Comparative kinetic characterization of rat thyroid iodotyrosine dehalogenase and iodothyronine deiodinase type 1

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Abstract

The initial characterization of a thyroid iodotyrosine dehalogenase (tDh), which deiodinates mono-iodotyrosine and di-iodotyrosine, was made almost 50 years ago, but little is known about its catalytic and kinetic properties. A distinct group of dehalogenases, the so-called iodothyronine deiodinases (IDs), that specifically remove iodine atoms from iodothyronines were subsequently discovered and have been extensively characterized. Iodothyronine deiodinase type 1 (ID1) is highly expressed in the rat thyroid gland, but the co-expression in this tissue

of the two different dehalogenating enzymes has not yet been clearly defined. This work compares and contrasts the kinetic properties of tDh and ID1 in the rat thyroid gland. Differential affinities for substrates, cofactors and inhibitors distinguish the two activities, and a reaction mechanism for tDh is proposed. The results reported here support the view that the rat thyroid gland has a distinctive set of dehalogenases specialized in iodine metabolism.

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Introduction

Iodine, the rate-limiting trace element in the biosynthesis of iodothyronines or thyroid hormones (THs), is actively recycled within the thyrocyte. This intrathyroidal iodine-salvage mechanism is primarily catalyzed by thyroid iodotyrosine dehalogenase (tDh). Operationally, tDh mediates the reductive deiodination of mono-iodotyrosine (MIT) and di-iodotyrosine (DIT) released by the lysosomal proteolysis of mature thyroglobulin (Larsen *et al.* 1998, Dunn & Dunn 2001). The initial description of tDh in several mammalian species (Roche *et al.* 1952) was followed by the demonstration that it is deficient in some patients with congenital goitrous hypothyroidism (Querido *et al.* 1956, Stanbury *et al.* 1956). Besides the thyroid of mammals and reptiles (Kusakabe & Miyake 1966, Chiu & Wong 1978), deiodination of MIT and DIT has been reported to occur in the liver, kidney and intestine of various mammals (Roche *et al.* 1952, Stanbury & Morris 1958). However, despite its clinical relevance and its initial identification as a flavoprotein from bovine thyroid microsomes (Rosenberg & Goswami 1979), the subsequent study of tDh was virtually abandoned. On the other hand, over the past two decades a different subset of reductive dehalogenases known as iodothyronine deiodinases (IDs) has been extensively studied. This family of seleno-enzymes includes at least three isotypes: ID1, ID2 and ID3. IDs catalyze the stepwise deiodination of iodothyronines and are pivotal in controlling local production of bioactive or

inactive intracellular THs in practically all vertebrate tissues (Köhrle 2000, Bianco *et al.* 2002). There seems to be important species-specific differences regarding the expression of IDs in the thyroid gland. Whereas rat thyroid gland expresses ID1 (Gereben *et al.* 2001), this isotype is absent in the gland of cattle and sheep (Beech *et al.* 1993, Solís-S 2004). Furthermore, and at variance with human, rat thyroid does not express ID2 activity (Salvatore *et al.* 1996, Gereben *et al.* 2001). On the other hand, and although both tDh and ID1 have been reported in the rat (Roche *et al.* 1952, Gereben *et al.* 2001), little is known about the biochemical and operational properties that distinguish between these two intrathyroidal dehalogenating enzymes. Therefore, this work was designed to characterize and compare tDh and ID1 activities in the rat thyroid gland. The results demonstrate that in terms of their biochemical and kinetic characteristics, rat tDh (rtDh) and ID1 are distinct catalytic entities.

