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Synthesis and Structural Analysis of the N-Terminal Domain of the Thyroid Hormone-Binding Protein Transthyretin

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Transthyretin (TTR) is a 55 kDa protein responsible for the transport of thyroid hormones and retinol in human serum. Misfolded forms of the protein are implicated in the amyloid diseases familial amyloidotic polyneuropathy and senile systemic amyloidosis. Its folding properties and stabilization by ligands are of current interest due to their importance in understanding and combating these diseases. To assist in such studies we developed a method for the solid phase synthesis of the monomeric unit of a TTR analogue and its folding to form a functional 55 kDa tetramer. The monomeric unit of the protein was chemically synthesized in three parts, comprising amino acid residues 1–51, 54–99 and 102–127, and ligated using chemoselective thioether ligation chemistry. The synthetic protein was folded and assembled to a tetrameric structure in the presence of the TTR's native ligand, thyroxine, as shown by gel filtration chromatography, native gel electrophoresis, TTR antibody recognition and thyroid hormone binding. In the current study the solution structure of the first of these fragment peptides, TTR(1–51) is examined to determine its intrinsic propensity to form β-sheet structure, potentially involved in amyloid fibril formation by TTR. Despite the presence of extensive β-structure in the native form of the protein, the N-terminal fragment adopts an essentially random coil conformation in solution. Clin Chem Lab Med 2002; 40(12):1221–1228

Key words: Peptide synthesis; Protein fragments; Transthyretin; Amyloid.

Abbreviations: DIEA, diisopropylethylamine; DMF, N,N-dimethyl formamide; ESMS, electrospray mass spectrometry; FAP, familial amyloidotic polyneuropathy; HBTU, O-benzotriazole-N,N,N′, N′-tetramethyluronium hexafluorophosphate; HF, hydrogen fluoride; NMR, nuclear magnetic resonance; RP, reverse phase; sTTR, synthetic transthyretin; T4, L-thyroxine; TFA, trifluoroacetic acid, TTR, transthyretin.

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Introduction

Transthyretin (TTR) is responsible for the transport of thyroid hormones such as 3,5,3′-triiodo-L-thyronine (T3) and L-thyroxine (T4) in both the bloodstream and the central nervous system (CNS). It is also involved in the transport of retinol-binding protein (1, 2). TTR is a 55 kDa tetrameric protein and its structure and ligand binding capabilities have been thoroughly characterized by X-ray crystallography (3–6). Each subunit contains extensive β-sheet structure and is arranged within a dimer of dimers to form a compact molecule with two funnel-shaped hormone ligand binding sites, each defined by a dimer-dimer interface. The thyroid hormones bind deeply within the hydrophobic binding channel, their iodine atoms residing in hydrophobic pockets at two different binding sites within the channel (7). Upon binding of one T4 ligand, the binding affinity for the second ligand is reduced substantially. Retinol-binding protein, by contrast, binds on the outer surface of TTR.

Figure 1 shows the structure of the TTR tetramer, highlighting the central hormone-binding channel that is formed at the dimer-dimer interface. One of the monomers is highlighted separately to illustrate the relative arrangement of monomers within the tetramer. Residues 1–10 are disordered and not defined in the crystal structure but it is clear that the structure is essentially all β-sheet apart from a small helical region near residues 74–82.

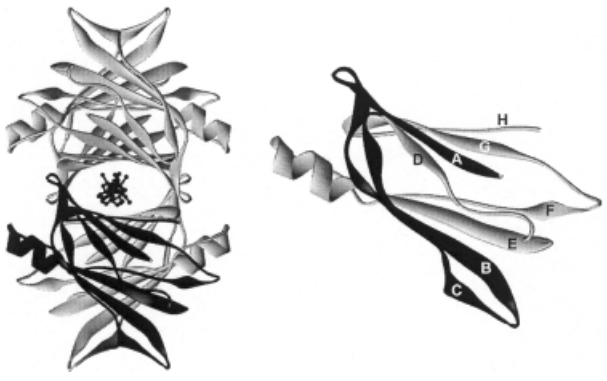

Figure 1 Three-dimensional structure of transthyretin highlighting the secondary structure elements (PDB accession number 1BM7). On the left is the structure of the tetramer, highlighting the binding channel, and showing bound thyroid hormone. On the right is a monomer unit, excluding the first 10 residues, which are disordered in the crystal structure. TTR(1–51) contains three strands of β-sheet, which are shown in a darker shade to the other strands in the monomer structure.

The TTR tetramer is inherently very stable (1, 8), but in some circumstances the protein undergoes a rearrangement to form amyloid fibrils. In diseases such as senile systemic amyloidosis (SSA) and familial amyloidotic polyneuropathies (FAP), native and mutant TTR, respectively, have been found to form long β-sheet-based fibrillar structures (9–11). Amyloid has been analyzed by X-ray diffraction and electron microscopy to reveal patterns indicative of stacked β-sheet structure with strands perpendicular to the fibril axis (11). These amyloid lesions accumulate in specific organs and are implicated in their dysfunction and ultimately the death of the patients. There are now approximately 80 separate mutations that appear to be associated with inherited amyloidosis (12–18). It is thought that the underlying mechanism for TTR fibril formation involves tetramer dissociation to a monomeric conformational intermediate, which self-assembles to form amyloid fibrils. Any mutation or cellular condition (such as low pH) that tends to destabilize the tetramer can result in an increased propensity of the protein to form amyloid fibrils (10, 13, 15).

