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

Bo Zhu*, Ashutosh Shrivastava*, Cristina Luongo, Ting Chen, John W Harney, Alessandro Marsili, Thuy-Van Tran, Anulika Bhadouria, Radhika Mopala, Amanda I Steen, P Reed Larsen and Ann Marie Zavacki

Division of Endocrinology, Diabetes and Hypertension, Thyroid Section, Brigham and Women's Hospital, Boston, Massachusetts 02115, USA

(Correspondence should be addressed to A M Zavacki who is now at Division of Endocrinology, Diabetes and Hypertension, Thyroid Section, Brigham and Women's Hospital, 77 Avenue Louis Pasteur, HIM 645A, Boston, Massachusetts 02115, USA; Email: azavacki@rics.bwh.harvard.edu)

*B Zhu and A Shrivastava contributed equally to this work

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 (rT3) is observed in intact cells, there was a significant loss of D1 activity in sonicates assayed from the same cells in parallel. This rT3-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 ≈50% decrease after 10 nM rT3 treatment. Inactivation of D1 was rapid, occurring after only half an hour of rT3 treatment.

D1 expressed in HEK293 cells was inactivated by rT3 in a similar manner. 75Se labeling of the D1 selenoprotein indicated that after 4 h rT3-mediated inactivation of D1 occurs without a corresponding decrease in D1 protein levels, though rT3 treatment causes a loss of D1 protein after 8–24 h. Bioluminescence resonance energy transfer studies indicate that rT3 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.


Introduction

The iodothyronine deiodinases are selenoenzymes that modulate tri-iodothyronine (T3) concentrations by catalyzing both its production and degradation (Bianco et al. 2002, Gereben et al. 2008). Thyroxine (T4) is activated via removal of an outer-ring iodine by the type 1 and 2 deiodinases (D1 and D2) to produce T3. Conversely, the type 3 deiodinase (D3), and under some conditions D1, can inactivate T3 and T4 by the elimination of an inner-ring iodine, generating T2 or reverse T3 (rT3). The deiodinases control circulating levels of thyroid hormone, with ~80% of the T3 produced daily in humans being derived extrathyroidally from T4 via D1 and D2 (Bianco et al. 2002). Notably, in patients with a hyperactive thyroid gland, the contribution of thyroidal D1 to T4 to T3 conversion becomes predominant, with up to two-thirds of the daily T3 production coming from this source (Laurberg et al. 2007). The deiodinases (primarily D2 and D3) also allow for the intricate regulation of intracellular T3 concentrations in a tissue-specific fashion independent of circulating concentrations of T4 or T3 (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 T4 (Steinsapir et al. 1998, 2000). Thus, as more T4 is converted to T3, the ubiquitination and proteasomal degradation of D2 also increase, balancing T3 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 rT3, and D1 activity in Reuber
FAO hepatoma cells was also reduced after rT₃ treatment, although supraphysiological concentrations of rT₃ 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 rT₃ is observed in intact cells, there is a significant loss (68%) of D1 activity in cell sonicates. Similar results were found using a HEK293 cell line, and notably, 75Se labeling of the deiodinase selenoprotein indicated that initially rT₃-mediated inactivation of D1 occurs despite stable D1 protein levels. Interestingly, bioluminescence resonance energy transfer (BRET) studies indicate that rT₃ treatment increases energy transfer between the D1 homodimer subunits, suggesting that rT₃ 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. 125I rT₃ (specific activity 4400 Ci/mM) was obtained from Perkin Elmer (Waltham, MA, USA), and 75Se 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% CO₂. 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 rT₃ 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 rT₃ and trace amounts of 125I rT₃ and 10 mM dithiothreitol (DTT), followed by TCA precipitation and counting of the 125I⁻ 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 rT₃ and trace amounts of 125I rT₃ in DMEM + 0-1% BSA for 20 h followed by TCA precipitation of media and counting of the 125I⁻ 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 YPF-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-CATTATGGGGGCTGTCCCAGCTATG-3′ and 5′-TAG-TGGATCCGGAAAATGAGGACATGTGTCC-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.

75Se 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 75Se labeled in 1 ml DMEM + 10% FBS + 50 nM Na₂SeO₃ and +2 µCi of Na₂⁷⁵SeO₃ as described previously (Curcio et al. 2001). After 20 h of labeling, cells were treated with 10 µM rT₃ 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′-ACATCGAAATCCTCAGAACCC-GTCA-3′ and 5′-CCAGAAGACGAGAACCTTTCTC-3′ and for human β-actin are 5′-GGCACCAACTTCTTAC-AATGAG-3′ and 5′-CCAGAGGGCTTACAGGATAGC-3′.
Results

The whole cell D1-mediated deiodination of 1 μM rT3 in the presence of trace amounts of 125I rT3 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 rT3 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 rT3-induced decrease in activity of the Ser128Pro D1 enzyme (Fig. 1D).

D1 activity in HepG2 sonicates was reduced by rT3 treatment in a dose-dependent manner, decreasing by ~50% after overnight treatment with 10 nM rT3, and by ~75% in the presence of 1 μM rT3 (Fig. 2A). This decrease in activity could not be explained by carryover of unlabeled substrate as only 1.7% of the radioiodinated 125I rT3 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 rT3 concentrations used this carryover would only account for ~2-8% of the final rT3 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 rT3 exposure (Fig. 2B). Importantly, D1 mRNA levels in HepG2 cells were unchanged after 20 h of rT3 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 rT3 treatment, although the amount of rT3 needed was approximately ten times more than that required to inactivate endogenous D1 (compare Figs 2A and 4A). rT3 treatment also significantly decreased the activity of transiently expressed D1 in a time-dependent fashion, with activity being decreased by ~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 75Se 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 rT3 exposure 75Se-labeled D1 is no longer apparent (Fig. 4C). Similar results were obtained

Figure 1 PTU treatment inhibits the rT3-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 rT3 and trace amounts of 125I rT3 ± 100 μM 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 rT3 ± 100 μM 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 ± 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.
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 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 μM rT3 for the indicated times and then D1 activity measured as in (A). The mean ± 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.

Figure 3: D1 mRNA levels in HepG2 cells after rT3 treatment. HepG2 cells were treated with 1 μM 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 ± 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.
Our results indicate that D1 activity in cells treated with rT3 is deceased 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 in rT3-treated groups 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 vitro, 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 T3 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

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**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.
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 µM 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 µM 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 T3-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 that wild-type D1; however, surprisingly, when increasing doses of rT3 were tested for their ability to confer inactivation, the Sec126Cys

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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 µM rT3 as indicated for 20 h, and D1 activity in sonicates was measured in the presence of 10 mM DTT. The mean ± 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 µM rT3 for 1 h as indicated. The mean ± 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<0.01, +P<0.05, by Student’s unpaired t-test.
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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