

Synthesis of an Analog of the Thyroid Hormone-binding Protein Transthyretin via Regioselective Chemical Ligation*

Received for publication, February 8, 2001, and in revised form, May 14, 2001
Published, JBC Papers in Press, May 14, 2001, DOI 10.1074/jbc.M101228200

Jackie A. Wilce‡, Stephen G. Love§, Samantha J. Richardson¶, Paul F. Alewood§,
and David J. Craik§||

From the ‡Department of Biochemistry/Chemistry, University of Western Australia, Nedlands Western Australia 6907, Australia, the §Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia, and the ¶Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria 3010, Australia

Transthyretin is an essential protein responsible for the transport of thyroid hormones and retinol in human serum and is also 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. Here we report the solid phase synthesis of the monomeric unit of a transthyretin analog (equivalent to 127 amino acids) using *t*-Boc chemistry and peptide ligation and its folding to form a functional 54-kDa tetramer. The monomeric unit of the protein was chemically synthesized in three parts (positions 1–51, 54–99, and 102–127) and ligated using a chemoselective thioether ligation chemistry. The synthetic protein was folded and assembled to a tetrameric structure in the presence of transthyretin's native ligand, thyroxine, as shown by gel filtration chromatography, native gel electrophoresis, transthyretin antibody recognition, and thyroid hormone binding. Other folding products included a high molecular weight aggregate as well as a transient dimeric species. This represents one of the largest macromolecules chemically synthesized to date and demonstrates the potential of protein chemical synthesis for investigations of protein-ligand interactions.

Transthyretin (TTR)¹ is a 54-kDa tetramer, which is present in human plasma (3.6 μ M tetramer) and transports thyroid hormones such as 3,5,3'-triiodo-L-thyronine and L-thyroxine (T4) as well as retinol-binding protein (1, 2). Its structure and ligand binding capabilities have been characterized by x-ray crystallography (3–5). 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 iodanyl moieties residing in hydrophobic pockets

* This work was supported by a grant from the Australian Research Council (to D. J. C. and J. A. W.). The Institute for Molecular Bioscience is a Special Research Center of the Australian Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

|| An Australian Research Council Senior Fellow. To whom correspondence should be addressed. Tel.: 61 7 3365 4945; Fax: 61 7 3365 2487; E-mail: d.craik@imb.uq.edu.au.

¹ The abbreviations used are: TTR, transthyretin; T4, L-thyroxine; sTTR, synthetic TTR; HPLC, high pressure liquid chromatography; RP-HPLC, reverse phase HPLC; ESMS, electrospray mass spectrometry.

at two different binding sites within the channel (6). Upon binding of one T4 ligand, the binding affinity for the second ligand is reduced significantly.

Although the TTR tetramer is inherently very stable (1, 7), in some circumstances transthyretin has a propensity to form amyloid fibrils. In diseases such as senile systemic amyloidosis and familial amyloidotic polyneuropathies, native and mutant TTR, respectively have been found to form long beta-sheet based fibrillar structures (8, 9). These amyloid lesions accumulate in specific organs and are implicated in their dysfunction and ultimately the death of the patients. More than 70 separate mutations that appear to increase the propensity of TTR to form amyloid structures underlying familial amyloidotic polyneuropathy have so far been identified (10–14). 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 (9, 11).

The ligand binding properties of TTR have become of major importance recently, with 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 (15, 16). They 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 nonsteroidal anti-inflammatory drugs are currently being investigated for their ability to inhibit and reverse amyloid formation (17). While crystallographic information has revealed the orientations of several of these drugs bound to TTR, there are many aspects of the ligand binding that cannot be probed using crystallographic techniques. These include dynamic aspects of protein-ligand binding in solution and in the presence of competitors or in the presence of other serum proteins, including the other thyroid hormone carriers albumin and thyroxine-binding globulin. For such studies, it is possible that NMR spectroscopic methods could be employed.

NMR can be used to observe the interaction of a ligand with particular sites in the protein. Such studies currently depend upon the assignment of the specific resonances that are perturbed by the ligand (18), which limits studies to systems in which the protein signal of interest is either fortuitously distinct or has been fully assigned using isotopic labeling and heteronuclear NMR techniques. In the latter case, this also requires that the protein is less than about 40 kDa due to the broad line widths of the NMR signals and large number of signals that have similar chemical shifts. The problem can be somewhat reduced by using chemical ligation strategies in

which just one portion of the protein is isotopically labeled (19). Of even greater value would be the ability to completely control the position of the isotopic label in a protein for probing the ligand interaction.

The current study thus presents the first stage in the development of a spectroscopic method for probing a protein-ligand interaction. The strategy involves the complete synthesis of a TTR analog using solid phase synthesis and chemical ligation techniques and refolding of the protein to a tertiary and quaternary structure that approximates the native form. Since the structure of TTR is known to atomic resolution from x-ray crystallographic studies, an NMR active probe (*i.e.* ^{15}N - or ^{13}C -labeled amino acid) may be incorporated at any strategic position to enable subsequent ligand binding studies to be manifest in NMR spectra.

The chemical synthesis of proteins of the size of the monomeric unit of TTR (127 residues) represents a significant challenge. In the past, long peptides were synthesized in a stepwise fashion, as exemplified by human immunodeficiency virus type 1 protease (20) and interleukin-8 (21), but significant purification problems resulted in low yields of protein. Currently, the synthesis techniques for proteins of this size rely on chemoselective ligation techniques, where two or more nonprotected peptides are joined through a highly selective chemical reaction.

There have been several notable protein syntheses described using chemoselective ligation techniques that incorporate thioester and thioether surrogate amide bonds. These include the synthesis of linked heterodimeric basic helix-loop-helix transcription factors (22) and Cpn10 (23), respectively. Recently, techniques for forming a native amide bond have been applied to a range of protein types including serine protease inhibitors (24–26), human II secretory phospholipase A2 (27), and barnase (28, 29).

