The oligomeric state of thyroid receptor regulates hormone binding kinetics

Suzana T Cunha Lima^{1,2} and Edson D Rodrigues^{1,3}

¹Diabetes Center, University of California, 513 Parnassus Avenue, 94143 San Francisco, California, USA

²Instituto de Biologia, Universidade Federal da Bahia, Rua Barão de Geremoabo 147, Campus de Ondina, 40170-290 Salvador, Bahia, Brazil

³Centro Universitário da Bahia - FIB, Rua Xingu, 179, 41770 Salvador, Bahia, Brazil

(Correspondence should be addressed to S T Cunha Lima at Instituto de Biologia, Universidade Federal da Bahia; Email: stcunhalima@ufba.br)

Abstract

We previously reported that mutations in the thyroid hormone receptor (TR) surface that mediates dimer and heterodimer formation do not alter affinity for cognate hormone (triiodothyronine (T₃)) yet dramatically enhance T₃ association and dissociation rates. This study aimed to show that TR oligomeric state influences binding and dissociation kinetics. We performed binding assays using marked hormone (¹²⁵I-T₃) and TRs expressed and purified by different methods. We find that T₃ associates with TRs with biphasic kinetics in solution; a rapid step (half-life ± 0.1 h) followed by a slower second step (half-life ± 5 h) and that purification of monomers suggests that biphasic kinetics are due to the presence of monomers and dimers in

our preparations. In support of this idea, incubation of TR ligand binding domain monomers with corepressor peptide induces dimer formation and decreases association rates and T₃ binds to, and dissociates from, a TR β mutant that only forms dimers (TR β D355R) with slow single-phase kinetics. In addition, heterodimer formation with retinoid X receptors also influences ligand binding kinetics. Together, these results suggest that the dimer/heterodimer surface is allosterically coupled to the hormone binding pocket and that different interactions at this surface exert different effects on ligand binding that may be relevant for TR actions in the cell.

Journal of Endocrinology (2011) **210**, 125–134

Introduction

The nuclear receptor (NR) family of transcription factors includes receptors for thyroid hormone (TH), steroids, vitamins A and D, and other small hydrophobic molecules (Laudet & Gronemeyer 2002). NRs play widespread roles in growth, development, and homeostasis, and NR signaling frequently becomes deregulated in disease. Consequently, TH receptors (TRs) are important targets for pharmaceutical development (Baxter & Webb 2009). Agonists selective for the TR β versus the TR α isoform reduce circulating levels of cholesterol, lipoprotein, and triglycerides and promote loss of adipose tissue in animal models, without eliciting harmful effects on heart, bone, and muscle. Thus, TR β -selective analogs might reduce heart disease and prevent obesity in humans. TR antagonists could be rapid acting treatments for hyperthyroidism and cardiac arrhythmias (Webb *et al.* 2002). Improved understanding of the fundamental mechanisms of TR ligand binding, and how ligand binding is coupled to alterations in TR function, will facilitate development of improved versions of these compounds that interact stably and selectively with TRs and other new drugs.

The nature of the TR binding site for cognate hormone (3,3',5-triiodo-L-thyronine, triiodothyronine (T₃)) is well

understood (Moore *et al.* 2010). TRs, like most NRs, are single polypeptide chains comprising three domains. T₃ binding is mediated by the C-terminal ligand binding domain (LBD) that contains coregulator binding sites and the major surface that mediates homodimer and heterodimer formation with the retinoid X receptor (RXR) and is linked to the other domains: the central DNA binding domain (DBD) and an N-terminal activation domain. X-ray structures of TR α and TR β LBDs in complex with T₃ (Webb *et al.* 2002), and structures of other NR LBDs obtained by other groups (Bleicher *et al.* 2008, Sonoda *et al.* 2008), reveal that agonists are buried in the core of the domain.

The major mechanism known to couple NR hormone binding to changes in gene expression is also well understood: ligand induces packing of the receptor LBD C-terminal helix against the body of the receptor, occluding part of a binding surface for corepressors and completing a coactivator binding site, with exchange of coregulators at hormone-regulated promoters (Glass & Rosenfeld 2000). Analysis of effects of TR mutants on gene expression and coregulator binding and a crystal of the TR LBD in complex with a short coactivator peptide confirms that this formulation applies to TRs (Darimont *et al.* 1998, Feng *et al.* 1998).

In contrast, factors that influence the stability of bound T_3 , and mechanisms involved in T_3 entry and exit from the pocket, are incompletely characterized. H12 plays a major role in stabilizing bound ligand in TRs and other NRs (Carlson *et al.* 1997, Huber *et al.* 2003b, Sandler *et al.* 2004, Nettles & Greene 2005), possibly by serving as a gate for a ligand entry/exit route or by stabilizing the ligand/receptor complex. Nevertheless, other regions of the TR β LBD surface are important for stable ligand binding. TR β mutations that arise in the human resistance to TH syndrome increase T_3 dissociation rates by destabilizing the loop between H1 and H3, on the opposite side of the LBD from H12 (Huber *et al.* 2003a,b) or by disrupting surface exposed clusters of charged amino acids on H7/H8 and H11, below the TR dimer/heterodimer surface at the junction of H10 and H11 (Togashi *et al.* 2005b). Finally, molecular dynamic simulations suggest that T_3 may dissociate from TRs in three ways: under H12 or through cavities that form between H1 and H3 or H8 and H11 (Martinez *et al.* 2005, 2006). Thus, different regions of the TR β LBD surface are required for stable T_3 binding and several regions of the TR surface could rearrange in response to hormone binding.

