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Thyroxine Binding to Transthyretin Met 119

Comparative Studies Of Different Heterozygotic Carriers And Structural Analysis

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The majority of the known transthyretin (TTR) variants are associated with amyloidosis, but there are also variants associated with euthyroid hyperthyroxinemia and others are apparently nonpathogenic. TTR Met 119 is a nonpathogenic variant found to be frequent in the Portuguese population. Previous studies on thyroxine (T_4) binding to semi-purified TTR from heterozygotic carriers of TTR Met 119, reported by us and other groups, revealed different results. Therefore, to further characterize T_4 binding to TTR Met 119 we performed T_4-TTR binding studies in homotetrameric recombinant TTR Met 119 variant and normal TTR. We also studied T_4 binding to TTR purified from serum of different heterozygotic carriers of TTR Met 119 including compound heterozygotic individuals carriers of a TTR mutation in the other allele. We observed an increased T_4 binding affinity to TTR Met 119 from heterozygotic individuals and compound heterozygotes and this effect of increasing T_4 binding affinity was consistent and independent from the mutation present in the other allele. Recombinant homotetrameric TTR Met 119 and heterotetrameric protein from heterozygotic carriers of TTR Met 119 presented similar T_4 binding affinity demonstrating the increased T_4 binding affinity of TTR Met 119. X-ray crystallography studies performed on the recombinant TTR Met 119 variant revealed structural alterations mainly at the level of residue Leu 110 allowing a closer contact between the hormone and the mutant protein. These results are consistent with the observed T_4 binding results.

Key Words: Transthyretin; TTR Met 119; thyroxine binding; structural analysis.

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Introduction

Transthyretin (TTR) is a thyroxine (T_4) binding protein, carrying about 20% of T_4 in serum. TTR is also a carrier of retinol binding protein (RBP). The TTR molecule is a tetramer of identical subunits with 127 amino acid residues each, coded by a single copy gene. About 50 different point mutations have been described in TTR *(1)*. Most of them are associated with familial amyloidotic polyneuropathy (FAP), a disease characterized by the deposition of TTR as amyloid; others are responsible for euthyroid hyperthyroxinemia and there are still some apparently non-pathogenic mutations. In addition to the high frequency of occurrence of the FAP associated TTR Met 30 variant in the Portuguese population, screening studies revealed also a high frequency of occurrence of some apparently nonpathogenic variants, in particular of TTR Ser 6 *(2)* and TTR Met 119 *(3)* in heterozygotic carriers and in compound heterozygotic carriers.

Some studies have already been performed with TTR from heterozygotic individuals carriers of each of these variants. TTR Ser 6 was first reported by Fitch et al. *(4)* and described in association with hyperthyroxinemia; however, recent studies on recombinant TTR Ser 6 have demonstrated that this variant has a normal T4 binding affinity *(5)*. TTR Met 30, the most studied variant, has low T_4 binding affinity in the heterozygotic form *(6)*, and the homozygotic TTR Met 30 has almost no affinity for T_4 *(7)*.

Concerning T_4 binding to TTR Met 119, its carriers were first described as presenting no differences in thyroid hormone levels as compared to normals *(8)*. Scrimshaw et al. *(9)* found also normal levels of free T_4 in carriers of TTR Met 119, but increased T_4 binding to TTR. A study by Alves et al. *(10)* reported normal T_4 levels in serum of TTR Met 119 carriers but an increased T_4 binding potential as compared to control, attributed to the relatively high levels of TTR in their serum. However, recently, Curtis et al. *(11)* described an increased T_4 binding affinity for TTR semi-purified from sera of heterozygotic TTR Met 119 carriers. To further characterize T_4 binding to

Table 1
Serum TTR Concentration (mg/dL) Determined by Rocket Immunoelectrophoresis

	N	N/Met 30	N/Met 119	Met 30/Met 119
	31.7	16.1	38	38
	33.3	22.4	39.5	46.5
	27	14.5	34.9	29.4
	26.3	24.7	40.3	29.4
	30.2	27	28.6	34.9
Mean ± SD	29.7 ± 3.0	20.9 ± 5.4	36.3 ± 4.8	35.6 ± 7.1
P*		0.02	0.04	0.145

P* - relative to values of N.