Native chemical ligation chemistry is only useful provided there are suitably positioned cysteines. In order to synthesize a protein like transthyretin without suitably located cysteines, it becomes necessary to use chemically modified amino acid substitutes or amino acid mutations. The thioether ligation strategy we use here introduces $-\text{NH}-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_2-\text{CO}-$, which mimics a two-amino acid subunit closely resembling a glycylglycine ($-\text{NH}-\text{CH}_2-\text{CO}-\text{NH}-\text{CH}_2-\text{CO}-$). While the thioether moiety closely resembles the spatial requirements for glycylglycine, it may lack potential hydrogen bond donation/acceptor behavior of the diamino acid unit, thereby potentially introducing some nonnative structural characteristics.

Here we demonstrate the total chemical synthesis of an analog of human TTR through the use of the thioether strategy for the sequential ligation of three peptides. We also show that this synthetic TTR (henceforth referred to as sTTR) may be successfully refolded and reconstituted to form a 54-kDa tetrameric structure able to bind the thyroid hormone T4. This represents one of the largest active proteins made synthetically and provides methodology for future protein-ligand investigations using NMR spectroscopic techniques.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Trifluoroacetic acid, dichloromethane, *N,N*-dimethyl formamide, and diisopropylethylamine were from Auspep (Melbourne, Australia). *O*-Benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate was from Richelieu Biotechnologies (St. Hyacinth, Quebec Canada). Acetonitrile was from BDH Laboratory Supplies (Poole, United Kingdom). Acetic acid and chloroacetic acid were from Ajax chemicals (Auburn, Australia), diethyl ether was from Fluka Biochemicals (Melbourne, Australia), and mercaptoethanol was from Sigma. Ethanolamine, *N,N*-diisopropylcarbodiimide, and bromoacetic acid were from Aldrich. Hydrogen fluoride (HF) was purchased from Boc Gases (Brisbane, Australia). The *N*-Boc-protected L-amino acids Ala, Gly, Ile, Leu, Phe, Pro, Val, Arg(*p*-toluenesulphonyl),

Asp(*O*-cyclohexyl), Asn(xanthyl), Glu (*O*-cyclohexyl), His(dinitrophenyl), Lys(2-chlorobenzoyloxycarbonyl), Ser(benzyl), Thr(benzyl), Trp(formyl), Tyr(2-bromobenzoyloxycarbonyl) were purchased either from NovaBiochem (La Jolla, CA) or Bachem (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 composed of a model 600 solvent delivery system 600E controller and model 484 detector. Vydac C18 columns, analytical (4.6×250 mm) at a flow rate of 1 ml/min, semipreparative (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 trifluoroacetic acid (solvent A) and 90% aqueous acetonitrile 0.09% trifluoroacetic acid (solvent B).

Mass spectral data were collected using a PerkinElmer Sciex (Toronto, Canada) API III biomolecular mass analyzer ion-spray mass spectrometer equipped with an ABI 140B solvent delivery system. Raw data were analyzed using the program MassSpec (PerkinElmer Sciex). Calculated masses were obtained using the program MACROMASS (Sunil Vemuri and Terry Lee, City of Hope, Durate, CA).

Enhanced Chemiluminescence kit was from Amersham Pharmacia Biotech, x-ray film was from Eastman Kodak Co., methylcellulose and activated charcoal (Norit PN.5) were from BDH, L-[^{125}I]thyroxine (1.2 Ci/mg) was from PerkinElmer Life Sciences (NEN Dupont), SepPak C-18 cartridges were from Millipore Corp., and thin layer chromatography plates were from Merck. All reagents were of analytical grade.

Native Human TTR—Native human TTR was isolated from serum using an adapted version of the method described by Dwulet and Benson (30).

Peptide Synthesis—Peptides were synthesized using the rapid manual *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate *in situ* neutralization synthesis technique (31) or using the same technique on a modified ABI 430A peptide synthesizer (32). The thioether resins were prepared according to the methods of Englebretsen *et al.* (33), initially on amino methyl resins and then subsequently Boc-amino acid-Pam resins (ABI). The bromoacetyl and chloroacetyl groups at the amino termini of peptides were coupled using the symmetrical anhydride formed from reaction with *N,N*-diisopropylcarbodiimide. The dinitrophenyl group was removed using 20% mercaptoethanol in 10% diisopropylethylamine/*N,N*-dimethyl formamide solution for two or three 30-min treatments. The Trp formyl deprotection was carried out using ethanolamine prior to HF cleavage.

Peptide resins were cleaved using HF with *p*-cresol and *p*-thiocresol as scavengers at -5 to 0°C for 1–2 h. The HF was removed *in vacuo*, the peptide product was triturated with cold diethyl ether (3×50 ml), and the precipitated peptide was collected and dissolved in 50% acetonitrile with 0.1% trifluoroacetic acid.

The crude peptides were purified by reverse phase (RP)-HPLC, and fractions were collected and analyzed by analytical RP-HPLC and electrospray mass spectrometry (ESMS). Fractions containing the purified peptide were combined and lyophilized.

Solid Phase Synthesis of Br-Ac-PRRYTIAALLSPYSYSTTAVVT-NPKE-OH (Bromoacetyl-102–127 TTR) (I)—Peptide I (see Fig. 2) was synthesized on a Boc-Glu(*O*-benzyl)-Pam-polystyrene resin (ABI) on a 0.5-mmol scale. Amino acid couplings averaged 99.5% efficiency. *N*-Boc deprotection and coupling of bromoacetic acid followed by HF cleavage gave the crude peptide, which was purified by preparative HPLC using a linear gradient of 0–70% B. The peptide was then analyzed by HPLC and ESMS. The purified peptide was characterized as the desired product (I) by ESMS (observed mass = 3021 ± 0.3 ; calculated for $\text{C}_{133}\text{H}_{210}\text{N}_{34}\text{O}_{41}\text{Br}_1 = 3021.24$ (average isotope composition)).