Materials and Methods

Reagents

L-DIT, L-MIT, dithionite, flavin adenine dinucleotide (FAD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), propylthiouracil (PTU) and reverse tri-iodothyronine (rT₃), were purchased from Sigma Chemical (St Louis, MO, USA); NaI¹²⁵ (specific activity: 17.4 Ci/mg) was from Amersham Pharmacia

Table 1 Kinetic parameters of rtDh and ID1

	Method	K_m (μM)	$V_{\max}$ (pmol ^{125}I /mg per h)	Catalytic efficiency ($V_{\max}/K_m$)
Substrate				
<i>rtDh</i>				
DIT	^{125}I release	1.5 ± 0.5	6190 ± 540	4180
	NADPH oxidation	1.1 ± 0.2	7645 ± 877^b	6950
MIT	^{125}I release ^a	1.1 ± 0.3	8145 ± 665	7140
	NADPH oxidation	1.0 ± 0.2	9553 ± 724^b	9553
NADPH	^{125}I release	27 ± 1.6	6617 ± 317	246
<i>ID1</i>				
rT ₃	^{125}I release	1.0 ± 0.2	7500 ± 560	7730
DTT	^{125}I release	1000 ± 300	5075 ± 450	5

Each value represents the mean $\pm$ S.E. ^aKinetic values for MIT were indirectly estimated based on the competitive inhibitory effect of MIT upon ^{125}I -DIT deiodination. ^b $V_{\max}$ values were determined assuming a NADPH : released iodide stoichiometry of 1 : 1. Assay conditions were as in Fig. 1.

(Buckinghamshire, UK). ^{125}I -labeled rT₃ (specific activity: 1174 $\mu\text{Ci}/\mu\text{g}$) was obtained from Perkin Elmer-NEN, Inc. (Boston, MA, USA). Resins AG 50W-X8 and AG 50W-X2 (both mesh size 100–200) were acquired from Bio-Rad Laboratories (Hercules, CA, USA). Dithiothreitol (DTT) was obtained from Calbiochem (La Jolla, CA, USA). 3,5-L-dinitrotyrosine (DNT) and 3,5-L-dibromotyrosine (DBT) were purchased from TCI of America (Portland, OR, USA). Radiolabeled substrates were purified prior to use by means of a SEP-PAK C18 cartridge from Millipore Waters Chromatography (Boston, MA, USA).

Animal and tissue handling

All animals included in the present study were taken from the exceeding breeding stocks of our animal house. Preliminary experiments revealed no significant gender or strain differences between rtDh and ID1 activities (data not shown). Thus, thyroid glands from male and female rats ($n=400$) of Wistar and Sprague–Dawley strains (350–650 g body weight) were pooled. Animals were killed by CO₂ aspiration, and thyroid glands were dissected and immediately stored at -70°C until used. Procedures regarding handling and euthanasia of animals were revised and approved by the Animal Welfare Committee of our Institute. Tissue was kept at 4°C while homogenizing with a Polytron (three to four strokes). The buffers used for rtDh and ID1 homogenization (1:5 w/v) were phosphate (0.5 M, pH 7.4) and HEPES (0.01 M, pH 7.0) respectively. Homogenates were centrifuged at 13 000 g for 5 min, 4°C to eliminate intact cells and cell debris. Protein content was measured by Bradford's method (Bradford 1976). To minimize inactivation by freeze–thaw cycles, the homogenate supernatants were frozen in aliquots of 200 μl . The enzyme suspension did not sediment upon standing.

Substrate labeling

^{125}I -labeled DIT was prepared by the exchange method (Cahnman 1972). Briefly, 100 μl 0.1 M L-DIT were mixed with 2 mCi of Na- ^{125}I and 20 μl of 0.05 M I₂ in methanol. This mixture was allowed to stand for 10 min with occasional shaking. The reaction was stopped by adding a drop of a saturated solution of sodium metabisulfate, and the mixture was applied to an AG 50W-X8 column equilibrated with 10% acetic acid. This column was washed with 10% acetic acid until no further radioactivity was detected in the eluate. Subsequently, ^{125}I -DIT was eluted with five successive volumes (2.5 ml each) of 1 M NH₄OH. Labeling efficiency varied from 20 to 30%. The aliquot containing maximal radioactivity was used in the assay mixture. The mean specific activity was $765 \pm 110 \mu\text{Ci}/\mu\text{mol}$.