TTR Met 119, we started X-ray diffraction studies on TTR Met 119 and at the same time a series of studies on the T_4-TTR binding properties. We present here T_4 binding assays in whole serum and isolated TTR from heterozygotic carriers of TTR Met 119 and from different compound heterozygotes, respectively: TTR Ser 6-TTR Met 30; TTR Ser 6-TTR Met 119; and TTR Met 30-TTR Met 119. So far only found in the Portuguese population, since in the case of heterozygotic individuals TTR may form hybrid heterotetrameric species composed of two kinds of TTR monomers present in different ratios, we compared the T_4 binding of serum heterotetrameric TTR with that of homotetrameric recombinant TTR. The structural modifications induced by the substitution of a methionine for a threonine at position 119 and their influence in the hormone binding site were further analyzed by X- ray crystallography of homozygous TTR Met 119 and compared with the known crystal structure of wild type TTR complexed with 3,3' diiodo-L-thyronine (T_2) *(12)*.

Results

T_4 Binding to TTR in Whole Serum

T_4 binding was first assayed in whole serum samples from heterozygotic carriers of TTR Met 30 and TTR Met 119 and also on serum from compound heterozygotic individual carriers of TTR Met 30-TTR Met 119. TTR concentration in serum was determined by rocket immunoelectrophoresis. The results obtained (Table 1) showed that, although within the normal range, TTR concentration was elevated in TTR Met 119 carriers sera, which is in agreement with previous reports *(10)*.

The results of binding assays are shown in Fig. 1. Figure 1, Panel I-A shows a competitive binding assay representing percentage of bound T_4 to total T_4 whereas Panel I-B is the Scatchard representation of the data in Panel l-A. Each curve represents the mean values obtained for five different serum samples, except for homozygous TTR Met 30 serum. The affinity constants (Ka) obtained for each variant (Table 2) are different, as inferred from the different slopes of the Scatchard analysis. Thus, concerning TTR Met 30, heterozygotic carriers (N-Met 30) presented low T_4-TTR binding affinity and the homozygotic carrier (Met 30-Met 30) seemed to have no specific T_4 binding, which is in accordance with previous reports *(6,7)*. On the contrary, TTR Met 119 heterozygotic carriers (N-Met 119) presented a T_4 binding affinity that is approx 1.5–2 times higher than the affinity of normal serum TTR, similarly to the results reported recently by Curtis et al. *(11)*. The results obtained for compound heterozygotic carriers of TTR Met 30-Met 119 indicate that T_4 affinity constant is slightly (but not statistically significant) lower than the affinity of TTR Met 119 heterozygotic individuals. Thus, the presence of Met 119 has an effect of increasing T_4 binding affinity constant, whereas TTR Met 30 has the opposite effect—it decreases T_4 binding affnity. These effects were also observed when we studied T_4 binding to TTR in serum of compound heterozygotic carriers of TTR Ser 6. Relative binding affinity for the heterozygotic TTR Ser 6 individual (N-Ser 6) was similar to normal serum (relative Ka 1.02), whereas for the compound heterozygotic individual carrier of TTR Ser 6–TTR Met 30 was lower (relative Ka 0.41) and for compound heterozygotic TTR Ser 6–Met 119 was higher (relative Ka 1.26).

T_4 Binding to Isolated TTR from Serum

The same binding studies were performed on purified proteins from the serum of heterozygotic carriers of TTR Met 30, TTR Met 119, and compound heterozygotes of TTR Met 30 -Met 119 (Fig. 1, Panels II-A and II-B). Although the binding affinity constants (Ka) (Table 2) were slightly lower than the ones obtained in the whole serum, the relative values were similar, i.e., TTR Met 119 variant increased T_4 binding affnity about twofold when compared to normal TTR and this effect was independent of the other TTR monomer present.

