditions of complete H/D exchange. Fig. 4B shows a representative curve fit for residues 93 and 110.

As reported previously (10), the rate of fibril dissolution is nonlinear; in other words, not all fibrils convert to NMR-detectable monomers at the same rate. In the case of a slowly dissociating amyloid the final value of protection must, as a consequence, be normalized against the actual monomeric concentration at the specific time point. To follow dissociation into a monomeric species a one-dimensional proton NMR spectrum was therefore collected prior to each two-dimensional \(^{15}\)N HSQC experiment. The intense signal of the nonexchangeable methyl resonances (unaffected by deuterium exchange) was used to monitor dissociation rates of the sample. The result shows that initially a fast dissociation occurred, and prior to the first spectrum 75% of the material was recovered into a detectable state. From here on a slower, approximately bieponential, phase of dissolution was observed with two different rate constants (0.038 min\(^{-1}\) and 0.0027 min\(^{-1}\)) (Fig. 4C). The degrees of protection were therefore normalized against the monomeric concentration at each specific time point.

The relative amide proton protection observed for assigned residues of the TTRY114C fibril is listed in Table I and mapped onto a ribbon structure of the native TTR monomer (Fig. 5). Note that the highest protection appears to coincide particularly well with the native \(\beta\)-strands B, E, F, G, and H, and to some extent \(\beta\)-strand A, whereas the N-terminal, loop regions and strands C and D show little or no protection.

Because the fibrillar fold differed significantly from the monomer dissolved in SDS, it was important to verify that the fast exchanging amide protons (listed as 0% protection in Table I) are actually substituted in the fibrillar state and not the result
of an exposed position within the SDS monomer. A second H/D experiment was therefore performed directly on the monomer dissolved in appropriate SDS solution. Through this approach all residues listed in Table I, with 0% protection in the fibril, were well protected within the SDS monomeric state (data not shown), confirming their solvent-exposed position within the fibril.

**DISCUSSION**

The difficulty of elucidating the amyloid architecture at atomic resolution has hampered the understanding of the mechanisms of amyloid formation for a long time, as well as any advancement within the field. Recently, we have devised a new and powerful method that provides detailed structural information about the amyloid fibril structure. It combines a pulsed H/D experiment to trap solvent exchange patterns of the fibrils followed by a rapid dissociation into its monomeric constituents and analysis using solution NMR spectroscopy. The method was applied successfully to the highly amyloidogenic Aβ(25–35) derived from the Alzheimer β-peptide (10). Within this work we have challenged our technique and probed the fibrillar core structure of transthyretin amyloid, a comparatively large protein associated with both inherited and idiopathic amyloidosis.

The mechanism by which the native fold of TTR may be converted into elongated fibrillar assemblies has been investigated extensively, and numerous studies have shown that dissociation of the native tetrameric structure into a partly unfolded monomeric species precedes amyloid formation (25–28). Mutational analysis of amyloidogenic TTR variants does not, however, reveal the mechanism by which amyloid forms. Although it is likely that each mutation has a different impact on the structure and stability of the protein, investigations have shown that the amyloidogenic propensity is controlled by the rate of tetrameric dissociation rather than its thermodynamic stability (27, 29).

The clinically relevant TTRY114C mutant protein may easily be converted into amyloid under near physiological conditions and was chosen as a model for a typical TTR amyloid fibril. In addition, TTRY114C fibrils dissociate rapidly into monomers in acidic SDS solution under conditions in which the solvent exchange rate is considerably reduced, thereby providing a convenient way of trapping H/D exchange patterns of the fibril and performing analysis by solution NMR spectroscopy. The time of incubation (fibrils in D$_2$O) was optimized to 10 min using mass spectrometry in an approach similar to that described by Kheterpal and co-workers (4). During this time span the bulk of fast exchanging amides, interpreted as surface-located residues, was exchanged (Fig. 4A). The remaining part, corresponding to ~45 protons, had a significantly slower exchange rate and was considered to constitute the fibrillar core. The optimized conditions were then employed in the following pulsed H/D exchange experiment and analyzed by two-dimensional 15N HSQC NMR experiments. The result showed a good correlation with mass spectrometry, and a partial protection of around 50 residues was detected.

From the sequence-specific resonance assignment procedure it was possible to assign 85 of 119 non-proline residues using standard triple-resonance NMR techniques and a reference sample of 15N,13C-labeled TTRY114C in 100 mM d-SDS at pH 3.05. Table I lists the assigned residues of TTRY114C for which the relative protection factors could be determined quantitatively. Fig. 5 shows the protection factors mapped onto the native monomeric structure.

