REVIEW

Iodothyronine deiodinase structure and function: from ascidians to humans

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Abstract

Iodothyronine deiodinases are important mediators of thyroid hormone (TH) action. They are present in tissues throughout the body where they catalyse 3,5,3′-triiodothyronine (T_3) production and degradation via, respectively, outer and inner ring deiodination. Three different types of iodothyronine deiodinases (D1, D2 and D3) have been identified in vertebrates from fish to mammals. They share several common characteristics, including a selenocysteine residue in their catalytic centre, but show also some type-specific differences. These specific characteristics seem very well conserved for D2 and D3, while D1 shows more evolutionary diversity related to its *K*m, 6-*n*-propyl-2-thiouracil sensitivity and dependence on dithiothreitol as a cofactor *in vitro*. The three deiodinase types have an impact on systemic T_3 levels and they all contribute directly or indirectly to intracellular T_3 availability in different tissues. The relative contribution of each of them, however, varies amongst species, developmental stages and tissues. This is especially true for amphibians, where the impact of D1 may be minimal. D2 and D3 expression and activity respond to thyroid status in an opposite and conserved way, while the response of D1 is variable, especially in fish. Recently, a number of deiodinases have been cloned from lower chordates. Both urochordates and cephalochordates possess selenodeiodinases, although they cannot be classified in one of the three vertebrate types. In addition, the cephalochordate amphioxus also expresses a non-selenodeiodinase. Finally, deiodinase-like sequences have been identified in the genome of non-deuterostome organisms, suggesting that deiodination of externally derived THs may even be functionally relevant in a wide variety of invertebrates.

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Introduction

Thyroid hormones (THs) are involved in a wide variety of processes in developing as well as adult vertebrates. The majority of these actions are mediated by nuclear TH receptors, which are ligand-dependent transcription factors. TH receptors have a high affinity for 3,5,3′-triiodothyronine (T_3) and a lower affinity for 3,5,3′,5′-tetraiodothyronine or thyroxine (T_4), which is the major TH secreted by the thyroid gland. T_4 can be converted into T_3 by a process called (mono-) deiodination, occurring predominantly in extra-thyroidal tissues. Evidence for this type of peripheral conversion was found already in 1950 following injection of radiolabelled T_4 into the blood stream of rats (Gross & Leblond 1950).

It took until the 1970s to study the reaction mechanisms of deiodination in more detail. These studies, performed mainly *in vitro* on rat tissues, eventually led to the characterisation of three different types of activity and the corresponding enzymes were defined as type 1, type 2 and type 3 iodothyronine deiodinase (D1, D2 and D3). Two of these enzyme types are selective: D2 only catalyses outer ring deiodination (ORD) while D3 only catalyses inner ring deiodination (IRD). The D1 enzyme is non-selective and catalyses both ORD and IRD (Fig. 1). Additional properties distinguishing D1 from D2 and D3 are its sensitivity to inhibition by 6-*n*-propyl-2-thiouracil (PTU), the relatively high *K*m for its preferred substrate T_3 (3,5,3′-triiodothyronine) and reverse T_3 or rT_3 (3,3′,5′-triiodothyronine) and the ping–pong kinetics of the reaction (Leonard & Visser 1986).

In the following years, a multitude of kinetic studies confirmed the presence of more or less similar types of deiodinating activities in other mammals as well as in birds and reptiles (Rudas 1986, Galton & Hiebert 1987, Rudas *et al.* 1993, Hugenberger & Licht 1999, Fenton & Valverde 2000, Shepherdley *et al.* 2002). The situation in amphibians and fish seemed to be different as no high *K*m D1-like

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Figure 1 Major pathways of thyroid hormone deiodination. D1, D2, D3, deiodinase types 1, 2 and 3.

activity could be detected in amphibians and most fishes studied at that time, with the exception of tilapia. Tilapia kidney catalysed ORD of rT_3 with a relatively high *K*m, but the activity did not seem to be inhibited by PTU (Mol *et al.* 1993).

Differences were also observed in the temperature dependence of the deiodinating activities between endotherm and exotherm vertebrates. The optimum for T_4 and/or rT_3 ORD was rather constant and high (around 37 °C) for mammals and birds but varied in fish from low (25–30 °C) for rainbow trout and turbot, living in cold water, up to high (37 °C) for tilapia and the catfish *Clarias gariepinus*, living in warm water. The optimum for IRD of T_3 was more uniform around 30–37 °C (Mol *et al.* 1997, 1998). ORD activities in the liver of reptiles remained stable over a broad temperature range, as found in the turtle *Trachemys scripta* (28–37 °C) and the lizard *Scleroporus grammicus* (15–42 °C) (Hugenberger & Licht 1999, Fenton & Valverde 2000).

It took until 1991 before the first iodothyronine deiodinase, rat D1, was cloned and identified as a selenocysteine (Sec)-containing protein (Berry *et al.* 1991). From that time onwards, a variety of deiodinases from different species have been cloned and some recombinant proteins have been functionally characterised. These data provide more insight into the evolution of the structure and function of this group of enzymes and have challenged part of our initial view on the differences between the three types of deiodinases. Although they can be found in all vertebrate classes, their structure and maybe more importantly their tissue distribution show some interesting variations that have an impact on their biological function. In the present review we will provide a comparative view on the structure and function of the iodothyronine deiodinases known so far. For clarity, we will use throughout the text *dio1*, *dio2* and *dio3* for the vertebrate deiodinase genes (always with lower case letters when referring to multiple species) and D1, D2 and D3 for the deiodinase proteins.

Structure and activity of deiodinases

Common characteristics of the three types of vertebrate deiodinases

All vertebrate deiodinases that have been cloned so far are selenoproteins with a Sec in their catalytic centre. Sec is encoded by UGA which normally functions as a stop codon. Therefore, all deiodinases contain a selenocysteine insertion sequence (SECIS) element in the 3′-UTR of the mRNA. The SECIS element forms a stem–loop structure necessary to interact with components of the translational machinery (selenocysteyl-tRNA and elongation factor; Berry *et al.* 1993). The structure and function of the SECIS elements present in deiodinases are discussed in more detail in other reviews, e.g. Bianco *et al.* (2002) and Gereben *et al.* (2008).

The three types of deiodinases belong to the thioredoxin fold superfamily and share a similar structural organisation. They are integral membrane proteins that function as homodimers, although probably only one monomer partner is required for catalytic activity (Bianco & Larsen 2005). The hydrophobic transmembrane region of the enzymes is located in the N-terminal domain. Their active site is oriented towards the cytoplasma and its core sequence is very well conserved in all known deiodinases (Gereben *et al.* 2008). A second highly conserved 16 amino acid (AA) region located in the C-terminal half of the protein, containing one of the two conserved His residues, seems to be important for the formation of the homodimers (Leonard *et al.* 2005). The Sec residue in the catalytic centre plays an essential role in deiodinase activity. Substitution of this Sec by Cys results in a drop of catalytic activity by two or three orders of magnitude, whereas substitution by Leu or Ala completely abolishes activity (Gereben *et al.* 2008). Information on the three-dimensional conformation of deiodinases is limited due to the lack of soluble, catalytically active protein for crystallisation purposes, but hydrophobic cluster analysis of the deiodinase sequences compared with other members of the thioredoxin superfamily allowed to propose a general model (Callebaut *et al.* 2003). It shows a protein composed of a single aminoterminal-anchoring segment, a short hinge region, and a thioredoxin fold-containing globular domain as described in more detail elsewhere (Callebaut *et al.* 2003, Bianco & Larsen 2005, Bianco & Kim 2006).

Structure and activity of D1

The biochemical characteristics of the D1 enzyme were originally determined using rat liver homogenates and microsomes. D1 was described as a high *K*m non-selective enzyme that catalysed both ORD and IRD reactions. Its efficiency for ORD of rT_3 was more than 100-fold higher than for ORD of T_4, while sulphation of T_4 and T_3 strongly increased IRD efficiency (Leonard & Visser 1986). *In vitro* deiodination depended on the presence of a reducing cofactor such as dithiothreitol (DTT). The enzyme showed ping–pong kinetics and was strongly inhibited by PTU (100% by

0·05 mM PTU). This inhibition was uncompetitive with substrate and competitive with cofactor. Enzyme activity was also inhibited by iodoacetate (IAc) and iopanoic acid (IOP) (Kobayashi *et al.* 1985, Leonard & Visser 1986).