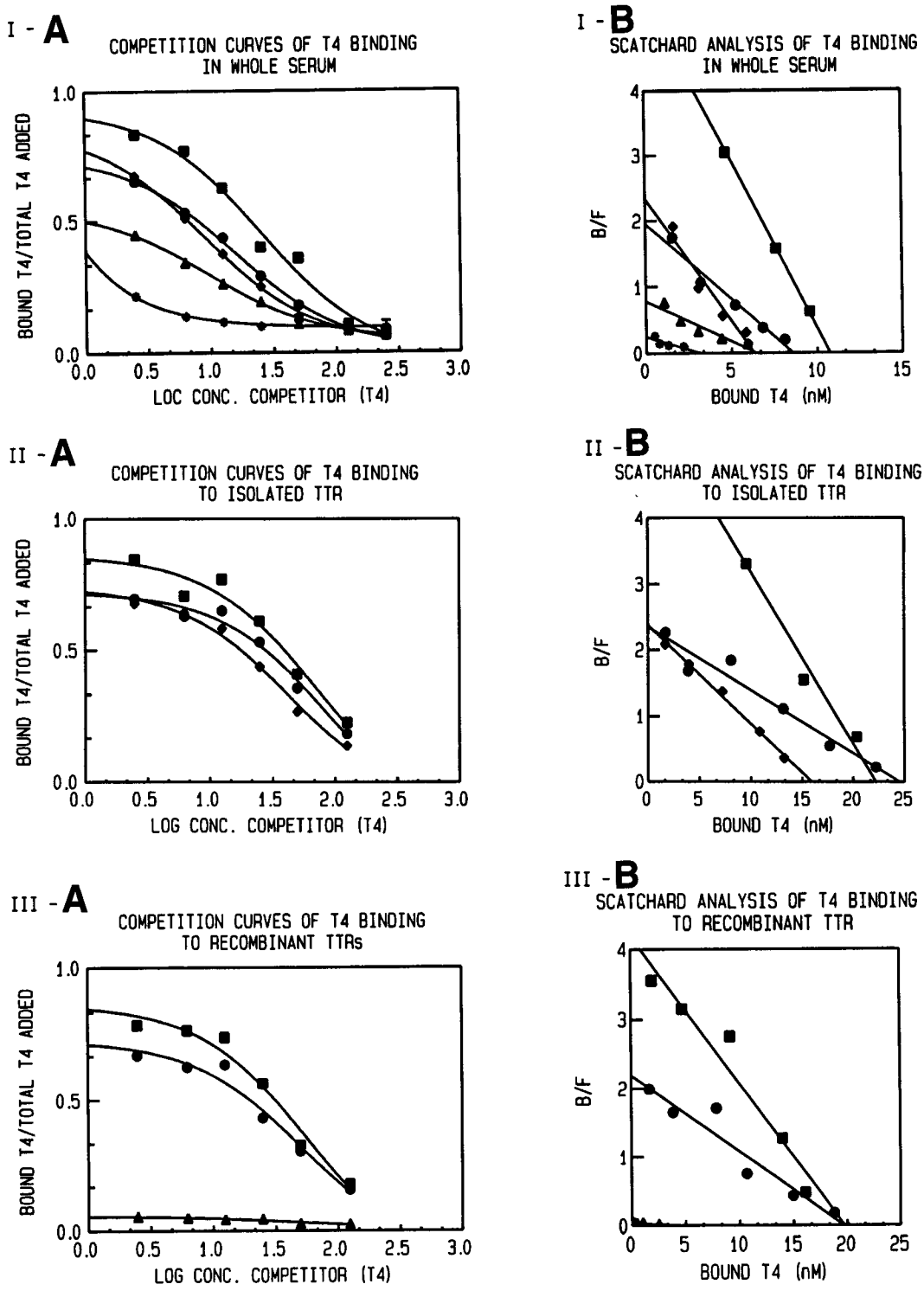

Fig. 1. T_4 binding to TTR in whole serum (panel I), to TTR isolated from serum (panel II) and to recombinant TTR (panel III). The panels in the left side **(A)** represent competition studies and panels on the right side **(B)** represent the Scatchard analysis. For panels I and II the symbols are: ●, N-N; ▲, N-Met 30; ■, N-Met 119; ◆, Met 30-Met 119; and *, Met 30-Met 30. For panel III, the symbols are: ●, Rec N; ▲, Rec Met 30; and ■, Rec Met 119.

T4 Binding to Synthetic TTR Variants

Serum TTR from heterozygotic and compound heterozygotic individuals is probably a complex mixture of heterotetramers composed by different proportions of mutated and normal TTR monomers. In order to evaluate the influence of each mutation individually, we studied T_4 binding to homotetrameric mutant proteins produced by recombinant bacteria. Thus, we studied T_4 binding to recombinant normal TTR, TTR Met 30 and TTR Met 119, as shown in Figure 1, Panels III-A and III-B. The values obtained for the affinity constants are also shown in Table 2. Recom-

Table 2
T_4 Binding Affinity to TTR Variants

Samples		Absolute affinity (Ka × 10^8 M^{-1})	Relative affinity
	Normal	2.4 ± 0.34	1
Whole	N - Met 30	1.58 ± 0.64	0.65
serum	Met 30-Met 119	4.03 ± 0.65	1.68
	N - Met 119	4.43 ± 1.63	1.85
	Met 30-Met 30	0.77	0.32
	Normal	1.18 ± 0.26	1
Isolated TTR	N-Met119	2.1 ± 0.41	1.78
	Met 30 - Met 119	2.14 ± 0.41	1.8
	Normal	1.1 ± 0.02	1
Recombinant	Met 119	2.3 ± 0.25	2.1
TTR	Met 30	0.12	0.11

binant TTR Met 30 has virtually no affinity for T_4, similarly to what was found for the protein from serum of the homozygous carrier of TTR Met 30. However, T_4 binding affinity for TTR Met 119 is twice the value of normal TTR.

Thyroid Hormone Binding Pocket in Variant TTR Met 119

The crystal structure of variant TTR Met 119 was solved with the molecular replacement method and then refined using X-PLOR. The initial model was that of wild type TTR refined to an R factor of 17% at 1.7Å resolution *(13)*. The substitution of methionine for threonine at position 119 was clear from the initial Fourier maps. Seven rounds of positional and B-factor refinement coupled with manual rebuilding continued until the R-factor converged. The actual refined model has a crystallographic R-factor of 18% and it consists of residues 10–125 for chains A and B and 136 solvent molecules.