The exchange data essentially showed that well protected residues were found within stretches corresponding to the native strands A, B, E, F, G, and H, suggesting that they constitute the core of the fibril. The presence of solvent-exposed residues found in strands C (residues 41, 46, 47, and 49) and D (residues 53 and 55) as well as in the connecting and following loops (residues 51, 52, 56, 57, and 60) clearly shows that this region is not part of the fibrillar core. This result is supported by several of our previous investigations on the structures of TTR fibrils where the protease fragmentation patterns both in vivo (22) and in vitro (30) suggest an increased exposure of strands C and D and their connecting loops. The hypothesis of a dislocated edge region is supported further by the successful design of two monoclonal antibodies specific for the amyloid state, which show a selective binding to the loop regions after strands C and D, respectively (31). The consensus view that the C and D strands become less structured and attain a more exposed position within the fibril is now confirmed by the H/D exchange data presented here.

A significant protection from solvent exchange can be observed for residues 25, 26, 28, 29, 30, 31, 32, 33, and 34, all part of strand B. Such a protection would be expected if the protein has a native β-sheet conformation, where strand B is packed antiparallel between strands C and E, forming stabilizing hydrogen bonds on both sides. However, as our experimental data show that strand C is highly unprotected, a novel hydrogen-bonding partner to strand B must be considered. Recently, TTR fibrils were investigated using electron paramagnetic resonance to measure distances between paramagnetic probes coupled to sulphydryl groups at various positions in the protein (19). The results of these authors support a model where two B strands align in an antiparallel manner centered on position 31 and form a novel dimer-dimer interface. This model is in good accordance with our data because the H/D solvent protection pattern for residues 28–34 has a maximum at the central residues.

In the native structure of TTR, strand E is located in a four-stranded β-sheet, positioned between strands B and F in an antiparallel manner. Several of the stabilizing hydrogen bonds within this sheet also appear to be preserved in the fibrillar state because a clear solvent protection is observed for residues 67, 72, and 74 in strand E as well as for residues 91, 93, 94, 96, and 97 in strand F. It is also likely that residues 67, 72, and 74 find their native hydrogen bonding partners, residues 97, 31, and 29, respectively, in the fibril. Taken together, the observed solvent protection supports a native or near native interaction among strands B, E, and F.

From the perspective of amyloid formation the native dimeric interface between strands F-F' and H-H' creates a symmetric subunit, and an attractive possibility is that a native-like interaction is also preserved within the fibrillar state. This hypothesis has been proposed by others previously, and from an electron paramagnetic resonance study on TTR fibrils a close proximity between two F strands was detected (18). In contrast to the relatively unprotected residues found in the dimer interface of the native structure (32), we observed a significant protection of residues 94 and 96 in the fibril state. Interestingly, enough residue 98 also shows a high degree of protection, contrary to its completely exposed position in the FG loop of the native structure (32). The adjacent residues, however, C-terminal of position 98 (positions 99, 100, and 101), exhibit 0% protection, suggesting that they still constitute part of the FG loop. This protection pattern indicates a strengthening of the subunit association, together with either a prolongation of the β-strand or a shift in register between the two F strands. The latter agrees particularly well with our data if the two strands are centered on position 93 or 94 instead of 92 as in the native structure.
Within the native structure an antiparallel arrangement of two H strands creates an interface between two monomers through intermolecular hydrogen bonding via residues 114, 116, 118, and 120. Our results also support the notion of a native-like interface between the two H strands within the fibril because a clear protection of residue 118 and 120 was observed. To our surprise residues 122 and 124 are protected in the fibril, but not so or only to a low extent in the native state, a fact that further strengthens the proposal of a shift in register between the \( \beta \)-strands in the interface. Centering the two H strands between position 118 and 119, instead of 117 as within the native structure, would best satisfy the protection pattern which was obtained.

The proposed shift in \( \beta \)-strand register between strand F-F' and H-H' in the dimer interface points to a dissociation of the dimer into monomers prior to the formation of the altered interface and would clarify the correlation between tetramer dissociation and fibril formation. A shift in register would further explain the finding presented by (33), where the native register was enforced by introducing disulfide bridges in either position 92–92' or 117–117' and which resulted in non-amyloidogenic proteins.

Further protection of the H strand is observed for residues 119, 121, and 123, which agrees well with the native structure where they are hydrogen-bonded to the adjacent strand G and residues 108, 106, and 104, respectively. For strand G, normally located between strands A and H, protection of residues 105, 109, and 110 is observed. This agrees with the situation in the native structure in which residues 105 and 109 form hydrogen bonds to residues 11 and 15 of strand A, respectively, whereas residue 110 is hydrogen-bonded to residue 117 of strand H. Residue 18 is protected in strand A and may be the result of a native-like interaction to residue 23.