Since then, D1 has been cloned from a wide variety of vertebrate species and presently information of partial/complete nucleotide coding sequences for D1 from ~70 vertebrate species is available from different databases. The structure of the gene encoding D1, *dio1*, has been described for mouse (Maia *et al.* 1995*a*) and human (Toyoda *et al.* 1996, Jakobs *et al.* 1997*a*). The gene consists of four exons, which seems to be the case for all species available in the Gene database at the NCBI website. The TGA (Sec) codon is located in exon 2, while the TAG (stop) codon and the SECIS element are located in exon 4. The 5′-flanking region (5′-FR) of the mouse and human *dio1* genes is GC rich and contains no TATA or CAAT boxes (Maia *et al.* 1995*a*, Jakobs *et al.* 1997*b*). The human gene also contains two thyroid hormone response elements (TREs), but, although mouse and rat D1 mRNAs are also increased by T_3, canonical TREs have not yet been identified in the 5′-FR of these genes (Bianco *et al.* 2002). Splice variants have been described for rat D1 mRNA differing in the length of the 3′-UTR (Navarro *et al.* 1997). Quite recently, a D1 mRNA splice variant has also been found in a fish, the walleye (*Sander vitreus*), containing an 11-bp insert downstream of the codon for Sec. The resulting protein, if translated, would however not be homologous to other D1s due to a frame shift (Picard-Aitken *et al.* 2007).

Comparison of the full coding sequence of different D1s shows that the D1 protein is fairly constant in length consisting of 244–257 AAs (Table 1). The Sec is situated around position 121–130. Functional characterisation of the recombinant D1 protein has only been done for a limited number of species. The data available for mammals show that the *K*m for ORD of rT_3 is up to two orders of magnitude higher in dog and cat compared with human, rat, pig and house shrew (Table 1). In contrast to the lower efficiency for ORD of rT_3, the efficiency of these enzymes for deiodination of T_4 and T_3 is quite similar to that in human or rat (Toyoda *et al.* 1997, Kuiper *et al.* 2003).

Sequence comparison of the proteins from human, rat, dog and cat shows that various differences are concentrated in the region between AA residues 40 and 70. Both in dog and cat the AA residues present at position 48–52, in human and rat D1s are missing. Other differences are found in the AA residues corresponding to positions 45–46 and 65–66 in the human sequence (Fig. 2). Mutagenesis of the dog and cat sequences showed that the insertion of Thr-Gly-Met-Thr-Arg (human 48–52) alone did not improve deiodination of rT_3. For the cat enzyme, a combination of mutations including the insertion of those five AA as well as AA substitutions placing Gly-Glu and Phe at the positions 45–46 and 65 was necessary to improve the ORD of rT_3 to the level typical for human D1. In dog, the most efficient mutation was the replacement of Leu-Tyr by Phe-Phe at the position corresponding to 65–66 in the human sequence. Opposite mutations in the human sequence indicated that deletion of the 48–52 sequence increased the *K*m for rT_3 ORD only twofold, while changing Phe at position 65 into Leu increased the *K*m tenfold (Toyoda *et al.* 1994, Kuiper *et al.* 2003). The conclusion of the comparative study of these enzymes was that the Phe at position 65 found in most D1 sequences (Fig. 2) is particularly important for ORD of rT_3 but not for ORD of iodothyronines with two iodines on the inner ring, possibly due to interaction of the aromatic ring of Phe (or Tyr in killifish) with the mono-substituted inner ring of rT_3 (Toyoda *et al.* 1997).

Only one full D1 coding sequence has been cloned from birds and none from reptiles. The chicken AA sequence shows 61% homology with the human sequence (Fig. 2) and functional studies of the recombinant protein in cell culture revealed identical substrate specificity and sensitivity to inhibitors as the human and rat enzyme (Van der Geyten *et al.* 1997).

The cloning of the first fish D1 from tilapia (*Oreochromis niloticus*) in 1997 was an important milestone as it showed that one of the major characteristics originally defined for D1, namely its high sensitivity to inhibition by PTU, is not a conserved feature (Sanders *et al.* 1997). Tilapia D1 shows 47% AA sequence homology with human D1 (Fig. 2); it also has a Sec in its active site and has a similar substrate preference. It is, however, insensitive to PTU (only 5% inhibition by 1 mM PTU) and also approximately tenfold less sensitive to IAc and aurothioglucose (ATG) than rat and human D1 (Sanders *et al.* 1997). A few years later, killifish (*Fundulus heteroclitus*) D1 was cloned and expressed, showing also low sensitivity to PTU (only 10% inhibition by 1 mM PTU; Orozco *et al.* 2003).

Characterisation of fish D1 proteins has led to more surprises. Sea bream (*Sparus auratus*) kidney and liver contain high *K*m rT_3 ORD activity that is inhibited by DTT, the artificial cofactor that stimulates *in vitro* activity of all other deiodinases characterised so far. In the absence of DTT, the *K*m for rT_3 is 5 μM, which is close to the 2 μM described for tilapia in the presence of DTT (Klaren *et al.* 2005). The cDNA cloned from sea bream kidney confirmed the presence of a D1 protein with a catalytic centre identical to that of tilapia. The Phe at position 65 and the Gly-Glu at position 45–46, important for ORD activity in mammals, are also conserved. It was suggested that the substitution of some conserved positively charged AA residues from the N-terminal hydrophobic domain of mammalian D1 by uncharged polar or non-polar residues in sea bream D1 may be linked to its divergent interaction with DTT (Klaren *et al.* 2005), but this hypothesis remains to be tested. Interestingly, the low level of rT_3 ORD measured in sea bream kidney homogenates in the presence of DTT is almost insensitive to PTU, while the higher activity level in the absence of DTT is about 68% inhibited by 0·1 mM PTU. This finding shows some similarity with the fact that PTU is only efficient in blocking mammalian or chicken D1 activity when there

Table 1 Vertebrate species for which the full coding sequence of D1 is available in GenBank. Information on PTU sensitivity and *K*m for ORD of rT_3 by the recombinant proteins has been taken from 1, Toyoda *et al.* (1994); 2, Berry *et al.* (1991); 3, Wassen *et al.* (2004); 4, Kuiper *et al.* (2003); 5, Rogatcheva *et al.* (2002); 6, Van der Geyten *et al.* (1997); 7, Kuiper *et al.* (2006); 8, Klaren *et al.* (2005); 9, Sanders *et al.* (1997); and 10, Orozco *et al.* (2003)

Species	**Common name**	**GenBank ID** (mRNA)	**Number of AAs**	**PTU sensitive**	***K*m** (ORD rT_3)
Mammals					
Homo sapiens	Human	NM_000792	249	+	0·32 μM (1)
Pan troglodytes	Chimpanzee	NM_001122651	249	+	
Pongo abelii	Orangutan	XM_002810800	249	+	
Macaca mulatta	Rhesus monkey	NM_001122652	249	+	
Oryctolagus cuniculus	Rabbit	NM_001099958	249	+	
Cavia porcellus	Guinea pig	NM_001257974	249	+	
Mus musculus	Mouse	NM_007860	257	+	
Rattus norvegicus	Rat	NM_021653	257	+	0·13 μM (2)
Cricetulus griseus	Chinese hamster	NM_001256759	257	+	
Sus scrofa	Pig	NM_001001627	249	+	0·17 μM (3)
Bos taurus	Cattle	NM_001122593	249	+	
Bubalus bubalis	Water buffalo	JQ791197	249	+	
Equus caballus	Horse	NM_001166452	244	+	
Canis familiaris	Dog	NM_001007126	244	+	9 μM (1)
Felis catus	Cat	NM_001009267	244	+	11 μM (4)
Suncus murinus	Asian house shrew	AB055517	257	+	0·14 μM (5)
Birds					
Gallus gallus	Chicken	NM_001097614	246	+	0·26 μM (6)
Amphibians					
Xenopus tropicalis	Western clawed frog	NM_001256297	252	−	
Xenopus laevis	African clawed frog	NM_001095667	252	−	0·3 μM (7)
Teleost fishes					
Paralichthys olivaceus	Japanese flounder	AB362421	248	−	
Sparus aurata	Gilthead sea bream	AJ619717	248	±	5 μM (8)[a]
Chrysiptera cyanea	Sapphire devil	GU583740	248	−	
Oreochromis niloticus	Nile tilapia	XM_003439801	248	−	2 μM (9)
Takifugu rubripes	Japanese pufferfish	NM_001136144	248	−	
Fundulus heteroclitus	Killifish/mummichog	AY184803	248	−	0·12 μM (10)
Danio rerio	Zebrafish	NM_001007283	254	−	

[a]*K*m for native protein.

is sufficient substrate turnover (Goswami & Rosenberg (1988) and own observations). It could mean that sea bream D1 can be classified as mildly PTU-sensitive, although less sensitive than mammalian D1s.

Although it had long been accepted that amphibians do not have D1, a D1 cDNA was finally cloned from the frog *Xenopus laevis* (Kuiper *et al.* 2006). The AA sequence shows 51% homology with the human D1 (Fig. 2), and the recombinant protein is a high *K*m enzyme with ORD and IRD activity. In contrast to the mammalian D1s, *Xenopus* D1 catalyses ORD of rT_3 and T_4 with equal efficiency, and sulphation does not stimulate the IRD of T_3 or the ORD of rT_3. Similar to tilapia D1, the enzyme is approximately tenfold less sensitive to inhibition by IAc and ATG than rat and human D1 and quite insensitive to PTU (only 15% inhibition by 1 mM PTU).