The substitution of Met 119 for Thr causes a movement of Leu 110, which is in the central region of the channel and normally anchors the T_2 molecule in the TTR:T_2 complex *(12)*. As shown in Fig. 2, the bulkier Met 119 side chain cannot be accommodated in the wild type structure (Fig.2A) without movement of Leu 110 to release electronic strain (Fig. 2 A and B). As a result, residues Leu 110 are shifted towards the T_2 molecule and a closer contact between the hormone and the protein occurs (Fig. 2C and D).

Discussion

In the present work, T_4 binding to the mutant TTR Met 119 was investigated both by ligand binding and structural studies. We compared T_4 binding to TTR Met 119 in heterozygotic individuals and compound heterozygotic individuals for this mutation and TTR Ser 6 and TTR Met 30. In parallel, and for comparative purposes, we tested binding to the homozygotic recombinant mutant. Conceming the natural variants, T_4 binding to TTR was studied using diluted whole serum and isolated, pure, serum TTR. Although the absolute values obtained for T_4-TTR binding affinity constants in both cases were different, the relative values were similar; these results demonstrate that T_4 binding to TTR can be studied in diluted whole serum using this methodology for comparative purposes, as previously described *(6,14)*. From the results obtained for the binding studies, we concluded that the occurrence of TTR Met 119 monomer consistently increases TTR-T_4 binding affinity independently from the other TTR monomer present in the tetramer, i.e., whether it is Ser 6 or Met 30. These results are in agreement with the data reported by Curtis et al. *(11)* that employed identical methodology using semipurified TTR and also found increased T_4 binding affinity in two heterozygotic TTR Met 119 carriers.