Deiodination assay

Both enzyme activities were measured in duplicate by the radiolabeled iodide release method under varying conditions. rtDh assays were performed in 0.5 M phosphate buffer at pH 7.4 by a modification of the original method (Rosenberg & Goswami 1984). Briefly, the assay mixture contained (except when stated otherwise): 2 μM DIT, 8 pmol ^{125}I -DIT (approximately 10 000 c.p.m.), 50 mM 2-mercaptoethanol, 200 mM KCl, 30 μM FAD, 1.5 mM methimazole and 30 μM NADPH in a final volume of 500 μl . In the case of ID1, the assay buffer was 0.01 M HEPES at pH 7.0, and the procedure was as described earlier (Valverde-R & Aceves 1989). Briefly, the assay mixture contained (except when indicated otherwise), 1 μM rT₃, 200 fmol ^{125}I -rT₃ (approximately 50 000 c.p.m.) and 5 mM DTT in a final volume of 100 μl . Both assays were incubated in a covered water bath with shaking for 60 min at 37°C and stopped by adding 50%

Figure 1 Double reciprocal plot of rtDh and ID1 deiodination rates as a function of substrate and cofactor concentrations. (A) rtDh in the presence of 0.25, 0.36, 0.7, 1 and 1.5 μM DIT and 15, 22, 30, 45 and 60 μM NADPH. (B) ID1 in the presence of 0.25, 0.5, 1 and 5 μM rT₃ and 0.25, 0.5, 1 and 5 mM DTT. Assay conditions: 200 μg and 20 μg protein, 8 pmol ¹²⁵I-DIT and 200 fmol ¹²⁵I-rT₃ for rtDh and ID1 respectively; incubating 1 h at 37 °C for both enzymes. Each point represents the mean of three independent experiments, each carried out in duplicate.

human plasma (complete fraction; 100 and 50 μl for rtDh and ID1 respectively), 10 mM PTU, and 10% trichloroacetic acid (500 and 100 μl, for rtDh and ID1 respectively). In both cases, the assay mixture was centrifuged (1300 *g* for 15 min), and the supernatant was decanted onto a 1 ml AG 50W-X2 column equilibrated in 10% acetic acid and eluted with 2 ml of 10% acetic acid (80% recovery of ¹²⁵I). In all cases less than 30% of the substrate was consumed during the reaction. The ¹²⁵I eluate, which is an index of enzyme deiodinating activity, was determined in a gamma scintillation counter (Cobra II, Auto-Gamma). Specific activity was calculated according to Pasos-Moura *et al.* (1991). Results for both enzymes are expressed as specific activity in picomoles of ¹²⁵I released/mg protein per hour. rtDh kinetic parameters for MIT and DIT were also assessed by means of a u.v. spectrophotometric procedure to monitor NADPH oxidation at 340 nm. The assay mixture and conditions were as described above, except for the absence of ¹²⁵I-DIT. To evaluate non-specific NADPH oxidation, each group of assays included a control containing the homogenate and all other assay components with no added substrate. NADPH concentrations were calculated using an absorption coefficient of 6.22 mM⁻¹ cm⁻¹.

Kinetic analysis of the data

Kinetic constants of both enzymes were determined by non-linear fit of the raw data to the Michaelis–Menten equation on the Microcal Origin 6.0 (Microcal Software, Northampton, MA, USA) iterative program. To determine the mode of inhibition and the inhibition constant (*K_i*) values experimental data were plotted according to the methods of Dixon (1/*v* against *i*) (Dixon 1953) and Cornish-Bowden (*s/v* against *i*) (Cornish-Bowden 1974).