TRs exist in many different oligomeric forms and T_3 regulates the distribution of these species (Yen 2001). TRs generally exist as heterodimers with the closely related RXR in living cells but also form monomers, dimers, trimers, and tetramers in solution in the absence of RXR. A low-resolution structure of TR dimers and tetramers (Figueira *et al.* 2007) showed that the holo TR β DBD–LBD construct forms a homodimer with LBD–DBD pairs in close contact, and the apo hTR β DBD–LBD construct forms tetramers that resemble bulged cylinders with pairs of LBD dimers in a head-to-head arrangement. RXR–TRs, TR monomers, and TR homodimers activate transcription from different TH response elements (TREs) in yeast and mammalian cells, indicating that each species is functionally important (Velasco *et al.* 2007). Nevertheless, even though RXR–TR and TR–TR homodimer formation involves a common hydrophobic surface at the junction of H10 and H11 (Ribeiro *et al.* 2001), fairly distant from the ligand binding pocket (LBP), T_3 selectively inhibits TR homodimer formation on DNA (Yen 2001, Togashi *et al.* 2005b). Thus, basic thermodynamic principles predict that oligomerization should selectively affect T_3 interactions with receptor. Recent hydrogen/deuterium (H/D) exchange experiments (Figueira *et al.* 2011) revealed hormone-dependent changes in the dimer surface at H10–H11, confirming allosteric coupling between the LBP and the dimer surface. Furthermore, we previously showed that T_3 binds TR β mutants that only form monomers (TR β P419R, L422R, and M423R) with similar affinity, yet associates with and dissociates from these mutants much more rapidly than wild-type TRs (Cunha Lima *et al.* 2009). In this study, we show that TR dimer/heterodimer interactions influence ligand association/dissociation kinetics and we propose that homodimer formation induces allosteric conformational changes in the TR–LBD that impair T_3

association and dissociation, whereas RXR–TR heterodimer formation allows rapid exchange of bound T_3 . These influences may be relevant for differential function of TR oligomers in the cell.

Materials and Methods

Plasmids

Expression vectors for full-length TR α and TR β (pCMX–TR β and pCMX–TR α) and TR β mutants (TR β P419R, L422R, M423R, and D355R) were previously described (Ribeiro *et al.* 2001). TR β –LBD and DBD–LBD (amino acids 202–461 and 102–461) were expressed as fusion proteins containing an N-terminal poly-histidine tag. The TR β LBD expression vector was previously described (Wagner *et al.* 2001). The DBD–LBD expression vector was created by PCR amplification using appropriate primers containing NdeI/BamHI restriction sites and cloning into pET28 (EMD Chemicals/Novagen, Gibbstown, NJ, USA). Expression vectors for TR β LBD L422R and D355R mutants were created using the QuickChange XL Site-Directed Mutagenesis Kit (Agilent Technologies/Stratagene, Santa Clara, CA, USA). Mutation of target sequences was verified by automated DNA sequence (Elim Biopharmaceuticals, Inc., Hayward, CA, USA).

Protein expression

Unless stated, TRs and TR truncations were expressed in TNT T7 Quick *in vitro* coupled transcription/translation kits, according to manufacturer's protocols (Promega). For some experiments, TR β LBDs and TR β LBD mutants were expressed in *Escherichia coli* (*E. coli*) BL21–DE3 strain (Huber *et al.* 2003a,b). Cultures (1 l) were grown overnight (12–18 h) at room temperature (20–23 °C) in 2X L-broth + antibiotic to OD₆₀₀ = 1.0. Protein production was initiated with isopropylthiogalactoside (Sigma, final concentration 1 mM) and cultures were incubated for a further 4–6 h at room temperature. Bacteria were pelleted by centrifugation at 2800 g for 10 min. The pellet was drained and frozen at –80 °C.

Purification of TR β LBDs from bacterial extracts

Frozen pellets of 1 l bacterial cultures were thawed slowly on ice and resuspended in 20 ml TST buffer (50 mM Tris with 150 mM NaCl, 0.1% monothioglycerol, and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 8.0; Glass & Rosenfeld 2000). Lysozyme was added to 10 µg/ml and the mix was shaken at 4 °C for 30 min. This suspension was sonicated for 60 s and centrifuged at 18 000 g for 30 min (Sorvall RC 5B Plus with SS-34 rotor, GMI Inc., Ramsey, Minnesota, USA). Supernatant containing histidine-tagged TR LBDs was mixed with 60 µl equilibrated Talon Metal Affinity Resin prepared according to manufacturer's

instructions (BD Biosciences, San Diego, CA, USA) and shaken for 1 h at 4 °C. Talon resin was pelleted and washed twice in ice-cold 50 mM NaPO₄ pH 7.5, 300 mM NaCl, 0.1% MTG, 1 mM PMSF, and 2 mM ATP. Histidine-tagged TR β LBDs were eluted at 4 °C in the same buffer (2 ml) + 200 mM imidazole. TR protein concentration in flow through was determined by standard colorimetric assay using Coomassie Plus protein reagent (Pierce, Rockford, IL, USA) versus a BSA curve and the integrity of protein was verified by analysis on 10% SDS-polyacrylamide gels. This method generally yielded 7–20 mg of pure (> 95%) TR LBD per liter of culture.

Where stated, TR β LBDs were further purified by gel filtration and 4 ml of TR β LBD solution (2 mg/ml) was concentrated in Amicon Ultra-4 filter (10 kDa cut) and applied to a Superdex 200-HR Gel-Filtration column (Pfizer/Pharmacia, New York, NY, USA) and eluted in 10 mM HEPES and 1 mM dithiothreitol. Fractions (250 μ l) were collected on ice. TR was detected by measuring absorbance at 280 nm and integrity was verified by SDS-PAGE analysis of each fraction.

Hormone binding and kinetics

T₃ binding affinities (K_d), association (K_{on}), and dissociation (K_{off}) were determined according to previous work (Suthers *et al.* 1976) with small modifications (Cunha Lima *et al.* 2009). Approximate amounts of expressed TRs were determined by measuring the amount of total T₃ binding activity in single-point binding assays: different dilutions of protein preparation were incubated overnight at 4 °C with 1 nM L-3,5,3'-[¹²⁵I]T₃ (Perkin Elmer/NEN Life Science, Waltham, MA, USA) in a 100 μ l binding buffer (400 mM NaCl, 20 mM KPO₄, pH 8, 0.5 mM EDTA, 1.0 mM MgCl₂, and 10% glycerol) containing 1 mM monothiolglycerol and 50 μ g calf thymus histones (EMD Biosciences/Calbiochem, Gibbstown, NJ, USA). Bound [¹²⁵I]-T₃ was isolated by gravity flow through a 2 ml course Sephadex G-25 column (Pfizer/Pharmacia) to separate bound from free ligand and bound ligand was quantified using a γ -counter (COBRA/Packard Instruments, Meriden, CT, USA). The approximate number of binding sites per unit volume in the starting mix was calculated from specific activity of radiolabeled T₃ (3824 c.p.m. = 1 fmol).