In addition, the three-dimensional structure of variant TTR Met 119 shows that the substitution of a methionine for a threonine at position 119 induces a movement of residue Leu-110, pushing it to a position closer to the hormone. This structural alteration may strengthen the binding interactions of TTR with the T_4 phenolic ring, leading to the observed increase in the binding affinity of the protein. However, in previous studies, concerning T_4 binding in whole serum from TTR Met 119 carriers by equilibrium-dialysis and stepwise saturation of iodothyronine binding sites *(10)*, we showed that when compared with parallel normal serum samples, TTR Met 119 carriers had an increased T_4 binding potential. This was attributed to an increased concentration of TTR in TTR Met 119 carriers as compared to normals—implying that the T_4-TTR affinity found was normal. Also, discrepant T_4 binding data were described for TTR Met 30 when tested in different conditions. Thus, homozygotic TTR Met 30 isolated from serum or in whole serum presented a very low T_4 binding affinity and has been reported to bind virtually no T_4 (*7*; and herein). However, isolated recombinant TTR Met 30 showed capacity to bind T_4 in qualitative assays *(15)*. In addition, Palha et al. *(16)* studied plasma T_4 levels in a mutant mouse strain carrying a null mutation at the TTR locus (TTR^-) and compared with those for a transgenic TTR^- mice carrying the human TTR Met 30 gene (TTR^-/hMet 30) finding a 30% higher plasma T_4 level for the transgenic TTR^-/hMet 30 mice. This result indicated that the human TTR Met 30 binds T_4 in vivo. These discrepancies of T_4 binding results found both for TTR Met 30 and TTR Met 119 could be due to the different methodologies used for the assays, namely when comparing in vivo and in vitro studies. Moreover, the different conditions of the assays namely the use of isolated proteins versus proteins in whole serum could be affected by other components in serum that may interfere with T_4 binding parameters. That could be the case of nonesterified fatty acids and lysolecithins that have been reported to influence T_4 binding to TBG and TTR *(17)*. In particular, arachi-

