The oligomeric state of thyroid receptor regulates hormone binding kinetics

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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 TR oligomeric state leads to 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 TRβ mutant that only forms dimers (TRβD355R) 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.

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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 TRβ versus the TRα 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, TRβ-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 TRα and TRβ 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 T3, and mechanisms involved in T3 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 TRβ LBD surface are important for stable ligand binding. TRβ mutations that arise in the human resistance to TH syndrome increase T3 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 T3 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 TRβ LBD surface are required for stable T3 binding and several regions of the TR surface could rearrange in response to hormone binding.

TRs exist in many different oligomeric forms and T3 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 TRβ DBD–LBD construct forms a homodimer with LBD–DBD pairs in close contact, and the apo hTRβDBD–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 TR 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), T3 selectively inhibits TR homodimer formation on DNA (Yen 2001, Togashi et al. 2005b). Thus, basic thermodynamic principles predict that oligomerization should selectively affect T3 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 T3 binds TRβ mutants that only form monomers (TRβP419R, 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 T3 association and dissociation, whereas RXR–TR heterodimer formation allows rapid exchange of bound T3. These influences may be relevant for differential function of TR oligomers in the cell.

Materials and Methods

Plasmids

Expression vectors for full-length TRα and TRβ (pCMX–TRβ and pCMX–TRα) and TRβ mutants (TRβP419R, L422R, M423R, and D355R) were previously described (Ribeiro et al. 2001). TRβ–LBD and DBD–LBD (amino acids 202–461 and 102–461) were expressed as fusion proteins containing an N-terminal poly-histidine tag. The TRβ 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 TRβ 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, TRβ LBDs and TRβ 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 TRβ LBDs from bacterial extracts

Frozen pellets of 11 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 phenylmethylsulphonyl 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 PMSE, and 2 mM ATP. Histidine-tagged TRβ 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, TRβ LBDs were further purified by gel filtration and 4 ml of TRβ LBD solution (2 mg/ml) was concentrated in Amicon Ultra-4 filter (10 kDa cut) and eluted in 10 mM HEPES and 1 mM dithiothreitol. Fractions (250 μl) 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 (Kd), association (Kon), and dissociation (Koff) 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-3,3',5'-[125I]T3 (Perkin Elmer/NEN Life Science, Waltham, MA, USA) in a 100 μl binding buffer (400 mM NaCl, 20 mM KPO4, pH 8, 0.5 mM EDTA, 1.0 mM MgCl2, and 10% glycerol) containing 1 mM monoethylglycerol and 50 μg calf thymus histones (EMD Biosciences/Calbiochem, Gibbstown, NJ, USA). Bound 125I-T3 was isolated by gravity through a 2 ml course Sephadex G-25 column (Pfizer/Pharmacia, New York, NY, USA) and eluted in concentrated in Amicon Ultra-4 filter (10 kDa cut) and eluted in 10 mM HEPES and 1 mM dithiothreitol. 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.

T3 association (Kon) and dissociation (Koff) rates were determined using similar methods to that described for saturation binding assays, with the following modifications. For Kon, unliganded TR preparations were added to binding buffer containing 1–5 nM 125I-T3 to a final concentration of 20 fmols TRs per 100 μl of buffer. Aliquots (100 μl) were then applied at various times to Sephadex G25 columns to separate bound from unbound T3. Binding curves and Kon 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 $Y = Y_{max} (1 - e^{-k_Xt})$, where $X$ denotes time and $Y$ specific binding. The variable $k$ in the exponential association equation is the observed rate constant, $k_{obs}$, expressed in units of inverse time. The two-phase exponential association equation used was $Y = Y_{max1} (1 - e^{-k_1 t_1}) + Y_{max2} (1 - e^{-k_2 t_2})$, $k_1$ being the first observed rate constant, $k_{obs1}$, and $k_2$ the second observed rate constant, $k_{obs2}$, according to Motulsky & Christopoulos (2003). The program identifies the best fit (one/two phase) for each curve.