Much recent interest has focused on the ligand binding properties of TTR because of the discovery that certain thyroid hormone competitors (*e.g.* 2,4,6-triiodophenol) are able to decrease the tendency of TTR to form amyloid fibrils (19, 20). Such ligands are reported to act by binding deeply within the TTR-binding channel at both ligand-binding sites and inhibiting the formation of amyloid by stabilizing the normal fold against the pathogenic conformational change. A range of non-steroidal anti-inflammatory drugs is currently being investigated for their ability to inhibit and reverse amyloid formation (21).

Solid phase peptide synthesis provides a methodology for studying the amyloidogenic propensity of different regions of TTR and for the analysis of ligand-binding to synthetic mutants. In a recent report (22) we demonstrated the total chemical synthesis of an analogue of human TTR using a thioether-based strategy for the sequential ligation of three peptide fragments of TTR. We showed that this synthetic TTR (henceforth referred to as sTTR) could be successfully refolded and reconstituted to form a 55 kDa tetrameric structure able to bind the thyroid hormone T4. This represents one of the largest active proteins made synthetically, and provides a methodology for future studies of TTR-ligand interactions using nuclear magnetic resonance (NMR) spectroscopic techniques. The approach also provides fragments of TTR that can be used for a variety of studies, including an evaluation of the intrinsic propensity of individual fragments of the protein to form a native secondary structure. Such studies have the potential to assist in understanding the folding properties of the protein and provide insight into possible pathways leading to amyloid deposition.

In the current study we focus on a large fragment corresponding to the N-terminal domain of TTR, referred to as TTR(1–51). NMR studies were used to examine the solution conformation of this fragment. The location of this fragment in the structure of TTR is highlighted in bold in Figure 1. The region corresponding to this peptide adopts exclusively a β-sheet structure in the native protein, with the C-terminus of the fragment being designed to occur at a natural turn in the structure. Since β-sheet is thought to be the major structural element in amyloid fibrils, and since the termini of proteins are the most likely to be first liberated during unfolding events, we hypothesized that an N-terminal region with a high intrinsic propensity for the formation of β-sheet structure would be interesting to study. Furthermore, this peptide encompasses a number of residues for which point mutations alter the amyloidogenic potential of TTR. In particular, the mutation of Val30 to Met is the most prevalent mutation associated with FAP (23).

A further motivation for the study of this peptide was based on the finding that a range of small synthetic peptides and proteolytically cleaved fragments of TTR have been identified as components of amyloid fibrils. For example, as noted by Hornberg *et al.* (6), the Lys-48-Thr49 peptide bond of TTR is a potential serine protease cleavage site, and both the N-terminal (Gly1-Lys48) and C-terminal (Thr49-Glu 127) proteolytic fragments of TTR have been found to be incorporated together with wild-type TTR in fibrils. TTR(1–51) is a slightly extended version of the former, raising the interest in examining its conformational properties.

This article is a contribution based in part on a presentation made at the 1st International Congress on Transthyretin in Health and Disease, in Strasbourg, April 2002. The focus of that presentation was on the chemical ligation strategy used to produce synthetic TTR. Accordingly, the current article provides a brief overview of that methodology, which has been described in full elsewhere (22), but focuses mainly on new experimental data on the solution properties of the N-terminal fragment peptide, TTR(1–51). It was of interest to determine whether this peptide would adopt a stable monomeric β-sheet structure or indeed any stable secondary structure in the absence of the remainder of the protein, and whether it may form fibrils by itself.

Materials and Methods

Chemicals and reagents

Trifluoroacetic acid (TFA), dichloromethane (DCM), N,N-dimethyl formamide (DMF), and diisopropylethylamine (DIEA) were from Auspep (Melbourne, Australia). O-benzotriazole-N,N,N′, N′-tetramethyl-uronium hexafluorophosphate (HBTU) was from Richelieu Biotechnologies (St. Hyacinth, Quebec, Canada). Acetonitrile was from BDH Laboratory Supplies (Poole, UK). Acetic acid and chloroacetic acid were from Ajax chemicals (Auburn, Australia), diethyl ether from Fluka Biochemicals (Melbourne, Australia) and mercaptoethanol from Sigma (St. Louis, MO, USA). Ethanolamine, N,N-diisopropylcarbodiimide (DIC) and bromoacetic acid were from Aldrich (Milwaukee, WI, USA). Hydrogen fluoride (HF) was purchased from Boc Gases (Brisbane, Australia). The following N-Boc protected L-amino acids Ala, Gly, Ile, Leu, Phe, Pro, Val, Arg (p-toluenesulphonyl), Asp (O-cyclohexyl; OChx), Asn (xanthyl;

Xanth), Glu (O-cyclohexyl; OChx), His (dinitrophenyl; DNP), Lys (2-chlorobenzyloxycarbonyl; ClZ), Ser (benzyl; Bzl), Thr (benzyl; Bzl), Trp (formyl; CHO), Tyr (2-bromobenzyloxycarbonyl; 2BrZ) were purchased either from NovaBiochem (La Jolla, CA, USA) or Bachem (Bubendorf, Switzerland). Human serum was supplied by the Red Cross Blood Bank, Melbourne, Australia.