Solid Phase Synthesis of Cl-Ac-ELHGLTTEEEFVEGIYKVEI-DTKSYWKALGISPFHEHAEEVFTAND-NH-CH₂-CH₂-SH (Chloroacetyl-54–99-NH-CH₂-CH₂-SH) (II)—The C-terminal thiol peptide II was manually synthesized using the thiol linker attached to Boc-Ala-Pam resin in the first synthesis and then Boc-Gly-Pam resin for the second. The average amino acid coupling for the syntheses was 99.5 and 99.6%, respectively. The dinitrophenyl protecting group was removed followed by *N*-Boc and formyl group removal. The chloroacetyl group was coupled, and then the peptide was cleaved by HF. The crude peptide was purified by preparative HPLC using a linear gradient of 0–70% B and then analyzed by HPLC and ESMS. The purified peptide was characterized as the desired product (II) by ESMS (observed mass = 5416.46 ± 1.1 ; calculated for $\text{C}_{246}\text{H}_{364}\text{N}_{58}\text{O}_{76}\text{S}_1\text{Cl}_1 = 5417.46$ (average isotope composition)).

Solid Phase Synthesis of H-GPTGTGESKAPLMVKVLDVAVRGSPA-INVAVHVRKAADDTWEPFASGKTSE-NH-CH₂-CH₂-SH (1–51-NH-CH₂-CH₂-SH) (III)—The C-terminal thiol peptide III was synthesized using machine-assisted synthesis. The peptide was synthesized using

the thiol linker attached to Boc-Gly-Pam resin. The average amino acid coupling was 99.6% (1st coupling) for the synthesis, which was routinely double coupled. The dinitrophenyl protecting group was removed, followed by *N*-Boc and then the formyl group. The peptide was cleaved from the resin, and the crude peptide was purified by preparative HPLC using a linear gradient of 0–70% B. The peptide was then analyzed by HPLC and ESMS. The purified peptide was characterized as the desired product (**III**) by ESMS (observed mass = 5355 ± 1.0; calculated for C₂₃₆H₃₇₉N₆₆O₇₂S₂ = 5357.14 (average isotope composition)).

Formation of Cl-Ac-ELHGLTTEEEFVEGIYKVEIDTKSYWK-ALGISPFHEHAEVVFTAND-NH-CH₂-CH₂-S-CH₂-CO-PRRYTIAALLSPYSYSTTAVVTNPKE-OH (Chloroacetyl-54–99-ψ-102–127) (IV)—The ligation reaction was initiated by mixing the two peptides, bromoacetyl-102–127 TTR (**I**) (5.44 mg, 1.8 mmol) and chloroacetyl-54–99-NH-CH₂-CH₂-SH (**II**) (6.84 mg, 1.26 mmol), in 1 ml of 6 M urea 0.1 M NaHCO₃, pH 8.3, under nitrogen. After mixing, the reaction mixture was left to stand at room temperature for 24 h. Samples were withdrawn at 0, 1, 2, and 20 h during this period for HPLC and ESMS analysis. The ligated peptide **IV** was isolated from the reaction mixture by semipreparative HPLC using a linear gradient of 0–70% B. The fractions were analyzed by HPLC and ESMS; the fractions containing the ligated peptide were then lyophilized. The purified peptide **IV** (6.01 mg) was characterized as the desired product **IV** by ESMS (observed mass = 8355 ± 1.2; calculated for C₃₇₉H₅₇₂N₉₂O₁₁₇S₁Cl₁ = 8356.8 (average isotope composition)).

Chloro-Iodo Exchange of (IV) to give I-Ac-ELHGLTTEEEFVEGIYKVEIDTKSYWKALGISPFHEHAEVVFTAND-NH-CH₂-CH₂-S-CH₂-CO-PRRYTIAALLSPYSYSTTAVVTNPKE-OH (Iodoacetyl-54–99-ψ-102–127) (V)—Peptide **IV** (5.46 mg, 0.654 mmol) was dissolved in 8 M urea 0.01 M NaOAc pH 7.5 (1 ml), and KI was added to saturation (~8 M). After placing under nitrogen, a sample was removed at 30 min, purified by HPLC, and analyzed by ESMS to check completion of the iodo exchange. The iodoacetyl peptide **V** was then purified by semipreparative HPLC and lyophilized to give 3.64 mg. The peptide was characterized as the desired product **V** by ESMS (observed mass = 8447 ± 1.0; calculated for C₃₇₉H₅₇₂N₉₂O₁₁₇S₁Cl₁ = 8448.2 (average isotope composition)).

Formation of H-GPTGTGESKAPLMVKVLDVAVRGSPAINVA-VHVRKAADDTWEPFASGKTSE-NH-CH₂-CH₂-S-CH₂-COELHGLTTEEEFVEGIYKVEIDTKSYWKALGISPFHEHAEVVFTAND-NH-CH₂-CH₂-S-CH₂-CO-PRRYTIAALLSPYSYSTTAVVTNPKE-OH (1–51-ψ-54–99-ψ-102–127 TR) (VI)—Ligation of the two peptides iodoacetyl-54–99-NH-CH₂-CH₂-SH **V** (3.46 mg, 0.43 mmol) and 1–51-NH-CH₂-CH₂-SH **III** (3.58 mg, 6.68 mmol) was initiated by mixing the two peptides in 6 M urea, 0.2 M NaHCO₃, pH 8.3 (500 ml) and placing under nitrogen. The reaction was mixed and then left to stand at room temperature for 5 h. Samples were withdrawn at 0, 1.5, and 4 h for HPLC and ESMS analysis. The ligated peptide **VI** was isolated from the reaction mixture by semipreparative HPLC using a linear gradient of 0–70% B. The fractions were analyzed by HPLC and ESMS; the fractions containing the ligated peptide were then lyophilized to give **VI** (2.76 mg). The purified peptide was characterized as the desired product **VI** by ESMS (observed mass = 13,676 ± 1.3; calculated for C₆₁₅H₉₄₉N₁₅₈O₁₈₉S₃ = 13,676.4 (average isotope composition)).

