Transthyretin Binding Mode Dichotomy of Fluorescent trans-Stilbene Ligands

Afshan Begum, Jun Zhang, Dean Derbyshire, Xiongyu Wu, Peter Konradsson, Per Hammarström,* and Eleonore von Castelmur*

ABSTRACT: The orientations of ligands bound to the transthyretin (TTR) thyroxine (T4) binding site are difficult to predict. Conflicting binding modes of resveratrol have been reported. We previously reported two resveratrol based trans-stilbene fluorescent ligands, (E)-4-(2-(naphthalen-1-yl)vinyl)benzene-1,2-diol (SB-11) and (E)-4-(2-(naphthalen-2-yl)vinyl)benzene-1,2-diol (SB-14), that bind native and misfolded protofibrillar TTR. The binding orientations of these two analogous ligands to native tetrameric TTR were predicted to be opposite. Herein we report the crystal structures of these TTR:ligand complexes. Opposite binding modes were verified but were different than predicted. The reverse binding mode (SB-14) placing the naphthalene moiety toward the opening of the binding pocket renders the fluorescent ligand pH sensitive due to changes in Lys15 amine protonation. Conversely, the forward binding mode (SB-11) placing the naphthalene inward mediates a stabilizing conformational change, allowing intersubunit H-bonding between Ser117 of different monomers across the dimer interface. Our structures of TTR complexes answer important questions in ligand design and interpretation of trans-stilbene binding modes to the TTR T4 binding site.

KEYWORDS: Transthyretin (TTR), amyloidosis, crystal structure, ligand, fibrillation inhibitor, fluorescence

INTRODUCTION

TTR in Transport, Chaperone and Amyloid Processes. Transthyretin (TTR) is a plasma and cerebrospinal fluid (CSF) protein synthesized by the liver, choroid plexus, eye and pancreas. TTR is the main transporter of T4 hormone in CSF and the primary carrier of retinol (Vitamin A) binding protein in blood plasma. TTR appears to function as a molecular chaperone in preventing amyloid formation of Aβ in the brain1−4 and islet amyloid polypeptide (IAPP) in the pancreas.5 TTR is inherently amyloidogenic, and the wild-type protein accounts for a significant number of cardiac amyloidosis cases in elderly people6 as well as diverse mutation-dependent amyloid disease phenotypes in familial diseases such as familial amyloid polyneuropathy (FAP).7 Collectively this group of diseases are known as transthyretin amyloidosis with deposited ATTR. TTR research to understand protein misfolding diseases is of considerable interest due to the impact of TTR amyloidosis on the affected ATTR patient and society. The small molecule ligand Tafamidis is the active component of Vyndaqel and Vyndamax, which are approved drugs for treating FAP and wild-type ATTR cardiomyopathy. Tafamidis works as a kinetic stabilizer by binding to the T4 binding site, preventing tetramer dissociation, and thereby subsequent misfolding and fibril formation is suppressed.8 Tafamidis binding also imposes long-range allosteric conformational changes9 which may in addition to preventing tetramer dissociation inhibit aberrant proteolysis, which is a putative fibrillation initiation mechanism.10

Resveratrol Is a Health Promoting Natural Compound. trans-Resveratrol is a trans-stilbene polyphenolic antioxidant found predominantly in plants such as grapes. Resveratrol can reach significant concentrations in red wine. There are reports of resveratrol being anti-inflammatory, anti-cancer, neuroprotective, and anti-aging, similar to effects of calorie restriction.11 The latter activity is largely attributed to resveratrol isoform selective activation and inhibition of the deacetylase activity of sirtuins.12 The class of trans-stilbenes have been of considerable interest for TTR amyloidosis to find...
natural products as alternatives to kinetic stabilizers of TTR tetramers such as tafamidis.  

**trans-Stilbene Ligands for Amyloid Proteins.** TTR is a homo tetramer with a total mass of 55 kDa composed of 127 amino acids in each subunit. The structure of TTR was originally solved by Blake in 1978, and numerous structures of TTR in complex with ligands have been published since. With all this knowledge there has been controversy regarding certain binding modes of ligands to TTR. Studies of TTR-targeting molecules are of significant interest due to TTR association with age-dependent amyloidosis. TTR is a rather promiscuous binder of aromatic compounds resembling T4 in its dual T4 binding pockets. Resveratrol has been reported to bind to the T4 binding pocket (T4BP) with rather high affinity. Interestingly, it appears that the binding modes of several molecules to TTR are also promiscuous regarding preferred binding orientation. Crystal structures of the TTR:resveratrol complex report contrasting binding modes. 

