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QUARTERLY

Structural basis of negative cooperativity in transthyretin★✪

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A comparison of the AC and BD binding sites of transthyretin (TTR) was made in terms of the interatomic distances between the Cα atoms of equivalent amino acids, measured across the tetramer channel in each binding site. The comparison of the channel diameter for apo TTR from different sources revealed that in the unliganded transthyretin tetramers the distances between the A, D and H β-strands are consistently larger, while the distances between the G β-strands are smaller in one site than in the other. These differences might be described to have a 'wave' character. An analogous analysis performed for transthyretin complexes reveals that the shape of the plot is similar, although the amplitudes of the changes are smaller. The analysis leads us to a model of the changes in the binding sites caused by ligand binding. The sequence of events includes ligand binding in the first site, followed by a slight collapse of this site and concomitant opening of the second site, binding of the second molecule and collapse of the second site. The following opening of the first, already occupied site upon ligand binding in the second site is smaller because of the bridging interactions already formed by the first ligand. This explains the negative cooperativity (NC) effect observed for many ligands in transthyretin.

Interest in thyroxine (T4) binding to human serum transthyretin (TTR) has been stimulated by studies of this protein and its relation to amyloidosis [1–3]. Transthyretin is a stable homotetramer with a central channel running through the molecule (Fig. 1). The tetramers of human transthyretin (hTTR) in an orthorhombic form or chicken TTR in the hex-

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Abbreviations: NC, negative cooperativity; NCS, non-crystallographic symmetry; rms, root mean square; T4, thyroxine; TTR, transthyretin; hTTR, human TTR

agonal form have D_2 symmetry with a crystallographic two-fold axis relating the pairs of monomeric subunits (A and A′, B and B′) forming each binding site [4–14]. In the monoclinic form of hTTR or in rat TTR, the four monomers are not related by any crystallographic symmetry [15–17].

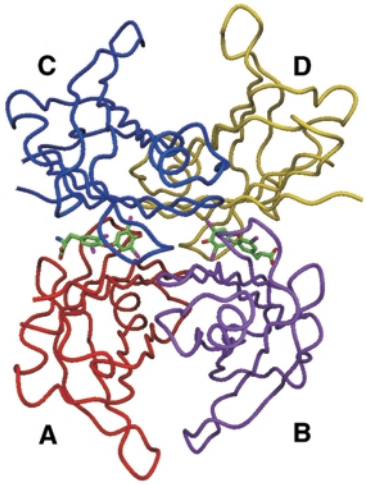

Figure 1. Tetramer of transthyretin (Cα trace) with two T4 molecules (atomic models) bound into central channel.

The monomers are labelled A, B, C and D, as in the structure of monoclinic polymorph of human TTR [15]. In the structures of the orthorhombic form, the two-fold-related C and D monomers are labelled A′ and B′, respectively.

Thyroxine (T4) and its various analogues and derivatives can be bound to the two binding sites of TTR. However, binding of the second ligand is much weaker than that of the first molecule. The TTR binding affinity for the ligand, as measured by equilibrium dialysis, revealed that the K_1 and K_2 association constants for the first and the second T4 molecule bound to TTR differ by a factor of about 100 ($K_1 = 1.0 \times 10^8$ M^{-1} and $K_2 = 9.5 \times 10^5$ M^{-1}) [18, 19]. This difference is explained by the negative cooperativity (NC) effect and has been described for several iodinated T4 analogues with similar binding constants [12, 20]. However, the fluorescent 8-anilino-1-naphtalenesulfonic acid (ANS) reveals a much smaller difference between the first and the second binding constants, with the K_1/K_2 ratio of 4.5 to 3.0 [18, 19]. An increase of the difference between the two binding constants with the decrease of pH was observed for 3-(4-hydroxy-3,5-diiodophenyl)-propionic acid (DIPA) [19]. This change was caused by a decrease of K_2, while K_1 remained almost unchanged. This observation was explained by the pH dependence of ionization of the DIPA phenolic hydroxyl group. These data also suggest that electrostatic interactions may contribute to the NC effect. A general mechanism can be introduced, which involves conformational changes upon binding of a ligand to one subunit, which alter the interactions between the TTR subunits and consequently affect the binding affinity of the second ligand molecule.

