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Crystallization and preliminary X-ray diffraction studies of isoform α1 of the human thyroid hormone receptor ligand-binding domain

F. M. Nunes,[a] R. Aparicio,[a]‡ M. A. M. Santos,[a] R. V. Portugal,[a] S. M. G. Dias,[a] F. A. R. Neves,[b] L. A. Simeoni,[b] J. D. Baxter,[c] P. Webb[c] and I. Polikarpov[a]*

[a]Instituto de Física de São Carlos, Universidade de São Paulo, Av. Trabalhador SãoCarlense 400, São Carlos, SP, 13560-970, Brazil, [b]Universidade de Brasília, Faculdade de Ciências da Saúde, Laboratório de Farmacologia Molecular, Brasília, DF, Brazil, and [c]University of California, Metabolic Research Unit, San Francisco, USA

‡ Current address: Universidade Estadual de Campinas, Instituto de Química, CP6154, Campinas, SP, 13084-862, Brazil

Correspondence e-mail: ipolikarpov@if.sc.usp.br

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Thyroid hormone receptors (TR) play critical roles in virtually all tissues. The TR ligand-binding domain (LBD) participates in important activities, such as transcriptional activation and repression, through conformational changes induced by hormone binding. Two crystal forms of isoform α1 of the human thyroid hormone receptor LBD (hTRα1) in complex with the thyroid hormones T3 and Triac were obtained. The hTRα1–T3 complex was crystallized in a previously unobserved crystal form (space group $P2_12_12_1$, a = 59.98, b = 80.80, c = 102.21 Å), with diffraction patterns extending to 1.90 Å resolution on a rotating-anode X-ray source, and in space group $C2$ (a = 117.54, b = 80.66, c = 62.55 Å, β = 121.04°), with data extending to 2.32 Å resolution. The hTRα1–Triac complex was also crystallized in the new space group $P2_12_12_1$, with unit-cell parameters a = 60.01, b = 80.82, c = 102.39 Å; its resolution limit extended to 2.20 Å on a home source. Phasing was carried out by the molecular-replacement method and structural refinement is currently in progress. The refined structures may provide insight into the design of new thyromimetics.

1. Introduction

Thyroid hormone receptors (TRs), members of a superfamily of eukaryotic transcription factors, are ligand-activated transcription factors that bind to thyroid hormone-response elements (TREs) in the regulatory region of target genes and mediate the biological effects of thyroid hormones. The receptors exhibit a modular structure with functionally separable domains. The most highly conserved domains are the DNA-binding domain (DBD) and the ligand-binding domain (LBD) (Evans, 1988; Laudet *et al.*, 1992). The LBD participates in several types of activity, including hormone binding, homo- and/or heterodimerization, molecular interactions with heat-shock proteins and transcriptional activation and repression (Tsai & O'Malley, 1994; Ribeiro *et al.*, 1995). Hormone binding induces conformational changes which control these properties and influence gene expression (Tsai & O'Malley, 1994; Ribeiro *et al.*, 1995). Therefore, the three-dimensional structure of a liganded LBD is critical for understanding the structural mechanisms of hormone action.

