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Ligand binding at the transthyretin dimer–dimer interface: structure of the transthyretin–T4Ac complex at 2.2 Å resolution

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The crystal structure of the complex of human transthyretin (hTTR) with 3,3′,5,5′-tetraiodothyroacetic acid (T4Ac) has been determined to 2.2 Å resolution. The complex crystallizes in the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 43.46, b = 85.85, c = 65.44 Å. The structure was refined to R = 17.3% and $R_{\rm free}$ = 21.9% for reflections without any σ-cutoff. T4Ac is bound in both the forward and the reverse mode in the two binding sites of hTTR. In the forward orientation, T4Ac binds in a position similar to that described for thyroxine (T4) in the orthorhombic hTTR–T4 complex. In this orientation, the iodine substituents of the phenolic ring are bound in the P3′/P2 halogen pockets. In the reverse orientation, which is the major binding mode of T4Ac, the ligand is bound deep in the TTR channel, with the carboxylic group bound in the P3′ pocket and forming simultaneous polar interactions with the residues constituting the two hormone-binding sites. Such interactions of a thyroxine-analogue ligand bound in the reverse mode have never been observed in TTR complexes previously.

1. Introduction

Transthyretin (TTR) is a 54 kDa transport plasma protein present in mammals, birds and reptiles. The protein circulates as a homotetramer and binds thyroxine (3,5,3′,5′-tetraiodo-L-thyronine; T4) and related compounds. The relative binding affinities of thyroid hormone analogues and metabolites to TTR reveal that specific structural characteristics enhance optimal hormone binding. Tetraiodothyroacetic acid (T4Ac), a thyroxine competitor, binds to TTR with 2.8 times the affinity of T4 (Jorgensen, 1978).

Structural data show that the TTR tetramer has molecular D_2 symmetry (Figs. 1 and 2), with four identical 127-residue subunits forming a central channel with two T4-binding sites (Blake *et al.*, 1978). Each monomer is a β-sandwich formed by eight strands labelled *A* to *H* organized into two antiparallel β-sheets, one forming the channel surface (strands *A*, *D*, *G* and *H*) and the other constituting the external surface of the tetramer (strands *B*, *C*, *E* and *F*). In the TTR tetramer, the two sterically equivalent binding sites differ in their hormone-binding affinity. The first T4 molecule binds with $K_a = 10^8\ M^{-1}$ and the second with $K_a = 10^6\ M^{-1}$ (Cheng *et al.*, 1977). A mechanism of negative cooperativity (NC) has been invoked to explain the differences in the binding affinity (Cheng *et al.*, 1977) and its structural basis has been proposed (Wojtczak, Neumann *et al.*, 2001; Neumann *et al.*, 2001).

Currently, more than 50 crystal structures of apo TTR from different sources and its complexes with T4 or its competitors have been determined by X-ray crystallographic methods (Wojtczak *et al.*, 1992, 1993, 1996; Wojtczak, Cody *et al.*, 2001;

Wojtczak, Neumann *et al.*, 2001; Cody *et al.*, 1991; Ciszak *et al.*, 1992; Hornberg *et al.*, 2000; Klabunde *et al.*, 2000; Morais-de-Sá *et al.*, 2004 and references cited therein). Most human TTR crystals have orthorhombic $P2_12_12$ symmetry with two TTR monomers (*A* and *B*) in the asymmetric unit (Figs. 1 and 2). The only exceptions are the monoclinic structures of wild-type hTTR with two tetramers in the asymmetric unit (Wojtczak, Neumann *et al.*, 2001), the TTR–diethylstilbestrol complex (Morais-de-Sá *et al.*, 2004) and the L55P variant (Sebastiao *et al.*, 1998). In orthorhombic hTTR, the twofold axis coincides with the axis of the protein channel. The lack of such symmetry in T4 or in its competitors results in disorder of the ligand when bound to hTTR. Consequently, the precise determination of the protein–ligand interactions is difficult.