Studies by ultraviolet difference spectroscopy and ultraviolet fluorescence methods [20–22] have suggested minor conformational changes of the protein or alteration of the H-bond network linking the two sites upon binding of thyroxine in the first site. The changes are coupled with quenching of the Thr-79 signal. Such changes should also induce conformational restrictions on the protein making the second site more rigid, so that transport of a second hormone molecule into the channel might be obstructed.

Our research on the crystal structure of monoclinic hTTR [15] provided a unique opportunity to compare the structure of apo hTTR and of its T4 complex at the same level of accuracy as the two forms coexist in one crystal. We were able to examine the conformational changes caused by T4 binding in the hTTR tetramers. During the structure determination process, two hTTR tetramers were located by the Molecular Replacement (MR) method as implemented in X-PLOR 3.851 [23] using orthorhombic hTTR tetramer [5] as a starting model and refined with CNS [24]. The structure consists of two hTTR tetramers with two T4 hormone molecules bound only to tetramer I. The absence of T4 molecules in tetramer II was confirmed by σ_A-weighted electron density maps [15].

To date no structural evidence of conformational changes induced by ligand binding in TTR has been found. To the best of our knowledge the size of the binding channel of TTR has never been discussed in the published literature. Therefore, the aim of this research was to describe the structural basis for the negative cooperativity effect in transthyretin by comparative analysis of the channel diameter in the structure of apo TTR and its complexes with different ligands.

METHODS

The analysis of the differences in the Cα positions [14] revealed limited differences in monomer geometry. However, such methods of comparison may be misleading if one looks for the differences in the geometry of two TTR binding sites. Therefore, we have calculated rms (root mean square) differences between the TTR dimers forming the two binding sites in the tetramer (AA′ *vs* BB′ in orthorhombic hTTR or chicken TTR, AC *vs* BD in rat TTR and monoclinic hTTR). The results of this comparison are listed in Table 1.

The ligands bound to TTR differ in the number of substituents (Fig. 2) and the pattern of binding interactions in the binding pockets. A comparative analysis of apo TTR and its complexes [5, 14] revealed the variable conformations of amino acids constituting the binding site, strongly influenced by the ligand architecture. Consequently, these changes preclude simple interpretation. Therefore we have selected the positions of Cα atoms of the polypeptide chains as a basis for the comparison. The analysis of the channel diameter was performed by calculating the Cα–Cα distances between the equivalent amino acids from the two monomers forming each binding site. The differences in the Cα–Cα distances between the two binding sites were plotted against the residue number for amino acids 14–18 (β-strand A), 53–56 (β-strand D) and 105–122 (β-strands G–H) involved in ligand binding.

RESULTS AND DISCUSSION

The least squares superposition of the two binding sites of TTR complexes calculated for 26 pairs of Cα from two monomers constituting each binding site gave the rms difference of 0.2–0.4 Å (Table 1). The largest rms difference between the two binding sites, calculated

Table 1. The rms differences [Å] in the Cα positions calculated for the BB′ binding site superimposed on the AA′ site of the TTR tetramer.

The *CA 218* symbol indicates the rms differences calculated for Cα atoms of residues 10–125, with the flexible loop residues 99–104 excluded from the comparison. The *CA 52* symbol denotes the rms differences calculated for the residues 14–18, 53–55 and 105–122 constituting the binding site.

PDB code	Reference	*CA 218* [Å]	*CA 52* [Å]
1tta	[10]	0.432	0.236
1tfp	[13]	1.086	0.442
1bmz	[2]	0.167	0.154
2rox	[5]	0.477	0.270
2roy	[5]	0.457	0.271
1tlm	[7]	0.405	0.188
1tha	[6]	0.462	0.211
1thc	[8]	0.566	0.353
1bm7	[2]	0.146	0.133
1ked	[26]	0.429	0.272

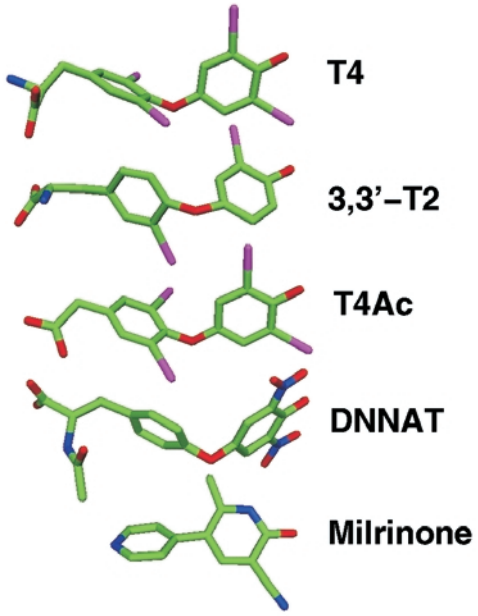

Figure 2. The ligand molecules bound in transthyretin in the compared complexes.