Formation of the Tetrameric Complex—The ligated peptide (1–51-ψ-54–99-ψ-102–127) (**VI**) (0.25 mg) was dissolved in 0.075 M NH₄HCO₃, pH 8.3 (100 μl) and then diluted with 100 μl of H₂O. To this solution was added 5 μl of thyroxine T4 (5 mg/ml in 0.1 M NaOH). After equilibrating at room temperature for 18 h, the tetrameric protein was isolated by gel filtration on a Superdex 75 column (HR10/30; Amersham Pharmacia Biotech; calibrated with phosphorylase, 97 kDa; bovine albumin, 66 kDa; native TTR, 54 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; soya bean trypsin inhibitor, 20.1 kDa, and α-lactalbumin, 14.4 kDa) with 0.075 M NH₄HCO₃ 10% CH₃CN as the eluent at 0.3 ml/min. The tetrameric protein was isolated at a retention time of 22 min as determined by its equivalent retention time to that of native TTR. It was kept in solution in the presence of excess T4 prior to further analysis.

Western Analysis of Synthetic TTR—2 μl of human serum, 25 μl (0.5 μg) of sTTR solution, and 50 μl (1.0 μg) of sTTR were separated in a 0.1% SDS-polyacrylamide gel, using a stacking gel of 4.5% acrylamide, pH 6.8, and a resolving gel of 15% acrylamide, pH 8.6 (34). Proteins were transferred onto a nitrocellulose membrane, following which the membrane was blocked. The primary antibody was 1:5000 antiserum raised in a rabbit against a mixture of TTRs purified from serum from human (*Homo sapiens*) wallaby (*Macropus eugenii*), and chicken (*Gallus gallus*), and the secondary antibody was 1:10,000 anti-rabbit Ig raised in sheep (*Silenus*), as described previously (35). Detection

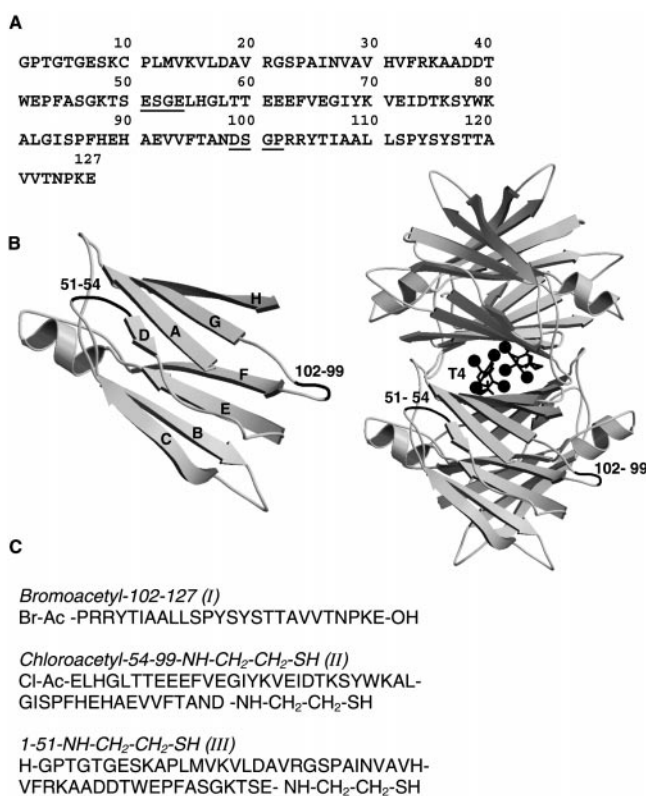


FIG. 1. A, sequence of human transthyretin 1–127 and depiction of ligation points within the monomeric sequence. The chemoselective thioether ligation sites are *underlined*. The cysteine at position 10 was changed to Ala in the current synthesis. B, ribbon diagram of the TTR monomer and tetramer showing the sites of chemical ligation in the folded molecule. C, names and sequences of the three peptides synthesized for the current study of sTTR.

achieved was using enhanced chemiluminescence against x-ray film.

Preparation of L-[¹²⁵I]Thyroxine—Commercially available L-[¹²⁵I]thyroxine was found to contain up to 5% ¹²⁵I[−] on the reference date. Therefore, [¹²⁵I]thyroxine was separated from ¹²⁵I[−] and other degradation products by reversed phase chromatography using a SepPak C-18 cartridge column (36). Purification was checked by thin layer chromatography followed by autoradiography (37).

Analysis of Thyroxine Binding to Synthetic Human TTR—Commercially purchased [¹²⁵I]thyroxine was purified from degradation products and ¹²⁵I[−] as described above. Methylcellulose charcoal was prepared as described by Chang *et al.* (38). In order to remove thyroxine from the solution containing sTTR, 40 μl of methylcellulose-charcoal (1% in Tris-HCl, pH 8.9, was centrifuged, and the supernatant was removed. The methylcellulose-charcoal was resuspended in a 160-μl solution containing 3.2 μg of sTTR. The mixture was kept at 4 °C for half an hour, with mixing each 10 min. The solution was centrifuged, and the supernatant was removed for analysis of [¹²⁵I]thyroxine binding to sTTR.

10 μl of human serum and 80 μl of solution containing sTTR (1.6 μg) were incubated with 1.1 fmol (2.4 nCi) of purified [¹²⁵I]thyroxine (room temperature for 1 h), and a second aliquot of 80 μl of solution containing sTTR (1.6 μg) was incubated with 4.4 fmol (9.6 nCi) of purified [¹²⁵I]thyroxine (room temperature for 1 h). 5 μl of human serum and the total amounts of sTTR solutions were analyzed by nondenaturing polyacrylamide gel electrophoresis, 10% acrylamide, 0.05 M Tris-HCl, pH 8.9, 4 °C (39) followed by autoradiography.