Wild-type transthyretin is able to self-assemble and to form amyloid fibrillar structures, producing neurotoxicity and organ dysfunction (Miroy *et al.*, 1996 and references cited therein). About 80 naturally occurring single-point mutations are responsible for onset of the TTR-associated amyloidosis called familial amyloid polyneuropathy (FAP; Hornberg *et al.*, 2004). These mutations do not affect the structure of the ligand-binding sites significantly, but rather destabilize the tetramer and facilitate the amyloidogenic intermediate formation by perturbing either the thermodynamics or kinetics of the folding/denaturation pathway (Hornberg *et al.*, 2004; Hammarström *et al.*, 2003). Electron paramagnetic resonance (EPR) data indicated that major conformational changes in the *CD* β-strand fragment are necessary for the reassembly of TTR into amyloid (Serag *et al.*, 2002). Research on aggregation of the TTR monomers revealed that amyloid formation is a downhill polymerization and the highest energy entity is a native TTR monomer (Hursman *et al.*, 2004). Calculations using continuum electrostatic techniques revealed the contribution of some residues to the TTR tetramer/dimer stability (Skoulakis & Goodfellow, 2003). Surprisingly, these results also showed an effect of Lys15 and Glu54, residues that are involved in ligand binding rather than tetramer formation. However, the mechanism of rearrangement and dissociation of TTR to form monomeric intermediates and subsequently to form amyloid deposits is still unclear. Thyroxine and other ligands are reported to stabilize the TTR tetramer and prevent the conformational changes leading to amyloid formation (Klabunde *et al.*, 2000; Miroy *et al.*, 1996). Two hotspots for TTR–ligand interactions were suggested by structure analysis. The first is located at the tetramer centre, near the loops *AB* and *FG* that form the dimer–dimer interface. The second region important for the tetramer stabilization consists of β-strands *A* and *D* and loop *CD*, which are shown to partly unfold and start the amyloidogenic transformations of the TTR tetramer (Serag *et al.*, 2002). The binding interactions of the ligand bridging the two surfaces of the binding sites also seem to be of great importance for tetramer stabilization. Research on TTR fibril formation or tetramer stabilization (Hursman *et al.*, 2004; Skoulakis & Goodfellow, 2003) has emphasized the impor-

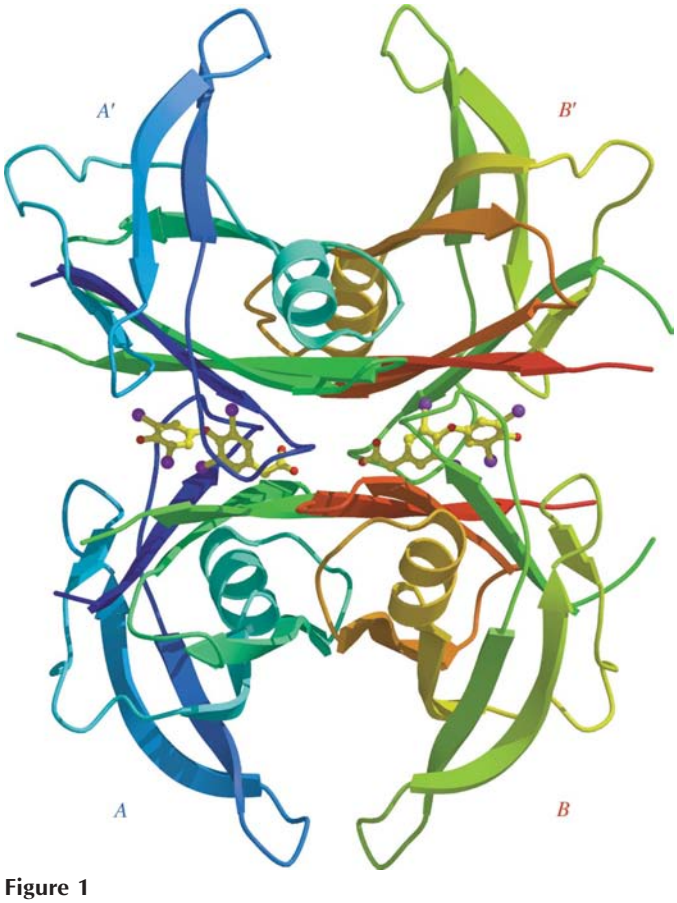

Figure 1
The hTTR–T4Ac structure. The whole TTR tetramer is generated by twofold symmetry from the dimer. In both binding sites the T4Ac ligand molecules are shown in a reverse orientation.