Equipment

Analytical and preparative HPLC was carried out using a Waters HPLC system comprised of model 600 solvent delivery system, 600E controller and model 484 detector. Vydac C18 analytical columns (4.6 × 250 mm) at a flow rate of 1 ml/min and semi-preparative (10 × 250 mm) at a flow rate of 3 ml/min and preparative (22 × 250 mm) at a flow rate of 8 ml/min were used. All peptides were purified using linear gradients of 0.1% aqueous TFA (solvent A), 90% aqueous acetonitrile 0.09% TFA (solvent B). Mass spectral data were collected using a Perkin Elmer Sciex (Toronto, Canada) API III Biomolecular Mass Analyzer ion-spray mass spectrometer equipped with an ABI 140B solvent delivery system.

Peptide synthesis

The TTR fragment was synthesized as described previously (22). It comprises the N-terminal 51 amino acids of TTR linked to an alkyl thiol, with Cys10 replaced by Ala. The peptide was synthesized using machine-assisted solid phase synthesis, with the thiol linker attached to Boc-Gly-Pam resin. The thioether resin was prepared according to the method of Englebretsen *et al.* (24) and the synthesis achieved using a rapid HBTU *in situ* neutralization protocol (25). The average amino acid coupling was 99.6% (1st coupling) for the synthesis, which was routinely double coupled.

Prior to HF cleavage, the DNP group of the histidine was removed using 20% mercaptoethanol in 10% DIEA/DMF solution for 2–3 × 30 min treatments and the Trp formyl group was removed using ethanolamine. The peptide resin was cleaved using HF with p-cresol and p-thiocresol as scavengers at −5 to −0 °C for 1–2 h. HF was removed in vacuo, the peptide triturated with cold diethyl ether (3 × 50 ml), and the precipitated peptide was collected and dissolved in 50% acetonitrile with 0.1% TFA. The crude peptide was purified by reverse phase (RP)-HPLC and fractions collected and analyzed by analytical RP-HPLC and electrospray mass spectrometry (ESMS). The purified peptide was characterized as the desired product by ESMS (observed mass = 5355 ± 1.0 calculated for $C_{236}H_{379}N_{66}O_{72}S_2$ = 5357.14 (average isotope composition)).

NMR spectroscopy

Samples for ^{1}H NMR measurements contained ~1 mM TTR(1–51) in 90% H_2O/10% ^2H_2O (v/v) at pH 4. ^2H_2O was obtained from Cambridge Isotope Laboratories (Woburn, MA, USA). Spectra were recorded at 25 °C and 35 °C on a Bruker ARX-500 spectrometer (Karlsruhe, Germany) equipped with a shielded gradient unit. 2D NMR spectra were recorded in phase-sensitive mode using time-proportional phase incrementation for quadrature detection in the t_1 dimension. The 2D experiments included TOCSY using a MLEV-17 spin lock sequence with a mixing time of 80 ms, and NOESY with mixing times of 200 ms and 250 ms. Solvent suppression for NOESY and TOCSY experiments was achieved using a modified WATERGATE sequence. Spectra were routinely acquired over 6024 Hz with 4096 complex data points in F_2 and 512 increments in the F_1 dimension.

Spectra were processed on a Silicon Graphics Indigo workstation using XWINNMR (Bruker) software. The t_1 dimension was zero-filled to 2048 real data points, and 90 ° phase-shifted sine bell window functions were applied prior to Fourier transformation. Chemical shifts were referenced to external DSS at 0.00 ppm.

Results

The peptide chosen for study is a thiolated derivative of the N-terminal domain of TTR that we synthesized originally as part of a strategy to make a synthetic variant of TTR. Figure 2 shows the general strategy used to synthesize sTTR based on the ligation of three fragment peptides using thioether linkages. The choice of ligation sites for the preparation of sTTR was based on both the amino acid sequence and the known tertiary structure of native TTR. The thioether linker spans a distance equivalent to two amino acids, is highly flexible and non-functionalized. Gly-Gly sequences are thus ideally suited as ligation points, but the TTR sequence contains no Gly-Gly. Instead, Ser-Gly sites, which are present at convenient positions, including two in loop positions within the TTR structure (Figure 1) were chosen as ligation points. Ser^{100}-Gly^{101} occurs in the loop between β-strands F and G, and Ser^{52}-Gly^{53} in the loop between β-strands C and D. Neither is close to the hormone-binding site or at points of inter-subunit contact. A third Ser-Gly site (Ser^{46}-Gly^{47}) occurs in the center of β-strand C and was ruled out as a potential ligation point due to the likely disruption of the β-sheet by a surrogate amide.

