Catalysis leads to posttranslational inactivation of the type 1 deiodinase and alters its conformation

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Abstract

Previously, it was shown that the type 1 deiodinase (D1) is subject to substrate-dependent inactivation that is blocked by pretreatment with the inhibitor of D1 catalysis, propylthiouracil (PTU). Using HepG2 cells with endogenous D1 activity, we found that while considerable D1-mediated catalysis of reverse tri-iodothyronine (rT_3) is observed in intact cells, there was a significant loss of D1 activity in sonicates assayed from the same cells in parallel. This rT_3 -mediated loss of D1 activity occurs despite no change in D1 mRNA levels and is blocked by PTU treatment, suggesting a requirement for catalysis. Endogenous D1 activity in sonicates was inactivated in a dose-dependent manner in HepG2 cells, with a $\sim 50\%$ decrease after 10 nM rT_3 treatment. Inactivation of D1 was rapid, occurring after only half an hour of rT_3 treatment.

D1 expressed in HEK293 cells was inactivated by rT_3 in a similar manner. ^{75}Se labeling of the D1 selenoprotein indicated that after 4 h rT_3 -mediated inactivation of D1 occurs without a corresponding decrease in D1 protein levels, though rT_3 treatment causes a loss of D1 protein after 8–24 h. Bioluminescence resonance energy transfer studies indicate that rT_3 exposure increases energy transfer between the D1 homodimer subunits, and this was lost when the active site of D1 was mutated to alanine, suggesting that a post-catalytic structural change in the D1 homodimer could cause enzyme inactivation. Thus, both D1 and type 2 deiodinase are subject to catalysis-induced loss of activity although their inactivation occurs via very different mechanisms.

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Introduction

The iodothyronine deiodinases are selenoenzymes that modulate tri-iodothyronine (T_3) concentrations by catalyzing both its production and degradation (Bianco *et al.* 2002, Gereben *et al.* 2008). Thyroxine (T_4) is activated via removal of an outer-ring iodine by the type 1 and 2 deiodinases (D1 and D2) to produce T_3 . Conversely, the type 3 deiodinase (D3), and under some conditions D1, can inactivate T_3 and T_4 by the elimination of an inner-ring iodine, generating T_2 or reverse T_3 (rT_3). The deiodinases control circulating levels of thyroid hormone, with $\sim 80\%$ of the T_3 produced daily in humans being derived extrathyroidally from T_4 via D1 and D2 (Bianco *et al.* 2002). Notably, in patients with a hyperactive thyroid gland, the contribution of thyroidal D1 to T_4 to T_3 conversion becomes predominant, with up to two-thirds of the daily T_3 production coming from this source (Laurberg *et al.* 2007). The deiodinases (primarily D2 and D3) also allow for the intricate regulation of intracellular T_3 concentrations in a tissue-specific fashion independent of circulating concentrations of T_4 or T_3 (Bianco *et al.* 2002, Gereben *et al.* 2008).

Ubiquitination and subsequent proteasomal degradation of D2 are important components of the D2-mediated feedback regulation of TSH (reviewed in Bianco *et al.* (2002) and Gereben *et al.* (2008)). Ubiquitination of D2 is substrate-dependent, increasing with catalysis of T_4 (Steinsapir *et al.* 1998, 2000). Thus, as more T_4 is converted to T_3 , the ubiquitination and proteasomal degradation of D2 also increase, balancing T_3 production. Additional layers of complexity are added to this scenario by the potential de-ubiquitination and reactivation of D2 by VDU1 (USP33), and the finding that ubiquitination drives apart the globular domain of the D2 homodimer, thus inactivating D2 yet also leaving the system primed for VDU1-mediated reactivation (Curcio-Morelli *et al.* 2003b, Sagar *et al.* 2007). Taken together, the substrate-mediated regulation of D2 activity provides a flexible mechanism to accurately regulate thyroid hormone production under a variety of physiological conditions.