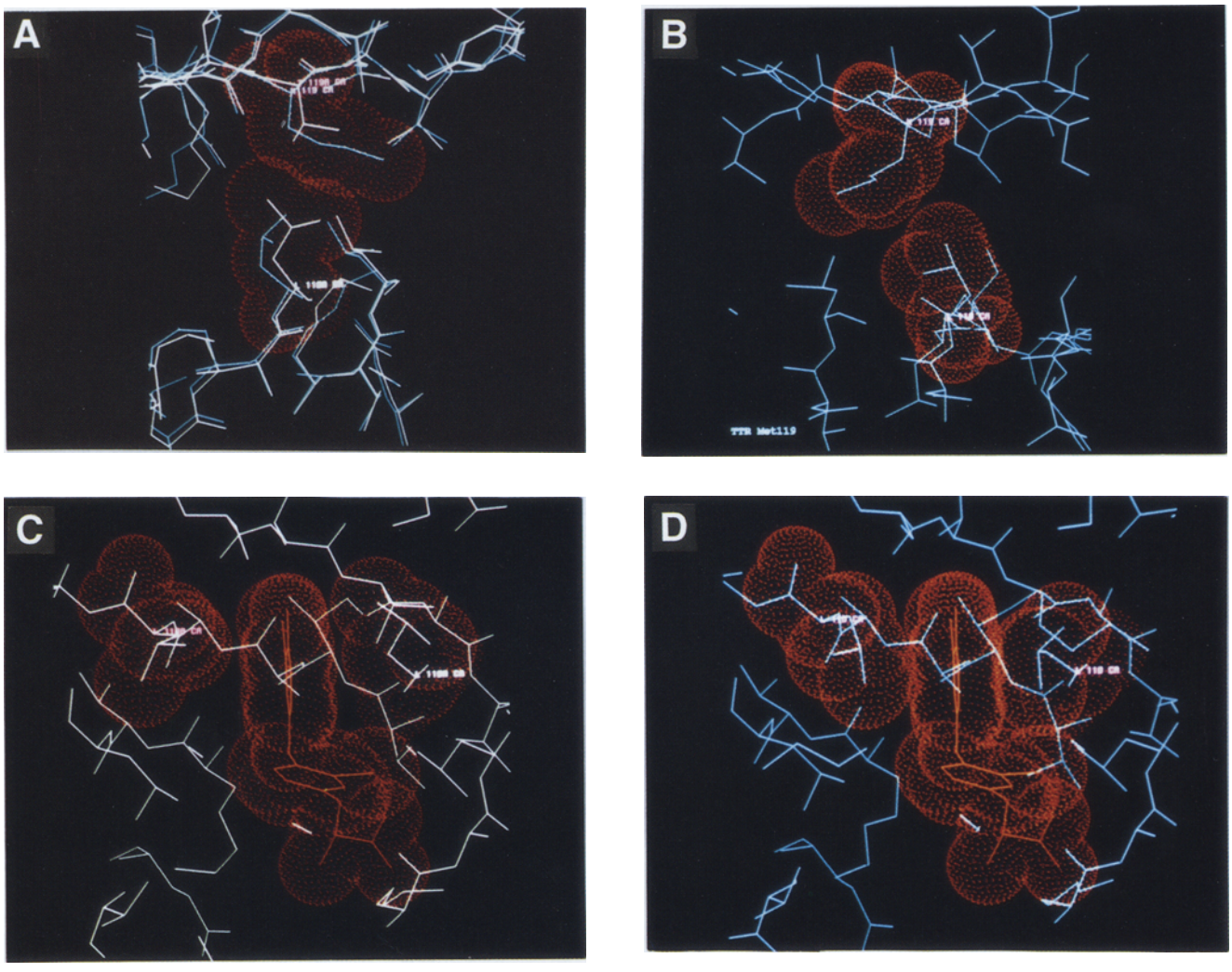

Fig. 2. The van der Waals surfaces represented for **(A)** residues Met 119 and Leu 110 in the wild type protein, as if the mutation occurred without any changes in the position of Leu 110 residues. This shows a close contact between these two amino acids and therefore the need for the movement of Leu 110 to accomodate the Met side chain (main chain and side chains are represented in blue for the mutant protein and in white for the wild type protein); **(B)** residues Met 119 and Leu 110 in the mutant protein, in a different orientation, showing that the close contact no longer exists; **(C)** the T_2 hormone and the two Leu 110 residues in the case of wild type protein, as reported by Wojtczak et al. *(12)*; and **(D)** the T_2 hormone and residues Leu 110 in variant TTR Met 119, showing a closer contact between the protein and the hormone when compared with wild type TTR.

donic acid has a 0.49% relative affinity of binding to TTR when compared to unlabeled T_4. Although the influence of these nonesterified fatty acids on the T_4 binding in serum should be overcome by the high affinity of binding to albumin, it is not known if in specific conditions they exert an effect on mutated TTR or TBG in serum.