The selection of Ser^{52}-Gly^{53} and Ser^{100}-Gly^{101} ligation sites required the synthesis of three peptides of 51, 46 and 26 residues, as indicated in Figure 2. These were then chemically joined using thioether linkers. The N-

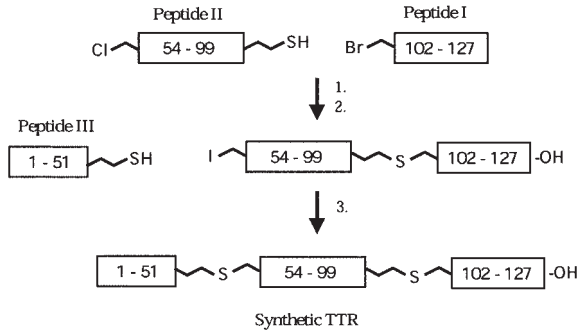

Figure 2 Ligation scheme for the synthesis of transthyretin showing the three-step process to give synthetic transthyretin. Peptide I, representing TTR residues 102–127 was prepared with an N-terminal bromoacetyl group and was used to form a thioester bond with the free thiol of Peptide II (Step1). Peptide II, representing TTR residues 54–99, was prepared with both a C-terminal free thiol, and N-terminal chloroacetyl group. Whilst the chloroacetyl group does not react with free thiols, it is readily exchanged to an iodoacetyl group (Step 2), which does. Thus, the first conjugated peptide could readily be ligated to Peptide III, TTR residues 1–51 synthesized with a C-terminal thiol, to create the complete sTTR (Step 3).

bromoacetylated 102–127 (peptide I) was first ligated to the N-chloroacetylated, C-thiolated 54–99 (peptide II). The purified chloroacetylated 54–127 peptide was then exchanged to the iodoacetylated form and ligated with C-thiolated 1–51 (peptide III) to produce fully ligated 1–127 synthetic TTR. The ligated 1–127 peptide was purified by RP-HPLC in excellent yield, and its identity confirmed by ESMS (22).

The synthetic TTR spontaneously folded to its tetramer complex in the presence of the ligand T4, in 0.075 M NH_4HCO_3 (22). Western analysis was employed to determine the subunit molecular mass and confirm the recognition of the sTTR by anti-TTR antiserum. Synthetic TTR gave rise to bands corresponding to the molecular masses of the sTTR monomer and dimer. The correct folding and formation of the tetramer was assessed by analyzing non-denaturing polyacrylamide gel migration combined with a ^{125}I-thyroxine-binding assay. The analysis of ^{125}I-thyroxine binding to proteins in human serum was used as the reference and revealed the presence of thyroxine-binding globulin, albumin and TTR. The position of migration was almost identical to that in serum, indicating that the tetrameric size, shape and charge distribution of sTTR was almost identical to native TTR.

These results showed that, when ligated, the synthetic peptide fragments could adopt a structure similar to the native form and exhibit functional thyroid hormone binding. The question we next wanted to address was whether the entire TTR sequence was required for native secondary structure, or whether the N-terminal domain alone would fold into native-like structure. Peptide III (now referred to as TTR(1–51)) was therefore resynthesized as described in the Materials and Methods. As was the case in the original studies, the only modification to the native TTR sequence was the replacement of Cys10 with Ala to remove the possibility of intermolecular disulfide bond formation. Cys10 is the only Cys residue in native TTR. The required peptide was produced in good yield and its identity was confirmed by mass spectrometry. The sequence is GPTGTGESKAPLMVKVLDAVRGSPAINVAV-HVFRKAADDTWEPFASGKTSE-(NH-CH_2-CH_2-SH), with the section in brackets corresponding to the thioether linker.

NMR studies on TTR(1–51)

TTR(1–51) was readily soluble in water at millimolar concentration and did not aggregate or precipitate over a period of days used to record the NMR spectra. The sample was prepared under a nitrogen atmosphere to avoid potential oxidation reactions associated with the terminal thiol moiety. A series of 1D, TOCSY and NOESY spectra of TTR(1–51) were recorded and were consistent with the presence of a single monomeric species in solution. The NMR peaks were assigned using the standard sequential assignment method in which amino acids were first assigned to residue type, mainly using the TOCSY spectrum, and then these were assigned to specific amino acids in the sequence by reference to the NOESY spectrum. The assigned chemical shifts are summarized in Table 1.

Figure 3 shows the fingerprint region of the TOCSY spectrum to illustrate the chemical shift dispersion of the amide and αH protons and to summarize the assignments. The narrow range of NH chemical shifts and the lack of substantially downfield or upfield shifted αH chemical shifts provided the first indication that the peptide was not highly structured. This was confirmed by calculating the secondary αH shifts by subtraction of random coil αH chemical shifts from the observed shift for each residue. Helical regions of peptides and proteins are characterized by a contiguous series of negative secondary shifts, while β-sheet structure is typified by a series of positive secondary shifts (26). Figure 4 shows that the measured secondary αH shifts for TTR(1–51) are smaller than 0.1 ppm, apart from a few residues.

Given this small range of αH secondary shifts it was clear that TTR(1–51) adopts a random coil conformation and that it was not justified to attempt a full three-dimensional structure calculation based on NOE data.

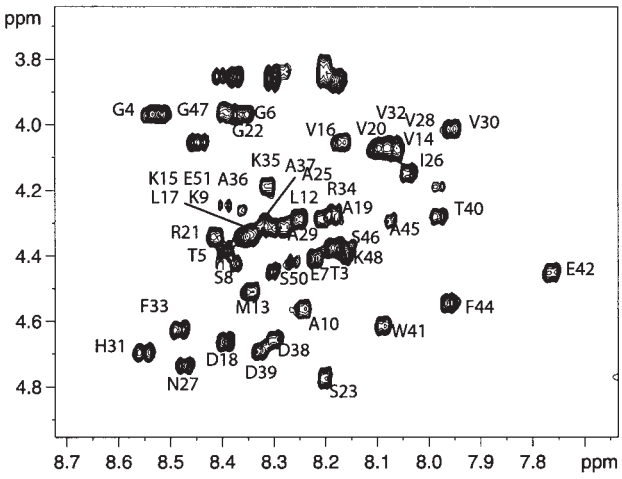

Figure 3 Fingerprint region of the TOCSY spectrum of TTR(1–51) recorded on a Bruker ARX 500 spectrometer 298 K. The $d_{\alpha N}$ peaks are labeled with the single letter amino acid code and residue number.