D1 activity is also regulated by substrate exposure, though the physiological significance and mechanism of this are yet to be defined. D1 activity in liver microsomes was decreased when rats were injected with rT_3 , and D1 activity in Reuber

FAO hepatoma cells was also reduced after rT_3 treatment, although supraphysiological concentrations of rT_3 were needed to achieve these effects (St Germain 1988a). Pretreatment with propylthiouracil (PTU) blocked the substrate-dependent loss of D1 activity in liver microsomes, suggesting that the loss of D1 activity was dependent on catalysis (St Germain & Croteau 1989). The objective of this study was to further analyze the mechanism by which D1 is inactivated after substrate exposure. Using a human cell line, HepG2, with endogenous D1 expression, we found that while considerable D1-mediated catalysis of rT_3 is observed in intact cells, there is a significant loss (68%) of D1 activity in cell sonicates. Similar results were found using a HEK293 system with transfected D1 and, notably, ^{75}Se labeling of the deiodinase selenoprotein indicated that initially rT_3 -mediated inactivation of D1 occurs despite stable D1 protein levels. Interestingly, bioluminescence resonance energy transfer (BRET) studies indicate that rT_3 treatment increases energy transfer between the D1 homodimer subunits, suggesting that rT_3 treatment either brings these monomers closer together, or stabilizes their interactions, and that this conformational change is associated with D1 inactivation.

Materials and Methods

Materials

Chemicals and reagents were from Fisher Scientific (Pittsburgh, PA, USA) or Sigma unless otherwise specified. ^{125}I rT_3 (specific activity 4400 Ci/mM) was obtained from Perkin Elmer (Waltham, MA, USA), and ^{75}Se was a generous gift from Dr Dolph Hatfield of the National Cancer Institute, Bethesda, MD, USA.

Cell culture and transfection

HepG2 or HEK293 cells were grown in DMEM + 10% FBS + 100 nM Se under 5% CO_2 . HEK293 cells were transfected with either a flag-tagged human D1 expression vector or a rat D1 expression vector where the serine at 128 was mutated to a proline and a vector expressing β -galactosidase for normalization as described using Lipofectamine Plus (Invitrogen; Callebaut *et al.* 2003, Curcio-Morelli *et al.* 2003a, Goemann *et al.* 2010). Cells were treated with rT_3 in DMEM + 0.1% BSA for the indicated time period. Transfected HEK293 cells were maintained in six-well dishes and treated with 1.5 ml media containing the indicated agents.

Deiodinase assays

Assessment of D1 activity in whole cells and in sonicates was performed as described previously (Goemann *et al.* 2010). For sonicates, 2–50 μg protein was incubated for 1 h at 37 °C in the presence of 1 μM unlabeled rT_3 and trace amounts of ^{125}I rT_3 and 10 mM dithiothreitol (DTT),

followed by TCA precipitation and counting of the $^{125}\text{I}^-$ released in the supernatant. Under these conditions ~50% of the D1 can be recovered from the D1–PTU complex from cells pretreated with PTU. Activity from transfected HEK293 cells was normalized by β -galactosidase activity to control for transfection efficiency. Nonspecific iodide release was determined in HepG2 cells by the addition of 10 mM PTU, and in HEK293 cells by the assay of lysates from untransfected cells. The whole cell D1 activity was measured in HepG2 cells by incubation of cells with 1 μM unlabeled rT_3 and trace amounts of ^{125}I rT_3 in DMEM + 0.1% BSA for 20 h followed by TCA precipitation of media and counting of the $^{125}\text{I}^-$ remaining in the supernatant. Nonspecific whole cell deiodination was determined by the addition of 100 μM PTU.

BRET measurement

Energy transfer between carboxyl-terminal tagged Renilla Luciferase-D1 and YFP-D1 fusion proteins was assessed as described previously (Sagar *et al.* 2007). Luciferase-D1 and yellow fluorescent protein (YFP)-D1 proteins where Sec126 was changed to Ala were created by amplifying BG132 (a D1 expression vector with Ala126 (Curcio-Morelli *et al.* 2003a)) with Pfu turbo polymerase (Agilent Technologies, Santa Clara, CA, USA) using the following oligos: 5'-ACGGATT-CATTATGGGGCTGTCCCAGCTATG-3' and 5'-TAG-TGGATCCCGGAAGTGGAGGCATGTGTCC-3'. This fragment was cut with EcoRI and BamHI and subcloned into the carboxyl-terminal tagged luciferase-D1 and YFP-D1 expression vectors. All constructs were sequenced to confirm mutations and fusion reading frame.