For Koff, 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 μM (1000-fold excess) the following morning and aliquots were taken at various times and applied to Sephadex G25 columns to determine how rapidly labeled ligand dissociates from TR. Binding curves and Koff values were calculated using the GraphPad Prism one-phase exponential decay model.

Non-denaturing PAGE

TRs (1 μg) diluted in 10 mM HEPES to a final volume of 10 μl were mixed with 10 μl loading buffer (final concentration 125 mM Tris, 20% glycerol, and 1 mM 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 μg BSA as a sizing and loading control. Where specifically mentioned, TRs were preincubated ± 10 nM T3 or 100 μM hybrid N-CoR interaction domain (ID) peptide (Marimuthu et al. 2002), sequence N-RKTTTIAANFEDIIRKALM-GSFDD-C, or vehicle for 10 min.
Results

**T₃ binds TRs with biphasic kinetics**

We first examined association of T₃ with in vitro translated full-length TRβ at 4°C (Kₘ₀, 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 TRα (Fig. 1B). T₃ bound rapidly to about 50% of TRs (Kₘ₀ T₁/₂ = 0.1 h, ranging from 0.06 to 0.15 h in different experiments) and more slowly to the remaining TRs (Kₘ₀ T₁/₂ = 4.7 h, ranging from 3.85 to 7.4 h). Equilibrium was generally only achieved after more than 8 h of incubation of T₃ with TR.

Biphasic T₃ association kinetics was also seen with versions of TRβ 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 TRβ expressed in Sf9 insect cells and preparations of TR DBD–LBD (Fig. 1D) and with purified preparations of TRs expressed in various TR truncations and found no evidence for multiple TR binding sites (Suthers et al. 1976, Nguyen et al. 2002, 2005, Cunha Lima et al. 2009). The data in Fig. 1E confirm that T₃ association was determined with (B) full-length TRα, (C) TRβ DBD–LBD and (D) TRβ LBD. (E) Saturation TRβ binding assays performed with in vitro translated full-length TRβ. (F) Dissociation of T₃ from TRβ. Radiolabeled T₃ was equilibrated with in vitro translated full-length TRβ overnight and TR–T₃ complex is challenged with unlabeled T₃. Note single-phase kinetics of the dissociation curve.

**TRs form monomers and dimers in solution and T₃ promotes dimer to monomer conversion**

The simplest explanation for results described above is that unliganded TRs comprised two populations with different T₃ binding properties and that T₃ 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 ± T₃.

Our data indicate that unliganded TRs form a mix of dimers and monomers in solution and T₃ promotes dimer to monomer conversion. Analysis of migration of purified TRβ-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 1](image_url)

**Figure 1** T₃ binds TR with biphasic kinetics but exhibits monophasic saturation binding and dissociation curves. (A) Association kinetics of radiolabeled T₃ with 20 fmols of full-length TRβ, in vitro translated material. Rapid and slow phases of association are marked with arrows and calculated half-lives (T₁/₂) on T₁/₂ = 0.06 h, T₁/₂ = 0.10 h, T₁/₂ = 0.12 h, T₁/₂ = 4.7 h, T₁/₂ = 7.6 h. (B) TR full length. (C) TRβ DBD LBD. (D) TRβ LBD. (E) Saturation T₃ binding assays performed with in vitro translated full-length TRβ. (F) Dissociation of T₃ from TRβ. Radiolabeled T₃ was equilibrated with in vitro translated full-length TRβ overnight and TR–T₃ complex is challenged with unlabeled T₃. Note single-phase kinetics of the dissociation curve.
defined molecular weight and pI (Fig. 2A, not shown). Incubation with saturating concentrations of T₃ 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 μM T₃ 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 T₃ yields a preparation that consists of monomers.

TR monomers exhibit rapid T₃ association rates

To investigate whether biphasic T₃ association kinetics is related to existence of dimers and monomers in our TR preparations, we determined rates of T₃ association with purified TRβ LBD monomers. T₃ bound rapidly to TRβ LBD monomers with single-phase kinetics. Passage of the TRβ–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 T₃ bound the input TRβ dimer/monomer mix with characteristic biphasic kinetics (Kₐ on T₁/₂ = 0.18 and 6.19 h), it associated with the purified TRβ monomer preparation with single-phase rapid kinetics (Kₐ on T₁/₂ = 0.06 h; Fig. 3B).