Several research groups have used site-directed mutagenesis in search of the factors determining PTU sensitivity. Comparison of the active site of the PTU-insensitive D1s from tilapia, killifish and *Xenopus* with that of PTU-sensitive D1s in mammals and chicken showed two consistent substitutions: Pro instead of Ser two positions downstream of Sec, and Ser instead of Asn six positions upstream from Sec (Fig. 2). A Pro128Ser mutation of tilapia D1 showed decreased ORD activity but remained resistant to PTU (Sanders *et al.* 1997). A Ser124Asn mutation in *Xenopus* D1 also did not influence PTU sensitivity, but in this species the Pro132Ser mutation strongly increased inhibition by PTU up to the level typical for mammalian enzymes. At the same time, this mutation increased the *K*m for ORD of T_4 and rT_3 about tenfold (Kuiper *et al.* 2006). Using the opposite approach, PTU resistance could be induced by the Ser128Pro substitution in human D1 without changing the *K*m for rT_3 or T_4 ORD (Callebaut *et al.* 2003). Taken together, these results strongly suggest that the Ser two positions downstream of Sec plays a role in the PTU sensitivity of D1. However, the above-mentioned results for sea bream D1, showing partial PTU sensitivity despite the presence of Pro at this position, suggest that rather than the type of AA *per se*, differences in substrate turnover rate could also be a determining factor, a possibility already discussed for the *Xenopus* Pro132Ser mutant protein (Kuiper *et al.* 2006).

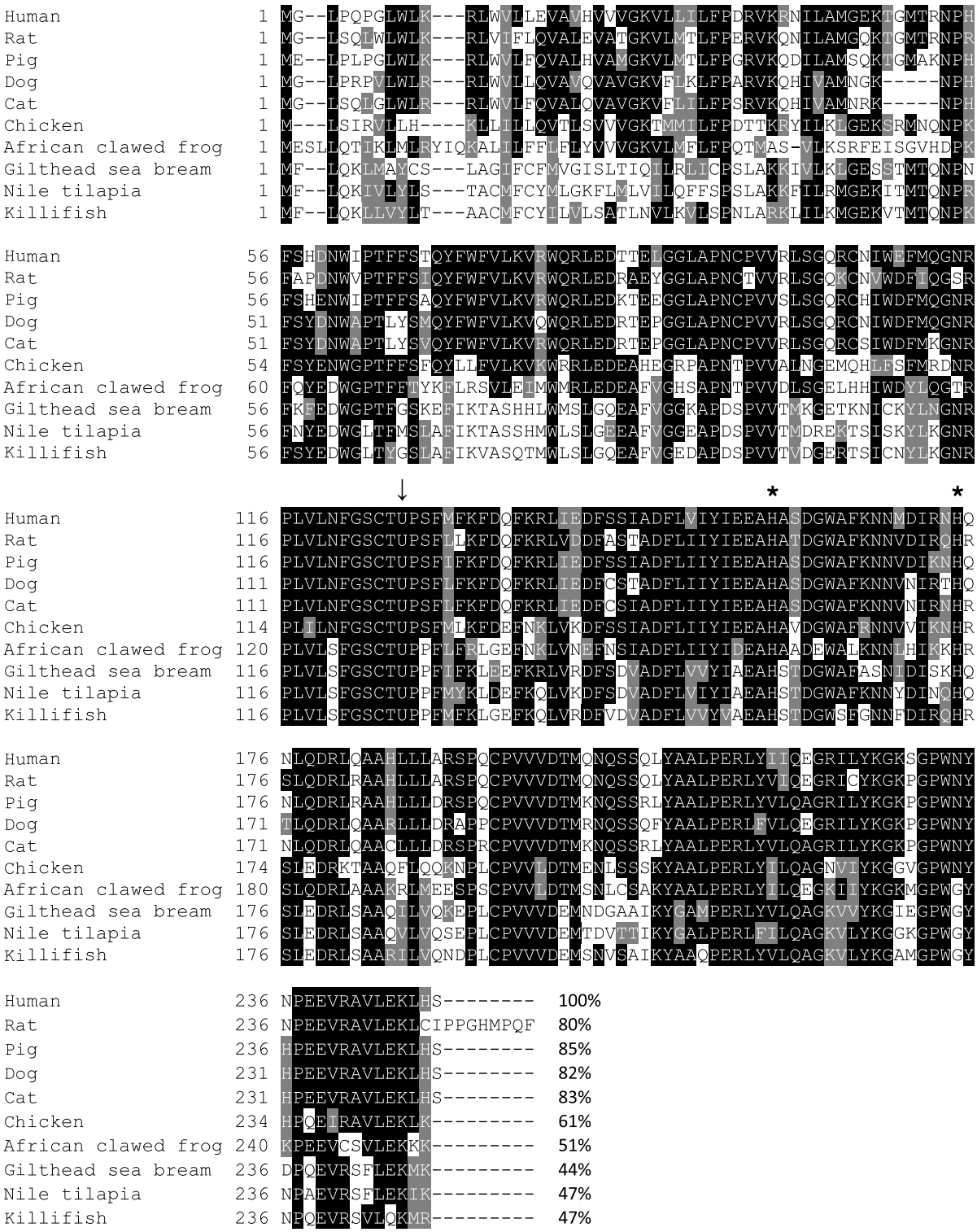


Figure 2 AA sequence alignment for some representative vertebrate D1 proteins. Identical AAs are indicated in black, similar AAs in grey. The catalytic Sec residue is indicated by the arrow and the two conserved His residues by the asterisks. Protein sequences were obtained from GenBank and aligned by ClustalW; the nucleotide accession numbers as well as the genus and species names are shown in Table 1. The numbers at the end of each sequence indicate the percentage of AA identical to the human sequence.

Structure and activity of D2

D2 was originally characterised from rat brain tissue as an obligate ORD enzyme (Fig. 1) with a *K*m for its preferred substrate T_4 in the low nanomolar range (Kaplan & Yaskoski 1980, Visser *et al.* 1981). The enzyme showed a sequential reaction mechanism and had a high need for DTT as a cofactor *in vitro*. The activity was not inhibited by PTU, weakly inhibited by IAc and strongly inhibited by IOP (Leonard & Visser 1986).

The first D2 was cloned in 1995 from the bullfrog *Rana catesbeiana*. The mRNA sequence contained an in-frame UGA codon and a putative SECIS element in the 3′-UTR. Expression of capped RNA transcripts in *Xenopus* oocytes resulted in ORD activity with typical D2 characteristics (Davey *et al.* 1995). The open reading frame of the partial rat and human D2 cDNAs cloned 1 year later showed the presence of two in-frame TGA codons, one located in a region very similar to the active site sequence of other known deiodinases, while the other one was located not far from an unambiguous TAG stop codon (Croteau *et al.* 1996). The structure of the *dio2* gene has been described for human (Celi *et al.* 1998), mouse (Davey *et al.* 1999), killifish (Orozco *et al.* 2002) and trout (Sambroni *et al.* 2001). The *dio2* gene typically consists of two exons, separated by a single intron. Exon 2 contains the TGA(Sec) codon located at the active site and the SECIS element (Valverde *et al.* 2004). Except for bullfrog, D2 mRNA is rather long, ranging from 4·7 kb in killifish up to 8·0 kb in mouse. This is due to an extended 3′-UTR with the SECIS element positioned close to the poly(A) tail (Valverde *et al.* 2004). The human, mouse and rat *dio2* 5′-FR all contain consensus TATA and CAAT elements and a functional CRE, but only human *DIO2* has thyroid transcription factor 1 (TTF1) binding sites (Bianco *et al.* 2002). In contrast to mammals, the killifish 5′-FR does not contain TATA or CAAT boxes and no CRE sequence is present within 1·3 kb upstream of the transcription start site, which could be phylogenetically relevant for the control of *dio2* gene transcription (Valverde *et al.* 2004). The rainbow trout *dio2* gene, on the other hand, has a CAAT box and two putative TATA boxes in the promoter region, suggesting that the transcription of the *dio2* gene might be differentially controlled amongst fish species (Sambroni *et al.* 2001).

The existence of multiple splice variants has been described for human D2 mRNA (Bartha *et al.* 2000, Ohba *et al.* 2001). The human gene possibly codes for four different proteins. One is the typical deiodinase (variant a) while a longer deiodinase (variant b), containing three Sec residues, may be generated by splicing in of an additional exon located between the two typical ones. Two other variants (c and d) are truncated proteins. The long D2 variant with three Sec residues may also be present in chimpanzee, orangutan, rhesus monkey and marmoset. D2 mRNA splice variants have also been described in chicken (Gereben *et al.* 2002) and in the Australian lungfish (Sutija *et al.* 2003). The chicken splice variant cannot express a functional protein due to a shift in the reading frame, while the lungfish splice variants only differ in their 3′-UTR and therefore would lead to identical proteins.