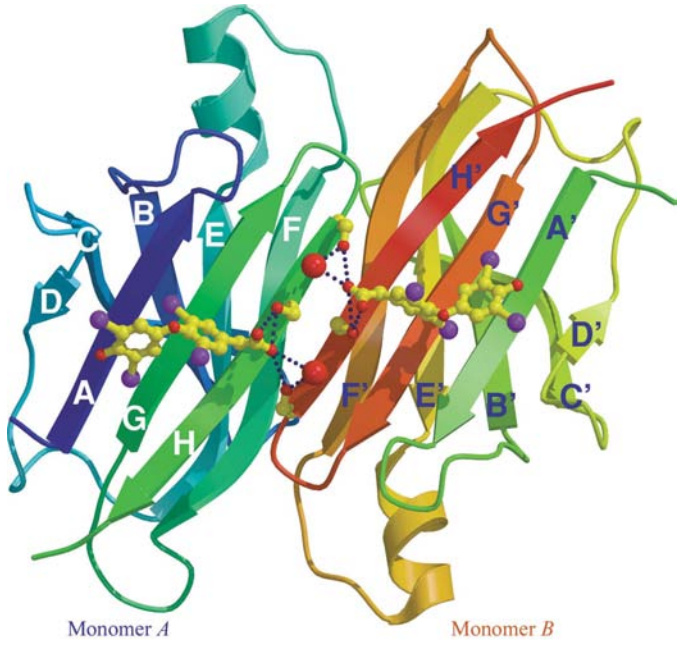

Figure 2
Monomers *A* and *B* forming an asymmetric unit of the structure, with the T4Ac ligands (in reverse mode) indicating the location of two binding sites. The dotted lines indicate the simultaneous interactions between the carboxylic group of the ligand molecules and amino acids constituting two binding sites of transthyretin.

tance of the ligand interactions in these regions. Many of the investigated ligands reveal a binding affinity to TTR that is higher than that of thyroxine. One such efficient TTR ligand is 3,5,3′,5′-tetraiodothyroacetic acid, with a binding affinity 2.8 times that of T4. Analysis of the differences in the binding interactions between thyroxine, which is known to inhibit TTR-related amyloidosis, and its potent competitor T4Ac might give an insight into subtle ligand effects on TTR stabilization.

We previously reported the structure of the T4Ac complex of rat TTR, which has 85% sequence homology with the human protein (Muzioł *et al.*, 2001). The goal of the present work is to determine the possible orientations of a high-affinity ligand in the human TTR-binding channel and to compare the results with the T4Ac binding in rat transthyretin. The determination of binding interactions of T4Ac may provide insight into the structural aspects of TTR stabilization and permit the design of new ligands with even greater affinity.

2. Experimental

Human transthyretin was purified as described previously (Wojtczak *et al.*, 1992). Protein was incubated for 5 d at 277 K with an excess of T4Ac, then crystallized using HANGMAN (Luft & DeTitta, 1992) and the hanging-drop vapour-diffusion method from 48% ammonium sulfate, 0.1 *M* phosphate buffer pH 5.5. The droplet contained 2 µl protein solution and 2 µl reservoir solution. The diffraction data were collected from a 0.3 × 0.5 × 0.5 mm orthorhombic crystal to 2.2 Å resolution at 293 (1) K on a Rigaku R-AXIS II imaging-plate system with a rotating-anode source generating Cu $K\alpha$ radiation (λ = 1.5418 Å). The completeness of the data within the 43.5–2.2 Å resolution range is 94.5% and in the highest resolution shell (2.3–2.2 Å) it is 79.2%. The mean $I/\sigma(I)$ ratio is 22.9 for all data and 3.2 for the highest resolution shell. The $R_{\rm merge}$ for the whole data set is 4.8%.

The structure is isomorphous with other orthorhombic hTTR structures. The model for initial phasing was based on the hTTR–T4 complex (PDB code 2rox; Wojtczak *et al.*, 1996). Refinement was carried out with the *CNS* program (Brünger *et al.*, 1998) and manual fitting to electron-density maps was performed with the *O* program (Jones *et al.*, 1991). Over 8% (1035) of the reflections were randomly selected for an $R_{\rm free}$ test set to monitor the refinement process (Kleywegt & Brünger, 1996). The refinement was performed against the maximum-likelihood (ML) target with bulk-solvent correction. The repulsive parameters for protein and water atoms, as included in the current release of *CNS*, were used. All electron-density maps used in manual fitting were $\sigma_{\rm A}$-weighted. The ligand-parameter and topology files were based on the crystal structure of 3,3′,5,5′-tetraiodothyroacetic acid (Cody *et al.*, 1977) and AM1 quantum-chemical calculations (Nowak & Wojtczak, 1997). The $F_{\rm o} - F_{\rm c}$ and $3F_{\rm o} - 2F_{\rm c}$ maps revealed density corresponding to T4Ac bound in the forward and reverse orientations in both binding sites. The position of iodine substituents of T4Ac was confirmed by the $\sigma_{\rm A}$-weighted ML omit maps (Fig. 3). The position of T4Ac in the binding sites was included in further refinement.