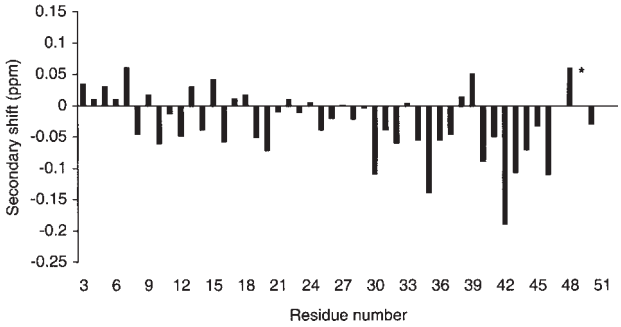

Figure 4 Secondary shift values for TTR(1–51) αH protons. Deviations were calculated by subtracting random coil values from the observed chemical shifts. Corrections for residues preceding Pro were applied. Only five residues have chemical shift deviations greater than 0.1 ppm, indicating a lack of ordered secondary structure. The asterisk indicates that signals for Thr48 were not assigned.

Table 1 Chemical shifts of TTR(1–51) at 298 K.

Residue	NH	αH	βH	Other
1 Pro	n/a			
2 Thr	8.19	4.39	4.27	γCH_3 1.20
3 Gly	8.53	3.97		
4 Thr	8.40	4.38	4.25	γCH_3 1.24
5 Gly	8.36	3.97		
6 Glu	8.22	4.41	2.12, 1.96	γCH_2 2.44
7 Ser	8.38	4.43	3.86	
8 Lys	8.35	4.34	1.42	γCH_2, δCH_2 1.68, εCH_2 2.97
9 Ala	8.24	4.56	1.35	
10 Pro		4.41	2.28, 1.89	γCH_2 2.01, δCH_2 3.76, 3.64
11 Leu	8.25	4.30	1.61	δCH_3 0.9
12 Met	8.34	4.51	2.02	2.57, 2.50
13 Val	8.09	4.08	1.94	γCH_3 0.84
14 Lys	8.35	4.36	1.45	γCH_2, δCH_2 1.68, εCH_2 2.97
15 Val	8.17	4.06	2.04	γCH_3 0.91
16 Leu	8.36	4.35	1.59	δCH_3 0.91, 0.86
17 Asp	8.40	4.66	2.86, 2.78	
18 Ala	8.17	4.27	1.36	
19 Val	8.07	4.05	2.07	γCH_3 0.93
20 Arg	8.41	4.33	1.85, 1.77	1.64, 3.19
21 Gly	8.37	3.97		
22 Ser	8.20	4.77	3.82	
23 Pro		4.43	2.29, 1.91	γCH_2 2.01, δCH_2 3.80, 3.70
24 Ala	8.31	4.28	1.22	
25 Ile	8.04	4.15	1.84	γCH_2 1.44, 1.16, γCH_3 0.88
26 Asn	8.47	4.74	2.80, 2.72	
27 Val	8.07	4.10	2.05	γCH_3 0.92
28 Ala	8.28	4.32	1.33	
29 Val	7.96	4.01	1.98	0.88
30 His	8.55	4.70	3.08	
31 Val	8.09	4.06	2.02	γCH_3 0.91
32 Phe	8.48	4.62	3.01	δH 7.23
33 Arg	8.21	4.29	1.74, 1.65	γCH_2 1.51, δCH_2 3.12
34 Lys	8.31	4.18	1.78,	1.37, 1.68, εCH_2 2.97
35 Ala	8.36	4.27	1.38	
36 Ala	8.31	4.28	1.36	
37 Asp	8.30	4.65	2.86	
38 Asp	8.32	4.69	2.83	
39 Thr	7.98	4.26	4.19	1.14
40 Trp	8.09	4.61	3.22	NH 10.08, HD1 7.22, HE3 7.51, HZ2 7.45, HZ3 7.09, HH2 7.19
41 Glu	7.76	4.45	1.83, 1.62	2.23
42 Pro		4.31	2.13, 1.69	γCH_2 1.84, δCH_2 3.48, 3.37
43 Phe	7.96	4.55	3.10, 3.03	δH 7.22
44 Ala	8.07	4.29	1.32	
45 Ser	8.18	4.36	3.87	
46 Gly	8.40	3.96		
47 Lys	8.16	4.38	1.85, 1.75	1.43 1.68, εCH_2 2.97
48 Thr	n/a			
49 Ser	8.30	4.44	3.85	
50 Glu	8.35	4.35	1.85, 1.72	2.00
51 Ser	n/a			

This was confirmed by the observation from the NOESY spectrum that few medium or long range NOES were present. The spectrum was dominated by only intraresidue NOEs and NOEs between sequentially adjacent residues.