The crystallographic structure of wild type TTR has been known for a long time *(18)*. Briefly, TTR is a tetramer composed by four identical polypeptide chains of 127 amino acid residues each. About 45% of the amino acids are organized in 8 β-strands designated from A to H. The β strands are six to nine amino acid residues long except strand D, which is only three amino acids long. The TTR monomers are associated in dimmers and these in tetramers; thus forming a central channel where two binding sites for the thyroid hormones are located. Although both binding sites are similar, they present different binding affinities for the thyroid hormones due to the negative cooperativity of binding. The negative cooperativity of binding was also evident in the present work when we compared the T_4-TTR affinity constants (Ka) obtained for TTR isolated from TTR Met 119 carriers with the Ka for recombinant homozygotic TTR Met 119 and did not find a cumulative effect of the higher affinity in the TTR Met 119 homotetramer. This result was similar to what was reported for homozygous and heterozygous TTR Thr 109, a TTR variant that also increases T_4-TTR binding affinity *(19)*.

In addition to thyroid hormones, other substances are known to bind TTR at the T_4 binding channel. Among those ligands are T_4 analogs, e.q., polyhalogenated biphenyls (PHBs) *(20)*, flavonoids *(21)*, and retinoids *(22)*. Therefore, the study of interactions between these com-

pounds and TTR variants may have some impact on the definition of the structural details involved in the binding of different ligands at the binding pocket. This may also contribute to understand the toxicity of these compounds and their influences on the binding and metabolism of other TTR ligands, such as retinol, retinoic acid *(23)* and pterins *(24)*.

The binding data reported here together with the crystallographic studies of mutant TTRs, both amyloidogenic and nonamyloidogenic, will also have an impact on the knowledge of structural features associated with amyloid formation. Although there is no relationship between the T_4 binding properties and amyloidogenicity, it seems likely that both are related with structural alterations induced by the mutation. In particular, McCutchen et al. *(25)* propose that amyloid formation, at least in vitro, may depend on the polymerization of a monomeric intermediate partially denatured, which is more readily formed by the presence of certain mutations. In this context, it is interesting to note that studies by Alves and Saraiva *(26)* on the Met 119 mutation indicate that, in opposition to most TTR variants, the Met 119 substitution may induce the stabilization of the tetrameric structure resulting on lower amyloidogenic potential *(27)*. This finding is consistent with the protective role concerning amyloidogenesis that the Met 119 mutation plays as indicated by the benign and relentless evolution of the disease in compound heterozygotes of TTR Met 30 -Met 119. Further crystallographic studies on the Met 119 protein will contribute to understanding the low amyloidogenic potential and binding properties of this mutant.

Materials And Methods

Samples

T_4 binding assays were performed in whole serum and/or isolated TTR from: heterozygotic individuals carriers of different TTR mutations namely TTR Ser 6, TTR Met 30 (five individuals), TTR Met 119 (five individuals); from a homozygous individual for TTR Met 30; and from compound heterozygotic individuals carriers of two different TTR mutations, one in each allele, namely TTR Ser 6-TTR Met 30, TTR Ser 6-TTR Met 119, TTR Met 30-TTR Met 119 (five individuals) *(3)*.

The same studies were carried out with recombinant proteins synthetically produced in an *E. coli* expression system: normal TTR, TTR Met 30, and TTR Met 119 *(15)*.

TTR Isolation

Plasma was dialyzed against phosphate buffer 50 m*M*, 77 m*M* NaCl, pH 7.6. The dialyzed plasma was passed through an ion exchange column of diethylaminoethyl (DEAE)-cellulose. The column was washed with the phosphate buffer, pH 7.6, and the fraction containing TTR was eluted by increasing the ionic strength of phosphate buffer from 50 to 600 m*M* NaCl. The TTR fraction was dialyzed, lyophilized, and chromatographed in a Blue-Sepharose column. Then TTR was isolated by preparative electrophoresis in a native Prosieve agarose (FMC, Rockland, ME) gel following instructions of the supplier. After electrophoresis, a slice of the gel was stained with Coomassie Blue to localize the TTR band. This band was excised from the gel and electro-eluted in a Elutrap system (Schleicher and Schuell, S & S, Dassel, Germany) in 5 m*M* Tris, 38 m*M* glycine pH 8.3 buffer for 3 h at 200 V or overnight at 50 V (4°C). The protein was further purified by gel filtration with high performance liquid chromatography (HPLC) using a Ultraspherogel (Beckman, Fullerton, CA) column in buffer 0.1 *M* KPO4, 0.1 *M* Na_2SO_4, 0.05% NaN_3.