Thyroid hormones, namely 3,5,3′-triiodo-L-thyronine (T3), 3,5,3′,5′-tetraiodo-L-thyronine (T4) and 3,5,3′-triiodothyroacetic acid (Triac), play critical roles in the differentiation, growth, metabolism and physiological function of virtually all tissues. Two major subtypes of thyroid hormone receptors (TRα1 and TRα2, and TRβ1 and TRβ2) are encoded by two different genes (Ribeiro *et al.*, 1995). TRα1, TRβ1 and TRβ2 are ligand-binding isoforms of TR, whereas TRα2 does not bind thyroid hormones. Differences in affinity towards thyroid hormones are observed amongst the different ligand-binding isoforms. TRs mediate distinct physiological effects owing to differences in tissue abundance and receptor-specific activity (Forrest & Vennström, 2000). Studies in patients with the syndrome of resistance to thyroid hormones, in which abnormal TRβ is present, and with TR$\alpha1^{-/-}$ mice suggest that TRα is the major TR regulating heart rate (Johansson *et al.*, 1998; Forrest & Vennström, 2000; Yen, 2001; Gloss *et al.*, 2001). TRβ is critical in controlling hepatic cholesterol metabolism and thyroid-stimulating hormone (TSH) suppression, which may be because of the high expression of TRβ in liver (70–80% of total TR) and pituitary (Schwartz *et al.*, 1992; Wikström *et al.*, 1998; Gloss *et al.*, 2001). In particular, TRβ1 is widely distributed in the tissues and regulates metabolic rate. Identifying thyromimetics that interact selectively with the isoforms TRα1 and TRβ1 may be crucial in treating important diseases such as obesity and lipid disorders.

The available TR structural data include rat TRα1 LBD in complex with T3 (Wagner *et al.*, 1995) and Triac (Wagner *et al.*, 2001) and human TRα1 LBD (hTRα1) in complex with a synthetic thyromimetic (Ye *et al.*, 2003). However, despite this previous work, the structural basis for the selectivity of the TR

isoforms in thyroid ligand binding is not yet well established. To further investigate the molecular mechanism of hTRα1 specificity, X-ray crystallographic studies were recently initiated. In this work, we report the purification, crystallization, data collection and molecular-replacement solutions obtained for hTRα1 in complex with T3 and Triac. Data to higher resolution than previously published (Ye *et al.*, 2003; Dow *et al.*, 2003) have been obtained, which is likely to be a consequence of a different molecular packing. These new studies may be important for better understanding of the structural basis of TR isoform selectivity and for the design of more potent isoform-specific thyromimetics.

Table 1
Crystal parameters and data-collection statistics.

Values in parentheses refer to the last resolution shell

	T3 complex		Triac complex
Space group	$P2_12_12_1$	$C2$	$P2_12_12_1$
Unit-cell parameters			
a (Å)	59.98	117.54	60.01
b (Å)	80.80	80.66	80.82
c (Å)	102.21	62.55	102.39
β (°)		121.04	
Solvent content (%)	68.3	69.1	68.4
ASU content (molecules)	1	1	1
Resolution range (Å)	31.47–1.90 (2.00–1.90)	19.92–2.32 (2.45–2.32)	48.22–2.20 (2.32–2.20)
No. images	260	77	291
No. observed reflections	323718	32461	267634
No. unique reflections	39272	18188	26000
Multiplicity	8.2 (7.8)	1.8 (1.7)	10.3 (11.0)
Completeness (%)	98.7 (98.9)	83.7 (87.3)	100 (100)
R_{sym} (%)	5.9 (38.3)	7.9 (37.5)	5.9 (37.5)
$\langle I/\sigma(I)\rangle$	7.8 (2.0)	7.7 (2.0)	11.2 (2.1)

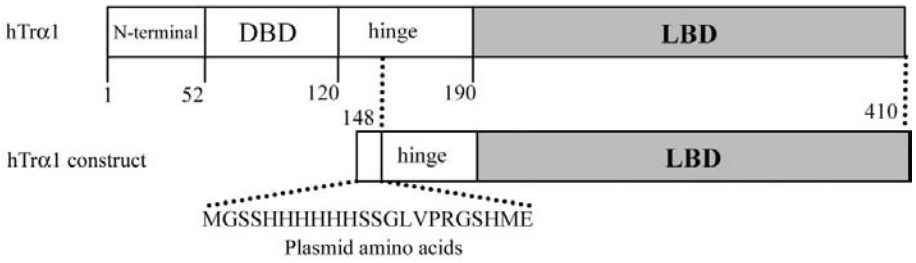

Figure 1
Schematic diagrams of human TRα1 (hTRα1) and the construct used in the crystallographic experiments (hTRα1 construct), showing the N-terminal, DBD (DNA-binding), hinge (connecting region) and LBD (ligand-binding) domains. Numbers indicate the amino-acid positions in the receptors. The amino acids of the plasmid pET28a(+) that is coexpressed with hTRα1 construct are shown.