RESULTS

Synthetic Strategy—The choice of ligation sites for the preparation of sTTR was based on both the amino acid sequence and the known tertiary structure of the TTR molecule (Fig. 1, A and B) and involved the ligation of three peptides (Fig. 1C). While the ligation of two peptides, each of 60–65 residues in length, is a possible alternative strategy, the degree of difficulty of preparing and purifying peptides of this length is comparable

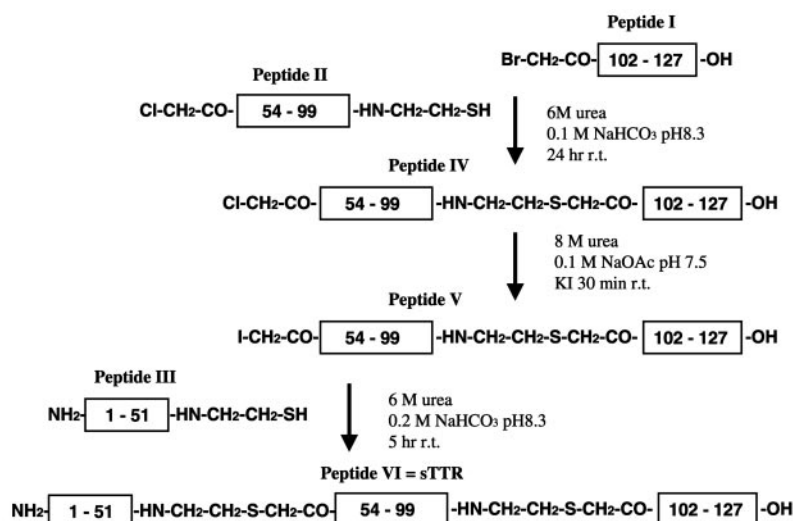


FIG. 2. Ligation scheme for the synthesis of transthyretin showing the three-step process to give synthetic transthyretin.

with the difficulty of a second ligation. In addition, since all of the residues considered for future labeling studies occur within the last 30 residues, it was desirable to prepare a relatively small C-terminal fragment. It was anticipated that this approach would increase the ease of preparing several sTTR molecules with selective labels, since only this fragment would need to be resynthesized.

The thioether linker spans a distance equivalent to two amino acids, is highly flexible, and is nonfunctionalized. While Gly-Gly sequences are thus ideally suited as ligation points, the TTR sequence contains no Gly-Gly site, although several Ser-Gly sites are present at convenient positions. Two of these Ser-Gly sites exist in loop positions within the TTR structure, which were considered to be potentially more tolerant of surrogate amide bonds than β -sheet or helical regions. Ser¹⁰⁰-Gly¹⁰¹ occurs in the loop between β -strands F and G, and Ser⁵²-Gly⁵³ occurs in the loop between β -strands C and D. Neither are close to the hormone binding site or at points of intersubunit contact. A third Ser-Gly site (Ser⁴⁶-Gly⁴⁷) 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 Ser⁵²-Gly⁵³ and Ser¹⁰⁰-Gly¹⁰¹ ligation sites required the synthesis of three peptides of 51, 46, and 26 residues. The ligation strategy for the three peptides is outlined in Fig. 2. The *N*-bromoacetylated 102–127 (peptide I) is ligated to the *N*-chloroacetylated, C-thiolated 54–99 (peptide II). The ligation proceeds preferentially between the thiol and the bromoacetyl groups, so that polymerization of the peptide II should not occur. The purified chloroacetylated 54–127 (peptide IV) may then undergo simultaneous or sequential exchange to the iodoacetylated form (peptide V) and ligation with C-thiolated 1–51 (peptide III) to produce fully ligated 1–127 (peptide VI).

In addition to nonnative bonds, one other modification was made to sTTR. Cysteine 10 was replaced with alanine in order to avoid any problems of competition for the iodoacetyl peptide fragment during the ligation reaction and to exclude the possibility of oxidation when the protein was folded. Alanine was chosen due to its similarity in bulk and hydrophobicity to cysteine. This was considered unlikely to have any detrimental effect on the folding or activity of TTR.

Peptide Synthesis and Purification—Trial syntheses of the peptides I, II, and III were carried out to see how each of the peptides would behave during the synthesis, cleavage, and ligation reactions. This preliminary work showed that all three

peptides could be readily synthesized, cleaved from the resin, and purified, excepting the middle fragment, which tended to retain the methylphenoxycetic acid (AMPA) linker at the C-terminal thiol after HF treatment. This was detected as a result of cleavage at the C-terminal amino acid attached to the resin on which the peptide-AMPA linker was synthesized (see “Experimental Procedures” for details). Despite this, the three target peptides were obtained in good yield and purity.

Ligation of Peptides I and II—Ligation of peptides I and II proceeded cleanly to give peptide IV (Fig. 2). The purification and monitoring of the ligation reaction was complicated by the fact that the ligated peptide IV co-elutes with the starting peptide II; thus, the use of an excess of peptide I was required to drive the reaction to completion. After 2 h, the starting peptide II could not be detected, and the ligated peptide IV was present in high yield as judged by HPLC and ESMS (Fig. 3, A and B). The ligation was left overnight for completion of the reaction. The broad HPLC profile of peptide II was attributed to the chloroacetyl and the thiol functionalities at the N and C termini of the peptide. The absence of the chloroacetyl group, in particular, significantly reduces the broadness of the peak shape.

Formation of the Iodoacetyl Peptide V—The initial chloroiodo exchange reaction and the subsequent ligation were carried out *in situ* (peptide III was added to peptide IV in saturated KI solution). Most of the thiol peptide III was found to form disulfide dimers, and the iodoacetyl peptide V also lost a small percentage of its iodofunctionality. In subsequent ligations, the iodoacetyl peptide V was either purified by RP-HPLC and the ligation reaction was carried out immediately after lyophilization, or it was purified by rapid desalting using a PD10 column (Amersham Pharmacia Biotech) followed by ligation.

Ligation of Peptides III and V to Give VI—After purification of the iodoacetyl peptide V, its ligation to peptide III (1–51 TTR) was carried out as rapidly as possible under nitrogen. Samples were removed from the ligation mixture and analyzed by HPLC and ESMS. The rate of the ligation reaction, affording the ligated 1–127 TTR VI, was monitored by a slow HPLC gradient (0.5%/min B) as the ligated product eluted very close to the two starting peptides (III and V) and the disulfide-linked peptide formed by the unreacted excess of peptide III (Fig. 4A). The ligated 1–127 peptide VI was easily purified by RP-HPLC in excellent yield, and its identity was confirmed by ESMS (Fig. 4B).