For saturation binding analyses, about 20 fmol of TR protein was incubated overnight at 4 °C with varying concentrations of [¹²⁵I]-T₃. The amount of [¹²⁵I]-T₃ was verified by precount in each aliquot of buffer, prior to addition of protein. Next morning, the amount of bound versus free [¹²⁵I]-T₃ was determined by passage over the Sephadex G-25 column, as above. K_d values were calculated by fitting saturation curves to the equations of Swillens using the GraphPad Prism program (GraphPad Software V3.03, San Diego, CA, USA). In these conditions, non-specific binding of [¹²⁵I]-T₃ to unprogrammed reticulate lysates was negligible, < 1% of that observed in the presence of 20 fmol

TRs, as was residual binding of 1 nM [¹²⁵I]-T₃ obtained in the presence of a 1000-fold excess of unlabeled T₃ (not shown). Labeled T₃ applied to the column in the absence of TRs only dissociates after several hours of repeated washing and does not contribute to measurements of bound T₃ (not shown). Thus, most (> 99%) of the labeled ligands that pass through the Sephadex G-25 column correspond to TR bound to T₃.

T₃ association and dissociation rates

T₃ association (K_{on}) and dissociation (K_{off}) rates were determined using similar methods to that described for saturation binding assays, with the following modifications.

For K_{on} , unliganded TR preparations were added to binding buffer containing 1.5 nM [¹²⁵I]-T₃ to a final concentration of 20 fmol TRs per 100 μ l of buffer. Aliquots (100 μ l) were then applied at various times to Sephadex G25 columns to separate bound from unbound T₃. Binding curves and K_{on} values were calculated by non-linear regression analysis using one- and two-phase association growth models with GraphPad Prism Software. The one-phase exponential association equation used was $Y = Y_{max} (1 - e^{-kX})$, where X denotes time and Y specific binding. The variable k in the exponential association equation is the observed rate constant, k_{ob} , expressed in units of inverse time. The two-phase exponential association equation used was $Y = Y_{max1} (1 - e^{-k_1X}) + Y_{max2} (1 - e^{-k_2X})$, k_1 being the first observed rate constant, k_{ob1} , and k_2 the second observed rate constant, k_{ob2} , according to Motulsky & Christopoulos (2003). The program identifies the best fit (one/two phase) for each curve.

For K_{off} , TRs were incubated overnight with saturating (1 nM) [¹²⁵I]-T₃ at 4 °C (Suthers *et al.* 1976, Glass & Rosenfeld 2000). Unlabeled T₃ was added to a final concentration of 1 μ M (1000-fold excess) the following morning and aliquots were taken at various times and applied to Sephadex G-25 columns to determine how rapidly labeled ligand dissociates from TR. Binding curves and k_{off} values were calculated using the GraphPad Prism one-phase exponential decay model.

Non-denaturing PAGE

TRs (1 μ g) diluted in 10 mM HEPES to a final volume of 10 μ l were mixed with 10 μ l loading buffer (final concentration 125 mM Tris, 20% glycerol, and 1 μ g bromophenol blue) and applied to a pre-cast 4–15% Tris-HCl gel (Bio-Rad). Proteins were separated in Tris-glycine running buffer SDS in the cold room for 1 h at 150 V and 4 °C versus 1 μ g BSA as a sizing and loading control. Where specifically mentioned, TRs were preincubated $\pm$ 10 nM T₃ or 100 μ M hybrid N-CoR interaction domain (ID) peptide (Marimuthu *et al.* 2002), sequence N-RGKTTITAANFIEDIIRKALM-GSFDD-C, or vehicle for 10 min.

Analytical ultracentrifugation

TR LBD (0.2 mg; purified from bacterial extracts by incubation with talon resin and eluted as described above) was subjected to analytical ultracentrifugation in the presence or absence of 25 μM T_3 in an Optima XLA (Beckman Coulter, Brea, CA, USA) equipped with spectrophotometric and interferometer optics. Samples were loaded into two-channel velocity sedimentation cells and placed into an An60Ti rotor to spin at 300 000 g at 4 °C. In total, 30 absorbance scans were collected at 280 nm in 10 min intervals. The data was analyzed with SedFit 8.9g Software (NIH) to model the $c(s)$ continuous distribution of Lamm equation solutions.

Gel shifts

Binding of TRs and RXR–TRs to DNA were assayed as described previously (Cunha Lima *et al.* 2009).

Results

T_3 binds TRs with biphasic kinetics

We first examined association of T_3 with *in vitro* translated full-length TR β at 4° (K_{on} ; Fig. 1A). Unexpectedly, we found that the association curve exhibited better fit with the biphasic kinetics than with typical monophasic kinetics. The same experiment was performed together with TR α (Fig. 1B). T_3 bound rapidly to about 50% of TRs (K_{on} $\text{T}_{1/2}$ = 0.1 h, ranging from 0.06 to 0.15 h in different experiments) and more slowly to the remaining TRs (K_{on} $\text{T}_{1/2}$ = 4.7 h, ranging from 3.85 to 7.4 h). Equilibrium was generally only achieved after more than 8 h of incubation of T_3 with TR.

Biphasic T_3 association kinetics was also seen with versions of TR β that contained only the DBD and LBD (Fig. 1C) or LBD (Fig. 1D) and with purified preparations of TRs expressed in different systems, including full-length TR β expressed in Sf9 insect cells and preparations of TR DBD–LBD and LBDs expressed in *E. coli* (not shown, and see below). This suggests that biphasic kinetics is not caused by contaminating activities contained within a particular expression system.

Others (and ourselves) have previously examined equilibrium binding of THs to, and dissociation from, TRs and various TR truncations and found no evidence for multiple T_3 binding sites (Suthers *et al.* 1976, Nguyen *et al.* 2002, 2005, Cunha Lima *et al.* 2009). The data in Fig. 1E confirm that T_3 binds *in vitro* translated full-length TR β with apparent equilibrium (K_d) of 108 pmol and the T_3 –TR saturation curve is characterized by a single component (one-phase exponential growth). Furthermore, T_3 dissociated from *in vitro* translated full-length TR β at 4° with monophasic kinetics (one-phase exponential decay; Fig. 1F; K_{off}). Thus, T_3 associates with TRs with biphasic kinetics yet dissociates with monophasic kinetics.

Figure 1 T_3 binds TR with biphasic kinetics but exhibits monophasic saturation binding and dissociation curves. (A) Association kinetics of radiolabeled T_3 with 20 fmols of full-length TR β , *in vitro* translated material. Rapid and slow phases of association are marked with arrows and calculated half-lives ($\text{T}_{1/2}$) of each phase are indicated. (B–D) As for Fig. 1A except that T_3 association was determined with (B) full-length TR α , (C), TR β DBD–LBD and (D) TR β LBD. (E) Saturation T_3 binding assays performed with *in vitro* translated full-length TR β . (F) Dissociation of T_3 from TR β . Radiolabeled T_3 was equilibrated with *in vitro* translated full-length TR β overnight and TR– T_3 complex is challenged with unlabeled T_3 . Note single-phase kinetics of the dissociation curve.