Our results support a model of the fibril core, in which the native conformation of the six \( \beta \)-strands A, B, E, F, G, and H are essentially preserved and able to self-associte into a dimeric (monomer-monomer) native-like conformation. Our data further suggest an interface region formed by an antiparallel arrangement of strands F-F' and H-H' with a strengthened subunit assembly, and a likely shift in register by 2–3 residues. Furthermore, the former strands C and D are dislocated from their native positions and become structurally less defined, leaving strands A and B exposed. Solvent protection of this novel edge region, as well as formation of a continuous \( \beta \)-Sheet, is then achieved by an intermolecular association via antiparallel strands A-A' and B-B'. An illustration of the model is given in Fig. 6, where the protected residues are color-coded in

![Proposed model for a TTR fibril.](https://example.com/fig6.png)
blue and found within the core of the fibril, whereas the solvent-exposed residues are depicted in red and found on the fibrillar surface.

Several different models of the TTR amyloid architecture have been proposed previously. Fiber diffraction studies of ex vivo isolated TTR amyloid have shown that a repetitive unit of 115 Å exists within the fibril (34). The data support a model in which 24 β-strands constitute a helical turn of the fibril, where reorganized monomers or dimers make up the subunits. This accords with our model, Fig. 6, in which eight reorganized monomers (strands B-E-F-A-G-H) create a continuous β-sheet sandwich forming a helical turn, with a length along the direction of the long fibril axis corresponding to ~24 β-strands or 115 Å. Furthermore, the radial distance of the fibrillar core is in good agreement with experimentally derived data of a protofilament (35).

Two additional models of TTR amyloid architecture have been based on crystal structures from highly amyloidogenic TTR variants. The first model was derived from TTR/P55 representing a highly amyloidogenic variant showing an interesting packing within the crystal lattice, where specific monomers form a propagating chain involving associations within the loop regions (36). Through this arrangement a superhelix of selected monomers shows a fibril with the β-strands arranged parallel to the fibril axis. The native fold of each monomeric subunit is retained within this structure. This model, however, is not in accordance with our results because the solvent protection pattern as well as CD analysis only partly support a native fold. The second model is based on a study in which we presented a crystal structure of the highly amyloidogenic TTRG53S/E54D/L55S protein (30), where a slip of strand D was shown (37). This structural change forms a novel loop region between strands C and D creating an intermolecular contact with the helix on an adjacent molecule within the crystal. A propagating unit of tetramers can be seen within the crystal packing, having a repetitive twist of 115 Å. This structure is also not in accordance with the solvent protection pattern from the result presented within this work. However, considering the model presented in Fig. 6, the structural alterations in the edge of TTRG53S/E54D/L55S may reflect an initial event for exposure of strands A and B and a trigger to form fibrils.

To elucidate the structural mechanisms preceding amyloid formation, the dynamics of tetrameric TTR has been investigated both under native and amyloid-forming conditions using H/D exchange in combination with NMR (32, 38, 39). These studies provide important information about the stability of TTR and its tendency to form amyloid. The results show that at pH 7 TTR exhibits a core of slowly exchanging residues located in strands A, B, E, G, and also within the loop connecting strands A and B (38). The H/D solvent protection pattern has also been investigated during amyloid forming conditions represented by brief incubations at low pH, an established method of inducing fibrils from TTR (39). The data show that the amide protons within the outer sheet, comprising strands C, B, E, and F, become more labile, although they are still protected. In addition, an increased solvent exposure was seen within the dimer-dimer interface, supporting the suggestion that a dissociation of the tetramer is important for the process of amyloid formation. The protection pattern described, and the destabilization for tetrameric TTR is in line with our proposed model and its structural reorganization.

The model suggests that strands C and D prevent exposure of strands A and B, thus furthering assembly via end-to-end associations. This implies a dimeric species, constituting a building block in the course of amyloid formation. However, at low pH a monomeric species is predominantly populated (26). Whether this is an intermediate in the path of fibril formation or represents an off-pathway under conditions where a dimeric species is rapidly converted into fibrils remains to be elucidated. A previous study does however support the notion of a dimeric intermediate, where the isolation of dimeric species has been facilitated using highly amyloidogenic TTR mutants having a low tetrameric stability under conditions (neutral pH and temperature) and where amyloid formation is favored (23). This dimeric species can further be aggregated into fibrillar structures, suggesting that it is in the pathway of amyloid formation. Future work will reveal whether the dimeric interface of the isolated intermediates involves the F and H strands or is constituted by novel contacts within strands A and B.

In conclusion, we have successfully probed and identified the fibrillar core structure of a TTR amyloid at a residue specific level and provided a structural model for a fibril as well as a possible mechanism for fibril propagation. The results provide novel structural information, which from a therapeutic point of view is particularly useful as it pinpoints target areas for the rational design of inhibitors against TTR amyloid formation.

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