By now full or partial cDNA sequence information is available for ∼80 vertebrate species. The length of the D2 protein typically varies between 257 and 279 AAs (Table 2), although additional longer forms may exist in primates as discussed earlier. The sequence alignment in Fig. 3A clearly illustrates that the protein structure has been highly conserved from fish up to human. One striking difference is found at the C-terminus where some sequences contain a second Sec while others do not. A more detailed comparison of the available C-terminal coding sequences clearly separates the teleost fish from the other vertebrates (Fig. 3B). At the position where the sequences of the other vertebrates (except pig and bullfrog) encode a Sec, the sequences of teleost fish have an unambiguous stop codon. This should, however, not influence their function because mutation of the second Sec codon in human D2 to a Cys codon or an unambiguous stop codon did not change the catalytic activity of the enzyme (Salvatore *et al.* 1999). It can therefore not be excluded that the primary function of the second UGA in the D2 sequence is to function as a stop codon has been recently suggested (Mariotti *et al.* 2012).

Structure and activity of D3

D3 was first characterised from rat brain tissue and human placenta as an obligate IRD enzyme (Fig. 1; Kaplan & Yaskoski 1980, Roti *et al.* 1981). D3 showed a substrate preference for T_3 in the low nanomolar range. Its *in vitro* activity required relatively high DTT concentrations, although very high concentrations (>200 mM) seemed to be detrimental. The enzyme showed sequential type reaction kinetics and was resistant to inhibition by PTU, while activity was inhibited by IAc and IOP (Leonard & Visser 1986).

The first D3 cDNA was cloned in 1994 from *X. laevis* tadpole tail (St Germain *et al.* 1994). The clone contained an in-frame Sec codon and had a SECIS element in the 3′-UTR. Expression of capped RNA transcripts in *Xenopus* oocytes confirmed that the protein catalysed IRD of T_3 with a *K*m of 2 nM and was resistant to PTU as well as to ATG (St Germain *et al.* 1994). One year later, two mammalian D3 cDNAs were identified from rat neonatal skin and human placenta (Croteau *et al.* 1995, Salvatore *et al.* 1995). By now partial or complete cDNA sequences are available for around 50 D3s, coding for proteins with variable length.

The AA sequence of D3 has been well conserved throughout vertebrate evolution, particularly around the active site containing the Sec (Fig. 4). The only striking difference is found at the N-terminus where most predicted mammalian D3 proteins are longer than those of non-mammalian vertebrates (Table 3). This difference is linked to the fact that the mammalian genomic *dio3* sequences include two putative transcription start sites. This may lead to proteins of respectively 304 or 278 AA long, the last one corresponding more closely to the length in non-mammalian

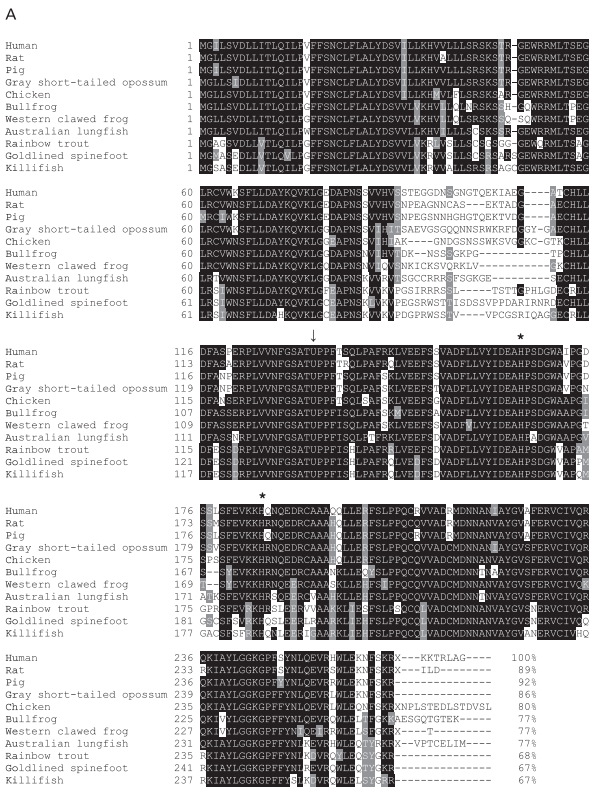
Table 2 Vertebrate species for which the full coding sequence of D2 is available in GenBank. Information on the *K*m for ORD of T_4 by the recombinant proteins has been taken from 1, Croteau *et al.* (1996); 2, Wassen *et al.* (2004); 3, Gereben *et al.* (1999); and 4, Orozco *et al.* (2002)

Species	**Common name**	**GenBank ID** (mRNA)	**Number of AAs**	***K*m** (ORD T_4)
Mammals				
Homo sapiens	Human	NM_000793	273 (isoform a)	1·2 nM (1)
Pan troglodytes	Chimpanzee	NM_001122638	273 (isoform 2)	
Pongo abelii	Orangutan	XM_002824993	309	
Macaca mulatta	Rhesus monkey	NM_001159294	309	
Callithrix jacchus	White-tufted-ear marmoset	XM_003734052	309	
Oryctolagus cuniculus	Rabbit	NM_001256300	269	
Cavia porcellus	Guinea pig	NM_001257977	269	
Mus musculus	Mouse	NM_010050	266	
Rattus norvegicus	Rat	NM_031720	266	0·7 nM (1)
Cricetulus griseus	Chinese hamster	NM_001256762	266	
Sus scrofa	Pig	NM_001001626	265	8 nM (2)
Bos taurus	Cattle	NM_001010992	269	
Equus caballus	Horse	NM_001166455	269	
Ailuropoda melanoleuca	Giant panda	XM_002919047	272	
Canis lupus familiaris	Dog	NM_001122645	269	
Monodelphis domestica	Grey short-tailed opossum	XM_003339485	268	
Birds				
Gallus gallus	Chicken	NM_204114	279	1 nM (3)
Meleagris gallopavo	Turkey	XM_003206645	264	
Reptiles				
Anolis carolinensis	Green anole	XM_003214362	266	
Amphibia				
Rana catesbeiana	Bullfrog	L42815	264	
Xenopus tropicalis	Western clawed frog	NM_001197232	258	
Xenopus laevis	African clawed frog	AF354707	262	
Lung fishes				
Neoceratodus forsteri	Australian lungfish	AF327438	269	
Teleost fishes				
Oncorhynchus mykiss	Rainbow trout	NM_001124268	264	
Paralichthys olivaceus	Japanese flounder	AB362422	271	
Siganus guttatus	Goldlined spinefoot	GU372962	270	
Chrysiptera cyanea	Sapphire devil	GU583739	271	
Takifugu rubripes	Japanese pufferfish	NM_001136145	270	
Fundulus heteroclitus	Mummichog/killifish	U70869	266	0·64 nM (4)
Oryzias latipes	Japanese medaka	NM_001136521	277	
Danio rerio	Zebrafish	NM_212789	257	

vertebrates. So far it remains unclear whether one or both protein variants occur *in vivo*. Interestingly, the sequence of wallaby, the only marsupial for which a full coding sequence is available, only shows a long variant but with a very different N-terminus compared with placental mammals (Fig. 4). Only a few recombinant D3 proteins have so far been expressed, yielding PTU-resistant, low *K*m IRD enzymes with very similar characteristics (Table 3).

All known *dio3* genes consist of a single exon, a characteristic that is rather uncommon in the eukaryotic kingdom (Hernandez *et al.* 1998, 1999). According to the NCBI Gene database, the rabbit *dio3* gene is an exception, having two putative exons, although this seems doubtful given the identity of the putative intron sequence with the missing part of the cDNA of other mammalian *DIO3* sequences. The promoter region of the mouse and human *dio3* gene contains consensus TATA, CAAT and GC elements (Hernandez *et al.* 1999). The *Dio3* gene seems to give rise to mRNA variants of different length in rat (Tu *et al.* 1999). Multiple D3 mRNA variants are also found in fish such as tilapia (Sanders *et al.* 1999) and trout (Bres *et al.* 2006), but in these species the variants may derive from two different genes. The genome databases of the pufferfishes *Takifugu* and *Tetraodon* each contain two *dio3* orthologues (Itoh *et al.* 2010) and this is probably also the case for other teleosts (Mariotti *et al.* 2012). Many genes are found to be duplicated in teleosts, a phenomenon that can be linked to a whole genome duplication that occurred in fish before the teleost radiation (Amores *et al.* 1998, Taylor *et al.* 2003).