The final model consists of residues 10–125 in monomer *A* and residues 8–125 in monomer *B*, as well as 86 water molecules and four T4Ac ligand molecules. Attempts to locate the missing residues at the N- and C-termini failed because of poorly defined electron-density maps, which is likely to be a consequence of high flexibility of these surface regions. Multiple side-chain conformations have been found for 12

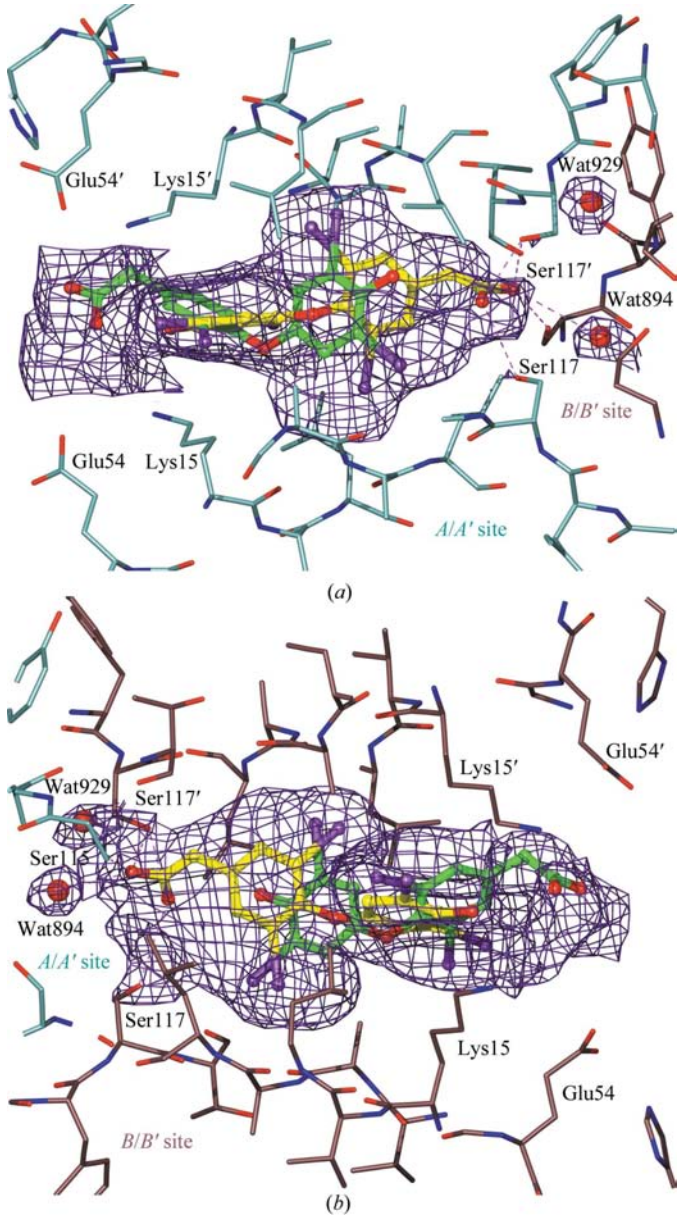

Figure 3
Omit $\sigma_{\rm A}$-weighted maximum-likelihood map at 1.5σ (blue) reveals the ligand orientation (*a*) in the *A*/*A*′ binding site and (*b*) in the *B*/*B*′ binding site. The T4Ac ligands in the forward and reverse orientation are coloured green and yellow, respectively. The electron-density maps correspond to the twofold symmetry of ligand binding in the channel, but the symmetry-related ligand molecules are not displayed for clarity. The water molecules mediating the T4Ac interactions in the reverse mode are labelled. Amino acids forming the *A*/*A*′ binding site are coloured blue, while those forming the *B*/*B*′ site are coloured brown.

Table 1
Refinement statistics for the hTTR–T4Ac complex.

Space group	$P2_12_12$
Unit-cell parameters (Å)	a = 43.462, b = 85.585, c = 65.443
Resolution range (Å)	43.5–2.2
Reflections measured (% of those possible)	11346 (94.5)
R_{merge}	0.048
Reflections ($F > 0.0\sigma$) used in refinement (% of those possible)	11295 (87.4)
R factor/No. of reflections used	0.173/10262
R_{free}/No. of reflections used	0.219/1033
Protein atoms	1863
Water molecules	86
B factor (Wilson) (Å^2)	26.60
B factor (average) (Å^2)	34.57
B factor (protein) (Å^2)	33.97
B factor (water) (Å^2)	42.14
R.m.s. deviations from ideality	
Bond distances (Å)	0.007
Angles (°)	1.411
Impropers (°)	0.945
Dihedrals (°)	23.60
Ramachandran statistics (most favoured/allowed) (%)	91.2/8.8
Positional Luzzati error (working/test set) (Å)	0.22/0.29
Highest resolution bin statistics	
Resolution range (Å)	2.30–2.20
Completeness (working + test sets) (%)	73.4
R value/No. of reflections in bin (working set)	0.233/1060
R value/No. of reflections in bin (test set)	0.313/102

residues (monomer *A* Cys10, Met13, Arg34, Asp39, Glu66, Asp74, Arg103, Arg104 and Ser112, and monomer *B* Lys35, Ser77 and Glu92) and their occupancies were refined. The final refinement gave R = 17.29% and R_{free} = 21.88% for 10 262 working and 1033 test reflections, respectively. The individual B factors of all atoms in the model were also refined. The bulk-solvent parameters were refined in *CNS* to 0.29 e Å^{-3} and a B factor of 42.14 Å^2. Conformational statistics calculated with the program *PROCHECK* (Laskowski *et al.*, 1993) show 91.2% of the residues to be in the most favored regions and the remaining 8.8% to be in additionally allowed regions of the Ramachandran plot. The coordinates and structure factors have been deposited in the Protein Data Bank (Berman *et al.*, 2000) with PDB code 1z7j. A summary of structure solution and refinement is given in Table 1.