2. Materials and methods

2.1. Expression and purification

The human TRα1 LBD construct including amino-acid residues Glu148–Val410 (NCBI protein accession No. A40917) fused in frame to the C-terminus of a polyhistidine (His) tag was expressed in *Escherichia coli* strain B834 harbouring a pET28a(+) plasmid (Novagen), as illustrated in Fig. 1. A Luria Broth [LB; 1.6%(*w*/*v*) tryptose, 1%(*w*/*v*) yeast extract, 0.5%(*w*/*v*) NaCl] starter culture was inoculated with a single colony of an LB-agar culture and grown overnight at 310 K. The initial culture was inoculated at 1% in a major LB culture and grown at 293 K in kanamycin medium until the $A_{600\,nm}$ reached 1.7. After this, isopropyl thio-β-D-galactoside (IPTG) was added to a concentration of 0.5 m*M* and culture growth was continued for 6 h at 293 K.

The cells were harvested by centrifugation and the pellet resuspended in 50 m*M* Tris–HCl buffer pH 7.5 containing 150 m*M* NaCl, 0.05% Tween-20, 1 m*M* phenylmethylsulfonylfluoride (PMSF) and 20 m*M* 2-mercaptoethanol. The culture was incubated on ice with 0.5 mg ml^{-1} lysozyme and disrupted by sonication. The lysate was centrifuged for 20 min at 14 000 rev min^{-1} in a Sorvall SS34 rotor at 277 K and the obtained supernatant was incubated for 30 min with a 20-fold molar excess of T3 (Sigma) or Triac (Sigma).

To purify the holo hTRα1, the supernatant was mixed with Talon Superflow Metal Affinity Resin (Clontech) and shaken at 277 K for 1 h. The resin was washed twice with 50 m*M* sodium phosphate buffer pH 8.0 containing 300 m*M* NaCl, 10% glycerol, 10 m*M* 2-mercaptoethanol, 25 m*M* imidazole, 1 m*M* PMSF and 0.05% Tween-20 and twice with the same solution without Tween-20. The protein was eluted in a single step with 50 m*M* sodium phosphate buffer pH 8.0 containing 300 m*M* NaCl, 10% glycerol, 10 m*M* 2-mercaptoethanol, 1 m*M* PMSF, 0.05% Tween-20 and 500 m*M* imidazole.

After the affinity column, the fractions were pooled and corrected for the conductivity of the initial phenyl solution. The protein was applied onto a Phenyl 5PW 8/75 (TosoHaas) column pre-equilibrated with 20 m*M* Na HEPES buffer pH 8.0 containing 0.5 m*M* EDTA, 700 m*M* $(NH_4)_2SO_4$, 3 m*M* dithiotreitol (DTT). The column was washed with the previous solution and eluted with a 90 min 0–100% gradient of 20 m*M* Na HEPES buffer pH 8.0 containing 0.5 m*M* EDTA, 20% glycerol, 10% acetonitrile, 3 m*M* DTT at 0.75 ml min^{-1}. After this step, the protein was loaded onto a HL Superdex 200 or 75 26/60 gel-filtration column (Amersham Bioscience) equilibrated with 20 m*M* Na HEPES buffer pH 8.0 containing 1 m*M* EDTA, 3 m*M* DTT, 0.01% Tween-20 and 200 m*M* NaCl. The protein recovered was concentrated by ultrafiltration (Amicon Ultra 10 kDa MWCO, Millipore), resuspended in a solution of 600 m*M* NaCl and 3 m*M* DTT and further concentrated to 10 mg ml^{-1}.

The protein content and purity of all chromatographic fractions were checked by Coomassie Blue-stained SDS–PAGE. The average yield of the protein was about 6 mg per litre of culture, with purity higher than 99%. Protein concentrations were determined using the Bradford dye assay (Bio-Rad) using bovine serum albumin as standard.