All reactions were performed in duplicate, and each experiment was repeated a minimum of three times.

rtDh and ID1 characterization

For both enzymes the following parameters were assessed: incubation time (0.16, 0.5, 1 and 1.5 h); pH (5–9); temperature (0, 6, 12, 22, 32, 37, 42 and 50 °C); protein concentration (2–1000 μg); substrate concentration for apparent Michaelis–Menten constants (MIT and DIT, 0.1–10 μM for rtDh and rT₃, 0.05–20 μM for ID1); cofactor concentration (NADPH, 0.025–3 mM for rtDh and DTT, 1 μM to 20 mM for ID1); and sensitivity to inhibitors (PTU, 1–10 mM, DNT, 0.1 μM to 1 mM and DBT, 1 nM to 10 μM for rtDh; and PTU, 1 μM to 10 mM and DBT, 0.5 μM to 1 mM for ID1).

Comparisons of rtDh and ID1 activities

In a series of parallel experiments, and working under optimal conditions for both enzymes, we assessed the differential affinities for substrate (DIT, MIT and rT₃) and cofactor (NADPH, DTT and dithionite) and sensitivity to inhibitors (PTU and DBT).

Results

Initial characterization

rtDh activity was linearly dependent on protein concentration from 100 to 550 μg (data not shown). For subsequent assays, a protein concentration of 200 μg/tube was chosen. In the case of ID1, the activity was linear from 2 to 25 μg of protein (data not shown). Thus, subsequent

Figure 2 Mechanism of DBT inhibition of rtDh activity. (A) Deiodination rates with increasing concentrations of DIT and DBT (0, 100, 300, 500 and 700 nM) at fixed NADPH concentration (30 μ M). (B) Deiodination rates using increasing concentrations of NADPH and DBT (0, 25, 50, 100 and 200 nM) at a fixed DIT concentration (2 μ M). Inset plot: Cornish-Bowden plot of the same data. Assay conditions were as in Fig. 1.

assays were carried out using a protein concentration of 20 μ g/tube. Optimal rates of deiodination were obtained at pH 7.4 and 7.0 for rtDh and ID1 respectively, and at 37 °C after incubating for 1 h for both enzymes (data not shown). For convenience, the above conditions were fixed for subsequent assays. Inter- and intra-assay coefficients of variation using the radioiodide released method were <10% and <5% for rtDh and ID1 respectively ($n=10$).

Cofactor and substrate kinetics

Table 1 summarizes the apparent kinetic parameters for both cofactors (NADPH and DTT) as well as for substrates (DIT, MIT and rT₃). In the case of rtDh, and on an

equimolar basis, NADPH was more than twice as effective as the inorganic reducing agent dithionite (data not shown). Furthermore, total rtDh activity increased as a function of NADPH concentration, reaching a maximum between 50 and 100 μ M. A direct competitive inhibitory effect of added unlabeled MIT on ¹²⁵I-DIT deiodination was observed, thus allowing an indirect estimation of kinetic parameters for MIT using the radiolabeled iodide release method. In addition and as shown in Table 1, the kinetic values obtained by this method were in close agreement with those obtained by the u.v. quantitation of NADPH. Moreover, in stoichiometric terms, the appearance of released iodine and the disappearance of NADPH was 1:1. rtDh activity was saturated by DIT and

Figure 3 The effect of 3,5-DBT on tDh activity when employing NADPH and dithionite as cofactors. Assay conditions were as in Fig. 1, employing 10 μ M DIT, 38 mM dithionite, 200 μ M NADPH and 1 μ M 3,5-DBT (0 in controls).

MIT at concentrations of 5 μ M. As judged by catalytic efficiency, MIT deiodination was favored over DIT. For ID1, maximal activity was achieved with a DTT concentration of 5 mM. The enzyme was saturated at 5 μ M rT_3 .

Catalytic mechanism

As depicted in Fig. 1A and B, the parallelism obtained in the linear regression analysis of the double reciprocal plots, as well as the resultant slopes of each line suggest a double displacement (ping-pong) bisubstrate reaction as the catalytic mechanism for both enzymes.