3. Results and discussion

3.1. Binding modes of 3,5,3′,5′-tetraiodothyroacetic acid (T4Ac) to hTTR

Statistical disorder of the ligand was detected in each binding site, with the T4Ac ligand bound in both the forward and the reverse orientation (Fig. 3). The ligand positions are almost identical in both binding sites. In all orientations, the ligand has a skewed conformation (Cody, 1980), similar to that found for thyroxine in its hTTR complex (Wojtczak *et al.*, 1996) or in the small-molecular structures of T4Ac and T4 (Cody *et al.*, 1977; Cody, 1981). In the forward ligand orientation the phenolic I3′ and I5′ atoms are positioned in the P3 and P2′ binding pockets (described in Wojtczak, Cody *et al.*, 2001) as in the thyroxine hTTR–T4 complex (Wojtczak *et al.*, 1996). The phenolic hydroxyl group of T4Ac does not form any polar interactions at the tetramer centre. It occupies a similar space in the internal P3 pocket as the CF_3 group of PHENOX (*N*-*m*-trifluoromethylphenyl-phenoxazine-4,6-dicarboxylic acid; Klabunde *et al.*, 2000) (Fig. 4). Analogous to the hTTR–T4 complex (Wojtczak *et al.*, 1996), the phenolic I atoms positioned in the P2′ pocket interact with the main-chain C=O and NH groups, the shortest distance being 2.82 Å for Ala109 O···I5′ in the *A*/*A*′ site and 3.02 Å for Ala109 O···I5′ in the *B*/*B* site. The T4Ac carboxylic group forms weak polar interactions with the β-strand *A* Lys15 side chain near the entrance to the binding channel. A 2.87 Å contact between the T4Ac carboxylic group and Glu54 was also found in the *A*/*A*′ site. The lack of electron density corresponding to the water molecule mediating this contact suggests the protonation of one of these groups. An alternative explanation might be the electrostatic compensation by the ammonium group of Lys15. Similar interactions of two negatively charged groups were observed in the hTTR–PHENOX complex (PDB code 1dvy; Klabunde *et al.*, 2000) in monoclinic and orthorhombic hTTR–T4 (PDB codes 1ict and 2rox; Wojtczak, Neumann *et al.*, 2001; Wojtczak *et al.*, 1996) as well as in rTTR–T4Ac (Muzioł *et al.*, 2001; PDB code 1kgi).

The structure also reveals reverse binding of T4Ac (Fig. 3), with the occupancy slightly higher in both binding sites than that for the forward mode. The T4Ac ligand is the first L-thyronine derivative for which reverse binding has been observed in the TTR complex. In this orientation, the phenolic O4′ atom near the channel entrance forms polar interactions with Lys15. In each binding site, the carboxylic group of the T4Ac ligand bound in the reverse mode is positioned deep in the channel in the P3 pocket. It forms polar interactions with

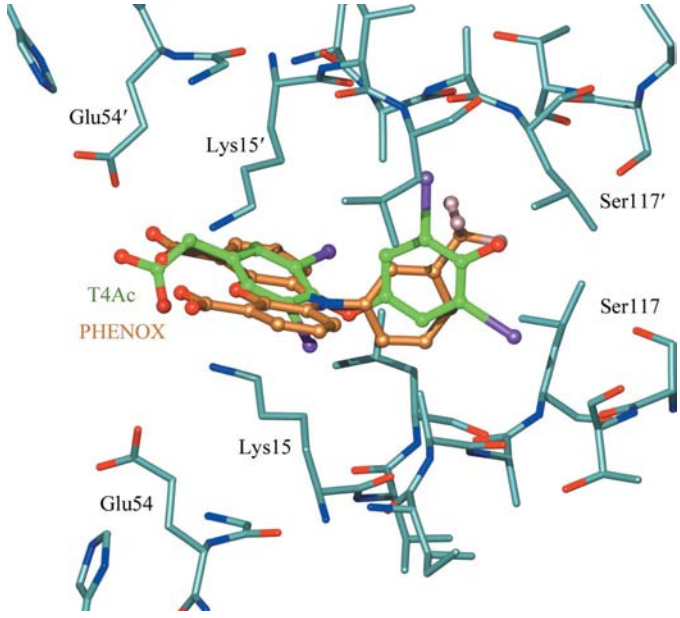

Figure 4
Superposition of hTTR–T4Ac in a forward mode (green) and PHENOX (gold) (Klabunde *et al.*, 2000). The carboxylic groups of both ligands interact with the side chains of Glu54 (β-strand *D*) and Lys15 (β-strand *A*).