The conclusion to emerge from these studies is that the peptide adopts an essentially random coil structure in solution and shows no signs of adopting the highly β-sheet structure seen in the corresponding region of the native protein. The various spectra were recorded over the course of 5–6 days and there was no obvious evidence of aggregation or fibril formation during this time. This suggests that the peptide does not have a strong intrinsic tendency to aggregate.

Discussion

In the current study we synthesized a fragment corresponding to the N-terminal domain region of TTR and examined its solution properties. TTR(1–51) is soluble at millimolar concentrations and forms an essentially random coil conformation in solution showing that the fragment itself does not have high intrinsic propensity to form β-sheet structure. It thus appears that the presence of an extensive β-sheet structure in the intact protein relies on cooperative interactions with other parts of the protein. The results also suggest that this fragment of the protein is unlikely to be a nucleating region for amyloid type structure as the fragment appeared to be relatively stable in solution and did not aggregate over the course of several days.

In previous studies the proteolytically derived N-terminal (Gly1-Lys48) fragment of TTR and other peptide fragments have been found to be incorporated together with wild-type TTR in fibrils (27, 28). The observation in the current study that the slightly longer TTR(1–51) fragment does not appear to strongly aggregate or form β-sheet structure suggests that the proteolytic fragment is not likely to be a "seeding" intermediate for fibril formation. This is in agreement with the suggestion by Hornberg *et al.* (6) that there are currently no structural data that support such a proposition.

Several shorter peptide fragments of TTR have been shown to assemble into amyloid fibrils *in vitro* (27). One of these, TTR(10–20), corresponds to just one of the β-strands of TTR(1–51). In early structural studies of this and other short peptides that line the pore of the thyroxine-binding channel Jarvis *et al.* found that the peptides adopt random coil conformations in 30% acetonitrile (29) but spontaneously form β-sheet fibrils in water (30). The dissociation of similar fibrils has more recently been examined using TTR(10–19) in the presence of denaturants and co-solvents (31). The presence of denaturants partially disrupted the fibrils to form protofilaments. High concentrations of TFE also dissociate TTR(10–19) fibrils to protofilaments but they also slowly dissociate to monomeric soluble peptides with extensive α-helical structure. The results suggest that predominantly hydrophobic interactions are involved in the assembly of TTR(10–19) into fibrils from protofilaments, whereas assembly of peptides into protofilaments involves both electrostatic and hydrophobic forces (31).

In another study of TTR fragments we examined the peptide spanning the only helical region in TTR, corresponding to residues 71–93. In the native structure of TTR this sequence contains a helical region (residues 74–82) flanked by two extended regions that form an antiparallel β-strand region within a β-sheet. While there was no propensity for β-strand formation in the peptide TTR(71–93), even when the ends were tethered with a non-native disulfide bond (32), there was a strong tendency for the helical region to form in aqueous solution (33). This suggests that while the helical region of the protein has an intrinsic tendency to form its native secondary structure, β-strand formation relies much more heavily on its position in the context of the whole protein.

These findings are consistent with comparable studies on other proteins. For example, extensive use of the fragment dissection approach has been made to determine nucleation sites for the folding of T4 lysozyme (34, 35). In that case it was found that peptide fragments corresponding to helical regions in the intact protein generally had an intrinsic tendency to be helical in the isolated fragments, but this was not the case for β-sheet regions. Different regions had differing intrinsic helical tendencies, allowing likely protein folding initiation sites to be proposed (34). Conclusions on folding initiation sites are much more difficult to draw for highly β-sheet proteins such as TTR.

As noted earlier, mutations in TTR exist that cause or suppress disease. The V30M mutation is the most prevalent cause of FAP in heterozygotes, whereas a T119M mutation on the second *TTR* allele protects V30M carriers from disease (23). Incorporation of one or more T119M TTR subunits into a predominantly V30M tetramer strongly stabilized the mixed tetramer against dissociation (23). The structural basis for the effects of the mutations is not yet understood. The tertiary structure of native sTTR and many of its disease-causing mutants has been determined, and structural differences seen between TTR variants in early studies (36–38) have been proposed to be significant for amyloid formation. However, a more recent study that derived a 1.5 Å resolution structure of wild-type TTR and compared it to a range of mutant structures proposed that the structural differences are not significant (6).

This recent finding and the current study are consistent with the hypothesis of Kelly (15) that amyloid formation by TTR may not be due to a major disruption of secondary structure but rather to subtle perturbations of structure which shift the subunit association equilibrium towards the monomer. The small ratio of monomeric TTR becomes involved in amyloid formation as a complete unit. Fragments of TTR do not necessarily have any intrinsic ability to form β-strands or partake in amyloid formation.

This also explains the basis for the design of drugs to decrease TTR amyloidosis. More than 50 small molecules have been found that bind to and stabilize tetrameric TTR, inhibiting amyloid fibril formation *in vitro* (39). The molecules with the greatest therapeutic potential are the non-steroidal anti-inflammatory drugs, currently under investigation for their ability to inhibit human TTR amyloid disease (21). These molecules, which include flufenamic acid, diclofenac and flurbiprofen, bind in the T4-binding site of TTR (40), forming similar polar and non-polar contacts, as do the natural ligands. These interactions are thought to stabilize the native quaternary structure of TTR against pH-mediated dissociation and conformational changes associated with amyloid formation (20). Analogues of diclofenac have recently been evaluated as TTR amyloid formation inhibitors with promising results (41). However, further study is required to establish the ef-

fectiveness of these analogues as therapeutics. Structure-based design has also been applied to the synthesis of N-phenyl phenoxazine derivatives. One derivative was found by analytical ultracentrifugation to block tetramer dissociation to the alternatively folded amyloidogenic monomer (42).