The recombinant proteins were isolated by the same process after osmotic shock of the bacteria *(15)*.

TTR Quantification

TTR in whole serum was quantified by rocket immunoelectrophoresis *(28)* using rabbit antihuman TTR with a titre of 450 mg/L in a concentration of 0.75 µL/cm2 (DAKO, Denmark). Isolated TTR variants were quantified by the Bio-Rad micro plate assay.

T4 Binding Assays

125-T_4 Purification:

Labeled L^{125}I-Thyroxine (^{125}I-T_4) (1300 µCi/µg) (Dupont NEN, Boston, MA) was purified using LH-20 Sephadex chromatography *(29)*. The column (1 mL bed vol) was washed with 0.1 *M* HCl to elute the free iodine, then washed with water and finally the purified ^{125}I-T_4 was eluted with a solution of 0.25% ammonium in methanol. Labeled T_4 was dried under nitrogen and resuspended in the appropriate buffer.

Competition Assays:

T_4 binding assays were based on a gel filtration procedure described by Somack et al. *(30)* with minor modifications *(31)*. Briefly, for the isolated proteins, 100 µl of TTR 60 n*M* in Tris buffer pH 8.0 (Tris 0.1 *M*, NaCl 0.1 *M* and ethylenediaminetetraacetic acid [EDTA] 0.001 *M*) were incubated with 100 µl of cold T_4 solutions of variable concentrations ranging from 0 to 1000 n*M* and with a constant amount of labeled ^{125}I-T_4 (~50,000 cpm). This solution was counted in a gamma (γ) spectrometer and incubated at 4°C overnight. Protein bound ^{125}I-T_4 and free ^{125}I-T_4 were separated by gel filtration through a 1 mL BioGel P6DG (Bio-Rad, CA) column. The protein bound fraction was eluted, by centrifugation at 500*g*, with Tris buffer in the two first 200 µL fractions, whereas free T_4 was retained on the BioGel matrix. The first eluates containing the bound T_4 were collected and counted. T_4 bound was expressed as a percentage of total T_4 added. Adsorption of the free hormone by the gel was nonsaturable. Unspecific binding of labeled T_4 was determined using a high concentration of T_4 (10 µM).

The same procedure was applied for the T_4 binding assays in whole serum. However, in this case, after TTR

quantification, the serum was diluted in saline to a TTR concentration of 20 n*M* (approx 400 times dilution).

Each assay was performed in duplicate or triplicate and was repeated at least twice. For analysis of the binding data, we used the GraphPAD InPlot computer program (version 3.15, San Diego, CA) and for statistical analysis of grouped values we used Student's *t*-test. $P < 0.05$ was considered statistically significant.

X-Ray Diffraction Work

Recombinant TTR Met 119 was used for the crystallization experiments. Crystals were grown at room temperature using the hanging drop vapor diffusion method. The drops contained equal volumes of 10 mg/mL protein solution and the reservoir solution was composed of 41% ammonium sulfate, 200 m*M* sodium citrate, pH 4.9.

The X-ray diffraction data to 1.75Å resolution were collected using synchrotron radiation on X11 beam line at EMBL Hamburg. The crystals were orthorhombic, space group $P2_12_12$ with cell dimensions a = 43.77Å, b = 86.177Å, and c = 65.227Å.

The determination of the crystal orientation and integration, merging and scaling of the reflections were performed with the DENZO and SCALEPACK programs *(32)*. A total of 25,112 unique reflections were collected with an $R_{merge} = 6.3\%$, representing 99% of the theoretically possible data.

The structure was solved by isomorphous replacement and refined using X-PLOR *(33)*. The Fourier syntheses were visualized using TURBO-FRODO *(34)*.

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