^{75}Se labeling of D1

Thirty-five millimetre dishes of HEK293 cells were transfected with 200 ng of a human D1 expression vector (Curcio-Morelli *et al.* 2003a), and ^{75}Se labeled in 1 ml DMEM + 10% FBS + 50 nM Na_2SeO_3 and + 2 μCi of $\text{Na}_2^{75}\text{SeO}_3$ as described previously (Curcio *et al.* 2001). After 20 h of labeling, cells were treated with 10 μM rT_3 in DMEM + 0.1% BSA for the indicated time period, lysed, and 20 μg of each sample were used for SDS-PAGE on a 10% gel followed by autoradiography.

mRNA preparation and quantitative real-time PCR

mRNA was harvested from HepG2 cells using Trizol (Invitrogen), and quantitative real-time PCR was performed as describe previously (Zavacki *et al.* 2005). Primer sequences for human D1 are 5'-ACATCAGAAATCACCAGAACC-TTCA-3' and 5'-CCAGAACAGCACGAACCTTCCTC-3' and for human β -actin are 5'-GGCACCACACCTTCTAC-AATGAG-3' and 5'-CCAGAGCGGTACAGGGATAGC-3'.

Results

The whole cell D1-mediated deiodination of 1 μM rT_3 in the presence of trace amounts of ^{125}I rT_3 was determined in HEPG2 cells overnight, with this activity being completely blocked by the addition of 100 μM PTU (Fig. 1A). However, when D1 activity was measured in cell sonicates from the same plates in the presence of 10 mM DTT, the activity in the PTU-treated sonicates was almost three times that of the sonicates from plates without PTU treatment (Fig. 1B). PTU treatment was also protective of D1 activity when HEK293 cells transfected with D1 were rT_3 treated, leading to a twofold greater activity in sonicates (Fig. 1C). A conserved serine that found two amino acids at position 128 downstream from the active site selenocysteine of D1 was essential for PTU sensitivity (Sanders *et al.* 1997, Callebaut *et al.* 2003, Kuiper *et al.* 2006). When this serine was changed to proline, as is found in the PTU insensitive D1, D2, and D3 enzymes of *Tilapia* and *Xenopus*, the activity of the Ser128Pro D1 was no longer inhibited by PTU (Sanders *et al.* 1997, Callebaut *et al.* 2003, Kuiper *et al.* 2006). Consistent with this loss of sensitivity, PTU no longer prevented the rT_3 -induced decrease in activity of the Ser128Pro D1 enzyme (Fig. 1D).

D1 activity in HepG2 sonicates was reduced by rT_3 treatment in a dose-dependent manner, decreasing by $\sim 50\%$ after overnight treatment with 10 nM rT_3 , and by $\sim 75\%$ in the presence of 1 μM rT_3 (Fig. 2A). This decrease in activity could not be explained by carryover of unlabeled substrate as only 1.7% of the radiolabeled ^{125}I rT_3 tracer was present in the sonicates. As only a small fraction of the total cell sonicate is used for deiodinase assays, even at the highest rT_3 concentrations used this carryover would only account for $\sim 2.8\%$ of the final rT_3 concentration used in the *in vitro* deiodinase reaction. The substrate-dependent inactivation of endogenous D1 in HepG2 cells was rapid, occurring within 30 min of rT_3 exposure (Fig. 2B). Importantly, D1 mRNA levels in HepG2 cells were unchanged after 20 h of rT_3 treatment, despite a 66% decrease in activity in plates treated in parallel (Fig. 3A and B).