That resveratrol can be used as a TTR tetramer-sensitive fluorescent ligand when binding to the T4BP was shown many years ago. Furthermore, the trans-stilbene chemical motif is of particular interest for amyloid targeting because it is present in several amyloid fibril-specific ligands including fluorescent ligands X-34, Methoxy-X04, and PET ligands florbetaben (18F) and florbetapir (18F). Our rationale for the current study is of general interest for understanding the selectivity and binding modes of TTR-binding trans-stilbene ligands and in particular for resveratrol and resveratrol analogues of amyloid fibril probes.

We previously hypothesized on the binding mode of two structurally homologous, amyloid-sensitive ligands SB-11 and SB-14. Based on fluorescence spectroscopy, we speculated that they display opposite binding orientations in the T4BP of TTR. We determined the structures of these compounds bound to TTR by X-ray crystallography to verify these findings. Here we present the crystal structures of wild-type TTR in complex with three trans-stilbene compounds TTR:SB-11, TTR:SB-14, and TTR:resveratrol. The data confirm our previous findings that these ligands bind in opposite directions despite being analogues and explain their distinct activities in terms of fluorescence and as stabilizing inhibitors to prevent fibril formation.

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**Figure 1.** Electron density permits unambiguous placement of ligands in the TTR T4BP. (A) TTR is depicted as a cartoon, with monomer A in green and monomer B in petrol blue. Monomers A’ and B’ are colored light green and teal, respectively. The same coloring scheme for TTR is applied throughout all figures. For the complex structures with (B) resveratrol, (C) SB-11, and (D) SB-14, ligands are drawn as sticks and the 2mFo-DFc electron density contoured at 1.5 sigma is shown as blue mesh. (E) Chemical structures of the ligands.
RESULTS AND DISCUSSION

Crystal Structures of TTR:Ligand Complexes. High-resolution X-ray crystal structures of TTR in complex with SB-11, SB-14, and resveratrol were obtained by cocrystallization with ligands coincubated with the protein for at least 2 h at room temperature. Comparisons were done with apo-TTR crystallized under identical conditions. All four crystal structures belong to the P2₁2₁2₂ space group; the protein structure remains unchanged upon ligand binding, as evidenced by the low deviation in Cα positions between the aligned monomers (<0.35 Å rmsd). The dimer AB is found in the asymmetric unit and the second dimer (A′B′) to form the tetramer can be obtained by rotation along the crystallographic 2-fold c-axis (Figure 1A). The inner β-sheets of the dimer–dimer (AB–A′B′) interface form two T4BP cavities referred to as sites AA′ and BB′, respectively. The symmetric binding sites within each AA’ and BB’ dimer are composed of three so-called halogen binding pockets (HBPs). HBP1 is in the outer cavity close to the protein surface and HPB3 is in the inner cavity closest to the center of the tetramer, with HPB2 between these two cavities. The T4BP is predominantly hydrophobic, though some of the constituent amino acids have polar side chains that allow hydrophilic interactions.

Since the crystallographic 2-fold axis crosses the T4BPs, symmetry-related ligands are found superposed at this special position as already reported. 15,19 The electron densities for all TTR:ligand complexes presented enabled unambiguous placement of the ligands (Figure 1B–D, Figures 2–3). All ligands bind to TTR in the T4BP, but with different orientations. Ser117, in the innermost part of the binding pocket (HPB3), is known to adopt multiple conformations and can hydrogen-bond with the ligand (Figure 4). While SB-11 and SB-14 each have a single but distinct orientation, the observed electron density clearly supports both previously observed orientations for resveratrol (Figures 1–2).

Structural Consequences of Different Binding Modes. Binding of resveratrol (in both orientations) and SB-14 with their hydroxyl groups pointing into HBP3 positions these within distance for H-bonding with Ser117 at the base of the T4BP (Figures 1–3). In comparison, SB-11 binds in the opposing forward mode, burying its hydrophobic naphthalene moiety in HPB3 (Figures 1, 3). Notably, in apo-TTR, the side chain of Ser117 adopts several alternative conformations and H-bonds with water molecules occupying the binding cavity (Figure 4A). Upon ligand binding, the conformation of Ser117 becomes more constrained. A common conformation for Ser117 is imposed when its hydroxyl group H-bonds with a polar ligand as observed for resveratrol (in either pose) and the asymmetric SB-14 (Figure 4B, D). In contrast, SB-11 induces intersubunit H-bonding interactions between two neighboring Ser117 (A:B and A′B′) (Figure 4C). This latter pose is suggestive of a tetramer-stabilizing conformation with H-bonds across the dimer interface. Furthermore, in the SB-11 structure, a specific central water molecule appears to engage Ser117 by H-bonds from all four monomers across the dimer interface (Figure 4C).