the Ser117 residues of two monomers forming the binding site and with Ser115 of the second binding site across the dimer–dimer interface. Such penetration of the interface between two binding sites is unique for the T4Ac ligand and has not been found for any other T4 derivative in TTR. In particular, the non-planar alanyl moiety of T4 or its analogues (Wojtczak *et al.*, 1992, 1996) may be too large to penetrate the narrow space available at the TTR tetramer centre and prevents such a binding mode for those ligands. The only ligand reported to bind as deep as T4Ac is diethylstilbestrol (DES), which also penetrates the interface between two sites and hydrogen bonds to the ligand molecule of the second site (Morais-de-Sá *et al.*, 2004). In the reverse orientation, the I atoms of T4Ac occupy both the P2/P2′ and P1/P1′ pockets and form several polar interactions with the main-chain nucleophilic groups of Ala109 and Leu110, analogous to those found for T4Ac in the rat TTR complex (Muzioł *et al.*, 2001). These interactions seem to be crucial for positioning the T4Ac ligand in the binding channel. The iodine interactions positioning the ligand are almost identical in all binding sites of human and rat TTR complexes and result in a similar orientation of T4Ac bound in the reverse mode.

The O8 atom of the carboxylic group of T4Ac is positioned similar to the hydroxyl or carbonyl O atoms of other ligands that are bound more deeply in the TTR channel than thyroxine, such as 3′,5′-dinitro-*N*-acetyl-L-thyronine (DNNAT; Wojtczak *et al.*, 1996) and diethylstilbestrol (Morais-de-Sá *et al.*, 2004) (Fig. 5), the stilbene derivative resveratrol (Klabunde *et al.*, 2000), 3,3′-diiodo-L-thyronine (3,3′-T2) and the inotropic agent milrinone (Cody *et al.*, 1991; Wojtczak *et al.*, 1992, 1993). In this way, T4Ac in a reverse mode forms a similar network of polar binding interactions to the OH group or its analogue in the molecules of the ligands mentioned above.

The polar interactions of the OH group mentioned above are not the decisive factor that contributes to efficient binding, since weak competitors for T4 binding to TTR, such as 3,3′-T2 and DNNAT, are bound as deeply in the channel as the efficient T4 competitors milrinone (Wojtczak *et al.*, 1993) or diethylstilbestrol (Morais-de-Sá *et al.*, 2004) and form similar hydrogen bonds to the pair of Ser117 residues at the tetramer centre. The most significant differences in the binding affinity may be caused by penetration of the dimer–dimer interface by the T4Ac ligand in the reverse mode as described above. These interactions at the interface also explain the much higher binding affinity of T4Ac compared with that of T4. The TTR–ligand interactions in the reverse mode are so efficient that this orientation is slightly more populated in each site, with the refined occupancy of T4Ac being 0.22 and 0.20 in the *A*/*A*′ and *B*/*B*′ sites, respectively. The occupancy of the ligand in the forward orientation is 0.18 and 0.17 for the *A*/*A*′ and *B*/*B*′ sites, respectively. The global ligand occupancy in the binding sites reflects the twofold symmetry of the channel and is 0.80 and 0.74 for the *A*/*A*′ and *B*/*B*′ sites, respectively, indicating saturation of both binding sites. The binding modes reported for diethylstilbestrol (Morais-de-Sá *et al.*, 2004) indicate interactions between the ligand molecules positioned in two binding sites, either directly or mediated by the water molecule. However, owing to the positioning of the aromatic core of this ligand along the channel axis, it only forms weak interactions with the amino acids of the second binding site. Therefore, the mechanism of signal transmission between two sites, which possibly triggers the negative cooperativity, could be either direct *via* ligand–ligand interactions (diethylstilbestrol) or indirect *via* alterations in the hydrogen-bond network, as reported here for T4Ac or observed for T4 complexes (Wojtczak *et al.*, 1996; Wojtczak, Cody *et al.*, 2001).