A similar substrate-mediated inactivation of D1 occurred when D1 was transiently expressed. D1 activity in the sonicates of HEK293 cells transfected with human D1 was also decreased by rT_3 treatment, although the amount of rT_3 needed was approximately ten times more than that required to inactivate endogenous D1 (compare Figs 2A and 4A). rT_3 treatment also significantly decreased the activity of transiently expressed D1 in a time-dependent fashion, with activity being decreased by $\sim 50\%$ after 4 h of treatment with a continued decrease thereafter (Fig. 4B; $P < 0.001$ by two-way ANOVA). Notably, when the levels of the D1 selenoprotein were monitored by ^{75}Se labeling, there was not a corresponding decrease in D1 protein after 4 h (Fig. 4B and C). However, D1 protein levels markedly decrease by 8 h of treatment, and after 24 h of rT_3 exposure ^{75}Se -labeled D1 is no longer apparent (Fig. 4C). Similar results were obtained

Figure 1 PTU treatment inhibits the rT_3 -mediated loss of D1 activity in HepG2 cells, and in D1-transfected HEK293 cells. (A) HepG2 cells were incubated for 20 h with 1 μM rT_3 and trace amounts of ^{125}I $\text{rT}_3 \pm 100 \mu\text{M}$ PTU, and whole cell D1-mediated deiodination was determined. (B) Cells from (A) were harvested and D1 activity measured in sonicates in the presence of 10 mM DTT. (C and D) HEK293 cells were transfected with vectors expressing either a wild-type (WT) D1 (C) or a Ser128Pro D1 (D) and β -galactosidase. Cells were treated with 3 μM $\text{rT}_3 \pm 100 \mu\text{M}$ PTU as indicated for 20 h, and D1 activity was measured in sonicates and normalized by β -galactosidase activity. Data are normalized to the vehicle-treated control group being 100% for (C) and (D). The mean $\pm$ s.e.m. of three wells/group is shown, with similar results being obtained in two to three experiments. *** $P < 0.001$, * $P < 0.05$ by Student's unpaired *t*-test, NS, non-significant.

Figure 2 Dose-response and time course of inhibition of D1 activity in HepG2 cells by rT₃ exposure. (A) HepG2 cells were treated with the indicated concentrations of rT₃ for 20 h and D1 activity in sonicates was measured in the presence of 10 mM DTT. (B) HepG2 cells were treated with 1 μ M rT₃ for the indicated times and then D1 activity measured as in (A). The mean $\pm$ S.E.M. of three wells/group is shown with similar results being obtained in three experiments. *** P <0.001, ** P <0.001, * P <0.05 by one-way ANOVA when compared with either the vehicle control (A) or time 0 control (B) group.

when the effect of rT₃ on transfected flag-tagged D1 was studied by western blotting using α -flag, with less D1 protein being apparent 8 and 24 h after rT₃ treatment (data not shown). Taken together, this suggests that rT₃ treatment is accelerating the degradation of the D1 protein and not merely causing selective loss of its selenium. Activity of another deiodinase family member, D2, is also subject to substrate-induced inactivation via ubiquitination and proteasomal degradation (Steinsapir *et al.* 1998, 2000). However, treatment of D1-transfected HEK293 cells with the proteasomal inhibitor MG132 did not prevent the loss of D1 activity after 24 h of rT₃ treatment, confirming the previous data that the mechanism for the loss of D1 activity is distinct from that of D2 (data not shown; Gereben *et al.* 2000). In addition, ubiquitination of D1 is further ruled out by the absence of an increase in molecular weight of the ⁷⁵Se-labeled D1 (data not shown).

To determine if there could be structural modifications of the D1 protein during catalysis that could affect its homodimerization, we determined the BRET between a D1 protein tagged at the carboxyl terminus with Renilla Luciferase and a D1 protein tagged at the carboxyl terminus

with YFP. We confirmed that rT₃ treatment leads to the inactivation of both D1 fusion proteins in cell sonicates (Fig. 5A and B). Notably, rT₃ treatment increases the BRET signal between the D1 dimers, suggesting that exposure to substrate results in a molecular rearrangement that either brings the luciferase and YFP moieties into closer proximity or stabilizes the interactions between the D1 dimers (Fig. 5C). Interestingly, the increase in BRET signal persists for at least an hour after cells are washed to remove the rT₃ (data not shown).