for the Cα positions of amino acids, was found for the chicken TTR structure 1TFP [13]. No significant differences between the two binding sites are observed for two structures (1BM7, 1BMZ) refined with tight non-crystallographic symmetry (NCS) restraints between the two independent monomers [12].

The channel diameter was calculated in each binding site as the Cα–Cα distances between equivalent amino acids from the two monomers. The analysis was performed in two separate classes: TTR complexes and apo TTR. The series of the TTR complex structures were divided into four groups. The first consisted of structures with the TTR monomers and the occupancy of the ligands in the complexes refined independently in both binding sites. In the complex structures belonging to the second group, identical ligand occupancy was assumed in both binding sites, but individual B factors were refined. The third group of complexes had identical occupancy and group B factors used during the refinement. The comparison of the transthyretin structures from different sources was based on structures refined with no NSC restraints or constraints used and with individual treatment of ligand occupancy. The last group consisted of TTR structures with tight NCS restraints or constraints imposed on the TTR monomers and the ligands during the refinement (Table 1).

The differences in the diameter of the two binding sites were plotted against the residue number (Figs. 3–4). We found that many of these differences are larger than 0.5 Å, and larger than three times the Luzzati error. In all cases we found similar trends, although the magnitude of the changes varied. Therefore the analysis described in this paper provides a valuable insight into the channel deformations.

Consistent way of comparing apo TTR

The orthorhombic structures of hTTR and its complexes give an opportunity to compare the orientation of the tetramers in the crystal lattice. The patterns of intermolecular interactions differ for monomers A and B. In particular, the residue 83 of monomer A participates in these interactions, while in most orthorhombic structures this residue from monomer B does not form any contact closer than 3.5 Å (a value used as a cutoff in the analysis). The comparison of apo transthyretin structures reveals that the channel diameter calculated for the strands A, D and H of 1E3F [25] was larger in AA′ than in BB′, while in 2PAB, 1TTA and 1BMZ the situation was reversed. This comparison revealed that a consistent way of comparing the channel geometry is not related to tetramer orientation in the crystal lattice (AA′ *vs* BB′), but only to the pattern of the diameter differences between the binding sites. Thus there is no simple relationship between the geometry of the binding channel and the network of inter-molecular interactions formed by the tetramers in the crystal lattice.

We chose tetramer II of monoclinic hTTR (apo hTTR) as the basis for the comparison of the channel shape. The pattern of the differences between sites AC–BD can be compared with the AA′–BB′ differences calculated for

1E3F and apo rat TTR 1GKE (AC–BD) and BB′–AA′ for other apo structures [10, 12, 13]. Consequently, for the analysis described in this paper, we base the comparison of the TTR tetramers for apo transthyretin structures [10, 12, 13, 15, 16, 25] on the similarity of the plotted differences (Fig. 3).

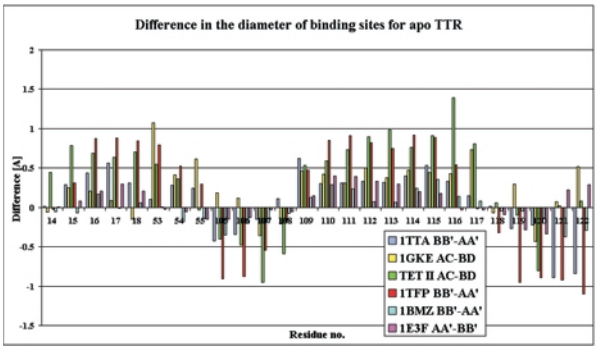

Figure 3. Differences in the channel diameter between the two binding sites for apo TTR from different sources.

The diameter of each site was calculated as the Cα–Cα distance between the two equivalent amino acids across the binding channel.