In the *B*/*B*′ site of the reported complex a water molecule (Wat894) has been found deep in the P3 pocket near the dimer–dimer interface. This water molecule interacts with the O9 atom of the carboxylic group of the T4Ac ligand bound in the *A*/*A*′ site in the reverse orientation and is hydrogen bonded to Ser115 of the *B*/*B*′ site. Therefore, the position of the carboxylic group of the *A*/*A*′-bound ligand at the interface is stabilized by the network of polar interactions formed between O9 and Ser117 of the *A*/*A*′ site and Ser115 and Wat894 of the *B*/*B*′ site (Fig. 3). The analogous water molecule (Wat929) was found in the *A*/*A*′ site. Superposition of the two monomers (calculated for the C^{α} atoms of residues 10–125) reveals that the positions of the water molecules (Wat894 and Wat929) are within a distance of 0.2 Å. These water molecules are equivalent to those found in the P3 pockets in rat TTR–T4 complexes (Wojtczak, Cody *et al.*, 2001). On the other hand, the positions of water molecules deep in the P3 pockets and at the dimer–dimer interface are similar to those reported for the low-temperature structure of apo hTTR determined at 1.5 Å resolution (Hornberg *et al.*, 2000). In this structure, the Ser117

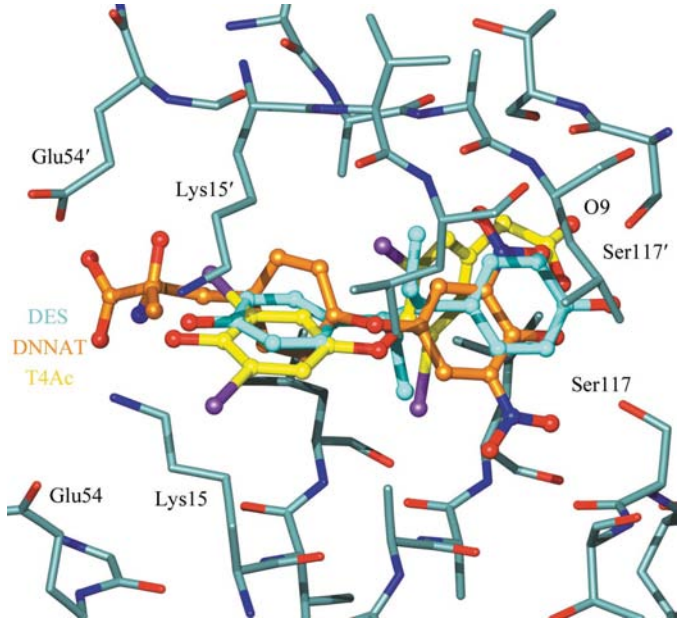

Figure 5
Binding of different ligands near Ser117 at the tetramer centre. Superposition of T4Ac in a reverse mode (yellow), DNNAT (gold; Wojtczak *et al.*, 1996) and diethylstilbestrol (cyan; Morais-de-Sá *et al.*, 2004). The O9 atom of T4Ac penetrating the interface between two binding sites is labelled.

residues at the tetramer centre reveal conformational disorder of the side chains, resulting in the network of hydrogen bonds formed along the interface and to the water molecules inside the P3 pockets. The polar groups of the ligand positioned deep in the binding channel determine the preferred position of the serine hydroxyl groups by formation of polar interactions. Therefore, it might be expected that it is the polar moiety of the ligand that triggers signal transmission between the sites by altering the hydrogen-bond network linking the two binding sites of transthyretin.

3.2. Comparison of T4Ac binding in human and rat TTR

In hTTR the ligand is bound in the alternative forward and reverse orientations in both binding sites. In the rat TTR complex the reverse mode of T4Ac binding was found only in the *A*/*C* binding site (Muzioł *et al.*, 2001). The conformation of the ligand molecule in the reverse orientation is similar in both human and rat complexes. The comparison reveals that the position of the acetate moiety of T4Ac differs in the two complexes. In human TTR, the carboxylic groups of the ligand form direct interactions with the Ser115 O^{γ} hydroxyl groups of other binding site, while in rat TTR complex this interaction is mediated by a water molecule (at a distance of 2.92 Å). This is a result of the different conformation of the Ser*B*115 side chain (χ^1 in human TTR is 170°, while in rat TTR it is 89°).

In the reported complex the position of the ligand in the forward mode, which is identical in both binding sites, is similar to that reported for thyroxine in the hTTR complex (Wojtczak *et al.*, 1996). The skewed conformation of the ether bridge of the T4Ac molecule is also identical in both sites (the φ/φ' torsion angles of the ether bridge are −93/−15°) and is similar to the reported conformation of the T4 ether bridge in the hTTR–T4 complex (Wojtczak *et al.*, 1996). However, in the rat TTR complex, the T4Ac ligand reveals different conformations reflecting the differences in positioning in the forward mode in the two sites (Muzioł *et al.*, 2001). In the *A*/*C* site its phenolic iodine substituents occupy the P3 and P3′ pockets and the ether-bridge φ/φ' torsion angles are −91/43°, respectively. The phenolic ring of T4Ac is positioned about 2 Å deeper than that found in the human TTR complex and the ligand molecule is tilted off the channel axis towards the channel wall. In the *B*/*D* site of the rat TTR complex two forward binding modes of T4Ac were reported. In one of them the orientation and conformation of the ligand (φ/φ' torsion angles of −91/−23°, respectively) is very similar to that found in the human TTR complex structure reported here. The second ligand (in a similar conformation) is shifted about 1.5 Å towards the channel entrance compared with the ligand in the hTTR complex. Since the binding sites of both rat and human transthyretin are identical, this proves the ability of TTR to bind the ligands in alternative positions and suggests multiple local energy minima for the TTR–ligand interactions. This observation is consistent with the reported variable positions of T4 and T3 bound to piscine TTR (Eneqvist *et al.*, 2004).