Effect of Br- and NO₂-containing tyrosines on rtDh activity

Both DNT and DBT effectively inhibited rtDh activity. However, on a molar basis DBT inhibition was sixfold

greater than that exerted by DNT (K_i 170 nM vs 1 μ M; data not shown). As depicted by the Dixon plots in Fig. 2A and B, DBT's mode of inhibition was non-competitive for DIT and either competitive or mixed for NADPH. However, when these data are plotted according to Cornish-Bowden (inset Fig. 2B) a mixed mechanism can be ruled out, thus confirming that DBT's mode of inhibition for NADPH is competitive. To further confirm this mode of inhibition of DBT, this substituted tyrosine was assayed in the presence or absence of NADPH. As Fig. 3 shows, DBT is able to inhibit tDh activity only in the presence of NADPH.

Comparisons of rtDh vs ID1 activities

As shown in Fig. 4A, rtDh activity was unaffected by the presence of rT_3 over a broad concentration range (nM to mM). Similarly, ID1 activity remained unchanged by the presence of DIT and MIT (Fig. 4B). DTT, the preferential *in vitro* cofactor for ID1, was unable to promote rtDh activity, and neither NADPH nor dithionite promoted ID1 activity (Table 2). Moreover, PTU, a classical *in vitro* inhibitor of ID1 activity, had no effect on DIT deiodination by rtDh, and rT_3 deiodination by ID1 was unaffected by DBT at concentrations ranging from 0.5 μ M to 1 mM.

Discussion

Although tDh and IDs were independently described over 70 and 20 years ago respectively, this is the first study that simultaneously characterizes and compares at these

Figure 4 Comparison of substrate specificity of rtDh and ID1 activities. (A) Percentage deiodination of ¹²⁵I-DIT under rtDh conditions in the presence of increasing concentrations of rT_3 (50 nM, 1 μ M and 1 mM), MIT and DIT (both: 50 nM, 2 μ M and 15 μ M). (B) Percentage deiodination of ¹²⁵I- rT_3 under ID1 conditions in the presence of increasing concentrations of rT_3 (50 nM, 1 μ M and 1 mM), MIT and DIT (both: 50 nM, 1 μ M and 1 mM). Assay conditions were as in Fig. 1, employing 30 μ M NADPH and 5 mM DTT for rtDh and ID1 respectively.

Table 2 Cofactor and inhibitor specificity of rtDh and ID1. Each value represents the mean of three independent experiments, each in duplicate ± s.e. Assay conditions were as in Fig. 1, except when the stated cofactor was studied

		rtDh (pmol ¹²⁵ I/mg per h)	ID1 (pmol ¹²⁵ I/mg per h)
Agent employed	Cofactor		
	DTT (5 mM)	Not detected	4431 ± 360
	NADPH (1 mM)	4093 ± 220	Not detected
Inhibitor	PTU (5 mM)	4031 ± 165	Not detected
	DBT (1 μM)	Not detected	4351 ± 160

thyroidal dehalogenases a kinetic level. Thus, we here confirm and extend previous information regarding the co-expression in the rat thyroid gland of a set of two distinct reductive dehalogenases. Both enzymes exhibit the same mechanism of reaction and similar kinetic parameters. However, as the present results demonstrate, there is a clear-cut distinction between the two dehalogenating activities in terms of their substrate, cofactor and inhibitor specificity. These operational differences may account for their distinct functions, i.e. while tDh activity is thought to be responsible for intrathyroidal iodine recycling, IDs catalyze TH bioactivation and/or inactivation.

Whereas the present results regarding ID1 basically confirm previous knowledge (Leonard & Visser 1986, Köhrle 2000, Bianco *et al.* 2002), our study on rtDh extends the up to now scarce knowledge about this important intrathyroidal dehalogenase. Indeed, there are two major contributions of the present study: the systematic analysis of the enzyme reaction mechanism, and the kinetics of DBT inhibition. As early as 1957, Stanbury (1957) reported that NADPH was the cofactor for tDh activity. This observation was confirmed and extended by the studies of Rosenberg & Goswami (1979), in which