To assess if catalysis of rT₃ was necessary to mediate an increase in BRET signal with rT₃ treatment, we evaluated the effects of changing the active center of the D1-YFP and D1-Renilla Luciferase to alanine. While BRET still exists between the subunits of the D1 dimer with alanine in the active center, there was no longer an increase in BRET after rT₃ treatment (Fig. 5D). Further, no increase in BRET was observed when even one of the BRET partners had the active center mutated to an alanine, suggesting both partners of the D1 dimer must be catalytically active for the increase in BRET signal observed with rT₃ treatment to occur (data not shown).

Figure 3 D1 mRNA levels in HepG2 cells after rT₃ treatment. HepG2 cells were treated with 1 μ M rT₃ for 20 h before mRNA harvest. (A) D1 activity was determined or (B) D1 mRNA levels were measured by quantitative real-time PCR and are shown normalized by the expression of the housekeeping gene β -actin. Plates for (A) and (B) were treated at the same time within the same experiment. The mean $\pm$ S.E.M. of three wells/group is shown, with similar results being obtained in three experiments. ** P <0.01 by Student's unpaired *t*-test, NS, non-significant.

Figure 4 D1 activity is inactivated by rT₃ exposure prior to a decrease in D1 protein in a transfected cell system. (A) HEK293 cells were transfected with vectors expressing human D1 and β-galactosidase. Cells were treated with the indicated concentration of rT₃ for 20 h, then D1 activity measured in sonicates in the presence of 10 mM DTT and is shown normalized by β-galactosidase activity. (B) HEK293 cells were transfected as in (A) and were treated with either vehicle or 10 μM rT₃ for the indicated time. D1 activity was measured as in (A). The mean ± s.e.m. of three wells/group is shown for (A) and (B), with similar results being obtained in two to three experiments. For (A) ****P* < 0.001 by one-way ANOVA when compared with the vehicle control. For (B) the effect of rT₃ treatment was significant (*P* < 0.001) over time by two-way ANOVA, followed by Student's *t*-testing between the vehicle- and rT₃-treated groups at each time with ****P* < 0.001, ***P* < 0.001, **P* < 0.05. (C) HEK293 cells were transfected as in (A) and were labeled with ⁷⁵Se for 19 h prior to treatment with either vehicle or 10 μM rT₃ for the indicated time. Cell lysates were run on a SDS-PAGE gel, which was then dried and used for autoradiography. All samples were collected within the same experiment and were run on two gels. Lysates from nontransfected (NT) cells are shown on the far right, and the band corresponding to D1 is indicated on the left by an arrow.

Discussion

Our results indicate that D1 activity in cells treated with rT₃ is decreased in a rapid and dose-dependent manner (Figs 2 and 4). Furthermore, assessment of whole cell-mediated deiodination in HepG2 cells indicated that prior to substrate-dependent inactivation of D1, substantial D1-mediated deiodination of rT₃ occurs, consistent with catalysis preceding inactivation (Fig. 1A and B). Our data agree with the previous work of St Germain (1988a), where D1 activity was decreased in rat microsomes and in Reuber FAO hepatoma cells after treatment with either IOP or rT₃. In addition, this effect was prevented by pretreatment with the D1 inhibitor PTU, also supporting a requirement for catalysis (St Germain & Croteau 1989). PTU blocks D1 activity by competing with an endogenous thiol cofactor for a putative selenenyl-iodine intermediate (E-Se-I) blocking a full cycle of catalysis, and thus protecting D1 from rT₃-mediated inactivation (Leonard & Visser 1986, St Germain & Croteau 1989). Further, after PTU treatment *in vivo*, D1 activity can be recovered by DTT

treatment *in vitro*, indicating that the D1–PTU interaction can be disrupted by DTT and an active regenerated enzyme (St Germain & Croteau 1989). Thus, in cells with rT₃ treatment alone, the D1 enzyme is inactivated, whereas in the rT₃- and PTU-treated groups (where full catalysis of rT₃ is prevented by PTU) D1 activity can be recovered as our deiodinase assays are performed in the presence of 10 mM DTT (Fig. 1B and C). Notably, when the PTU-insensitive Ser128Pro D1 mutant is treated with rT₃ ± PTU, PTU treatment no longer blocks the rT₃-mediated decrease in D1 activity, further supporting that PTU's inhibitory effects on D1 catalysis are necessary for its protective effects (Fig. 1D; Callebaut *et al.* 2003).