2.2. Crystallization

Initial crystallization conditions were screened at temperatures of 277 and 291 K by the sparse-matrix method (Jancarik & Kim, 1991) using the macromolecular crystallization reagent kits Crystal Screens I and II (Hampton Research). In each trial, a hanging drop of 1 µl of protein solution containing either T3 or Triac (see §2.1) was mixed with 1 µl precipitant solution and equilibrated against a reservoir containing 500 µl precipitant solution. At both temperatures, evidence for crystals was found in Hampton Crystal Screen I solution No. 07, formulated with 1.4 *M* sodium acetate trihydrate (NaH_3OAc) and 0.1 *M* sodium cacodylate (NaCac) pH 6.5. For both the T3 and Triac complexes, further optimization at 291 K led to crystallization condi-

tions similar to those reported for human TRβ LBD complexes (Wagner *et al.*, 2001). A reservoir solution containing 1.0 *M* NaCac and 0.1 *M* NaH_3OAc pH 7.2 was mixed with protein solution in equal amounts and equilibrated against reservoir solution at 291 K. Well formed crystals grew within 12–24 h.

Two different crystal forms of hTRα1 in complex with T3 have been obtained (Table 1). Crystals were grown under the same crystallization conditions and exhibited similar morphology (Fig. 2). In the case of the hTRα1–Triac complex, only crystals belonging to the previously unobserved space group $P2_12_12_1$ were obtained, with unit-cell parameters similar to those of the corresponding crystal form of the hTRα1–T3 complex; however, they had a somewhat different morphology (Table 1 and Fig. 3).

2.3. Data collection

X-ray diffraction experiments were performed with a MAR Research MAR345dtb image-plate detector mounted on a Rigaku UltraX 18 rotating-anode X-ray generator providing Cu $K\alpha$ radiation (1.5418 Å), operated at 50 kV and 100 mA and equipped with Osmic confocal MaxFlux optics.

To prevent radiation damage, cryocrystallographic techniques (Garman & Schneider, 1997) were employed. Crystals were briefly soaked in a cryoprotectant solution containing 1.0 *M* NaCac, 0.1 *M* NaH_3OAc pH 7.2 and 20%(*v*/*v*) ethylene glycol and rapidly cooled in a gaseous nitrogen stream (Oxford Cryosystems). Data were collected by the oscillation method from single crystals maintained at 100 K during data collection. In all cases, the oscillation range was 1°, with exposure times of 6 min (T3 complex, space group $P2_12_12_1$), 15 min (T3 complex, space group $C2$) and 20 min (Triac complex) per image (Fig. 4).

A single data set was collected for each crystal form of hTRα1–T3. X-ray data for the orthorhombic crystal were collected with a crystal-to-detector distance of 150 mm, giving an outer edge resolution of 1.86 Å. For the monoclinic form, the crystal-to-detector distance was set to 200 mm, with a maximum resolution of 2.21 Å at the detector edge. The hTRα1–Triac data set was collected using a crystal-to-detector

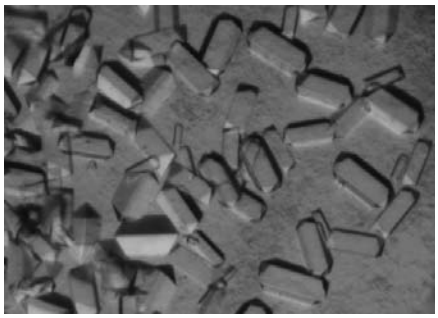

Figure 2
Single crystals of hTRα1 in complex with T3. Typical dimensions are 0.5 × 0.4 × 0.3 mm.