TRs form monomers and dimers in solution and T_3 promotes dimer to monomer conversion

The simplest explanation for results described above is that unliganded TRs comprised two populations with different T_3 binding properties and that T_3 converts TRs to a single species with uniform ligand binding and dissociation properties (see Discussion). To investigate this hypothesis, we examined the composition of our TR preparations $\pm \text{T}_3$.

Our data indicate that unliganded TRs form a mix of dimers and monomers in solution and T_3 promotes dimer to monomer conversion. Analysis of migration of purified TR β –LBD preparations on non-denaturing gels revealed two species, which were the approximate size of dimers and monomers, based on comparison with similar proteins of

Figure 2 TR LBDs comprised dimers and monomers and T_3 promotes conversion of dimers to monomers. (A) Unliganded TR LBDs comprised two species and short T_3 incubations enhance the mobility of the faster migrating species. An image of a non-denaturing polyacrylamide gel used to analyze purified TR β LBDs $\pm$ short (10 min) T_3 treatment. Proteins are visualized by Coomassie blue staining. (B) Sedimentation coefficients of unliganded and liganded LBDs. The panel shows results of an analytical ultracentrifugation experiment. Unliganded TRs form two species (50 kDa and 25 kDa), dimers, and monomers, whereas liganded TRs exist as one species (24 kDa), monomers.

defined molecular weight and pI (Fig. 2A, not shown). Incubation with saturating concentrations of T_3 at room temperature for short times (10 min) increased the proportion of the rapidly migrating species and also specifically increased its mobility.

Analytical ultracentrifugation experiments confirm that unliganded TR–LBDs comprised two forms (Fig. 2B). Furthermore, analysis of sedimentation coefficients of these species (4.0S and 2.18S) confirms that they exhibit expected molecular weights of dimers and monomers (MW: 50 kDa, 25 kDa; Fig. 2B). Addition of 25 μ M T_3 before the

experiment (5 h) converted the dimer/monomer mix to a single monomeric species (2.09S, 24 kDa). Thus, incubation of the TR dimer/monomer mix with saturating concentrations of T_3 yields a preparation that consists of monomers.

TR monomers exhibit rapid T_3 association rates

To investigate whether biphasic T_3 association kinetics is related to existence of dimers and monomers in our TR preparations, we determined rates of T_3 association with purified TR β LBD monomers. T_3 bound rapidly to TR β LBD monomers with single-phase kinetics. Passage of the TR β –LBD monomer/dimer mix over a sizing column (Superdex 200HR gel filtration) yields monomers, as judged both by elution time relative to molecular weight standards (Fig. 3A) and migration on non-denaturing gels relative to wild-type TRs (Fig. 3A inset). Although T_3 bound the input TR β dimer/monomer mix with characteristic biphasic kinetics (K_{on} $T_{1/2}$ = 0.18 and 6.19 h), it associated with the purified TR β monomer preparation with single-phase rapid kinetics (K_{on} $T_{1/2}$ = 0.06 h; Fig. 3B).

We have previously shown (Cunha Lima *et al.* 2009) that T_3 associates with three TR β mutants that impair dimer/heterodimer formation with rapid kinetics (TR β L422R, M423R, and P419R), reinvestigation of these association curves reveals single-phase kinetics, and that the actual rate of T_3 association with these TR mutants was similar to, or even faster than, the first phase of T_3 association with wild-type TRs (K_{on} $T_{1/2}$ = 0.02–0.14 h).

T_3 associates slowly with a TR mutant with enhanced dimerization

Next, we explored whether T_3 associates slowly with TR dimers. We have been unable to obtain purified wild-type TR dimers devoid of monomers, so we examined T_3 association with a TR mutant (TR β D355R) that exhibits

Figure 3 Purified TR β LBD monomers bind T_3 rapidly. (A) Elution curve obtained after a TR β LBD dimer/monomer mix is purified over a Superdex gel filtration on HPLC, yielding monomers. Inset shows an image of a non-denaturing polyacrylamide gel, after staining with Coomassie blue, confirming that purified input TR β consists of dimers and monomers and that the eluate consists of TR monomers. (B) Determination of T_3 association kinetics with TR β LBD monomer/dimer mix (input) and purified TR β monomers (eluate), experiment performed as in Fig. 1A.

Figure 4 TRβD355R mutation that enhances dimer formation reduces T₃ association rates. Determination of T₃ association with wild-type purified TRβ LBD and TRβ LBD D355R, as in Fig. 1A. T₃ associates slowly with the TRβ D355R mutant, but with single-phase kinetics. Inset shows early time points on larger scale.

enhanced dimer formation on DNA in the presence of T₃ (Togashi *et al.* 2005b) and uses an unusual dimer interface that comprises the loop between H10 and H11, near the typical dimer interface at the H10–H11 junction, with auxiliary mutant-specific contacts between H8 and H6 (Jouravel *et al.* 2009).

TRβD355R exhibits enhanced dimer formation on DNA and in solution, as judged by non-denaturing gel electrophoresis and sizing columns (Togashi *et al.* 2005b, Jouravel *et al.* 2009 and not shown). Analysis of association rates revealed that TRβD355R bound T₃ with single-phase kinetics characterized by slow on rate (K_{on} $T_{1/2}$ = 0.92 vs 0.06 h for purified LBD monomers, Fig. 4 and Table 1). This represents further evidence that biphasic association kinetics is only seen in the presence of TR dimers and monomers and further suggests that the slower component of these biphasic on rates reflects T₃ association with dimers.

Table 1 Kinetics of binding of thyroid hormone receptor expressed and purified by different methods, containing or not a point mutation, or in heterodimer with retinoid X receptor (RXR)

Receptor	K_d (10^{-10} M)	Association first half-life (h)	Association second half-life (h)	Dissociation half-life (h)	Characteristic
WT FLTRβ	2.0 ± 0.34	0.10 ± 0.03	4.8 ± 2.6	6.7 ± 1.0	Monomers and dimers
His-tag purified LBD	2.3 ± 0.65	0.17 ± 0.07	6.1 ± 1.1	6.2 ± 1.2	Monomers and dimers
Purified LBD	1.6 ± 0.39	0.15 ± 0.12	None	5.6 ± 0.46	Monomers
P419R	3.5 ± 0.41	0.14 ± 0.23	None	3.1 ± 0.50	Monomers
L422R	1.8 ± 0.4	0.03 ± 0.009	None	1.5 ± 0.38	Monomers
M423R	1.7 ± 0.45	0.02 ± 0.005	None	0.2 ± 0.05	Monomers
D355R	1.3 ± 0.29	0.92 ± 0.14	None	10.8 ± 1.20	Dimers
TR:RXR	1.2 ± 0.66	0.23 ± 0.04	None	1.4 ± 0.49	Heterodimers

K_d , dissociation constant; WT FLTRβ, wild-type full-length thyroid hormone receptor beta; His-tag, histidine tagging; LBD, ligand binding domain.