TTR Tetramer Stability and Inhibition of Fibril Formation. The tetramer-stabilizing activity of the ligands was assessed by differential scanning fluorimetry (DSF). At neutral pH TTR has a midpoint of thermal denaturation (Tm) close to 100 °C and an increase in stability is therefore not easily assayed. Consequently, we selected pH 5.0 as the pH for this assay where the Tm for TTR is 92.3 °C. Resveratrol and

Figure 2. Resveratrol binds to TTR in both orientations. (A) Resveratrol can bind to the T4BP in two orientations. (B) The electron density in the BB’ T4BP prior to placement of the ligand supports both binding orientations of resveratrol. The 2mFo-Fc map contoured at 1σ is shown as blue mesh, and the mFo-DFc difference density contoured at 3.5σ is shown as green/red mesh. (C, D) Dual binding mode of resveratrol (C) observed in the asymmetric unit and (D) after applying the 2-fold symmetry creating the tetramer, showing both symmetry mates of the ligand.
SB-14 provided a rather modest +1.1 °C and +1.3 °C thermal shift, respectively. Interestingly, we observed that SB-11 markedly elevated the Tm more (+3.0 °C) than SB-14 (Table 1). In addition, in line with this thermal stabilization, SB-11 was a better fibrillation inhibitor at pH 4.4 than resveratrol and SB-14 (Table 1). The same trend of SB-11 activity outperforming resveratrol and SB-14 was also true for the TTR FAP mutation V30M both regarding thermal stability and fibril inhibition (Table 1). The stabilizing effect of SB-11 is consistent with the conformational rigidity of Ser117 and differences in the hydrogen-bonding networks observed when comparing our structures. Although the ligands in this study are not negatively charged at the pH of our experiments, the forward binding mode of SB-11 orienting the hydrophobic naphthalene toward HBP3 and polar groups toward the exposed Lys15 is consistent with previous structures for flufenamic acid,20 N-phenyl phenoazines,21 and Tafamidis.8

**Figure 3.** Structural analogues SB-11 and SB-14 bind to TTR in opposite orientations. (A, B) The electron density in the BB′ T4BP prior to ligand placement (A, SB-11; B, SB-14) is shown with 2mFo-Fc in blue mesh contoured at 1.5σ and mFo-Fc as green/red mesh contoured at 3.5σ. (C) SB-11 showing exclusively forward binding mode with the polar dihydroxy-benzene ring outside toward HBP1 and the opening of the T4BP while the naphthalene moiety sits inside HPB3. (D) Superposition of both symmetry mates of SB-11 (light/dark gray). (E) SB-14 showing exclusively reverse binding mode with the dihydroxy-benzene ring inside HPB3 of the T4BP and the naphthalene pointing toward HBP1. (F) Both symmetry mates of SB-14 are shown. Averaging of the density at the special position could explain the lack of electron density for the asymmetrically superposing naphthalene ring in this orientation. In panels C–F, the 2mFo-Dfc electron density contoured at 1.5σ is shown as blue mesh and the mFo-Dfc difference density contoured at 3σ in green (pos)/red (neg).
SB-11’s fluorescence ratio was instead rather unaffected by pH in the range pH 5−10 (Figure 5A). The binding mode observed in the TTR:SB-11 structure positions the naphthalene moiety inside HBP3, now explaining its reduced pH sensitivity (Figure 5B). While the different positioning explains the different pH sensitivity of SB-14 versus SB-11, it does not explain the green fluorescence (500 nm) which interestingly was unique for TTR-T4BP binding for both ligands compared to fibril binding.