We have previously shown (Cunha Lima et al. 2009) that T₃ associates with three TRβ mutants that impair dimer/heterodimer formation with rapid kinetics (TRβL422R, M423R, and P419R), reinvestigation of these association curves reveals single-phase kinetics, and that the actual rate of T₃ association with these TR mutants was similar to, or even faster than, the first phase of T₃ association with wild-type TRs (Kₐ on T₁/₂ = 0.02–0.14 h).

Figure 3 Purified TRβ LBD monomers bind T₃ rapidly. (A) Elution curve obtained after a TRβ 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 TRβ consists of dimers and monomers and that the eluate consists of TR monomers. (B) Determination of T₃ association kinetics with TRβLBD monomer/dimer mix (input) and purified TRβ monomers (eluate), experiment performed as in Fig. 1A.
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 biphase association kinetics is only seen in the presence of TR dimers and monomers and further suggests that the slower component of these biphase 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 retinoïd X receptor (RXR)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>K_{d} (10^{-10} M)</th>
<th>Association first half-life (h)</th>
<th>Association second half-life (h)</th>
<th>Dissociation half-life (h)</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT FLTRβ</td>
<td>2.0 ± 0.34</td>
<td>0.010 ± 0.03</td>
<td>4.8 ± 0.26</td>
<td>6.7 ± 1.0</td>
<td>Monomers and dimers</td>
</tr>
<tr>
<td>His-tag purified LBD</td>
<td>2.3 ± 0.65</td>
<td>0.17 ± 0.07</td>
<td>6.1 ± 1.1</td>
<td>6.2 ± 1.2</td>
<td>Monomers and dimers</td>
</tr>
<tr>
<td>Purified LBD</td>
<td>1.6 ± 0.39</td>
<td>0.15 ± 0.12</td>
<td>None</td>
<td>5.6 ± 0.46</td>
<td>Monomers</td>
</tr>
<tr>
<td>P419R</td>
<td>3.5 ± 0.41</td>
<td>0.14 ± 0.23</td>
<td>None</td>
<td>3.1 ± 0.51</td>
<td>Monomers</td>
</tr>
<tr>
<td>L422R</td>
<td>1.8 ± 0.4</td>
<td>0.03 ± 0.009</td>
<td>None</td>
<td>1.5 ± 0.38</td>
<td>Monomers</td>
</tr>
<tr>
<td>M423R</td>
<td>1.7 ± 0.45</td>
<td>0.02 ± 0.005</td>
<td>None</td>
<td>0.2 ± 0.05</td>
<td>Monomers</td>
</tr>
<tr>
<td>D355R</td>
<td>1.3 ± 0.29</td>
<td>0.92 ± 0.14</td>
<td>10.8 ± 1.20</td>
<td>1.4 ± 0.49</td>
<td>Dimers</td>
</tr>
<tr>
<td>TRβRXR</td>
<td>1.2 ± 0.66</td>
<td>0.23 ± 0.04</td>
<td>None</td>
<td>1.4 ± 0.49</td>
<td>Heterodimers</td>
</tr>
</tbody>
</table>

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

**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.

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 biphase 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 biphase 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 after 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.
**RXR–TRs bind and release T3 rapidly**

As TRs form heterodimers with RXR, it is relevant to examine binding of T3 to this species. We prepared in vitro translated TRs and RXR–TR mixtures, and association rates of ligand with each preparation. T3 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 (Fig. 6B).