Folding of Synthetic TTR to Give the 54-kDa Tetrameric

FIG. 3. A, HPLC traces showing the peptide ligation mixture for peptides I and II at time 0 (top) and 2 h (bottom). The resultant ligated peptide IV elutes at the same position that the starting peptide II elutes. B, the mass spectrum of the peak for peptide IV, showing the formation of the ligated peptide. *, daughter ions of starting peptide II still present after 2 h. Inset, mass spectrum reconstruct showing the correct mass for the ligated peptide IV.

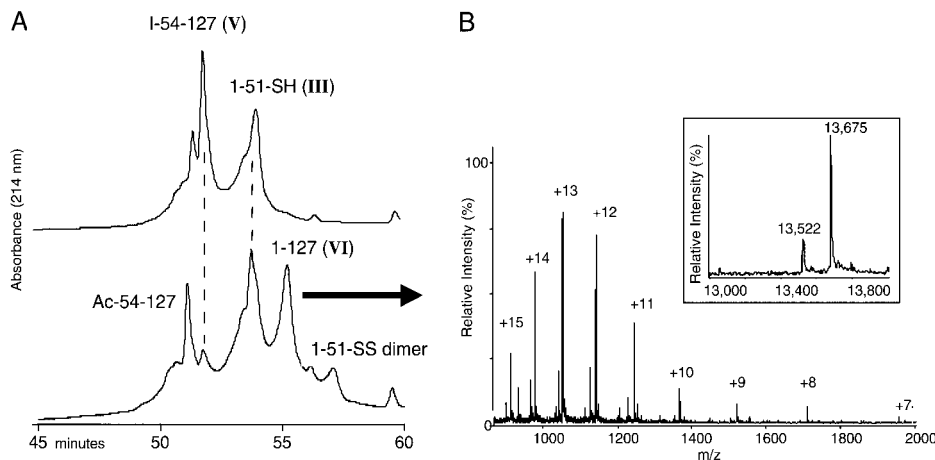
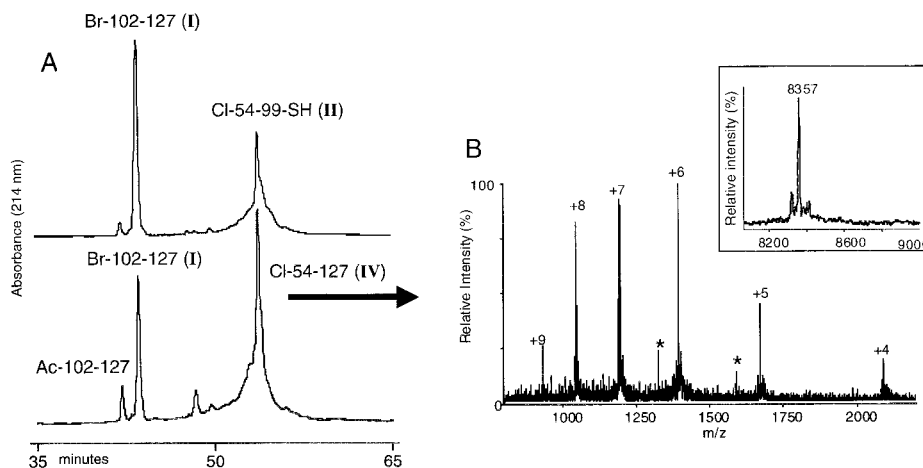


FIG. 4. A, HPLC traces showing the peptide ligation mixture for peptides III and V at time 0 (above) and 2 h (below). B, the mass spectrum of a sample of the peak for the ligated peptide VI, 1-127. Inset, mass spectrum reconstruct showing the correct mass for the ligated synthetic TTR, peptide VI (13,675 Da), as well as the presence of a deletion product (13,522 Da).

Complex—The sTTR spontaneously folded to its tetramer complex in the presence of the ligand T4, in 0.075 M NH_4HCO_3 . In addition to the formation of tetramer (as initially determined by its equivalent retention time to that of native TTR upon size exclusion chromatography), two other products were also formed. These corresponded to species with an intermediate molecular weight, likely to be a dimeric form of sTTR, as well as a high molecular weight aggregated sTTR (Fig. 5, top trace). The ratio of these products altered over time in the refolding buffer, with a gradual accumulation of the high molecular weight aggregate. After isolating the tetrameric complex, it was equilibrated at 37 °C for 48 h in the presence of excess T4 ligand. Some reequilibration between the monomeric, tetrameric, and high molecular weight aggregates occurred in the initial 2 h, but the ratios of tetramer to monomer and the high molecular weight aggregates remained constant after that time (Fig. 5, bottom trace).

Western Analysis of Synthetic TTR—Western analysis was employed to determine the subunit molecular mass and confirm the recognition of the sTTR by anti-TTR antiserum (Fig. 6). An aliquot of human serum was analyzed as a positive control. TTR has a subunit molecular mass of ~15 kDa, as estimated by SDS-polyacrylamide gel electrophoresis (39). The interactions between monomers to form the dimer are very strong, and even after boiling in the presence of 2% SDS for 20 min, some TTR still exists as a dimer (38). Thus, bands with molecular masses of about 15 and of about 34 kDa correspond to the TTR monomer and dimer, respectively. Bands are also apparent at higher molecular masses. These result from non-specific binding of the antibodies to other proteins in serum as is commonly observed (35).