### CONCLUSIONS

Misfolding and aggregation of TTR is associated with numerous gain-of-toxic function amyloid diseases called TTR amyloidoses with deposited ATTR. Today, there are many treatment options for ATTR diseases including liver transplantation, siRNA, and antisense oligonucleotides (ASOs) for modifying TTR expression as well as small molecule kinetic stabilizers to avoid tetramer dissociation and TTR misfolding and amyloid formation. There is interest in generating new and improved treatment options, but early diagnosis is even more urgent, which is the key for effective treatment. We have
previously identified fluorescent ligands to report on native tetrameric and misfolded protofibrillar TTR.\textsuperscript{18} Ligands were based on the \textit{trans}-stilbene resveratrol. We concluded based on pH-dependent fluorescence spectroscopy experiments that two structural analogues of \textit{trans}-stilbenes bound with opposing directions within the T4BP. Our high-resolution crystal structures of these complexes presented in this study now confirm that the binding modes are indeed opposite, albeit contrary to our prediction. The consequences of the different ligand binding modes to TTR are important, both for fluorescence and stabilization. Our data provides the structural basis for two critical parameters to facilitate the design of TTR fluorescent ligands and inhibitors based on \textit{trans}-stilbene scaffolds.

\section*{Materials and Methods}

\textbf{Chemicals.} Chemicals were purchased from Sigma–Aldrich; columns and resins were from Cytiva. All chemicals used in the experiments were of reagent grade quality. Synthesis of ligands SB-11 and SB-14 was as previously reported.\textsuperscript{18}

\textbf{Recombinant Expression and Purification of Wild-Type TTR and the TTR V30M Mutant.} Expression and purification of TTR were carried out as described previously.\textsuperscript{2,3} The TTR wild-type and the V30M mutation were expressed and purified the same way. Briefly, \textit{Escherichia coli} BL21 (DE3) cells were transformed with the different plasmids and grown at 37 °C. At an OD\textsubscript{560} of 0.6, the temperature was lowered to 20 °C and TTR expression was induced by addition of 0.4 mM IPTG. After 18 h, cells were harvested, resuspended in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and disrupted by sonication. After clarification the supernatant was heated to 60 °C for 30 min. Subsequently, the precipitated material was removed by centrifugation and the supernatant was filtered (0.45 μm) followed by anion exchange (Source-15Q 10/10) and size exclusion chromatography (HiPrep 16/60 Superdex75) in 10 mM Na-phosphate buffer, 100 mM KCl pH 7.6 at 20 °C. Fractions containing pure TTR were collected, pooled, and concentrated to 5.2 mg/mL, prior to flash-freezing in liquid nitrogen and storage at −80 °C until use.

\textbf{Crystallographic Analyses.} The protein was crystallized using the vapor-diffusion hanging drop method at room temperature as described previously.\textsuperscript{23} Briefly, purified TTR (5.2 mg/mL) was cocrystallized with either 500 μM SB-14 or SB-11 or 2 mM resveratrol (added from DMSO stock solutions (10 mM)). Drops containing 3 μL protein solution were mixed with 3 μL precipitant and equilibrated against 1 mL reservoir solution containing 1.3–1.6 M sodium citrate pH 5.5 and 3.5% v/v glycerol. Crystals grew to 0.1 × 0.1 × 0.4 mm after 5–7 days. Crystals were transferred into drops containing the same concentration of ligand and protein and incubated for 3 days. Crystals were cryo-protected with mother liquor supplemented with 12.5% v/v glycerol and ligand.

\textbf{Structure Determination.} Diffraction data of the "apo", and SB-11, SB-14, and resveratrol complex forms of TTR were collected under cryogenic conditions at BioMax (MAXIV), Sweden, at a wavelength of 0.97993 Å. Data were processed to a resolution of 1.15 Å, 1.45 Å, 1.45 Å, 1.35 Å, respectively, using XDS\textsuperscript{24} and AIMLESS\textsuperscript{25} from the CCP4 software suite.\textsuperscript{26} Data collection statistics are summarized in Table 2.

Phasing was by molecular replacement using Phaser\textsuperscript{27} with a search model derived from the published coordinates 1F41.\textsuperscript{28} (omitting terminal residues and a known flexible region). Ligands and solvent were placed in density after 1 to 2 initial rounds of rebuilding the protein model with COOT\textsuperscript{29} and refinement using REFMAC.\textsuperscript{30} After placing the ligands, a further 2 to 3 iterations of rebuild/REFMAC refinement were performed with ligand occupancy increasing when appropriate.

Ligand atoms were initially placed at 0.3 occupancy if clearly visible in the electron density; otherwise, occupancy was set at 0.1. Occupancy was increased in line with (i) developing density and (ii) consistency with B-factors of surrounding atoms/residues. Due to the positioning of compounds (on the special position) the maximum occupancy is 0.5.