An important discovery made in 2002 was that mouse *Dio3* is an imprinted gene (Hernandez *et al.* 2002). Genomic imprinting is an epigenetic process that causes genes to be expressed according to their parental origin. In vertebrates, its emergence is associated with the evolution of the placenta and therefore it does not occur in oviparous species (Edwards *et al.* 2008). The *dio3* gene is located in



B

Species	DIO2 sequence	translation
Mammals		
Human	GAGAAGAATTTCAGCAAGAGATGAAAGAAAACTAGATTAGCTGGTTAA	Sec
Chimpanzee	GAGAAGAATTTCAGCAAGAGATGAAAGAAAACTAGATTAGCTGGTTAA	Sec
Orangutan	GAGAAGAATTTCAGCAAGAGATGAAAGAAAACTAGATTAGCTGGTTAA	Sec
Rhesus monkey	GAGAAAAATTTCAGCAAGAGATGAAAGAAAACTAGATTAGCTGGTTAA	Sec
White-tufted-ear marmoset	GAGAAGAATGTCAGCAAGAGATGAAAGAAAACTAGATTAGCTGGTTAA	Sec
Rabbit	GAGAAGAATTTCAGCAAGAGATGAAATCTAGATTAG	Sec
Guinea pig	GAGAAGAATTTCCACAAGAGATGAAATCTAGATTAG	Sec
Mouse	GAGAAGAATTTCAGCAAGAGATGAATTCTAGATTAG	Sec
Rat	GAGAAGAATTTCAGCAAGAGATGAATTCTAGATTAG	Sec
Chinese hamster	GAGAAGAACTTCAGCAAGAGATGAATTCTAGATTAG	Sec
Pig	GAGAAGAATTTCAGCAAGAGATAA	Stop
Cattle	GAGAAGAATTTCAGCAAGAGATGAAGCTAGATTAG	Sec
Horse	GAGAAGAATTTCAGCGAGAGATGAAATCTAAGTTAG	Sec
Giant panda	GAGAAGAATTTCAGCAAAAGATGAAATCTAGGTTAG	Sec
Dog	GAGAAGAATTTCAGCAAAAGATGAAATCTAGATTAG	Sec
Gray short-tailed opossum	GAAAAGAACTTCAGCAAGAGATGA...	Sec (1)
Birds		
Chicken	GAACAAAACTTCAGCAAAAGATGAAATCCATTGTCTACAGAAGATTTGTCAACAGATGTGTCCCTTTAA	Sec
Turkey	GAGCAAAACTTCAGCAAAAGATGA...	Sec (1)
Reptiles		
Green anole	GAGCAGAATTTTAGGAAGAGATGA...	Sec (1)
Amphibians		
Bullfrog	GAGCTCACATTTGGAAAGAAAGCGCAAAGTGGCACAACTGGCACAGAGAAATAA	Alanine
Western clawed frog	GAACTTAGCTTTGGGAAGAGATGACGTAG	Sec
African clawed frog	GAACTTAGCTTTGGGAAGAGATGACATAG	Sec
Lung fishes		
Australian lungfish	GAACAGACGTACAGGAAACGATGAGTTCCAACATGTGAACTGATTATGTGA	Sec (2)
Teleost fishes		
Rainbow trout	GAACAGAGCTACGGCAAGAGATAG	Stop
Japanese flounder	GAACAGAGCTATGGTAAACGGTAG	Stop
Goldlined spinefoot	GAACAAACCTATGGTAAACGGTAG	Stop
Sapphire devil	GAACAGAGCTACGGTAGACGGTAG	Stop
Japanese pufferfish	GAACAGAGCTACGGCAAGCGGTAG	Stop
Killifish	GAGCTGAGTTACGGCAGACGATAG	Stop
Japanese medaka	GAACAGttacggcagacggtagGACcaaatacagaggagtga	(3)
Zebrafish	GAGAAGTGCTACGGAAACGGATAA	Sec-Stop

Figure 3 (A) AA sequence alignment for some representative vertebrate D2 proteins. Identical AAs are indicated in black, similar AAs in grey. The catalytic Sec residue is indicated by the arrow and the two conserved His residues by the asterisks. The numbers at the end of each sequence indicate the percentage of AA identical to the human sequence. (B) Nucleotide sequence alignment of the N-terminal region of different vertebrate *dio2*. The TGA codon for Sec is highlighted in green, the TAG/TAA stop codon in yellow and the divergent GCA codon for Ala in bullfrog in red. 1) End of the predicted mRNA sequence; 2) the accepted stop codon (underlined) is also present as TGA; 3) a 2 bp frameshift occurred after the underlined codon. The continuing mRNA sequence (in non-capitalised letters) remains highly similar to the other teleost sequences, but the typical stop codon lies out of frame. Protein sequences were obtained from GenBank and aligned by ClustalW; the nucleotide accession numbers as well as the genus and species names are shown in Table 2.

the dlk1–dio3 domain and the three proteins encoded within this domain are preferentially expressed from the paternal chromosome. The organisation and imprinting of this domain is highly conserved in humans, mice and sheep, but the domain is not imprinted in non-eutherian mammals (Edwards *et al.* 2008, da Rocha *et al.* 2008). Imprinted genes are involved in a range of developmental processes, and it has been shown that the correct dosage of *Dio3* expression in particular tissues, such as brown adipose tissue, is critical for survival in neonatal mice (Charalambous & Hernandez 2012, Charalambous *et al.* 2012).

Structure and activity of deiodinases in lower chordates

Over the years convincing data have been gathered showing that TH signalling is not restricted to vertebrates. Several invertebrate chordates have TH receptors and are capable of synthesising THs (Ogasawara *et al.* 1999, Paris *et al.* 2008*a*). Therefore, it is not surprising that they also express deiodinases. *In vivo* deiodination of T_4 was shown in the ascidian *Phallusia mammillata* already in 1989 (Leloup & Seugnet 1989). Approximately 10 years later, *in vitro* studies using low nanomolar substrate concentrations showed the presence of T_4 ORD, T_4 IRD and T_3 IRD in a primitive vertebrate, the sea lamprey (*Petromyzon marinus*) and in the invertebrate Atlantic hagfish (*Myxine glutinosa*) (Eales *et al.* 1997, 2000, McLeese *et al.* 2000). For both species the highest activity was found in the intestine. Interestingly, T_4 IRD in hagfish was completely inhibited by 0·01 mM PTU and T_4 ORD was 60% inhibited by 0·1 mM PTU, while T_3 IRD was unaffected by 1 mM PTU (McLeese *et al.* 2000). Unfortunately, no deiodinases have yet been cloned from hagfishes, which are the closest living relatives to the present vertebrates, or from lampreys, which are considered the most primitive vertebrates as shown in the simplified evolutionary tree of the Deuterostomia in Fig. 5. However, the sea lamprey genome contains at least one deiodinase-like sequence, showing the strongest homology with vertebrate D3.

Several deiodinase-like sequences can also be found in the genomes of the cephalochordate amphioxus

(*Branchiostoma floridae*) and the urochordate ascidians *Ciona intestinalis*, *Ciona savignyi* and *Halocynthia roretzi*. Only one ascidian deiodinase has been fully cloned and characterised (Shepherdley *et al*. 2004). Its sequence contains a region very similar to the active centre of D1, D2 and D3, including a UGA codon, and a SECIS element is present in the 3′-UTR. The recombinant selenoprotein (259 AA long) catalysed ORD of rT_3 and T_4 with an apparent *K*m around 3 μM but showed minimal IRD activity. The enzyme activity followed ping–pong type reaction kinetics, was stimulated by DTT and