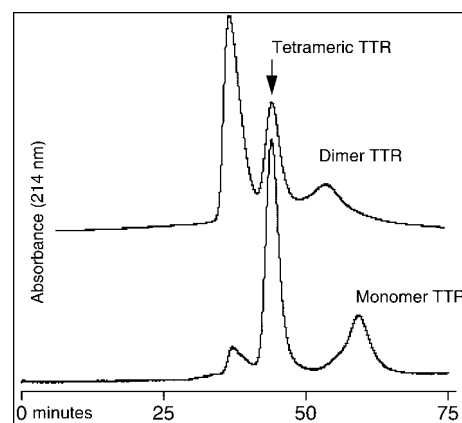


FIG. 5. Gel filtration profiles of the solution used for folding the ligated synthetic transthyretin VI. An Amersham Pharmacia Biotech Superdex 75 10/30 column used 0.075 M NH_4HCO_3 , pH 8.3, with 10% CH_3CN and a flow rate of 0.3 ml/min. The protein was detected at 214 nm. The top trace shows the protein VI (3.65 μM) after 18 h at room temperature in 0.375 M NH_4HCO_3 with 0.5 equivalents of T4 (excess T4 precipitates from solution). The bottom trace shows the isolated synthetic transthyretin VI tetrameric complex after reequilibration at 37 °C for 48 h. Monomeric and dimeric species, as ascertained by their retention time, are indicated.

Synthetic TTR gave rise to bands corresponding to the molecular masses of the sTTR monomer and dimer (Fig. 6, lanes 2 and 3). The bands were discrete, and no indication of partially synthesized or partially degraded sTTR was apparent.

Analysis of Thyroxine Binding to Synthetic TTR—The correct folding and formation of the tetramer was assessed by

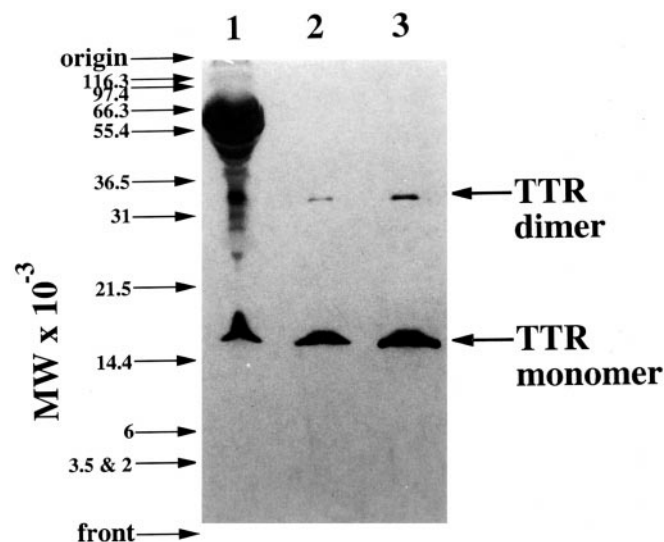


FIG. 6. Western analysis for the presence of transthyretin in human serum and in solution containing synthetic transthyretin. Samples were separated in an SDS-polyacrylamide gel, and then proteins were transferred onto a nitrocellulose membrane and probed with antiserum against a mixture of transthyretins purified from human, wallaby, and chicken sera (raised in a rabbit), and the secondary antibody was anti-rabbit Ig (raised in sheep). Detection was performed using enhanced chemiluminescence (see "Experimental Procedures" for details). Lane 1, 2 μ l of human serum; lane 2, 25 μ l of solution containing 0.5 μ g of synthetic transthyretin; lane 3, 50 μ l of solution containing 1.0 μ g of synthetic transthyretin. Molecular weight markers ($\times 10^{-3}$) were "Mark 12" from Novex: β -galactosidase, 116.3; phosphorylase b, 97.4; serum albumin, 66.3; glutamic dehydrogenase, 55.4; lactate dehydrogenase, 36.5; carbonic anhydrase, 31; trypsin inhibitor, 21.5; lysozyme, 14.4; aprotinin, 6; insulin B chain, 3.5; and insulin A chain, 2. The positions of the origin and migration of transthyretin dimer, transthyretin monomer, and the electrophoretic front are indicated.

analyzing nondenaturing polyacrylamide gel migration combined with a [¹²⁵I]thyroxine binding assay. The analysis of [¹²⁵I]thyroxine binding to proteins in human serum was used as the reference. This revealed the presence of thyroxine-binding globulin, albumin, and TTR (Fig. 7, lanes 1 and 4). The binding of [¹²⁵I]thyroxine by sTTR was clearly demonstrated in both the aliquots of 80 μ l of solution containing 1.6 μ g of sTTR, following incubation with both 1 μ l (1.1 fmol) and 4 μ l (4.4 fmol) of [¹²⁵I]thyroxine (Fig. 7, lanes 2 and 3, respectively). The position of migration was almost identical to that in serum, indicating that the tetrameric size, shape, and charge distribution of sTTR were almost identical to native TTR. There was no evidence for the existence of aggregates.

DISCUSSION

We have shown that it is possible to chemically synthesize and correctly fold a transthyretin analog (sTTR) from its monomeric unfolded state to produce the 54-kDa tetrameric quaternary structure in the presence of TTR's strongest binding native ligand, T₄. This is probably due to the stabilizing effect of T₄, which has previously been reported to stabilize native TTR against acid denaturation, leading to the formation of amyloid fibrils (15). 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.

Competing folding pathways involving the formation of high molecular weight aggregate as well as the appearance of smaller molecular weight species, possibly corresponding to dimeric and monomeric sTTR, were also apparent. The former pathway was expected, considering the predisposition for TTR