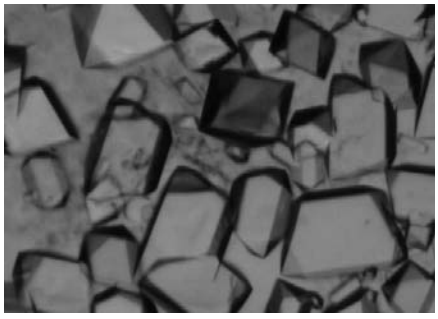

Figure 3
hTRα1–Triac crystals measuring approximately ∼0.5 mm in the longest dimension.

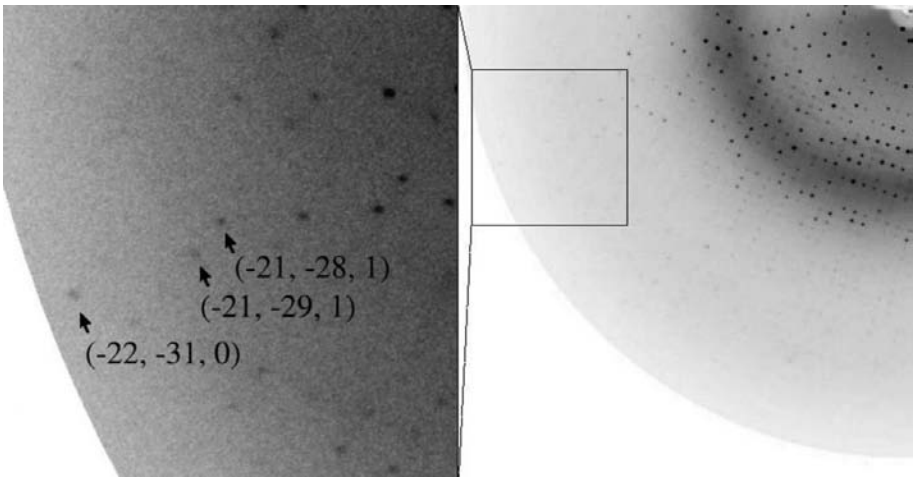

Figure 4
A 1° oscillation frame from a cryocooled hTRα1–T3 crystal. Diffraction spots with *hkl* indices (−21, −28, 1), (−21, −29, 1) and (−22, −31, 0) were marked at resolutions of 2.03, 2.00 and 1.88 Å, respectively. The resolution at the edge of the image is 1.87 Å.