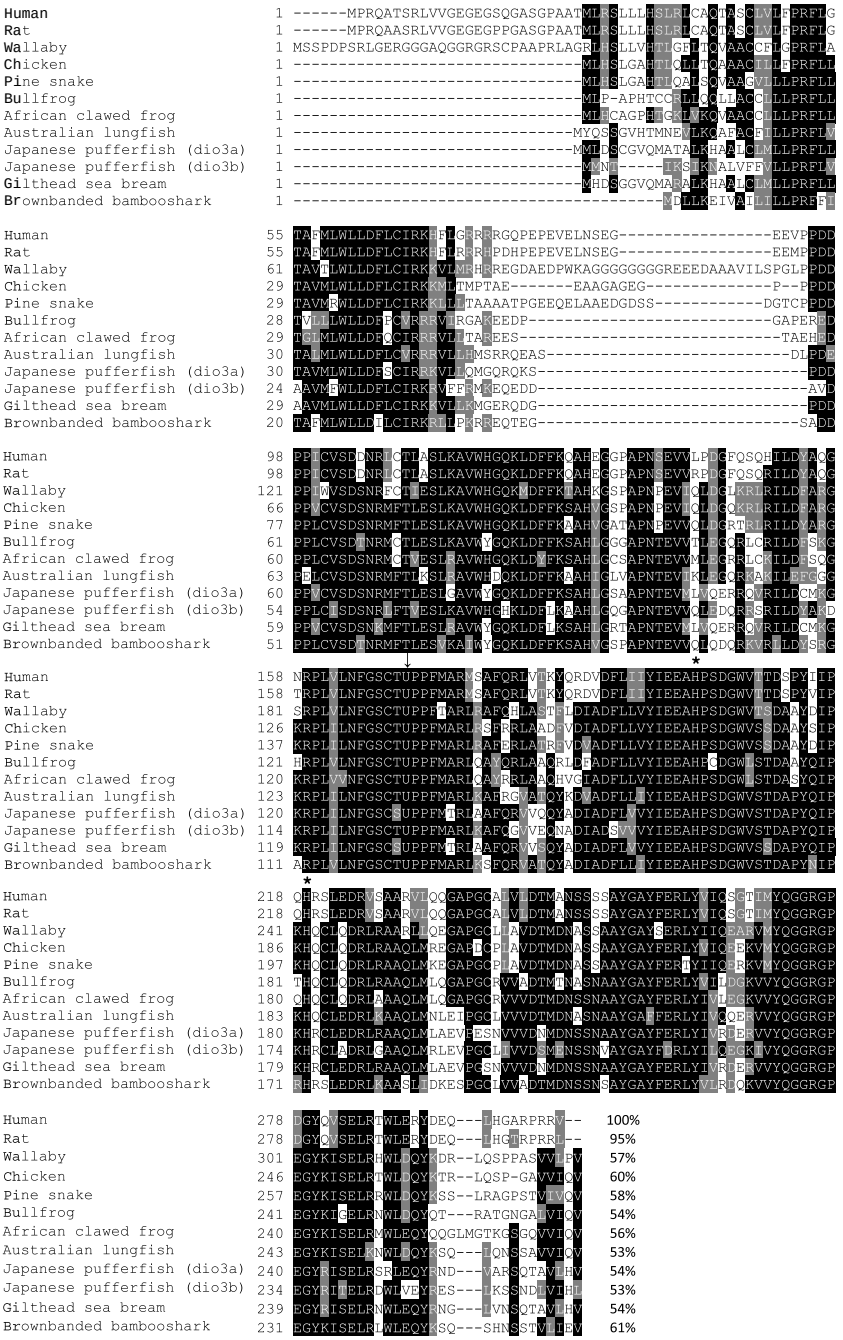


Figure 4 AA sequence alignment for some representative vertebrate D3 proteins. Identical AAs are indicated in black, similar AAs in grey. The catalytic Sec residue is indicated by the arrow and the two conserved His residues by the asterisks. Protein sequences were obtained from GenBank and aligned by ClustalW; the nucleotide accession numbers as well as genus and species names are shown in Table 3. The numbers at the end of each sequence indicate the percentage of AA identical to the human sequence.

Table 3 Vertebrate species for which the full coding sequence of D3 is available in GenBank. The two values for the number of AAs in most mammalian proteins are related to the presence of two putative translation start sites. The first number is the total length as indicated presently by GenBank and the shorter one (between brackets) the length if the translation starts at the position similar to the translation start site in non-mammalian species. Information on the *K*m for IRD of T_3 by the recombinant proteins has been taken from 1, Salvatore *et al.* (1995); 2, Croteau *et al.* (1995); 3, Villalobos *et al.* (2010); 4, St Germain *et al.* (1994); 5, Sanders *et al.* (1999); 6, Martinez *et al.* (2008); and 10, Orozco *et al.* (2003)

Species	**Common name**	**GenBank ID** (mRNA)	**Number of AAs**	***K*m** (IRD T_3)
Mammals				
Homo sapiens	Human	NM_001362	304 (278)	1·2 nM (1)
Pongo abelii	Orangutan	XM_002825118	304 (278)	
Macaca mulatta	Rhesus monkey	NM_001122649	304 (278)	
Callithrix jacchus	White-tufted-ear marmoset	XM_003734077	304 (278)	
Oryctolagus cuniculus	Rabbit	XM_002721708	281 (255)	
Mus musculus	Mouse	NM_172119	304 (278)	
Rattus norvegicus	Rat	NM_017210	304 (278)	1 nM (2)
Cricetulus griseus	Chinese hamster	NM_001256784	304 (278)	
Sus scrofa	Pig	NM_001001625	305 (278)	
Bos taurus	Cattle	NM_001010993	301 (278)	
Ovis aries	Sheep	NM_001122650	278	
Ailuropoda melanoleuca	Giant panda	XM_002926734	304 (278)	
Canis lupus familiaris	Dog	NM_001164188	304 (278)	
Macropus eugenii	Wallaby	EU919199	330	
Ornithorhynchus anatinus	Platypus	EU919198	298	
Birds				
Gallus gallus	Chicken	NM_001122648	274	
Reptiles				
Pituophis deppei	Pine snake	GQ862344	286	11 nM (3)
Amphibians				
Rana catesbeiana	Bullfrog	L41731	269	
Xenopus tropicalis	Western clawed frog	NM_001113667	271	
Xenopus laevis	African clawed frog	NM_001087863	271	2 nM (4)
Lung fishes				
Neoceratodus forsteri	Australian lungfish	AY339982	271	
Teleost fishes				
Paralichthys olivaceus	Japanese flounder	AB362423	259	
Siganus guttatus	Goldlined spinefoot	GU385469	268	
Sparus aurata	Gilthead sea bream	DQ888896	267	
Halichoeres trimaculatus	Three-spot wrasse	GU385468	248	
Chrysiptera cyanea	Sapphire devil	GU583741	267	
Oreochromis niloticus	Nile tilapia	Y11111	267	20 nM (5)
Takifugu rubripes	Japanese pufferfish	NM_001136146	268 (Dio3a)	
		NM_001136147	262 (Dio3b)	
Carassius auratus	Goldfish	EF190704	274	
Danio rerio	Zebrafish	NM_001177935	269 (dio3)	
		NM_001256003	264 (dio3a)	
Cartilaginous fishes				
Chiloscyllium punctatum	Brownbanded bamboo shark	EU275162	259	10 nM (6)

was insensitive to inhibition by PTU up to 1 mM. This mixture of characteristics does not agree with one of the three known vertebrate deiodinases, and the sequence was named hrDx. The genomes of *C. intestinalis* and *C. savignyi* both contain a partial sequence homologous to this hrDx (ciDx/csDx), as well as a second homologous sequence that was named ciDy/csDy and is not present in *H. roretzi* (Shepherdley *et al.* 2004).

Several of the deiodinase-like sequences identified from the amphioxus (*B. floridae*) genome contain a UGA codon as well as putative SECIS elements, suggesting that amphioxus expresses several selenodeiodinases (Paris *et al.* 2008*a*). Recently three partial amphioxus deiodinase-like sequences were compared, showing that one of them (bfDy) contained a UGC(Cys) codon instead of the UGA(Sec) codon present in bfDt and bfDx (Klootwijk *et al.* 2011). The full cDNA for this bfDy has been cloned. No SECIS element was found in the 3′-UTR, and UGA functions as a true stop codon at the end of the coding sequence. The resulting enzyme (266 AA long) did not deiodinate T_4 or T_3 but, surprisingly, catalysed IRD of 3,5,3′-triiodothyroacetic acid (Triac) and 3,5,3′,5′-tetraiodothyroacetic acid (Tetrac) with a *K*m of respectively 6·8 and 68 nM. The enzyme was stimulated by DTT, was not inhibited by PTU or IAc and was weakly inhibited by IOP (Klootwijk *et al.* 2011). Although the enzyme did not deiodinate T_4 and T_3, both hormones can probably bind to its

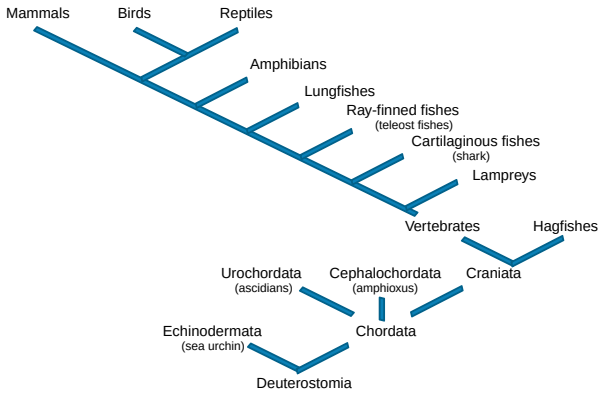


Figure 5 Evolutionary relationship between the major groups of Deuterostomia based on information from the taxonomy page of NCBI (http://www.ncbi.nlm.nih.gov/taxonomy). The position of some specific species/subdivisions discussed in this review is shown between brackets.

active centre as they were capable of inhibiting IRD of Triac. This is different from the situation in the sea squirt *H. roretzi* where neither Triac nor Tetrac could compete for the ORD of rT_3 (Shepherdley *et al.* 2004). The high affinity of bfDy for Triac is very interesting from an evolutionary point of view, as it has been shown that the amphioxus homologue of the vertebrate TH receptors is stimulated by Triac and not by T_3, suggesting that Triac could be an ancient bioactive TH (Paris *et al.* 2008*b*, Klootwijk *et al.* 2011).

Comparison of the deduced AA sequence of the deiodinase-like sequences of amphioxus and the ascidians with that of deiodinases from some representative vertebrate species shows that the urochordate sequences cluster together in a group, clearly distinct from the three clusters of typical vertebrate D1, D2 and D3, as well as distinct from the cephalochordate sequence (Fig. 6A). Despite this clear difference in overall sequence, the core AA sequence is well conserved (Fig. 6B).