Corepressor induces TR LBD dimer formation and biphasic T₃ association kinetics

Next, we examined effects of a short N-CoR peptide corresponding to an NR ID that is known to stabilize the unliganded TRβ LBD (Pissios *et al.* 2000, Webb *et al.* 2000), on TR monomer/dimer equilibrium and T₃ association kinetics. Figure 5A shows that incubation of purified unliganded TRβ LBD monomers with the N-CoR peptide increased the amount of TR dimers. This was accompanied by conversion of the single-phase association kinetic curve to a biphasic curve. Similar effects, on conversion of monomers to dimers or T₃ association, were not seen with a mutant (TRβL422R) that only forms monomers indicating that both effects require the dimer interface at the junction of H10 and H11 (Fig. 5B). This data supports the notion that biphasic association kinetics are related to the presence of TR monomers and dimers in solution and that T₃ exhibits decreased rates of binding to TR dimers.

TR mutants that influence dimer formation alter T₃ dissociation rates

We previously reported that TR mutations that block (L422R, M423R, and P419R; Cunha Lima *et al.* 2009) or enhance (TRβD355R; Togashi *et al.* 2005b) TR dimer formation do not significantly alter affinity of TR for T₃ (Ribeiro *et al.* 2001). As some of these TR mutants exhibit enhanced rates of T₃ association relative to wild-type TRs and TR monomers (Fig. 5), basic thermodynamic principles predict that the mutations should alter T₃ dissociation rates. Consistent with this formulation, TRβ mutants that block dimer formation exhibit higher rates of T₃ dissociation than wild-type TRs and the magnitude of these effects paralleled effects of the same mutations on T₃ association (Table 1). In particular, TRβL422R and TRβM423R exhibited very high association and dissociation rates relative to wild-type TRs. Moreover, T₃ dissociated from the TR mutant that only forms dimers (TRβD355R) nearly twice as slowly as from wild-type TRs and up to 50-fold more slowly than the TRβ mutants that only form monomers.

Figure 5 A corepressor (N-CoR) peptide induces TR LBD monomer to dimer conversion in solution and inhibits T_3 association. (A) The inset shows an image of a Coomassie blue-stained non-denaturing polyacrylamide gel, as in Fig. 3A, used to compare purified TR β LBD monomers and a similar TR β LBD monomer preparation after incubation for 30 min with a corepressor ID peptide. Note that this preparation consists of monomers and dimers. The main panel shows T_3 association with purified TR LBD monomers or TR LBD + N-CoR (monomer/dimer mix), as in Fig. 1A. (B) As for Fig. 6A, except that the experiment is performed with a TR β LBD L422R mutant that can only form monomers.

RXR–TRs bind and release T_3 rapidly

As TRs form heterodimers with RXR, it is relevant to examine binding of T_3 to this species. We prepared *in vitro* translated TRs $\pm$ RXRs and examined association rates of ligand with each preparation. T_3 bound to RXR–TRs with rapid single-phase kinetics similar to those observed with TR monomers (Fig. 6A). Gel shift experiments confirmed that RXR–TR mixtures comprised heterodimers, as judged by increased mobility of the complex relative to that of TR homodimers on a TRE oligonucleotide (DR-4; Fig. 6B).

T_3 also dissociated rapidly from RXR–TRs, even faster than from wild-type TRs or purified preparations of TR–LBD monomers ($T_{1/2}$ for T_3 dissociation of 1.4 h for heterodimer versus 6.7 h for wild-type TRs and 5.6 h for the TR β LBD monomer (Table 1)). These results emphasize that TR exhibits major differences in ligand association and dissociation kinetics when it is a monomer or homodimer versus a heterodimer.

Discussion

In this study, we have shown that the oligomeric state of the TR influences T_3 binding kinetics. Our investigation was prompted by our previous observation that mutations in the TR LBD surface that mediates dimer and heterodimer formation increase association rates without altering affinity of TRs for T_3 (Cunha Lima *et al.* 2009). Our analysis of T_3 association with TRs reveals better fit of the curves with biphasic kinetics rather than single-phase kinetics: T_3 binds TRs with a rapid first phase (half-life ± 0.1 h) followed by a slower second phase (half-life ± 5 h). This is a fundamental property of T_3 association with TR–LBDs; it is seen with both TR isoforms and TR truncations overlapping the DBD–LBD and LBD prepared in a variety of systems.

In principle, biphasic association kinetics could be explained in several ways, including existence of multiple ligand binding sites on the target protein, but two lines of evidence suggest that our TR preparations comprised two populations with different T_3 binding properties. First, our

Figure 6 RXR–TR Heterodimers bind and release T_3 rapidly. (A) Determination of T_3 association rates with *in vitro* translated TRs or RXR–TR mix, as in Fig. 1A. Note that RXR–TR heterodimer formation enhances rates of T_3 binding compared with TR only. (B) Incubation of *in vitro* translated TR β with *in vitro* translated RXR converts TR homodimers to RXR–TR heterodimers, as judged by reduced mobility of complex formed with a radiolabeled DR-4 element in a gel shift assay.

Figure 7 Model for T_3 binding to TRs. A schematic representation of T_3 binding to TRs. T_3 is represented by small black ovals, TRs by large white circles, and RXRs by shaded circles. T_3 binds rapidly to monomers and slowly to dimers and subsequently converts dimers to monomers. Thus, T_3 association measurements reflect contributions of k_1 and k_2 , whereas dissociation measurements primarily reflect k_{-1} .

TR X-ray crystal structures indicate that there is only a single high-affinity hormone binding pocket (Wagner *et al.* 2001, Sandler *et al.* 2004). Next, previous analyses of T_3 equilibrium binding and dissociation kinetics (Suthers *et al.* 1976, Nguyen *et al.* 2002, 2005, Cunha Lima *et al.* 2009), confirmed here, had revealed no evidence for multiple T_3 binding sites. Thus, the simplest explanation that reconciles our findings is that unliganded TRs comprised two distinct species that bind T_3 at different rates and that T_3 converts TRs to a single species with uniform ligand binding and dissociation properties.