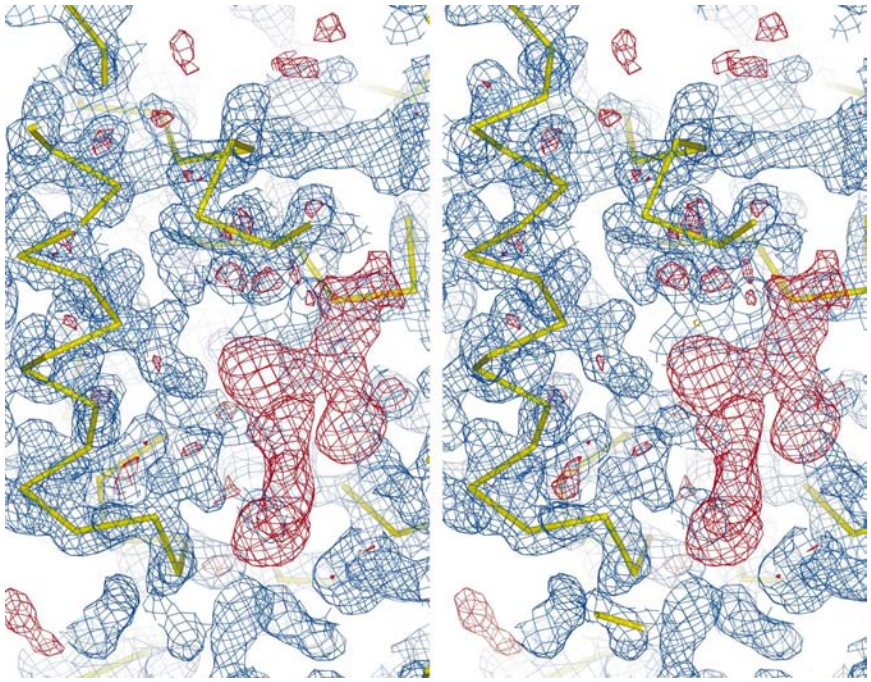

Figure 5
Stereoview of the hTRα1–T3 complex ligand-binding region. Initial $2F_{obs} - F_{calc}$ (blue) and $F_{obs} - F_{calc}$ (red) electron-density maps were contoured at the 1.0σ and 3.0σ levels, respectively, around the model (C^{α} trace, coloured yellow). The figure unequivocally indicates the presence of the ligand. The drawing was prepared using *PyMOL* (DeLano Scientific, San Carlos, CA, USA; http://www.pymol.org).

distance of 170 mm, with an outer edge resolution of 2.00 Å.

3. Results and discussion

Data reduction was performed using *MOSFLM* and *SCALA* (Collaborative Computational Project, Number 4, 1994; Winn *et al.*, 1997). Crystal parameters and data-collection statistics are summarized in Table 1. The solvent content was calculated using the total molecular weight of the hTRα1 amino-acid sequence in addition to 21 amino acids from the initiation codon, yielding a polypeptide of 284 amino acids (MW = 32 205 Da; Table 1). The correct number of molecules present in the asymmetric unit (ASU) was determined during the molecular-replacement procedure.

Primary sequence search and sequence alignments were performed using *ENTREZ* and *BLAST* (Altschul *et al.*, 1997). A sequence identity of 83%, with 92% similarity, resulted from the alignment of hTRα1 (Nakai *et al.*, 1988) and human TRβ (Weinberger *et al.*, 1986; Sakurai *et al.*, 1990). Thus, chain *A* of the corresponding dimeric X-ray structure (PDB code 1bsx; Darimont *et al.*, 1998) was used as a search model (waters, the T3 molecule and a peptide fragment of GRIP1, a coactivator, were excluded from the search model). It is worth noting that the recently reported thyroid receptor structures (PDB codes 1nav and 1nax; Ye *et al.*, 2003) could equally well be used in this case.

Molecular-replacement calculations were carried out using a resolution range of 10.0–4.0 Å and default parameters in the program *AMoRe* (Navaza, 1994; Collaborative Computational Project, Number 4, 1994; Winn *et al.*, 1997). In all cases, clear solutions were obtained for one molecule in the ASU. A search for a second molecule was not successful. After fitting, correlation coefficients of 60.2, 62.9 and 62.4% resulted for hTRα1–T3 in the orthorhombic crystal form, hTRα1–T3 in the monoclinic form and hTRα1–Triac, respectively. The corresponding *R* factors were 41.7, 39.5 and 41.9%, respectively.

The I atoms present in the T3 and Triac molecules provided significant anomalous signal, except in the case of the monoclinic form of hTRα1–T3 complex, the data set of which contains a smaller number of images. Thus, to certify whether the ligands were bound to the active sites in the three structures, the following procedure was employed. For each complex, an initial model was built by application of the suitable molecular-replacement solution (*PDBSET*; Collaborative Computational Project, Number 4, 1994; Winn *et al.*, 1997), from which structure factors were derived using the program *SFALL* (Collaborative Computational Project, Number 4, 1994; Winn *et al.*, 1997). Electron-density maps ($2F_{obs} - F_{calc}$ and $F_{obs} - F_{calc}$) were calculated and extended around the initial model. The known ligand-binding region was assessed by visual inspection using the program *O* (Kleywegt *et al.*, 2001), contouring the $2F_{obs} - F_{calc}$ and $F_{obs} - F_{calc}$ maps at the 1.0σ and 3.0σ levels, respectively. This approach was successful in clearly identifying the presence of the ligand in all three initial structures, as expected. Fig. 5 illustrates this result for the hTRα1–T3 complex in the orthorhombic crystal form. Structural refinement is in progress.

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