In a seemingly analogous manner the native TTR ligand, T4, was required in our study for the correct folding of sTTR from its monomeric unfolded state to produce the 55 kDa tetrameric structure. T4 has previously been reported to stabilize native TTR against acid denaturation leading to the formation of amyloid fibrils (19). The integrity of the final product was confirmed by native TTR antibody recognition and ligand-binding studies. Since the binding site for thyroid hormone ligands is formed only upon the formation of the tetrameric species, ligand binding also demonstrated tetramer formation. It is not known, however, whether the sTTR tetramer forms and then is stabilized by T4 or the tetramer spontaneously forms about the T4.

In conclusion, the chemical synthesis of a TTR analogue has provided insight into the folding propensity of the monomeric chain as well as an N-terminal fragment of the molecule. It appears that the spontaneous formation of the correct secondary and tertiary structure of sTTR is possible, even in the presence of the flexible regions necessarily incurred at the ligation sites. Correct folding occurs despite the fact that a major region of the molecule (*i.e.* residues 1–51) shows no intrinsic secondary structure formation. It was necessary, however, for a strong TTR-binding ligand to be present for the formation of the folded sTTR tetramer – reflecting the inherent instability of the TTR monomer.

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References

1. Nilsson SF, Peterson PA. Studies on thyroid hormone-binding proteins. I. The subunit structure of human thyroxine-binding globulin and its interaction with ligands. J Biol Chem 1975; 250:8543–53.
2. Monaco HL, Rizzi M, Coda A. Structure of a complex of two plasma proteins: transthyretin and retinol-binding protein. Science 1995; 268:1039–41.
3. Blake CC, Geisow MJ, Oatley SJ, Rérat B, Rérat C. Structure of prealbumin: secondary, tertiary and quaternary interactions determined by Fourier refinement at 1.8 Å. J Mol Biol 1978; 121:339–56.
4. Ciszak E, Cody V, Luft JR. Crystal structure determination at 2.3-A resolution of human transthyretin-3′,5′-dibromo-2′,4,4′,6-tetrahydroxyaurone complex. Proc Natl Acad Sci USA 1992; 89:6644–8.
5. Wojtczak A, Luft J, Cody V. Mechanism of molecular recognition. Structural aspects of 3,3′-diiodo-L- thyronine binding to human serum transthyretin. J Biol Chem 1992; 267: 353–7.
6. Hornberg A, Eneqvist T, Olofsson A, Lundgren E, Sauer-Eriksson AE. A comparative analysis of 23 structures of the amyloidogenic protein transthyretin. J Mol Biol 2000; 302:649–69.
7. Wojtczak A, Cody V, Luft J, Pangborn W. Structures of human transthyretin complexed with thyroxine at 2.0 Å resolution and 3′,5′-dinitro-N-acetyl-L-thyronine at 2.2 Å resolution. Acta Crystallogr D Biol Crystallogr 1996; 52:758–65.
8. Branch WT, Robbins J, Edelhoch H. Thyroxine-binding prealbumin. Conformation in urea and guanidine. Arch Biochem Biophys 1972; 152:144–51.
9. Saraiva MJ. Molecular genetics of familial amyloidotic polyneuropathy. J Peripher Nerv Syst 1996; 1:179–88.
10. Lai Z, Colon W, Kelly JW. The acid-mediated denaturation pathway of transthyretin yields a conformational intermediate that can self-assemble into amyloid. Biochemistry 1996; 35:6470–82.
11. Sunde M, Serpell LC, Bartlam M, Fraser PE, Pepys MB, Blake CC. Common core structure of amyloid fibrils by synchrotron X-ray diffraction. J Mol Biol 1997; 273:729–39.
12. Jacobson DR, Buxbaum JN. Genetic aspects of amyloidosis. Adv Hum Genet 1991; 20:69–123.
13. McCutchen SL, Lai Z, Miroy GJ, Kelly JW, Colon W. Comparison of lethal and nonlethal transthyretin variants and their relationship to amyloid disease. Biochemistry 1995; 34:13527–36.
14. Benson MD, Uemichi T. Transthyretin amyloidosis. Amyloid 1996; 3:44–56.
15. Kelly JW. The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways. Curr Opin Struct Biol 1998; 8:101–6.
16. Damas AM, Saraiva MJ. Review: TTR amyloidosis-structural features leading to protein aggregation and their implications on therapeutic strategies. J Struct Biol 2000; 130:290–9.
17. Hamilton JA, Benson MD. Transthyretin: a review from a structural perspective. Cell Mol Life Sci 2001; 58:1491–521.
18. Saraiva MJ. Transthyretin amyloidosis: a tale of weak interactions. FEBS Lett 2001; 498:201–3.
19. Miroy GJ, Lai Z, Lashuel HA, Peterson SA, Strang C, Kelly JW. Inhibiting transthyretin amyloid fibril formation via protein stabilization. Proc Natl Acad Sci USA 1996; 93: 15051–6.
20. Peterson SA, Klabunde T, Lashuel HA, Purkey H, Sacchettini JC, Kelly JW. Inhibiting transthyretin conformational changes that lead to amyloid fibril formation. Proc Natl Acad Sci USA 1998; 95:12956–60.
21. Klabunde T, Petrassi HM, Oza VB, Raman P, Kelly JW, Sacchettini JC. Rational design of potent human transthyretin amyloid disease inhibitors. Nat Struct Biol 2000; 7:312–21.
22. Wilce JA, Love SG, Richardson SJ, Alewood PF, Craik DJ. Synthesis of an analog of the thyroid hormone-binding protein transthyretin via regioselective chemical ligation. J Biol Chem 2001; 276:25997–6003.
23. Hammarstrom P, Schneider F, Kelly JW. Trans-suppression of misfolding in an amyloid disease. Science 2001; 293: 2459–62.
24. Englebretsen DR, Garnham BG, Bergman DA, Alewood PF. A novel thioether linker: chemical synthesis of a HIV-1 protease analogue by thioether ligation. Tetrahedron Lett 1995; 36:8871–4.
25. Schnolzer M, Alewood P, Jones A, Alewood D, Kent SB. In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. Int J Pept Protein Res 1992; 40:180–93.
26. Wishart DS, Sykes BD, Richards FM. Relationship between