Some recent papers also report the existence of deiodinase-like sequences in non-chordate animals. One of them is a deuterostome (Fig. 5), the sea urchin *Strongylocentrotus purpuratus*, where one sequence was found containing the catalytic domain with a putative Sec (Paris *et al.* 2008*a*). An evolutionary study of eukaryotic selenoproteomes even identified a selenodeiodinase homologue in the unicellular slime mold *Dictyostelium discoideum* (Lobanov *et al.* 2007). Sequences possibly coding for non-selenodeiodinases have been identified in the genome of the sea anemone *Nematostella vectensis* (Klootwijk *et al.* 2011). The complete cloning and functional characterisation of these proteins, as well as of possible other non-chordate deiodinase-like proteins, will hopefully help to clarify the possible existence and function of (iodothyronine) deiodinases in these animals. It has been shown that several non-chordate invertebrates contain THs, probably from exogenous food sources, and this allows one to hypothesise that TH signalling may not be restricted to chordates but could indeed occur in a wide variety of animals (Heyland & Moroz 2005).

Localisation and biological role of the deiodinases

The fact that mammalian liver and kidney predominantly expressed D1, while D2 expression was mainly found in brain has originally favoured the hypothesis that D1 is a T_3 'exporting' enzyme that is responsible for most of the circulating T_3, while D2 is responsible for the production of T_3 that is used in the cells where it is produced. Early data on the distribution of D3 in mammalian tissues led to the initial idea that this enzyme has little impact in adult life and is only important during development, to protect the embryo/foetus from premature T_3 exposure. However, several decades of more profound and comparative research have substantially increased our insight into the distribution and functioning of the three types of deiodinases in vertebrates, and have led to a far more refined view on their biological role. It also showed that several functions have been conserved, while others apparently have changed during vertebrate evolution.

Control of circulating T_3 levels

Several of the more recent reviews suggest that the major role of D1 may be to clear rT_3 and sulphated iodothyronines from the circulation. As such, it functions as a scavenger enzyme to remove inactive iodothyronines and recycle iodine within the organism (Bianco *et al.* 2002, St Germain *et al.* 2009, Maia *et al.* 2011). This does not, of course, exclude a role for D1 in the peripheral production of circulating T_3. In postnatal mammals, D1 is typically the major deiodinating activity present in the liver and kidney (Bates *et al.* 1999, Wassen *et al.* 2004), two organs that have a high blood perfusion rate. Subcellular localisation studies have shown that D1 is integrated in the plasma membrane in contrast to D2, which is localised in the endoplasmic reticulum (Baqui *et al.* 2000). This localisation could certainly contribute to the rapid equilibration of the T_3 produced by D1 in liver and kidney with plasma T_3. Recently, some polymorphisms in the *DIO1* gene in humans were also found to be correlated with changes in plasma T_3 (de Jong *et al.* 2007). However, there are also arguments against a predominant role of D1 in peripheral T_3 production. The major one is that D1 is extremely inefficient in converting T_4 into T_3 when compared with D2, which has a 700-fold greater catalytic efficiency (Maia *et al.* 2011).

It is clear by now that in mammals both D1 and D2 contribute to the production of plasma T_3, but their relative contribution varies amongst species. In euthyroid rat, where ~50% of plasma T_3 derives from extrathyroidal T_4 to T_3 conversion (Chanoine *et al.* 1993), D1 (mainly from liver and kidney) and D2 (mainly from skeletal muscle) may equally contribute to peripheral T_3 production (Nguyen *et al.* 1998). In euthyroid man, where as much as 80% of T_3 is produced

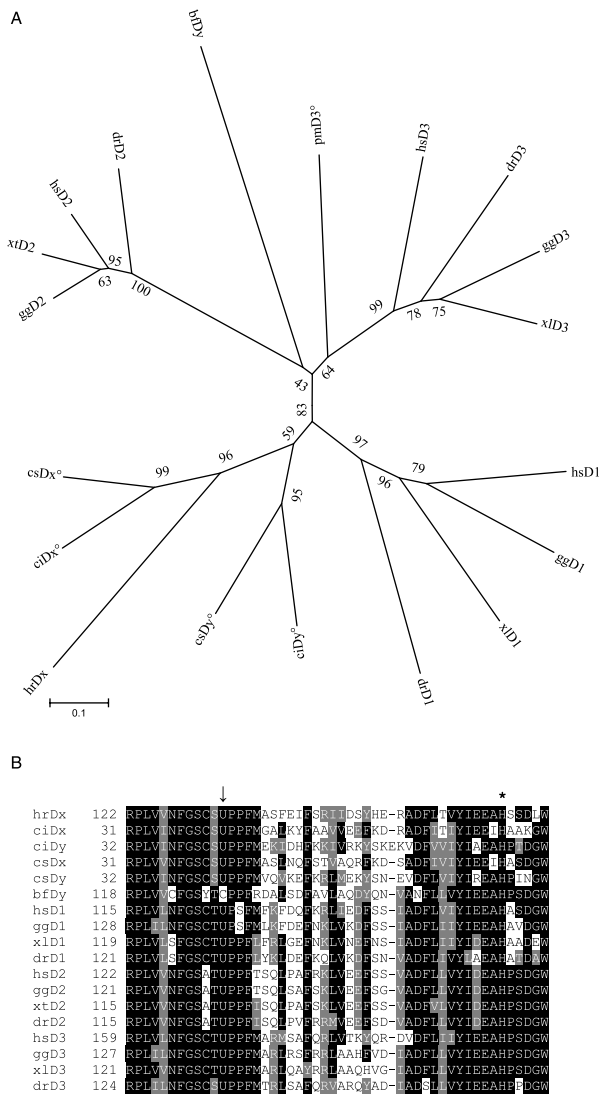


Figure 6 (A) Phylogenetic tree of invertebrate and representative vertebrate deiodinases AA sequence information using the Neighbour-joining method with 1000 bootstrap replications. The percentage of replicate trees in which the associated taxa are clustered together is indicated next to the branches. The tree presented is unrooted. Sequences marked by 'open circle' are only partially known sequences. (B) Alignment of the core amino acid sequences of invertebrate and representative vertebrate deiodinases. Identical AAs are indicated in black, similar AAs in grey. The catalytic Sec residue is indicated by the arrow and the conserved His residue by the asterisk. Vertebrate sequence information was obtained from GenBank, invertebrate sequences are available in literature (Shepherdley *et al.* 2004, Klootwijk *et al.* 2011). Bf, *Branchiostoma floridae*; ci, *Ciona intestinalis*; cs, *Ciona savigny*; dr, *Danio rerio*; gg, *Gallus gallus*; hs, *Homo sapiens*; hr, *Halocynthia roretzi*; pm, *Petromyzon marinus*; xl, *Xenopus laevis*; xt, *Xenopus tropicalis*.

peripherally (Engler & Burger 1984), D2 from skeletal muscle could be the major source of plasma T_3 (Maia *et al.* 2005).

In several birds and reptiles, high D1 activity is found in liver and kidney, while some peripheral organs such as liver, lung, and intestine express low levels of D2 (Freeman & McNabb 1991, Darras *et al.* 1992, Gereben *et al.* 1999, Fenton & Valverde 2000, Shepherdley *et al.* 2002). It can therefore be assumed that in these vertebrate classes too, both enzymes are important for the peripheral production of the T_3 that is released into the circulation. By contrast, D1 does not seem to play a role for plasma T_3 in amphibians. D1 mRNA is present in *Xenopus* tadpoles, mainly in the head region (Morvan Dubois *et al.* 2006), but so far D1 activity has not been detected in any adult amphibian, where peripheral tissues only show D2 and D3 activity (Galton 1988, Darras *et al.* 2002). In the case of fish it does not seem possible to generalise. Substantial D2 expression/activity has been found in peripheral organs, including liver and kidney, but the amount of D1 expression/activity varies widely amongst species (Leatherland *et al.* 1990, VanPutte *et al.* 2001, Orozco *et al.* 2003, Picard-Aitken *et al.* 2007). In euthyroid tilapia, D1 activity is found in kidney but not in liver (Mol *et al.* 1993), and fasting and re-feeding experiments suggested that D2 is the major enzyme regulating circulating T_3 levels (Van der Geyten *et al.* 1998).

The amount of T_3 in plasma is controlled not only by T_3 production but also by T_3 degradation. D3 plays a major role in this process, especially during development, and this role seems to be conserved throughout vertebrate evolution. Inverse correlations between decreasing D3 activity and increasing plasma/whole body T_3 levels have been observed in developing vertebrates (Galton & Hiebert 1988, Darras *et al.* 1992). In chicken embryos, an acute decrease in hepatic D3 expression following growth hormone or glucocorticoid injection rapidly increased circulating T_3 concentrations (Van der Geyten *et al.* 2001*a*). D3 also contributes to the control of plasma T_3 in adult life. This is most evident in situations where peripheral D3 expression is increased, such as illness and food deficiency. Partial food restriction strongly increased D3 activity in liver of chicken and rat, resulting in a drop in circulating T_3 without concomitant change in hepatic D1 activity (Darras *et al.* 1995). Increased D3 activity in liver and skeletal muscle has also been linked to the reduction in plasma T_3 in critically ill humans and rabbits (Peeters *et al.* 2003, Debaveye *et al.* 2005).