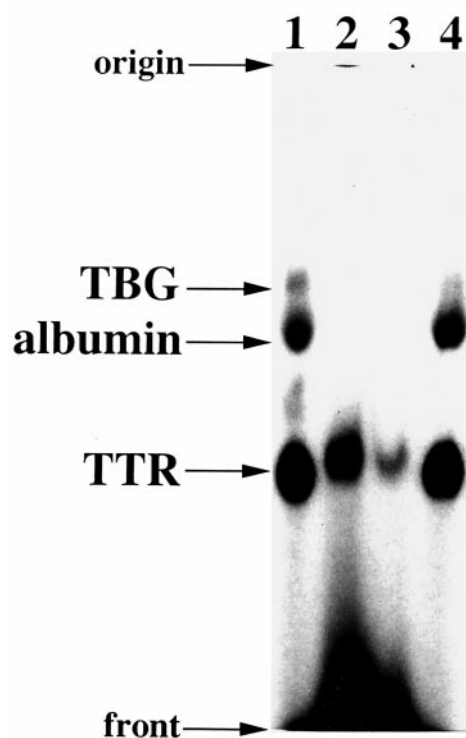


FIG. 7. Analysis of thyroxine binding to proteins in human serum and to synthetic transthyretin. Aliquots of human serum (10 μ l) and of synthetic TTR solution (80 μ l containing 1.6 μ g) were incubated with [¹²⁵I]thyroxine prior to separation in a nondenaturing polyacrylamide gel, pH 8.6. The gel was dried and then exposed to autoradiographic film for 7 days (see "Experimental Procedures"). Lane 1, 5 μ l of human serum; lanes 2 and 3, 80 μ l of solution containing 1.6 μ g of synthetic transthyretin with 1.1 and 4.4 fmol of [¹²⁵I]thyroxine respectively; lane 4, 5 μ l of human serum. The positions of migration of human thyroxine-binding globulin (TBG), albumin, and transthyretin (TTR) are indicated. The positions of the origin and front of the gel are also indicated.

to form amyloid fibrils and, in particular, the increased propensity of many mutant forms of TTR to do so. The appearance of dimeric-like species of sTTR, however, was unexpected, since this species has never previously been reported. The current model of the unfolding pathway of TTR involves a transition between correctly folded TTR to its monomeric form via a perturbed tetramer and an extremely transient dimeric form (40). The monomeric TTR is thought to be in equilibrium with a molten globule-like monomeric structure or to be sequestered to irreversible amyloid formation. Our refolding studies of sTTR have revealed a species that appears to have the molecular weight of the dimeric form of sTTR. It was not possible to isolate this dimeric species for further identification. Rechromatographing the species using gel diffusion chromatography only gave rise to the high molecular weight aggregate and some of the monomeric species. This highly unstable form may be unique to sTTR or represent an alternative species that could be the basic unit of TTR-based amyloid fibrils.

It is not surprising that the sTTR construct has a high propensity to form aggregates. Investigations into mutant forms of TTR have shown that even in the absence of apparent perturbations to the tertiary and quaternary structure of TTR, most mutant forms of TTR display a higher propensity to form amyloid fibril than native TTR (41–43). The mutations, rather than underlying an alternative TTR conformation, are thought to cause subtle perturbations to the equilibrium between the different forms of TTR. Any such movement toward the monomeric form of TTR thus results in the increased opportunity for TTR monomer to be irreversibly incorporated into amyloid.

As observed for native TTR, the tetrameric form of sTTR is its most stable form. Early studies of recombinant TTR and TTR isolated from serum showed that it is very difficult to dissociate the tetrameric structure into its monomeric subunits. TTR was reported as stable in chaotropic solutions of 8 M urea and 6 M guanadinium hydrochloride (7). More recent studies of the denaturation pathway of recombinant TTR showed that the tetrameric unit does not begin to dissociate into unfolded monomer until the chaotrope concentration exceeds 4 M guanadinium hydrochloride (44). Fully unfolded recombinant TTR (denatured in 7 M guanadinium hydrochloride) does not refold until the denaturant concentration has dropped well below 2 M guanadinium hydrochloride, showing that the tetrameric TTR structure is kinetically highly stable.

In the current study, sTTR could not be refolded by slowly decreasing the denaturant concentration by dialysis. This only led to the formation of high molecular weight aggregates. sTTR did, however, fold to the tetrameric structure at a low salt concentration in the presence of the native T4 ligand. Folding the protein using ligands more soluble than T4 such as 3,5,3'-triiodothyronine, 3',5',3-triiodothyronine, diiodothyronine, and thyronine did not give rise to the tetrameric form of the protein. This may have been due to the lower K_d of these ligands to TTR relative to the T4 ligand, thereby not stabilizing the sTTR complex to the same degree as the T4 ligand.

It is not known whether the sTTR tetramer forms and then is stabilized by T4 or the tetramer spontaneously forms about the T4. The stabilization of sTTR by a high affinity ligand, however, appears analogous to the stabilization observed for TTR in the presence of several nonsteroidal anti-inflammatory drugs currently under investigation for their ability to inhibit human transthyretin amyloid disease (17). These molecules, which include flufenamic acid, diclofenac, and flurbiprofen, bind in the T4 binding site of TTR (45), forming similar polar and nonpolar 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 (16). It may be that some of these drugs would also promote the folding of sTTR from its unfolded monomeric state.

In conclusion, the current study reports a methodology for the total chemical synthesis of a TTR analog (sTTR) through use of a thioether strategy for the sequential ligation of three peptides and successful folding and formation of the sTTR tetramer in the presence of the native ligand T4. It is remarkable that this macromolecule can be synthesized, however, since other multimeric forms of sTTR also formed readily, the construct may not be ideal for biophysical studies. It is possible that other TTR analogs made using thioether linkages placed in alternative positions may provide closer mimics of native structure. Such constructs potentially allow the incorporation of ^{15}N or ^{13}C isotopic labels at TTR ligand binding sites and may facilitate future TTR-ligand studies using NMR spectroscopy. In particular, the kinetics of the interaction between sTTR and TTR-binding drugs of current interest for their anti-amyloidogenic capacity may be carried out in the presence of other competitive binding proteins to provide a more complete picture of their mode of action in serum.

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Synthesis of an Analog of the Thyroid Hormone-binding Protein Transthyretin via Regioselective Chemical Ligation

Jackie A. Wilce, Stephen G. Love, Samantha J. Richardson, Paul F. Alewood and David J. Craik

J. Biol. Chem. 2001, 276:25997-26003.

doi: 10.1074/jbc.M101228200 originally published online May 14, 2001

Access the most updated version of this article at doi: [10.1074/jbc.M101228200](https://doi.org/10.1074/jbc.M101228200)

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