No change in D1 mRNA levels was observed in rT₃-treated HepG2 cells, indicating that the observed loss of D1 activity is not due to decreased *DIO1* expression or accelerated mRNA degradation (Fig. 3). Consistent with the posttranscriptional nature of these effects, rT₃ treatment also decreased D1 activity in a transfected cell system where D1 was expressed from a heterologous promoter (Fig. 4A and B). Approximately ten times more rT₃ was required to decrease D1 activity in a transfected system, and this difference persisted even when transfection conditions were adjusted such that enzyme activity levels were decreased by fourfold (data not shown; Fig. 4A). However, we cannot rule out that this difference might be due to the increased levels of D1 enzyme in a transient vs endogenous expression system. Alternatively, rT₃ can also be specifically transported inside the cell via thyroid hormone transporters and thus potential differences in transporter expression between the HepG2 and the HEK293 cells used for these experiments might also contribute to the observed differences (van der Deure *et al.* 2008a,b, Kinne *et al.* 2010).

Our results show that the mechanism of D1 inactivation by rT₃ is posttranslational, because even at 4 h after rT₃ treatment, when D1 activity is decreased by 50%, no corresponding decrease in ⁷⁵Se D1 protein was evident (Fig. 4B and C). The D1 enzyme has a long half-life that is estimated to be more than 6 h, and this was significantly decreased by rT₃ treatment in Reuber FAO hepatoma cells (St Germain 1988a, Baqui *et al.* 2003). Our data also indicate that rT₃ accelerates the degradation of D1 protein, because after 24 h ⁷⁵Se-labeled D1 can still be detected in the vehicle-treated group, whereas none is apparent in the group treated with rT₃ in parallel (Fig. 4C). A rT₃-enhanced loss of D1 protein over time is consistent with previous results indicating that the substrate-inducible loss of D1 is reversible at early time points after treatment, but becomes only partially reversible after longer periods (St Germain & Croteau 1989).

The ubiquitination and subsequent degradation of D2 is substrate dependent, increasing with catalysis of T₄ to provide a flexible system where T₃ production is counterbalanced by a loss of D2 protein (Steinsapir *et al.* 1998, 2000). Unlike D2, D1 does not appear to be regulated by ubiquitination. Using a transient expression system with a D1 enzyme where the active site selenocysteine was converted to a cysteine to

Figure 5 rT₃ treatment increases BRET signal between D1 dimers. (A and B) HEK293 cells were transfected with the D1-Renilla Luciferase (Luc) (A) or D1-YFP (B) expression plasmids as indicated, along with a β -galactosidase expression vector. Cells were treated with 3 μ M rT₃ as indicated for 20 h, and D1 activity in sonicates was measured in the presence of 10 mM DTT. The mean $\pm$ S.E.M. of three wells/group is shown, with similar results being obtained in two experiments. (C) BRET between D1-Renilla Luc and D1-YFP with cysteine substituted in the active center or (D) alanine substituted in the active center was measured after treatment with 10 μ M rT₃ for 1 h as indicated. The mean $\pm$ S.E.M. of triplicate determinations is shown for each treatment group, with similar results being obtained in two to three independent experiments. *** P < 0.001, ** P < 0.001, by Student's unpaired t -test.

facilitate overexpression, we did not observe any higher molecular weight forms of D1 by western blotting indicative of ubiquitination (Gereben *et al.* 2000). ⁷⁵Se-labeled wild-type D1 enzyme also did not display the characteristic ladder of higher molecular weight forms associated with ubiquitination, nor did MG132 treatment with the proteasomal inhibitor block the substrate-dependent decrease in D1 activity, further supporting that D1 is not regulated by the ubiquitin-proteasomal system.