nuclear magnetic resonance chemical shift and protein secondary structure. J Mol Biol 1991; 222:311–33.
27. Gustavsson A, Engstrom U, Westermark P. Normal transthyretin and synthetic transthyretin fragments form amyloid-like fibrils in vitro. Biochem Biophys Res Commun 1991; 175:1159–64.
28. Thylen C, Wahlqvist J, Haettner E, Sandgren O, Holmgren G, Lundgren E. Modifications of transthyretin in amyloid fibrils: analysis of amyloid from homozygous and heterozygous individuals with the Met30 mutation. EMBO J 1993; 12:743–8.
29. Jarvis JA, Kirkpatrick A, Craik DJ. 1H NMR analysis of fibril-forming peptide fragments of transthyretin. Int J Pept Protein Res 1994; 44:388–98.
30. Jarvis JA, Craik DJ, Wilce MC. X-ray diffraction studies of fibrils formed from peptide fragments of transthyretin. Biochem Biophys Res Commun 1993; 192:991–8.
31. MacPhee CE, Dobson CM. Chemical dissection and reassembly of amyloid fibrils formed by a peptide fragment of transthyretin. J Mol Biol 2000; 297:1203–15.
32. Wilce JA, Salvatore D, Wade JD, Craik DJ. 1H-NMR structural studies of a cystine-linked peptide containing residues 71–93 of transthyretin and effects of a Ser84 substitution implicated in familial amyloidotic polyneuropathy. Eur J Biochem 1999; 262:586–94.
33. Jarvis JA, Munro SL, Craik DJ. Structural analysis of peptide fragment 71–93 of transthyretin by NMR spectroscopy and electron microscopy: insight into amyloid fibril formation. Biochemistry 1994; 33:33–41.
34. Najbar LV, Craik DJ, Wade JD, McLeish MJ. Identification of initiation sites for T4 lysozyme folding using CD and NMR spectroscopy of peptide fragments. Biochemistry 2000; 39:5911–20.
35. Najbar LV, Craik DJ, Wade JD, Salvatore D, McLeish MJ. Conformational analysis of LYS(11–36), a peptide derived from the beta-sheet region of T4 lysozyme, in TFE and SDS. Biochemistry 1997; 36:11525–33.
36. Hamilton JA, Steinrauf LK, Liepnieks J, Benson MD, Holmgren G, Sandgren O, *et al.* Alteration in molecular structure which results in disease: the Met-30 variant of human plasma transthyretin. Biochim Biophys Acta 1992; 1139: 9–16.
37. Terry CJ, Damas AM, Oliveira P, Saraiva MJ, Alves IL, Costa PP, *et al.* Structure of Met30 variant of transthyretin and its amyloidogenic implications. EMBO J 1993; 12:735–41.
38. Hamilton JA, Steinrauf LK, Braden BC, Liepnieks J, Benson MD, Holmgren G, *et al.* The X-ray crystal structure refinements of normal human transthyretin and the amyloidogenic Val-30→Met variant to 1.7-Å resolution. J Biol Chem 1993; 268:2416–24.
39. Purkey HE, Dorrell MI, Kelly JW. Evaluating the binding selectivity of transthyretin amyloid fibril inhibitors in blood plasma. Proc Natl Acad Sci USA 2001; 98:5566–71.
40. Munro SL, Lim CF, Hall JG, Barlow JW, Craik DJ, Topliss DJ, *et al.* Drug competition for thyroxine binding to transthyretin (prealbumin): comparison with effects on thyroxine-binding globulin. J Clin Endocrinol Metab 1989; 68:1141–7.
41. Oza VB, Smith C, Raman P, Koepf EK, Lashuel HA, Petrassi HM, *et al.* Synthesis, structure, and activity of diclofenac analogues as transthyretin amyloid fibril formation inhibitors. J Med Chem 2002; 45:321–32.
42. Petrassi HM, Klabunde T, Sacchettini JW, Kelly JW. Structure-based design of N-phenyl phenoxazine transthyretin amyloid fibril inhibitors. J Am Chem Soc 2000; 122: 2178–92.

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