The data reported in the paper support this formulation and indicate that biphasic association kinetics reflects rapid T_3 binding to TR monomers and slow T_3 binding to dimers (Fig. 7). Analyses of purified unliganded TRs on non-denaturing gels and by analytical ultracentrifugation reveal a mix of TR monomers and dimers (Fig. 2). Furthermore, several lines of evidence indicate that T_3 rapidly binds to TR monomers and slowly to dimers (Figs 3–5, Table 1). 1) Short T_3 incubations selectively increase TR monomer mobility in non-denaturing gels, indicative of rapid hormone association with this species and not with dimers. 2) T_3 binds purified TR LBD monomers with rapid single-phase kinetics ($T_{1/2} = 0.03\text{--}0.27$ h; comparable to the first phase of T_3 association with TRs, $T_{1/2} = 0.07\text{--}0.13$ h). 3) The TR β D355R mutant (Togashi *et al.* 2005b) only forms dimers and binds T_3 with slow single-phase kinetics ($T_{1/2} = 0.92$ h, 6–15 times slower than TR LBD monomers and up to 46-fold slower than TR β mutants that only form monomers). 5) An N-CoR peptide promotes dimer formation within preparations of purified TR LBD monomers and restores biphasic ligand binding kinetics.

Our data are also consistent with another prediction of our model that T_3 converts TRs to a single species with uniform ligand binding properties. Analytical ultracentrifugation (Fig. 2) and sizing chromatography analysis (not shown)

reveal that T_3 converts TR dimers to monomers. Furthermore, T_3 dissociates from wild-type TRs at a comparable rate ($T_{1/2} = 5\text{--}7$ h) to purified LBD monomers ($T_{1/2} = 5.6$ h), consistent with the prediction that measurements of TR ligand dissociation kinetics reflect T_3 release from monomers.

Heterodimer formation also influences T_3 binding kinetics. We find that RXR–TRs exhibit unique properties that are distinct from TR–TR homodimers or TR monomers. RXR–TRs bind T_3 with rapid single-phase kinetics, comparable to monomers (Fig. 7; Table 1), but release T_3 more rapidly than TR monomers ($T_{1/2} = 1.4$ h for RXR–TRs versus 5.6 h for TR LBD monomers).

Together, our data suggest that interactions at the TR dimer/heterodimer surface influence the overall LBD conformation to regulate T_3 binding kinetics. Thus, we propose that 1) TR homodimer formation alters LBD conformation to slow the rate of T_3 entry to the pocket. 2) T_3 remodels the TR–LBD to prevent homodimer formation; although it was previously known that T_3 binding inhibits TR homodimer formation on DNA (Togashi *et al.* 2005b, Jeyakumar *et al.* 2008), our data represent direct proof that T_3 impairs dimer formation via effects on LBDs. 3) RXR–TR formation induces a novel TR conformation distinct from homodimers or monomers, with RXR–TRs able to bind T_3 more rapidly than TR–TR homodimers and release T_3 more rapidly than TR monomers. The fact that the N-CoR ID peptide, which stabilizes the unliganded TR LBD by binding to the H3–H5 region (Hu & Lazar 1999, Marimuthu *et al.* 2002), induces that homodimer formation through H10–H11 on the opposite side of the domain represents further evidence that the dimer surface is allosterically coupled to other parts of the LBD and recent H/D exchange data (Figueira *et al.* 2011) confirms that hormone binding results in conformational alterations that affect the entire domain including the dimer surface.

Analysis of dimerization and kinetics of binding has also been performed with estrogen receptors (ERs) and results link dimerization to stabilization of the ligand and decreasing in kinetics (Tamrazi *et al.* 2002). Another study with ER demonstrated that WT protein and mutants Y537S and Y537E exhibited biphasic dissociation kinetics, which, according to the author, was also due to the presence of dimers and monomers (Zhong & Skafar 2002).

What structural elements couple the dimer surface to changes in T_3 binding kinetics? We previously proposed that homodimer and heterodimer surfaces are partially overlapping and that T_3 blocks dimer formation but not RXR–TR heterodimer formation, by remodeling homodimer-specific portions of the LBD surface, including the charge cluster that is target for the D355R mutation used here (Togashi *et al.* 2005a). Thus, one possibility is that TR homodimer and heterodimer formation influences T_3 binding kinetics by altering the conformation of these homodimer- and heterodimer-specific regions of the dimer interface. Another possibility is that TR dimer and heterodimer partners could alter the conformation of the region that comprises the

common homodimer/heterodimer interface at the H10/H11 junction to alter TR LBD hormone binding properties. A piece of evidence that the H10–H11 region could influence ligand binding kinetics is that mutations in this region that inhibit dimer and heterodimer formation (TR β P419R; TR β L422R, and TR β M423R) exhibit differences in the magnitude of their effects on T₃ association and dissociation rates (Cunha Lima *et al.* 2009). Furthermore, HD exchange experiments (Figueira *et al.* 2011) confirm that hormone binding results in remodeling of the dimer surface.

We emphasize that enhanced dimer formation by the TR β D355R mutant involves novel surfaces in the H10–H11 loop and H8–H6 that lie close to the classic dimer surface at H10–H11 but are not identical to this interaction surface (Jouravel *et al.* 2009). The fact that dimer formation through an unusual surface affects hormone binding kinetics in a similar manner to native dimer formation raises the possibility that effects of dimerization on ligand binding kinetics may be related partly to physical occlusion of ligand entry/exit routes as well as allosteric connections between the LBP and the classic dimer face. Indeed, the TR β D355R dimer blocks one of the predicted ligand exit routes identified by molecular dynamics at H8 (Martinez *et al.* 2005). Interestingly, this is similar to predictions made from molecular dynamics simulations with ER monomers and dimers (Sonoda *et al.* 2008), which suggests that dimer interactions at H8 specifically occlude a major estrogen exit route.