While the T₄-mediated decrease in D2 activity has been shown to occur under physiologically relevant conditions, the lowest concentration of rT₃ where substrate-dependent inactivation of D1 can be observed in HepG2 cells is 10 nM rT₃, or 1 μ M rT₃ in a transfected cell system (Fig. 2; Silva & Larsen 1982, Leonard *et al.* 1984, St Germain 1988b, Steinsapir *et al.* 1998). Total rT₃ concentrations range from 0.14 to 0.34 nmol/l in normal individuals, indicating that the concentrations needed to inactivate D1 are several orders of magnitude above circulating amounts, and thus the physiological implications of D1 inactivation are currently unclear (Peeters *et al.* 2003). However, notably, another D1 substrate, T₄, has circulating concentrations that are several orders of magnitude greater than rT₃. Remarkably, a similar paradigm can be observed for D3, with exposure to high amounts of substrate (10 μ M T₃) resulting in D3 inactivation in a transient expression system (B Zhu, P R Larsen, and A M Zavacki, unpublished data).

The deiodinase enzymes are homodimers, and BRET studies have shown that T₄-dependent ubiquitination separates the globular domains of the D2 homodimer, resulting in a loss of D2 activity (Leonard *et al.* 2001, Curcio-Morelli *et al.* 2003a, Sagar *et al.* 2007, 2008). In contrast, we found that BRET signal between D1-YFP and D1-luciferase homodimers is increased after rT₃ exposure, suggesting either a conformational change that positions the YFP and luciferase moieties in closer proximity, or a stabilization of homodimerization, resulting in increased signal (Fig. 5C). This correlates with the loss of D1 activity, suggesting that a conformational change in D1 homodimerization results in the loss of D1 activity after rT₃ treatment. When the active center of D1 was changed to alanine, rT₃ treatment no longer caused an increase in BRET, indicating that catalysis is required to induce this conformational change (Fig. 5D).

Notably, the substrate-dependent inactivation of D1 occurs despite clearly present D1 protein (Fig. 4C). However, no observable change in D1 mobility was observed under reducing SDS-PAGE conditions, suggesting that no covalent modifications are associated with this loss (Fig. 4C). The loss of D1 activity could be observed using both a wild-type D1 enzyme and an enzyme where the active site selenocysteine had been changed to cysteine (Figs 2, 4 and 5), indicating that the loss of D1 activity is not dependent on the presence of a selenocysteine residue in its active center. The Sec126Cys D1 has been shown to have a lower affinity for rT₃ than wild-type D1; however, surprisingly, when increasing doses of rT₃ were tested for their ability to confer inactivation, the Sec126Cys

D1 enzyme was somewhat more sensitive to rT₃-mediated loss of D1 activity (data not shown) indicating that substrate-dependent inactivation cannot be solely predicted by the affinity of the substrate in the presence of DTT *in vitro* (Berry *et al.* 1992).

Our results provide further support for a requirement for catalysis in the substrate-dependent inactivation of D1. Previously, it has been suggested that exposure to saturating substrate concentrations oxidizes the D1 enzyme, although it is puzzling as to why the D1 enzyme cannot be adequately regenerated by endogenous co-factors within the cell (St Germain 1988b, St Germain & Croteau 1989, Croteau *et al.* 1998, Wajner *et al.* 2011). Our results support that whatever the change in the D1 enzyme, this inactivation occurs despite persistent D1 protein levels and is accompanied by a conformational change, and that this modification further results in increased degradation of the D1 enzyme over time. While it has been well established that D2 activity is decreased by exposure to substrate, these data and our preliminary studies with D3 indicate that this appears to be a general mechanism applicable to all three deiodinases.

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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Author contribution statement

B Z, A S, C L, P R L, and A M Z designed experiments; A M, P R L, and A M Z analyzed data and wrote the manuscript; B Z, A S, C L, J W H, T C, J W H, T-V T, A B, R M, and A S performed experiments.

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