3.3. Interactions stabilizing the structure of the TTR tetramer

Interactions formed by the phenolic OH (reverse mode) or carboxylic group (forward mode) to Lys15 or Glu54 may play a role in preventing the conformational changes in the 42–59 fragment of each monomer. This fragment, corresponding to β-strands *C* and *D*, has been suggested as the element of the monomeric β-sandwich that initiates the conformational changes leading to TTR aggregation (Hammarström *et al.*, 2003; Serag *et al.*, 2002). A small-molecule ligand, diclofenac, has been approved by the US Food and Drug Administration (FDA) as a TTR-associated amyloidosis inhibitor (Hammarström *et al.*, 2003). Diclofenac binds in a similar reverse orientation as T4Ac in the reported complex. However, it is positioned closer to the binding-channel entrance than T4Ac. The diclofenac binding pattern consists of interactions with Ser117 and hydrophobic interactions with Lys15, as well as the Leu and Ala side chains flanking the halogen-binding pockets of TTR (Klabunde *et al.*, 2000). It has been shown that another TTR-associated amyloidosis inhibitor, resveratrol, does not interact in the halogen-binding pockets of TTR (Klabunde *et al.*, 2000). However, by forming a hydrogen bond to Ser117 at the tetramer centre and bridging two monomers of a binding site *via* the pair of water-mediated hydrogen bonds to Lys15 side chains, it remains an efficient inhibitor (Klabunde *et al.*, 2000; Hammarström *et al.*, 2003). The T4Ac ligand seems to more rigorously satisfy the requirements of a good binder by interacting within the pockets (iodine substituents), with the amino acids of strands *A* and *D* at the channel entry and the polar side chains of strand *H* at the TTR centre. In all orientations the hydrophobic core of T4Ac interacts with Leu17 and Leu110 and Ala108 of the two TTR subunits forming each binding site. These interactions across the channel stabilize the TTR dimer constituting each binding site. The simultaneous interactions with Ser117 and Lys15/Glu54 correspond to those reported for resveratrol or diclofenac. The exceptional interactions of the carboxylic group of T4Ac in a deep reverse orientation are part of the forces stabilizing the whole TTR tetramer. This network of ligand interactions might therefore prevent tetramer dissociation, a process that is required for transformation between the functional protein and the amyloid aggregate.

4. Conclusions

These results provide a structural basis for understanding the high binding affinity of T4Ac to hTTR. In the forward mode, T4Ac has a skewed conformation and is bound in an orientation similar to that of T4 in the hTTR complex. However, the acetate group of T4Ac forms a different network of binding interactions to the alanyl moiety of T4, which results in the exceptional reverse-binding mode detected in the reported T4Ac complex. This reverse-binding mode is, in our opinion, responsible for the higher binding affinity of the T4Ac ligand (binding constant 2.75-fold higher than that of T4). The larger alanyl moiety of thyroxine or its analogues prevents binding in such an orientation without significant

conformational changes of the protein. The interactions of the carboxylic group of T4Ac with the amino acids of the β-strands *C* and *D*, the fragment that has been proposed to initiate the conformational changes in amyloid fibril formation, might stabilize the tetramer. In the reverse orientation, the carboxylic group of T4Ac forms polar interactions with Ser115 across the dimer–dimer interface. T4Ac is the first TTR ligand for which such unique interactions have been found. The penetration of the dimer–dimer interface by the carboxylic moiety of T4Ac in the reverse mode is possible because of the flexibility and relatively small size of this group. The exceptional interactions of the carboxylic group of T4Ac in a deep reverse orientation, bridging the two binding sites of TTR, also seem to be a part of the forces stabilizing the whole TTR tetramer. This network of ligand interactions, which are even more extensive than those reported for diclofenac or resveratrol, might therefore prevent tetramer dissociation, the step that is required for transformation between the functional protein and amyloid fibre. The unique interactions formed by T4Ac in the reverse orientation may also be important for disabling signal transmission between the two sites.

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