Overall, the fact that TR oligomer formation influences ligand binding kinetics lends support to the concept that TR and NR LBDs influence the conformation and activities of their dimer and heterodimer partners and raise the possibility that these effects could be important in living cells (Schueler *et al.* 1990, Ribeiro *et al.* 2001). For example, TR does not usually allow activation of RXR heterodimer partner by its cognate ligand (9-*cis* retinoic acid), whereas other NRs, such as the liver X receptor, do permit RXR activation *in vivo* (Shulman *et al.* 2004). Thus, effects of TR oligomer formation on ligand binding kinetics could be important for TR action in living cells. As RXR–TRs bind and release T₃ faster than wild-type TRs, we expect that this species should respond more rapidly to alterations in intracellular T₃ than other TR species. Furthermore, the fact that TR dimers are resistant to T₃ binding is consistent with previous proposals that this species is important for gene regulation in the absence of T₃ (Williams *et al.* 1992). Improved understanding of influences of homodimer and heterodimer formation on ligand binding, and vice versa, could improve our ability to develop drugs to selectively bind and modulate activities of particular TR oligomers.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by National Institute of Health (grants DK41482 and DK51281).

Acknowledgements

We thank Prof. John D Baxter and Dr Paul Webb for giving us the opportunity to develop this work at the Diabetes Center of University of California San Francisco.

References

- Baxter JD & Webb P 2009 Thyroid hormone mimetics: potential applications in atherosclerosis, obesity and type 2 diabetes. *Nature Reviews. Drug Discovery* **8** 308–320. (doi:10.1038/nrd2830)
- Bleicher L, Aparicio R, Nunes FM, Martinez L, Gomes Dias SM, Figueira AC, Santos MA, Venturelli WH, da Silva R, Donate PM *et al.* 2008 Structural basis of GC-1 selectivity for thyroid hormone receptor isoforms. *BMC Structural Biology* **8** 1–13. (doi:10.1186/1472-6807-8-8)
- Carlson KE, Choi I, Gee A, Katzenellenbogen BS & Katzenellenbogen JA 1997 Altered ligand binding properties and enhanced stability of a constitutively active estrogen receptor: evidence that an open pocket conformation is required for ligand interaction. *Biochemistry* **36** 14897–14905. (doi:10.1021/bi971746l)
- Cunha Lima ST, Nguyen NH, Togashi M, Apriletti J, Nguyen P, Polikarpov I, Scanlan TS, Baxter JD & Webb P 2009 Differential effects of TR ligands on hormone dissociation rates: evidence for multiple ligand entry/exit pathways. *Journal of Steroid Biochemistry and Molecular Biology* **117** 125–131. (doi:10.1016/j.jsmb.2009.08.003)
- Darimont BD, Wagner RL, Apriletti JW, Stallcup MR, Kushner PJ, Baxter JD, Fletterick RJ & Yamamoto KR 1998 Structure and specificity of nuclear receptor-coactivator interactions. *Genes and Development* **12** 3343–3356. (doi:10.1101/gad.12.21.3343)
- Feng W, Ribeiro RC, Wagner RL, Nguyen H, Apriletti JW, Fletterick RJ, Baxter JD, Kushner PJ & West BL 1998 Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. *Science* **280** 1747–1749. (doi:10.1126/science.280.5370.1747)
- Figueira AC, Neto Mde O, Bernardes A, Dias SM, Craievich AF, Baxter JD, Webb P & Polikarpov I 2007 Low-resolution structures of thyroid hormone receptor dimers and tetramers in solution. *Biochemistry* **46** 1273–1283. (doi:10.1021/bi061698h)
- Figueira AC, Saidenberg DM, Souza PC, Martinez L, Scanlan TS, Baxter JD, Skaf MS, Palma MS, Webb P & Polikarpov I 2011 Analysis of agonist and antagonist effects on thyroid hormone receptor conformation by hydrogen/deuterium exchange. *Molecular Endocrinology* **25** 15–31. (doi:10.1210/me.2010-0202)
- Glass CK & Rosenfeld MG 2000 The coregulator exchange in transcriptional functions of nuclear receptors. *Genes and Development* **14** 121–141. (doi:10.1101/gad.14.2.121)
- Hu X & Lazar MA 1999 The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. *Nature* **402** 93–96. (doi:10.1038/47069)
- Huber BR, Desclozeaux M, West BL, Cunha Lima ST, Nguyen HT, Baxter JD, Ingraham HA & Fletterick RJ 2003a Thyroid hormone receptor-beta mutations conferring hormone resistance and reduced corepressor release exhibit decreased stability in the N-terminal ligand-binding domain. *Molecular Endocrinology* **17** 107–116. (doi:10.1210/me.2002-0097)
- Huber BR, Sandler B, West BL, Cunha Lima ST, Nguyen HT, Apriletti JW, Baxter JD & Fletterick RJ 2003b Two resistance to thyroid hormone mutants with impaired hormone binding. *Molecular Endocrinology* **17** 643–652. (doi:10.1210/me.2002-0095)
- Jeyakumar M, Webb P, Baxter JD, Scanlan TS & Katzenellenbogen JA 2008 Quantification of ligand-regulated nuclear receptor corepressor and