T3 also dissociated rapidly from RXR–TRs, even faster than from wild-type TRs or purified preparations of TR–LBD monomers (T1/2 = 0.14 h for heterodimer versus 0.4 h for wild-type TRs and 0.8 h for the TRβ LBD monomer). 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 T3 binding kinetics. Our investigation was prompted by our previous observation that mutations in the TR LBD surface that mediate dimer and heterodimer formation increase association rates without altering affinity of TRs for T3 (Cunha Lima et al. 2009). Our analysis of T3 association with TRs reveals better fit of the curves with biphasic kinetics rather than single-phase kinetics: T3 binds TRs with a rapid first phase (half-life ≥ 0.1 h) followed by a slower second phase (half-life ≥ 0.3 h). This is a fundamental property of T3 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 T3 binding properties. First, our

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**Figure 5** A corepressor (N-CoR) peptide induces TR LBD monomer to dimer conversion in solution and inhibits T3 association. (A) The inset shows an image of a Coomassie blue-stained non-denaturing polyacrylamide gel, as in Fig. 3A, used to compare purified TRβ LBD monomers and a similar TRβ 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 T3 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 TRβ LBD L422R mutant that can only form monomers.

**Figure 6** RXR–TR Heterodimers bind and release T3 rapidly. (A) Determination of T3 association rates with in vitro translated TRs ± RXRs and examined association rates of ligand with each preparation. T3 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).

T3 also dissociated rapidly from RXR–TRs, even faster than from wild-type TRs or purified preparations of TR–LBD monomers (T1/2 = 0.14 h for heterodimer versus 0.4 h for wild-type TRs and 0.8 h for the TRβ LBD monomer). These results emphasize that TR exhibits major differences in ligand association and dissociation kinetics when it is a monomer or homodimer versus a heterodimer.

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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 T3 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 T3 binding sites. Thus, the simplest explanation that reconciles our findings is that unliganded TRs comprised two distinct species that bind T3 at different rates and that T3 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 T3 binding to TR monomers and slow T3 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 T3 rapidly binds to TR monomers and slowly to dimers (Figs 3–5, Table 1). 1) Short T3 incubations selectively increase TR monomer mobility in non-denaturing gels, indicative of rapid hormone association with this species and not with dimers. 2) T3 binds purified TR LBD monomers with rapid single-phase kinetics (T1/2 = 0.03–0.27 h; comparable to the first phase of T3 association with TR monomers, T1/2 = 0.07–0.13 h). 3) The TRBD355R mutant (Togashi et al. 2005b) only forms dimers and binds T3 with slow single-phase kinetics (T1/2 = 0.92 h, 6–15 times slower than TR LBD monomers and up to 46-fold slower than TRβ 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 T3 converts TRs to a single species with uniform ligand binding properties. Analytical ultracentrifugation (Fig. 2) and sizing chromatography analysis (not shown) reveal that T3 converts TR dimers to monomers. Furthermore, T3 dissociates from wild-type TRs at a comparable rate (T1/2 = 5–7 h) to purified LBD monomers (T1/2 = 5–6 h), consistent with the prediction that measurements of TR ligand dissociation kinetics reflect T3 release from monomers.

Heterodimer formation also influences T3 binding kinetics. We find that RXR–TRs exhibit unique properties that are distinct from TR–TR homodimers or TR monomers. RXR–TRs bind T3 with rapid single-phase kinetics, comparable to monomers (Fig. 7; Table 1), but release T3 more rapidly than TR monomers (T1/2 = 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 T3 binding kinetics. Thus, we propose that 1) TR homodimer formation alters LBD conformation to slow the rate of T3 entry to the pocket. 2) T3 remodels the TR–LBD to prevent homodimer formation; although it was previously known that T3 binding inhibits TR homodimer formation on DNA (Togashi et al. 2005b, Jeyakumar et al. 2008), our data represent direct proof that T3 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 T3 more rapidly than TR–TR homodimers and release T3 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, Mariimuthu 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 T3 binding kinetics? We previously proposed that homodimer and heterodimer surfaces are partially overlapping and that T3 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 T3 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 (TRβP419R; TRβL422R, and TRβM423R) 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 TRβ 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 TRβD355R dimer blocks one of the predicted ligand exit routes identified by molecular dynamics at H8 (Martínez 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.

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