Consistent way of comparing complexes: occupancy, B-factors or the pattern of the differences

A comparison of TTR complexes may be based on the determination of the major and minor sites (primary and secondary). The primary sites were identified according to the criteria of ligand treatment during the refinement, as described above for the three groups of complexes. The ultimate criterion for comparison would be the shape of the difference plot. The comparison has been performed for a series of seven wild type hTTR complexes [2, 5–8, 26].

In most of the orthorhombic structures, the applied criteria indicate domain AA′ as the primary site. The only complex not conforming to these criteria is hTTR–T4 structure (PDB code: 2ROX), in which the BB′ site appears to be the primary one. This conclusion is based on the average B-factor which is 5 Å^2 larger for the ligand bound in the AA′ site. However, the pattern of the diameter differences based on this assumption is opposite to that for the other complexes. Therefore, the AA′ site in hTTR–T4 [5] was treated as the primary site for the comparison. The diameter differences plot is similar to that for apo TTR structures (Fig. 4) but the amplitude of differences between the sites is decreased. The analysis reveals, however, that the differences between the primary and secondary sites have opposite signs to those calculated for the apo TTR structures.

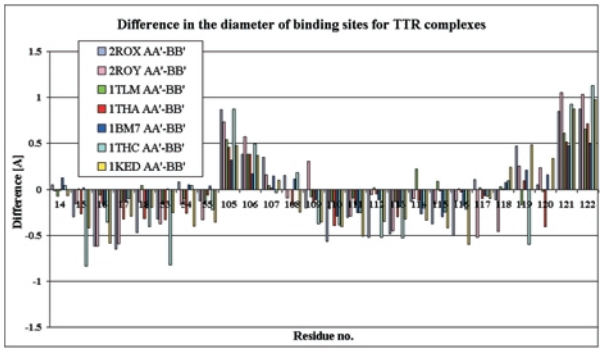

Figure 4. Differences in the channel diameter between the major and minor binding sites for TTR complex structures.

The diameter of each site was calculated as the Cα–Cα distance between the two equivalent amino acids across the binding channel.

The analysis of the differences in the channel shape caused by ligand binding should be based on a comparison of the same sites of liganded and free TTR. The difference between complex and apo site diameter should be similar to the difference between major and minor sites of each complex structure. We have performed such an analysis for the monoclinic form of hTTR [15] and identified the AC site as the primary site of the apo TTR (tetramer II). An analogous analysis was performed for other complex structures with apo hTTR determined to 1.7 Å resolution (1TTA) as the reference structure [10]. The fit of the complexes to that structure gave a non-random distribution of the differences in one ori-

entation only, with the AA′ site of the complexes compared to the BB′ site of apo hTTR and the complex BB′ site compared to the AA′ site of apo hTTR (Fig. 5). Consequently, the

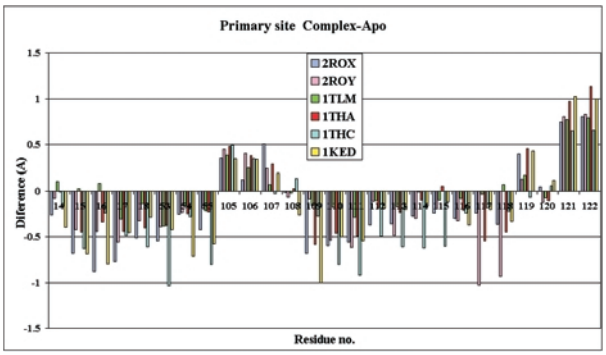

Figure 5. The difference of primary sites diameter calculated between hTTR complexes and apo hTTR [10].

The comparison reveals channel collapse upon ligand binding.

other pair of the binding sites was compared and the diameter changes plotted (Fig. 6). Our proposal of a possible mechanism of the negative cooperativity effect is based on this comparison of the difference pattern. Such an alignment of all the compared structures is consistent with the comparison of the tetramers of hTTR–T4 and apo hTTR from the monoclinic structure [15]. It has to be mentioned that this method of comparison is consistent with the comparison of the AA′ sites of the complexes and the 1E3F apo TTR structure [25]. The consistency of these results permitted us to unambiguously identify the primary site of transthyretin. In all the structures of apo transthyretin the binding site with the larger diameter at residues 15–17 (strand A) and 54–56 (strand D) binds the ligand with higher occupancy and therefore becomes the primary site.