- coactivator binding, key interactions determining ligand potency and efficacy for the thyroid hormone receptor. *Biochemistry* **47** 7465–7476. (doi:10.1021/bi800393u)
- Jouravel N, Sablin E, Togashi M, Baxter JD, Webb P & Fletterick RJ 2009 Molecular basis for dimer formation of TRbeta variant D355R. *Proteins* **75** 111–117. (doi:10.1002/prot.22225)
- Laudet V & Gronemeyer H 2002. *The Nuclear Receptor Facts Book*. Factsbook Series. edn 1, pp 462. London: Academic Press.
- Marimuthu A, Feng W, Tagami T, Nguyen H, Jameson JL, Fletterick RJ, Baxter JD & West BL 2002 TR surfaces and conformations required to bind nuclear receptor corepressor. *Molecular Endocrinology* **16** 271–286. (doi:10.1210/me.16.2.271)
- Martinez L, Sonoda MT, Webb P, Baxter JD, Skaf MS & Polikarpov I 2005 Molecular dynamics simulations reveal multiple pathways of ligand dissociation from thyroid hormone receptors. *Biophysical Journal* **89** 2011–2023. (doi:10.1529/biophysj.105.063818)
- Martinez L, Webb P, Polikarpov I & Skaf MS 2006 Molecular dynamics simulations of ligand dissociation from thyroid hormone receptors: evidence of the likeliest escape pathway and its implications for the design of novel ligands. *Journal of Medicinal Chemistry* **49** 23–26. (doi:10.1021/jm050805n)
- Moore TW, Mayne CG & Katzenellenbogen JA 2010 Minireview: not picking pockets: nuclear receptor alternate-site modulators (NRAMs). *Molecular Endocrinology* **24** 683–695. (doi:10.1210/me.2009-0362)
- Motulsky H & Christopoulos A 2003 Fitting models to biological data using linear and nonlinear regression. A practical guide to curve fitting. edn 4, pp 351. Oxford University Press.
- Nettles KW & Greene GL 2005 Ligand control of coregulator recruitment to nuclear receptors. *Annual Review of Physiology* **67** 309–333. (doi:10.1146/annurev.physiol.66.032802.154710)
- Nguyen NH, Apriletti JW, Cunha Lima ST, Webb P, Baxter JD & Scanlan TS 2002 Rational design and synthesis of a novel thyroid hormone antagonist that blocks coactivator recruitment. *Journal of Medicinal Chemistry* **45** 3310–3320. (doi:10.1021/jm0201013)
- Nguyen NH, Apriletti JW, Baxter JD & Scanlan TS 2005 Hammett analysis of selective thyroid hormone receptor modulators reveals structural and electronic requirements for hormone antagonists. *Journal of the American Chemical Society* **127** 4599–4608. (doi:10.1021/ja0440093)
- Pissios P, Tzamelis I, Kushner P & Moore DD 2000 Dynamic stabilization of nuclear receptor ligand binding domains by hormone or corepressor binding. *Molecular Cell* **6** 245–253. (doi:10.1016/S1097-2765(00)00026-5)
- Ribeiro RC, Feng W, Wagner RL, Costa CH, Pereira AC, Apriletti JW, Fletterick RJ & Baxter JD 2001 Definition of the surface in the thyroid hormone receptor ligand binding domain for association as homodimers and heterodimers with retinoid X receptor. *Journal of Biological Chemistry* **276** 14987–14995. (doi:10.1074/jbc.M010195200)
- Sandler B, Webb P, Apriletti JW, Huber BR, Togashi M, Cunha Lima ST, Juric S, Nilsson S, Wagner R, Fletterick RJ *et al.* 2004 Thyroxine-thyroid hormone receptor interactions. *Journal of Biological Chemistry* **279** 55801–55808. (doi:10.1074/jbc.M410124200)
- Schueler PA, Schwartz HL, Strait KA, Mariash CN & Oppenheimer JH 1990 Binding of 3,5,3'-triiodothyronine (T₃) and its analogs to the *in vitro* translational products of c-erbA protooncogenes: differences in the affinity of the alpha- and beta-forms for the acetic acid analog and failure of the human testis and kidney alpha-2 products to bind T₃. *Molecular Endocrinology* **4** 227–234. (doi:10.1210/mend-4-2-227)
- Shulman AI, Larson C, Mangelsdorf DJ & Ranganathan R 2004 Structural determinants of allosteric ligand activation in RXR heterodimers. *Cell* **116** 417–429. (doi:10.1016/S0092-8674(04)00119-9)
- Sonoda MT, Martinez L, Webb P, Skaf MS & Polikarpov I 2008 Ligand dissociation from estrogen receptor is mediated by receptor dimerization: evidence from molecular dynamics simulations. *Molecular Endocrinology* **22** 1565–1578. (doi:10.1210/me.2007-0501)
- Suthers MB, Pressley LA & Funder JW 1976 Glucocorticoid receptors: evidence for a second, non-glucocorticoid binding site. *Endocrinology* **99** 260–269. (doi:10.1210/endo-99-1-260)
- Tamrazi A, Carlson KE, Daniels JR, Hurth KM & Katzenellenbogen JA 2002 Estrogen receptor dimerization: ligand binding regulates dimer affinity and dimer dissociation rate. *Molecular Endocrinology* **16** 2706–2719. (doi:10.1210/me.2002-0250)
- Togashi M, Borngraeber S, Sandler B, Fletterick RJ, Webb P & Baxter JD 2005a Conformational adaptation of nuclear receptor ligand binding domains to agonists: potential for novel approaches to ligand design. *Journal of Steroid Biochemistry and Molecular Biology* **93** 123–137. (doi:10.1016/j.jsbmb.2005.01.004)
- Togashi M, Nguyen P, Fletterick R, Baxter JD & Webb P 2005b Rearrangements in thyroid hormone receptor charge clusters that stabilize bound 3,5',5-triiodo-L-thyronine and inhibit homodimer formation. *Journal of Biological Chemistry* **280** 25665–25673. (doi:10.1074/jbc.M501615200)
- Velasco LF, Togashi M, Walfish PG, Pessanha RP, Moura FN, Barra GB, Nguyen P, Rebong R, Yuan C, Simeoni LA *et al.* 2007 Thyroid hormone response element organization dictates the composition of active receptor. *Journal of Biological Chemistry* **282** 12458–12466. (doi:10.1074/jbc.M610700200)
- Wagner RL, Huber BR, Shiau AK, Kelly A, Cunha Lima ST, Scanlan TS, Apriletti JW, Baxter JD, West BL & Fletterick RJ 2001 Hormone selectivity in thyroid hormone receptors. *Molecular Endocrinology* **15** 398–410. (doi:10.1210/me.15.3.398)
- Webb P, Anderson CM, Valentine C, Nguyen P, Marimuthu A, West BL, Baxter JD & Kushner PJ 2000 The nuclear receptor corepressor (N-CoR) contains three isoleucine motifs (I/LXXII) that serve as receptor interaction domains (IDs). *Molecular Endocrinology* **14** 1976–1985. (doi:10.1210/me.14.12.1976)
- Webb P, Nguyen NH, Chiellini G, Yoshihara HA, Cunha Lima ST, Apriletti JW, Ribeiro RC, Marimuthu A, West BL, Goede P *et al.* 2002 Design of thyroid hormone receptor antagonists from first principles. *Journal of Steroid Biochemistry and Molecular Biology* **83** 59–73. (doi:10.1016/S0960-0760(02)00270-4)
- Williams GR, Harney JW, Moore DD, Larsen PR & Brent GA 1992 Differential capacity of wild type promoter elements for binding and trans-activation by retinoic acid and thyroid hormone receptors. *Molecular Endocrinology* **6** 1527–1537. (doi:10.1210/me.6.10.1527)
- Yen PM 2001 Physiological and molecular basis of thyroid hormone action. *Physiological Reviews* **81** 1097–1142.
- Zhong L & Skafar DF 2002 Mutations of tyrosine 537 in the human estrogen receptor- α selectively alter the receptor's affinity for estradiol and the kinetics of the interaction. *Biochemistry* **41** 4209–4217. (doi:10.1021/bi0121095)

Received in final form 26 March 2011

Accepted 20 April 2011

Made available online as an Accepted Preprint
20 April 2011