mammalian tDh was identified as a flavoprotein. Moreover, these authors postulated that the full reduction of flavin mononucleotide, the prosthetic group, is a prerequisite for tDh activity. In a series of elegant and systematic studies, these authors (Goswami & Rosenberg 1979, 1981) demonstrated that while the inorganic reducing compound dithionite directly activates the enzyme, NADPH acts through an intermediary reducing agent. In the present study and using the endogenous cofactor, rtDh activity was measured by two different analytical procedures: the radioiodide release method and the quantitation of NADPH oxidation. Results show that rtDh catalytic activity follows simple Michaelis–Menten kinetics with a typical ‘ping-pong’ bisubstrate reaction mechanism, and that the enzyme selectively deiodinates MIT and DIT. These data add further support to the proposal that a single enzyme deiodinates both iodotyrosines (Stanbury & Morris 1958). Kinetic values for MIT and DIT were very similar to those reported by Stanbury (1960) in rat thyroid, and by Bastomsky & Rosenberg (1966) and Green (1968) in sheep thyroid. In addition, the present findings confirm that DNT and DBT are potent and selective inhibitors of rtDh activity (Green 1968, Rosenberg & Goswami 1984). However, our results show that DBT is a more potent

Figure 5 Proposed model for ID1 (A) and rtDh (B) mechanisms of reaction and their interaction with inhibitors. These models are based on a ‘ping-pong’ reaction. For the sake of simplicity, the release of the last two products is assumed here to be a one-step process. In this model rT₃ binds to ID1 in the reduced state, and NADPH binds to tDh in the inactive state. The NADPH-mediated rtDh reduction intermediary is not represented. ESeH, reduced enzyme; ESeH.rT₃, reduced enzyme–rT₃ complex; ESe.I*, intermediary enzyme–selenocysteinyl–iodide complex; ESe.PTU, enzyme–PTU complex; DTTred, DTT in reduced state; E.I.DTT, enzyme–I–DTT complex; DTTox, DTT in oxidized state; E, enzyme; E.NADPH, enzyme–NADPH complex; E*, enzyme in activated state; NADP, NADPH in oxidized state; E.DIT, enzyme–DIT complex; E.DBT, enzyme–DBT complex; I⁻ iodide.

inhibitor than DNT (K_i in the nM and μ M range respectively), and that DBT inhibition was non-competitive with respect to DIT and competitive with respect to NADPH. We know of only one study addressing the inhibitory mechanism of both DNT and DBT on tDh activity (Green 1968). According to this author, DNT was a more potent inhibitor than DBT, and a competitive mechanism with respect to DIT was suggested. However, as shown by Rosenberg & Goswami (1984) and confirmed in the present study, neither DNT nor DBT inhibit tDh activity in the absence of NADPH, i.e. when dithionite is used as the reducing cofactor. These data together with the results presented in this study strongly suggest that neither DNT nor DBT competes directly with DIT. Instead, these substituted tyrosines inhibit rtDh activity by competing with NADPH and blocking the cofactor-mediated enzyme reduction. In this context, Fig. 5A illustrates the now classical model for PTU inhibition of ID1 activity (Leonard & Visser 1986, du Mont *et al.* 2001) and a proposed DBT inhibitory model for tDh (Fig. 5B). It is noteworthy that, other than the polycyclic iodine-free phenols or phenol-carboxylic acids, all substrate competitive inhibitors of IDs are iodinated. The replacement of iodine by either Br or NO_2 in the iodothyronine scaffold significantly decreases the inhibitory potency of all derivatives tested (Leonard & Visser 1986, Leonard & Köhrle 2000). In marked contrast, the only competitive inhibitor for DIT dehalogenation (K_i 380-fold lower than substrate K_m) so far described is a non-halogenated methylated pyridonyl analogue (Kunishima *et al.* 1999).

The thyroid is the major iodine reservoir in all vertebrates. By demonstrating the co-expression of two distinct dehalogenating systems in the gland, the present work highlights the importance of undertaking the systematic study of tDh for a more comprehensive understanding of iodine metabolism.

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