Control of intracellular T_3 availability

Part of the T_3 present in cells is taken up directly from the circulation while another part is produced within the cell. The ratio of plasma derived vs locally produced T_3 varies amongst different tissues. Early studies in rat injected with radiolabelled T_4 and T_3 showed that the percentage of locally produced T_3 bound to the nuclei was low for tissues with predominant D1 activity (14% in kidney and 28% in liver) and high for tissues with predominant D2 activity (55% in brown

adipose tissue and 65–75% in cerebral cortex; Silva *et al.* 1978, Crantz *et al.* 1982, van Doorn *et al.* 1985, Bianco & Silva 1987). Together with the finding that D2 is located in the endoplasmic reticulum (Baqui *et al.* 2000) this led to the idea that the T_3 generated by D2 in a given cell stays within that cell and binds to the nuclear receptors, unless it is degraded by D3. Later evidence, however, showed that this is not necessarily the case. The T_3 produced by D2 in peripheral tissues of amphibians and fish is an important source of plasma T_3 and a study in tilapia suggested that the type of tissue expressing ORD activity (in that case liver) and not necessarily the type of deiodinase *per se* (in that case D2) determines whether the T_3 is released into the plasma (Van der Geyten *et al.* 1998).

The combined presence of D2 and D3 in many tissues and the coordinated changes in their activities during ontogeny allow these tissues to control intracellular T_3 availability in part independently from the level in circulation. Perhaps the best example is found during amphibian metamorphosis, where at a given stage different tissues undergo opposite changes. The expression patterns of D2 and D3 found in metamorphosing *R. catesbeiana* tadpoles were clearly tissue-specific and linked to the different timing of metamorphic changes. Interestingly, in tissues where both genes were expressed, the expression profiles changed in a parallel way, indicating a potential for really tight control of intracellular T_3 levels (Becker *et al.* 1997). This does not, however, imply that D2 and D3 are necessarily expressed within the same cell. This has become very clear from studies in brain, where the cellular distribution pattern of D2 and D3 has been studied at the mRNA and protein level. The general picture emerging from these studies in rat, mouse and human is that D2 expression is restricted to glial cells while neurons mainly express D3 (Guadano-Ferraz *et al.* 1997, Tu *et al.* 1999). Neurons therefore cannot convert T_4 into T_3 and depend on T_3 produced by neighbouring glial cells or taken up into the brain via the cerebrospinal fluid or the blood capillaries present throughout the brain. As cellular uptake and efflux of THs are regulated by a variety of TH transporters (Visser *et al.* 2008, 2011), it is now clear that intracellular T_3 availability in neurons (and any other cell type) is tightly controlled by the combined action of TH transporters and deiodinases (Horn & Heuer 2010). D2 and D3 are present in all non-mammalian vertebrate brains studied and can therefore work together in controlling intracellular T_3 levels. D2 is clearly expressed in several glial cell types of chicken brain and D3 has been found in neurons (Gereben *et al.* 2004, Verhoelst *et al.* 2005). Interestingly, an immunohistochemical study in embryonic chicken cerebellum suggested that both D2 and D3 are transiently expressed in Purkinje cells, although not at the same time (Verhoelst *et al.* 2005). More detailed data on the cellular distribution of both deiodinases during the ontogeny of different species are certainly needed to draw general conclusions on the presence or absence of D2 in vertebrate neurons.

It remains unclear whether D1 plays a role in regulating intracellular T_3 availability in brain. D1 mRNA has been detected in brain of several mammals, birds and fish, and even in *Xenopus* tadpoles (Bates *et al.* 1999, Chan *et al.* 2002, Morvan Dubois *et al.* 2006, Johnson & Lema 2011, Van Herck *et al.* 2012). This mRNA is translated at least in part into D1 protein as shown in chicken, where the amount of D1 protein in cerebellum was influenced by thyroid status (Verhoelst *et al.* 2004). However, only minimal D1 activity could be found in brain tissue so far.

Response of deiodinases to thyroid status

The exact molecular mechanisms by which deiodinase expression is regulated at the transcriptional level remains unclear, because only the human *DIO1* gene has been shown to contain TREs in the 5′-FR (Toyoda *et al.* 1995). It is nevertheless not surprising that all three deiodinases are highly responsive to thyroid status given their important role in regulating intracellular T_3 availability. Hypothyroidism increases D2 mRNA expression and decreases D3 mRNA expression, while hyperthyroidism has the opposite effects. These typical responses occur in different tissues and have been demonstrated in a wide variety of vertebrates (St Germain *et al.* 1994, Bianco *et al.* 2002, Orozco & Valverde 2005, Rudas *et al.* 2005, Johnson & Lema 2011). In addition to transcriptional regulation, D2 is also strongly regulated by T_4 at the posttranslational level via a process of ubiquitination (Gereben *et al.* 2008, Arrojo & Bianco 2011).

The TH-dependent regulation of D1 seems to be more complex. It has been known for a long time that D1 activity in liver and kidney is increased in hyperthyroid rat and decreased in hypothyroid rat and this was confirmed later at the mRNA level (Kaplan & Utiger 1978, Berry *et al.* 1990). Similar changes have been observed in mouse and human (Maia *et al.* 1995*b*, Zhang *et al.* 1998, Friedrichsen *et al.* 2003). A study in pig with an anti-D1 antiserum showed that the amount of D1 protein was strongly reduced in microsomes prepared from hypothyroid liver or kidney, while there was no or only a slight increase of D1 in microsomes from hyperthyroid animals (Wassen *et al.* 2004). In house musk shrew, hypothyroidism did not reduce hepatic D1 mRNA and activity, while administration of a high dose of T_3 resulted in a modest but significant decrease in D1 mRNA and activity (Rogatcheva *et al.* 2002).

The few data available from birds (chicken) show some variation, but in most studies hyperthyroidism seemed to increase hepatic D1 activity, while hypothyroidism decreased hepatic D1 activity and also lowered D1 protein in brain (Decuypere *et al.* 1987, Verhoelst *et al.* 2004, Darras *et al.* 2006). Data from fish studies show a different response of D1 to thyroid status. A 24-h exposure to T_4, T_3 or 3,5-T_2 decreased D1 mRNA levels in killifish liver but without affecting enzyme activity (Garcia *et al.* 2004). Long-term T_3 supplementation in the food in rainbow trout also did not change hepatic D1 activity (Finnson & Eales 1999). In tilapia, long-term T_3 supplementation had no impact on hepatic or renal D1 activity, while T_4 supplementation strongly

increased hepatic D1 activity (Van der Geyten *et al.* 2005). Long-term hypothyroidism strongly induced D1 mRNA and activity in tilapia liver, while D1 activity in kidney was reduced or remained unchanged (Mol *et al.* 1999, Van der Geyten *et al.* 2001*b*). By contrast, hypothyroidism increased D1 activity in kidney of hypothyroid rainbow trout (Burel *et al.* 2000). In striped parrotfish, neither T_3 nor methimazole exposure changed D1 mRNA levels in liver, brain or gonads (Johnson & Lema 2011). Taken together, these data suggest that the divergence in D1 response in fish might be related not only to species or tissue differences but also to the way hypo- or hyperthyroidism is induced.

Conclusion

The presence of iodothyronine deiodinases in animals as diverse as ascidians and humans suggests that this type of enzyme was already present in the common ancestor of all chordates and possibly even earlier in evolution. The comparative study amongst vertebrates has shown that the structure of each of the three typical types of deiodinases (D1, D2 and D3) has been highly conserved. This is also true for most of their biochemical properties, with the clear exception of the PTU sensitivity of D1 and possibly also its DTT dependence. Although many full coding sequences have been identified by now, only a limited number of D1 and even fewer D2 and D3 cDNAs have been translated into recombinant proteins for functional characterisation. More efforts in this direction, combined with site-directed mutagenesis, would certainly help to further unravel the specific interactions between the enzyme and different substrates/inhibitors that are responsible for the typical differences in activity between the three enzyme types. A more detailed elucidation of the 3D structure of deiodinases would also be very helpful in this context. Another remaining challenge for the future is the identification of the natural cofactor(s) for these enzymes as their functional characteristics, typically determined in the presence of the artificial reductant DTT, may certainly differ *in vivo*.

Studies from fish to mammals have shown that D2 and D3 are expressed and active in all investigated species and react to thyroid status in a consistent way. The presence of D1 activity seems more variable and its response to thyroid status more divergent. As a result, the relative contributions of D1 and D2 to circulating T_3 levels also show considerable fluctuations. Comparative studies of the 5′-FR of the deiodinase genes from a wider variety of vertebrates, as well as further research on possible posttranscriptional regulation, are needed to increase our understanding of the molecular mechanisms controlling deiodinase expression.

Declaration of interest

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

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