Model of the ligand-induced changes in the transthyretin binding channel

From the analysis of the diameter differences between the two binding sites in apo TTR and in TTR complex structures [2, 5–8, 10, 13, 15–16, 26], we were able to propose a mechanism explaining the negative cooperativity effect.

The comparison of the TTR channel diameter reveals that the two hormone binding sites are not identical (Figs. 3 and 4). One site has a slightly larger channel diameter when measured between equivalent amino acids belonging to β-strands D, A and H, while the diameter for residue pairs 105–109 of strands G is smaller. The second binding site has the opposite characteristics and is narrower in both the outer and the inner part of the channel, while the middle of this binding site is wider.

The first hormone molecule binds in the site that has a larger diameter near the channel entrance (strands D and A). In the monoclinic hTTR structure [15] this is the A/C binding site of tetramer II. The site of such characteristics is identified as a primary binding site in apo transthyretin (Fig. 5).

Binding of the first hormone molecule in the primary site causes an increase of the site diameter measured between pairs of residues 105–109 (strands G). The outer part (strands D and A) and the inner part of the binding site (strand H) collapse because of interactions with the ligand.

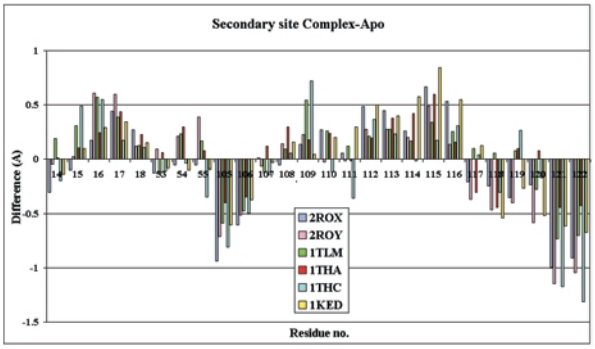

Figure 6. The difference of secondary sites diameter between hTTR complexes and apo hTTR [10].

The calculation reveals an expansion of the site caused by ligand binding.

The conformational changes caused by the binding of the first ligand molecule in the pri-

mary site trigger the changes in the secondary site, probably due to the extensive network of hydrogen bonds in the antiparallel β-sheet structure of the channel. The second binding site becomes wider in both the outer and inner parts of the channel (strands D, A and H), while the middle part (strand G) collapses. The resulting characteristics of the second binding site is similar to those of a primary binding site. This is consistent with Gonzalez suggestion that NC may be transmitted by changes in the network of hydrogen bonds linking two no-contiguous binding sites [21] and with the comparison of rat TTR and its T4 complex [17].

Upon binding of the second ligand, the alterations in both sites occur, which are reverse to those described above. However, the amplitude of the changes in the primary binding site are relatively small because of the binding interactions that are already formed by the first ligand. Therefore the collapse of the second site is not as significant as of the first site (Fig. 3), and the second hormone molecule is bound less tightly. The proposed changes are based on structural data and are consistent with fluorescence spectroscopy studies [27] showing that quenching of tryptophanyl emission is caused mostly by the first ligand molecule bound to the hTTR tetramer.

The slight conformational changes caused by the binding of two hormone molecules to the TTR tetramer differ in amplitude. Therefore the diameters of both binding sites are different. The diameter of the primary binding site in the complex structures is smaller in the outer and larger in the middle part of the channel than the diameter of the secondary site.

CONCLUSIONS

The analysis of the channel geometry revealed that the two binding sites of TTR differ in their diameter. The difference patterns observed for both apo transthyretins and the TTR complexes are similar. The distances between the A, D and H β-strands are consistently larger, while the distances between the G β-strands are smaller in one site than in the other. The amplitude of these differences in the apo TTR structures is larger than that found in the TTR complexes. The analysis of the ligand occupancies in the TTR complexes and the comparison of apo TTR and the TTR–T4 tetramers in the structure of monoclinic hTTR allowed the identification of the primary site, which binds the ligand with higher affinity. This site of TTR is larger near the channel entrance and at the tetramer center. The model of ligand induced changes in the transthyretin binding sites was formulated based on that conclusion. This model explains the negative cooperativity effect observed in TTR and is consistent with the results of ultraviolet difference spectroscopy and ultraviolet fluorescence methods.

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