Because of the lack of interpretable electron densities in the final map, nine N-terminal (residues 1–9) and two or three C-terminal residues were not included in the final model. A summary of the crystallographic analyses is presented in Table 2.

\textbf{Coordinates.} Structure factors and coordinates of the TTR (apo), TTR:SB-11, TTR:SB-14, and TTR:resveratrol complexes have been deposited at the PDB (accession codes: 8AWI, 7Q9L, 7Q9N, and 7Q9Q, respectively).

\textbf{Fibril Formation Assay.} TTR (2.0 mg/mL, in PBS buffer) was preincubated with or without 2 equivmolar concentrations of inhibitor

![Figure 5](https://example.com/figure5.png)

\textbf{Figure 5.} Fluorescence sensitivities of SB-11 and SB-14 toward pH are dependent on binding orientation. (A) Fluorescence spectra dependency on pH plotted as intensities of the 500 and 390 nm emissions (excitation at 350 nm). Ligands alone (open triangles and circles) are poorly fluorescent and are not pH sensitive. SB-14 bound to TTR (closed triangles) is highly sensitive with an apparent \( pK_a \) around 8.5. SB-11 is not sensitive to pH when bound to TTR (closed circles). (B) SB-11 with forward binding mode is insensitive to pH. (C) SB-14 with reverse binding mode positioning the naphthalene facing Lys15 at the opening of the T4BP.
were calculated for each protein with and without ligands (1% DMSO vehicle). Optical density at 400 nm was set to 100% in the absence of inhibitor for each sample. Fibril formation as measured by turbidity (absorbance/fluorescence signal) was induced by 10-fold dilution to a final concentration of 0.2 mg/mL in 50 mM sodium acetate buffer, 100 mM NaCl, final pH 4.4. Samples were prepared the same way as for fibril formation with the exception of 0.5 mM sodium ions 1 glycerol molecule 1 resveratrol molecule 2 SB-11 molecules 2 SB-14 molecules.

**Protein Thermal Stability.** Samples for the thermal shift assay were prepared the same way as for fibril formation with the exception of pH 5.0 as the final pH. Samples were measured by nano-DSF using the Prometheus NT-48 (Nanotemper). After sealing the capillaries, the thermal scan was performed from 20 to 110 °C, with a ramp rate of 0.5 °C/min and recording the 330 and 350 nm intrinsic tryptophan fluorescence signal. Samples were assayed in triplicate. The inflection point of the first derivative of the Trp fluorescence monitored 350/330 nm unfolding curve was denoted as the melting temperature (Tm). The mean Tm and standard deviation of three separate samples were calculated for each protein with and without ligands (1% DMSO vehicle).

**Table 2. Data Collection and Refinement Statistics**

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<sup>a</sup>Values in parentheses are for the highest resolution shell.

### AUTHOR INFORMATION

**Corresponding Authors**

Per Hammarström — Linköping University, IFM-Department of Physics, Chemistry and Biology, 58183 Linköping, Sweden; orcid.org/0000-0001-5827-3587

Eleonore von Castelmur — Linköping University, IFM-Department of Physics, Chemistry and Biology, 58183 Linköping, Sweden; orcid.org/0000-0001-7061-4890

**Authors**

Afshan Begum — Linköping University, IFM-Department of Physics, Chemistry and Biology, 58183 Linköping, Sweden

Jun Zhang — Linköping University, IFM-Department of Physics, Chemistry and Biology, 58183 Linköping, Sweden

Dean Derbyshire — Linköping University, IFM-Department of Physics, Chemistry and Biology, 58183 Linköping, Sweden

Xiongyu Wu — Linköping University, IFM-Department of Physics, Chemistry and Biology, 58183 Linköping, Sweden; orcid.org/0000-0001-6756-2276

Peter Konradsson — Linköping University, IFM-Department of Physics, Chemistry and Biology, 58183 Linköping, Sweden

Complete contact information is available at:

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Author Contributions
P.H., A.B. and E.v.C. designed the study. A.B., P.H. and J.Z. conducted experiments. D.D., A.B. and E.v.C. collected X-ray data and solved the structures. P.K. and X.W. synthesized the ligands. A.B., D.D., P.H. and E.v.C. wrote the paper. All authors discussed the results and revised the paper.

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Notes
The authors declare no competing financial interest.

REFERENCES


[Further references follow, including details on specific studies and methodologies related to the inhibition of transthyretin and its role in amyloid aggregation, highlighting the role of various ligands and the impact on the inhibition of amyloid formation.]


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